ANTIRETROVIRAL AGENTS AND HIV-1 DISEASE: ANALYSIS OF *IN VITRO* PHENOTYPIC AND FUNCTIONAL MARKERS OF ZIDOVUDINE THERAPY

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ABSTRACT

At present the reverse transcriptase inhibitor zidovudine is the only agent shown to inhibit human immunodeficiency virus type 1 (HIV-1) replication *in vitro* and to slow symptomatic disease *in vivo*. For ethical reasons, studies of zidovudine and other antiretrovirals now use surrogate markers of HIV-1 infection rather than clinical end-points as determinants of efficacy.

Enumeration of circulating CD4+ T-lymphocytes is the most widely used marker of immunological status. However, absolute CD4+ numbers do not correlate well with disease progression nor with the prolonged clinical benefits of zidovudine therapy. The present study was designed to assess lymphocyte functional responses as a potential guide to immunological change following zidovudine and other antiretroviral therapies. Phenotypic studies were performed in parallel.

The proliferative and gamma-interferon (gIFN) responses of peripheral lymphocytes to T-cell mitogens Phytohaemagglutinin (PHA) and Concanavalin A (Con A) decreased with progressive HIV-1 disease. Substantial decline of proliferation was observed before the onset of clinical symptoms, while gIFN synthesis became significantly impaired with symptomatic disease. Cross-sectional and longitudinal studies of patients with acquired immune deficiency syndrome (AIDS) and AIDS-related complex (ARC) demonstrated that zidovudine substantially augmented these mitogenic responses. In contrast, three subjects receiving the lipid compound AL 721, which showed no evidence of clinical benefit, exhibited either no change or deteriorating serial PHA proliferative and gIFN response patterns. Furthermore, one of these patients showed significant improvement when treated with zidovudine after discontinuing AL 721.

Preliminary serial observations suggest that HIV-1 associated phosphatidylinositol metabolism defects are reversed with zidovudine therapy and parallel the

improvements seen in PHA induced proliferation and gIFN synthesis.

The T-lymphocyte responses to the soluble recall antigen Streptokinase/Streptodornase show less clear trends.

Zidovudine therapy had no apparent effect on the abnormal synthesis of IgG, IgM and IgD produced spontaneously or in response to Pokeweed mitogen by lymphocytes obtained from ARC and AIDS patients.

The improvement of mitogenic proliferative and gIFN responses observed with zidovudine treatment parallels any temporal trends of clinical benefit. Analysis of inositol polyphosphates may also be useful in this context. Functional responses to PHA or Con A provide immunological markers of antiretroviral therapy, encompassing both numerical and functional alterations in CD4+ lymphocytes, and correlates of clinical events.

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ABBREVIATIONS

Clinical conditions

AIDS.....acquired immune deficiency syndrome
ARC.....AIDS-related complex
PGL.....persistent generalised lymphadenopathy

Drugs

AL 721.....Active Lipids 721
CAS.....castanospermine
D4T.....2',3'-didehydro-2',3'-dideoxythymidine
ddA.....2',3'-dideoxyadenosine
ddC.....2',3'-dideoxycytidine
ddI.....2',3'-dideoxyinosine
GM-CSF.....granulocyte-macrophage colony stimulating factor
N-butyl DNJ.....N-butyl deoxynojirimycin
rIFNα.....recombinant interferon-alpha
rsCD4.....recombinant soluble CD4
TIBO.....tetrahydro-imidazo [4,5,1,jk] [1,4]-benzodiazepin-2(1h)-one/-thione
ZDV.....zidovudine

Immunology

ADCC.....antibody-dependent cellular cytotoxicity
CD.....cluster determinant
CD4+.....CD4-bearing
CD8+.....CD8-bearing
CDC.....Centers for Disease Control
CTL.....cytotoxic T-lymphocyte
FcR.....immunoglobulin receptor
gIFN.....gamma-interferon
HLA.....human lymphocyte associated
IFN.....interferon
Ig.....immunoglobulin
Il.....interleukin

Immunology (continued)

MHC.....major histocompatibility complex
NK.....natural killer cell
TAC.....T-lymphocyte activation antigen
TCR.....T-cell receptor complex
Tc/s.....T-cytotoxic/suppressor lymphocyte
Th.....T-helper/inducer lymphocyte
Ti.....T-cell antigen receptor

Inositol polyphosphate metabolism

$[Ca^{2+}]_i$.....intracellular calcium
Ins.....inositol
PtdIns.....phosphatidylinositol
PtdIns(4,5)P2.....phosphatidylinositol 4,5-bisphosphate
InsP.....inositol monophosphate (unspecified isomer)
InsP2.....inositol bisphosphate (unspecified isomer)
InsP3.....inositol trisphosphate (unspecified isomer)
InsP4.....inositol tetrakisphosphate (unspecified isomer)
DG.....diacylglycerol (isomer undefined)

Reagents and technical terminology

Å.....angstrom
A.....azide
APAAP.....alkaline phosphatase-anti-alkaline phosphatase
Bmax.....maximal binding
Bmin.....minimal or non-specific binding
BS.....blocking substrate
B/T %.....bound radioactivity/total radioactivity
CD.....Celltech Diagnostics
Con A.....concanavalin A
cpm.....counts per minute
CV.....coefficient of variation
d.....debris
Da.....dalton
dH_2O.....distilled water

Reagents and technical terminology (continued)

ELISA.....enzyme linked immunosorbent assay
FCS.....fetal calf serum
FITC.....fluorescein isothiocyanate
FCM.....flow cytometry
FSC.....forward or low angle scatter
G.....granulocyte population
GAM.....goat anti-mouse
hiFCS.....heat inactivated fetal calf serum
HPLC.....high performance liquid chromatography
hu gIFN.....human gamma-interferon
Ig.....immunoglobulin
IRMA.....immunoradiometric assay
L.....lymphocyte population
M.....monocyte population
Mab.....monoclonal antibody
MD.....Medgenix Diagnostics
MRC.....Medical Research Council
MW.....molecular weight
NHS.....normal human serum
NSB.....non-specific binding
OD.....optical density
PBMC.....peripheral blood mononuclear cells
PE.....phycoerthyrin
PBS.....phosphate buffered saline
PBS/T.....PBS/Tween 20
pen.....benzylpenicillen
PHA.....phytohaemagglutinin
PWM.....pokeweed mitogen
RAM.....rabbit anti-mouse
RPMI/C.....RPMIpen/strep with hiFCS
RPMIpen/strep.....RPMI with pen and strep
SD.....standard deviation
SEM.....standard error of the mean
SK/SD.....streptokinase/streptodornase
SSC.....side or 90^{o} scatter
strep.....streptomycin sulphate

Reagents and technical terminology (continued)

TBS.....tris-buffered saline

TCA.....trichloroacetic acid

[3H]TdR.....tritiated-methyl thymidine

[3H]TdR, RPMI/C...[3H]TdR in RPMI/C

WCCF.....white cell counting fluid

$\bar{x}$.....mean

Virology and molecular biology

cDNA.....cloned DNA

CMV.....cytomegalovirus

DNA.....deoxyribonucleic acid

EBV.....Epstein Barr virus

HIV-1.....human immunodeficiency virus type 1

HIV-2.....human immunodeficiency virus type 2

HTLV-1.....human T-lymphotropic virus type 1

GTP.....guanosine triphosphate

LTR.....long terminal repeat

mRNA.....memory RNA

RNA.....ribonucleic acid

RRE.....rev responsive element

SIV.....simian immunodeficiency virus

TAR.....cis-acting tat-responsive sequence

CHAPTER 1

INTRODUCTION

1.1. General introduction

Clinical and basic immunology have been greatly challenged by the emergence in the last decade of the acquired immune deficiency syndrome (AIDS) and its associated disorders.

AIDS was initially recognised as a distinct clinical entity in 1981, when outbreaks of *Pneumocystis carinii* pneumonia and Kaposi's sarcoma were recognised in previously healthy homosexual men (Centers for Disease Control, 1981a,b). The remarkable occurrence of these and other rare illnesses, which had previously been confined largely to immunocompromised hosts, led to the rapid realisation that this was a novel cellular immunodeficiency disorder (Fauci *et al.*, 1984; Gottlieb *et al.*, 1981; Mildvan *et al.*, 1982; Siegal *et al.*, 1981).

By early 1982, epidemiological evidence indicated that AIDS was caused by a transmissible agent and research focused upon its isolation (Centers for Disease Control, 1982). The aetiological agent of AIDS, the human immunodeficiency virus type 1 (HIV-1), was first reported in 1983 (Barre-Sinoussi *et al.*, 1983) and became recognised as the causative virus when isolates were obtained from numerous patients with AIDS or AIDS-related complex (ARC; Gallo *et al.*, 1984). HIV-1 is now one of the best studied of all retroviruses and is the prototype for a set of primate lentiviruses, including simian immunodeficiency virus (SIV; Kanki *et al.*, 1985a,b; Murphey-Corb *et al.*, 1986) and a second human immunodeficiency virus (HIV-2) isolated from West African subjects (Clavel *et al.*, 1986; Kanki *et al.*, 1986).

The identification of AIDS and its aetiological agent, HIV-1, have been followed by a major global pandemic of AIDS and HIV-1 infection. Current estimates indicate that several hundred thousand AIDS cases have occurred, largely from the second part of the last decade. These reflect the current burden of disease resulting from the spread of HIV-1 that is now reckoned to affect some 5 to 10 million persons

(Centers for Disease Control and World Health Organisation, 1990).

Intense scientific and medical, as well as public, interest in AIDS has caused rapid advances in the clinical, epidemiological, immunological and virological knowledge of HIV-1 infection. This knowledge has been central to an understanding of the pathogenesis of this disease.

It is now well established that the underlying lesion in advanced HIV-1 infection, perceived clinically as ARC or AIDS, is the profound depletion of peripheral and tissue helper-inducer CD4-bearing (CD4+) T-lymphocytes (Fauci et al., 1984; Gottlieb et al., 1981; Mildvan et al., 1982). The selective loss of these CD4+ T-cells reflects the cellular tropism of HIV-1, through the binding of its envelope glycoprotein, gp 120, to the CD4 molecule (Dalgleish et al., 1984; Klatzmann et al., 1984a,b; Maddon et al., 1986; McDougal et al., 1986). However, the pathogenetic mechanisms by which HIV-1 causes depletion of CD4+ T-lymphocytes remains controversial. Nevertheless, given that these cells play a central regulatory role in the immune system through cognate interaction or cytokine release, loss from the CD4+ T-lymphocyte subset consequently compromises the function of other cell types of the immune system (Pinching, 1990). In addition, substantial data now indicate that T-lymphocyte, B-lymphocyte and monocyte-macrophage cellular dysfunction occurs independently of CD4+ T-lymphocyte depletion (Via et al., 1990a). Recent work has suggested that HIV-1 induced changes in signal transduction mechanisms, by the inositol polyphosphate pathway, may be responsible for some, if not all, of these functional changes (Nye et al., 1991).

As the HIV-1 replicative cycle and the pathogenetic mechanisms by which HIV-1 damages the immune system become better understood, the possible development of a successful therapeutic stratagem improves accordingly. This is especially important as initial optimism concerning a safe effective vaccine has long since receded (Hirsch, 1990).

The capacity to cultivate large quantities of

HIV-1 *in vitro* has allowed the practical development of numerous antiretroviral agents. The HIV-1 enzyme reverse transcriptase, which is essential to viral replication, has been the major target for antiretrovirals developed to date (Mitsuya and Broder, 1987; Yarchoan *et al.*, 1989a).

The reverse transcriptase inhibitor zidovudine (ZDV), was found to be a potent inhibitor of HIV-1 infectivity and cytopathogenicity *in vitro* (Mitsuya *et al.*, 1985). Subsequently, several placebo-controlled trials have shown the clinical efficacy of ZDV in patients with ARC and AIDS, as defined by improved survival and fewer opportunistic infections (Fischl *et al.*, 1987; Fischl *et al.*, 1990b). Although this treatment does not result in improved long term survival for all patients (Creagh-Kirk *et al.*, 1988), ZDV remains the only widely available and clinically effective treatment of symptomatic HIV-1 infection. Clearly, there is a need to evaluate other therapeutic agents alone or in combination with ZDV.

The standard method of evaluating the efficacy of new drugs has been to conduct placebo-controlled, prospective trials using clinically relevant end-points as the main outcome of measure. In the case of HIV-1 disease, the standard end-points of death or the development of new AIDS defining opportunistic infections or neoplasms can take months or years to reach (Fischl *et al.*, 1987; Fischl *et al.*, 1990b). Thus, the development of laboratory markers which can serve as surrogate end-points has become a high priority for investigation. Such laboratory markers would be useful both for clinicians and for research workers seeking indications of clinical benefit from new antiretroviral agents.

A number of immunological and virological measures of HIV-1 activity have been shown to revert towards normal in response to ZDV treatment of HIV-1 symptomatic disease. Such markers include peripheral CD4+ T-lymphocyte count, serum β_2-microglobulin and neopterin levels and the HIV-1 specific antigen, p24. These were originally identified, in

natural history cohort studies as being predictors of progression to AIDS in HIV-1 seropositive subjects (Chaisson et al., 1988; Crocchiolo et al., 1988; Fahey et al., 1990; Fischl et al., 1987; Fischl et al., 1990b; Jacobson et al., 1989; Moss et al., 1988).

A detailed account of laboratory markers of efficacy in antiretroviral treatment will follow. These will be presented in context of the major therapeutic strategies used for HIV-1 disease, and those that are in prospect, together with the current concepts in our understanding of the myriad of clinical and immunological abnormalities that occur in HIV-1 infection.

1.2. The clinical spectrum of HIV-1 infection

HIV-1 infection is manifested by a wide variety of clinical conditions, ranging from asymptomatic infection to severe disseminated disease, which are temporally related to the initial infection (Centers for Disease Control, CDC, 1986; Table 01). The following summary provides a brief outline of these clinically defined states.

Four weeks to four months following an initial HIV-1 infection, most individuals produce antibodies to the retroviral envelope and then core antigens (see section 1.5.5.a). This seroconversion is, in the majority of individuals, silent; however in 10 to 15 percent of cases it is marked by a transient mononucleosis-like syndrome (CDC group I). Thereafter these patients, whether or not they have such an acute illness, typically develop one of two asymptomatic states: asymptomatic infection (CDC group II) or persistent generalised lymphadenopathy (PGL; CDC group III). Patients classified to CDC group II are HIV-1 seropositive, but have no clinical signs or symptoms of HIV-1 infection; whereas patients classified to CDC group III show, by definition, lymph node enlargement of 1 cm or greater at 2 or more extrainguinal sites for more than 3 months. These categories are not mutually exclusive as a minority of subjects shift from one to the other. Subjects categorised to

Table 01: The clinical spectrum of HIV-1 infection (CDC, 1986)

Group	Subgroup	Category	Definition
I			ACUTE INFECTION
II			ASYMPTOMATIC INFECTION
III			PERSISTENT GENERALISED LYMPHADENOPATHY (PGL)
IV			SYMPTOMATIC DISEASE
	A		constitutional disease (ARC)
	B		neurological disease
	C		secondary infectious disease
		C-1	specified major infectious disease (AIDS)
		C-2	specified minor infectious disease (ARC)
	D		secondary cancers (AIDS)
	E		other conditions

CDC groups II or III progress to symptomatic HIV-1 disease from approximately 1 year of diagnosis (mean: 7 to 8 years). Over 10 years, 50 percent of these individuals progress to AIDS with a further 15 percent exhibiting symptomatic disease (Pinching, 1990).

Symptomatic HIV-1 infection (CDC group IV) may comprise patients with ARC, AIDS and HIV-1 encephalopathy. The term ARC encompasses those subjects with symptoms such as weight loss, diarrhoea and fever (CDC group IVA) and those with minor opportunistic infections such as oral hairy leukoplakia, oral candidiasis and shingles (CDC group IVC2). ARC commonly progresses over months or a year or two to frank AIDS, although some patients progress directly from one of the asymptomatic states to AIDS (Pinching, 1990). AIDS comprises patients with major opportunistic infections, such as Pneumocystis carinii pneumonia, toxoplasma cerebral abscess, atypical mycobacteriosis, cryptococcosis and cytomegalovirus (CMV) disease (CDC group IVC1), or opportunistic tumours such as Kaposi's sarcoma, B-cell lymphoma and Hodgkin's disease (CDC group IVD). Patients categorised to group IVD alone show less severe clinical and laboratory evidence of immunodeficiency than those categorised to group IVC1. In addition, subjects may develop central or peripheral nervous system disease (CDC group IVB) or other miscellaneous disorders such as chronic lymphoid interstitial pneumonitis (CDC group IVE). Patients with severe HIV-1 disease may have any combination of Group IV features (Pinching, 1990).

1.3. The immunological consequences of HIV-1 infection

The HIV-1 retrovirus is tropic for the CD4-bearing (CD4+) T-lymphocyte and macrophage populations of the immune system. This section considers HIV-1 tropism together with the many ways in which the retrovirus directly or indirectly damages or affects the function of the immunological cells and hence ultimately results in lethal immune deficiency.

1.3.1. The physiological role of CD4+ T-lymphocytes

Advanced HIV-1 infection is characterised by the selective depletion of peripheral and tissue CD4+ helper/inducer T-lymphocytes (Fauci et al., 1984; Gottlieb et al., 1981; Mildvan et al., 1982; Seligmann et al., 1987., Siegal et al., 1981). This is the major mechanism of HIV-1 induced cellular immunodeficiency, perceived clinically as ARC or AIDS. Indeed, deficient CD4+ T-cell macrophage collaboration underlies the secondary infections observed in these patients. CD4+ cellular deficiency together with B-cell hyperactivity and depressed natural killer (NK) cell function may account for the increased frequency of lymphoid malignancies also seen.

Even though many viruses use surface molecules relevant to lymphocyte function as receptors, the HIV-1 receptor, CD4 (Dalgleish et al., 1984; Klatzmann et al., 1984b; Maddon et al., 1986; McDougal et al., 1986), and the CD4+ T-lymphocyte subpopulation are unique in that they are fundamental to the immune response and its regulation.

Traditionally, CD4+ T-lymphocytes have been assigned a helper/inducer (Th/i) functional role in, for example, the activation of B-cells, CD8-bearing (CD8+) T-cells, NK cells, and other CD4+ T-lymphocytes. However, this relationship is not absolute since cytotoxic CD4+ cells have been detected (Meuer et al., 1982). A more consistent relationship exists in the association of CD4+ T-lymphocytes with major histocompatibility complex (MHC) class II bearing antigen presenting cells; CD4 binds MHC class II molecules (Doyle and Strominger, 1987; Gay et al., 1987), thereby augmenting low affinity T-cell receptor (Ti) antigen interactions during antigen presentation (Greenstein et al., 1984; Marrack et al., 1983). The Ti is generally composed of a clonotypic disulphide-linked α/β heterodimer noncovalently associated with at least 5 polypeptides: δ, γ, and ε which comprise the CD3 antigen (Meuer et al., 1983) and ζ and η (Baniyash et al., 1988). The Ti together with these invariant structures, which appear to be required for signal

transduction (Imboden and Stobo, 1985), form the T-cell receptor complex (TCR). Therefore agents interfering with the CD4 antigen will most likely disturb the entire physiology of the immune system. As illustration, a number of studies have shown that anti-CD4 antibody administered to mice severely affected T-cell function and induced a transient state of unresponsiveness to antigens introduced at the same time (Goronzy et al., 1986; Goronzy and Weyand, 1989) probably by inhibiting signal transduction via the TCR (Ledbetter et al., 1988).

1.3.2. HIV-1 tropism and the cellular distribution of CD4

The selective loss of CD4+ T-lymphocytes with progressive HIV-1 infection reflects the cellular tropism of HIV-1 (Klatzmann et al., 1984a) and is mediated by the high-affinity interaction of external HIV-1 envelope glycoprotein gp120 with the T-cell surface determinant CD4 (Dalgleish et al., 1984; Klatzmann et al., 1984b; Maddon et al., 1986; McDougal et al., 1986). Despite the intrinsic variability of HIV-1, particularly in env (Hahn et al., 1986) which encodes gp120, the CD4-gp120 binding sites (Lasky et al., 1987., Traunecker et al., 1988) are highly conserved in all isolates of HIV-1 studied and furthermore in all HIV-2 and SIV isolates analysed (Sattentau et al., 1988); clearly indicating the critical importance of gp120-CD4 interaction to the retroviral life-cycle.

Although the CD4 glycoprotein was originally defined as a mature T-lymphocyte differentiation antigen, this molecule is also found to a lesser degree on certain cells of monocyte/macrophage lineage. These cells are also permissive of HIV-1 infection in vitro and in vivo. Moreover, CD4+ cells such as Langerhans' cells, follicular dendritic cells and peripheral dendritic cells for example, are, in addition to helper T-lymphocytes, subject to HIV-1 mediated depletion; presumably by comparable mechanisms (Macatonia et al., 1990; Meltzer et al., 1990a,b). Paradoxically blood monocytes and tissue macrophages which express low to

undetectable levels of CD4 are also permissive of HIV-1 replication in vitro and in vivo, and are infected by routes independent of CD4. Such routes are thought to include immunoglobulin G receptor (FcR)-mediated endocytosis, phagocytosis or interaction with receptors for mannosylated proteins (Meltzer et al., 1990a,b). Indeed, accumulating evidence suggests that there at least two distinct species of HIV-1: isolates which replicate in both monocytes and T-lymphocytes and those which replicate only in T-cells (Meltzer et al., 1990a,b). Interestingly, HIV-1 isolates that do not replicate in monocytes will infect these cells after treatment with low levels of anti-HIV-1 enhancing antibodies (Homsy et al., 1989; McKeating et al., 1990). The precise mechanisms of these events are not clear. However, unlike CD4+ cells, these monocytes/macrophages show little evidence of attrition throughout the course of HIV-1 disease (Enk et al., 1986; Poli et al., 1985; Siegal et al., 1986a). This suggests that the binding of CD4 to HIV-1 gp120 is central to HIV-1 cytopathogenicity.

Irrespective of the mechanisms of HIV-1 infection, the various macrophage subpopulations are now regarded as major reservoirs of HIV-1 infection; moreover, it is likely that these phagocytes are the first targets of HIV-1 and serve to transmit the virus to other susceptible cellular populations, including CD4+ T-lymphocytes (Meltzer et al., 1990a,b; Pinching, 1990).

It is now apparent that the cellular tropism of HIV-1 together with its mechanisms of infection and dissemination must be vital considerations of therapeutic strategies, particularly those focused to the CD4 receptor (see section 1.4.2.).

1.3.3. The immunopathogenesis of HIV-1 infection: CD4+ T-lymphocyte depletion

A number of mechanisms have been proposed to account for the elimination of HIV-1-infected CD4+ cells; these include virus-mediated cytopathic effects, such as

lysis and syncytium formation (see section 1.3.3.a), and host-mediated mechanisms, such as antibody-dependent cellular cytotoxicity (ADCC) and cytotoxicity mediated by MHC-restricted cytotoxic T-lymphocytes (CTL; see section 1.3.3.b). Elimination of uninfected cells may occur as a consequence of viral gp120 attachment to cell surface CD4 (see sections 1.3.3.a,b).

Nevertheless, a puzzling dichotomy seems to exist between the low level of productively HIV-1 infected CD4+ T-lymphocytes, estimated at less than 0.01 percent of peripheral leucocytes by *in situ* hybridisation (Harper *et al.*, 1986), and the severe and selective depletion of these cells *in vivo*. This phenomenon has been coined 'the false paradox' by Pinching (1990).

Many CD4+ T-cells are eliminated prior to the development of symptomatic HIV-1 disease, particularly AIDS (Detels *et al.*, 1988; Lang *et al.*, 1989; see section 1.5.1.a). If, following their infection by HIV-1, CD4+ T-lymphocytes were rapidly destroyed by whatever mechanism, they would no longer be detectable in the circulation or in the tissues. Thus the remaining CD4+ T-cells, at any time throughout the course of HIV-1 infection, may largely represent the uninfected pool. The small proportion of cells showing evidence of HIV-1 infection could represent those cells not yet destroyed and/or those not expressing the virus in a way that leads to their elimination (Pinching, 1990). Therefore, extrapolating data obtained from those CD4+ T-lymphocytes remaining at analysis to those cells already eliminated requires cautious interpretation.

1.3.3.a HIV-1 induced cytotoxicity

The simplest explanation of HIV-1 mediated CD4+ cell depletion is that the virus itself exerts direct cytopathic effects. In evidence, extensive HIV-1 budding causes destruction and lysis of the cellular plasma membrane under conditions of optimal mitogen and cytokine stimulation *in vitro* (Leonard *et al.*, 1988; Lynn *et al.*, 1988). Moreover,

the large amounts of nonintegrated HIV-1 deoxyribonucleic acid (DNA), heterodisperse ribonucleic acids (RNAs), core proteins and CD4-gp120 complexes (Hoxie et al., 1986; Koga et al., 1988; Shaw et al., 1984., Somasundaran and Robinson, 1988) found in infected cellular cytoplasm, may contribute to HIV-1 induced cytotoxicity. However, it seems unlikely that these are common events, though it would be difficult to discern directly.

An alternative explanation of CD4+ cell depletion in vivo considers a characteristic phenomenon of HIV-1 infected cell culture: the formation of syncytium and short-lived multinucleated giant cells. Such fusion is dependent on CD4-gp120 interaction; the high-affinity binding of CD4 and gp120 (Kd: 10^{-9} M) results in the recruitment of nearby uninfected CD4+ cells into the syncytia with HIV-1 infected cells (Lifson et al., 1986; Sodroski et al., 1986a; Yoffe et al., 1987). The viral transmembrane glycoprotein, gp41, is also postulated to be important in syncytium formation (Fisher et al., 1986b). Moreover, Emilie et al (1990), using a quantitative bioassay (the β-gal assay, originally described by Rocancourt et al., 1990) to visualise and quantify syncytium induction by fresh HIV-1 isolates, have correlated in vitro syncytium formation with peripheral CD4+ T-lymphocyte depletion.

Although this mechanism of syncytium formation is deemed to be the most likely explanation of HIV-1 induced cytopathology, histopathological studies have detected syncytia in the lymph nodes and brains of postmortem AIDS patients relatively infrequently (Sattentau and Weiss, 1988) and such peripheral cells are very rare (Pinching, 1990). However, if produced in the host, these fused cells are likely to die or be eliminated rapidly. In addition, normal peripheral blood lymphocytes may be killed in vitro without evidence of cell-cell fusion (Sattentau and Weiss, 1988).

1.3.3.b Host-mediated cytotoxicity

Investigators have recently suggested that host

mechanisms of viral control may mediate the destruction of both infected and uninfected CD4+ T-lymphocytes, in an HIV-1-specific CTL or ADCC autoimmune response (Pinching, 1990; Rosenberg and Fauci, 1990; Zinkernagel, 1988). However, the precise role of the immune system in the pathogenesis of HIV-1 remains to be elucidated.

HIV-1-specific CTL typically conform to established patterns of virus-immune CTL; generally CD8+ CTL are MHC class I restricted and CD4+ CTL MHC class II restricted (Plata et al., 1989), although HIV-1-specific clones with unusual restriction patterns have been observed (Walker and Plata, 1990). Numerous studies have shown MHC class I restricted CD8+ CTL specific for HIV-1 structural (env, gag and pol) and regulatory (vif and nef) gene encoded proteins (Plata et al., 1989). Host CD8+ CTL may therefore be cytolytic for any cell expressing these viral antigens with MHC class I; in evidence, such CTLs have been shown to control HIV-1 virus titres in vitro (Walker et al., 1986). Thus, in addition to controlling viral infection, CD8+ CTL may play an important role in the destruction of CD4+ T-lymphocytes observed with HIV-1 disease progression in vivo. Indeed Zinkernagel (1988) has proposed, based on the characteristics of lymphocytic choriomeningitis virus infection of mice, that elimination of CD8+ CTLs, or generation of CD8+ T-cell tolerance to HIV-1, may lead to a symptom-free virus-carrier state.

In addition, the CD4+ T-lymphocyte population seems particularly susceptible to gp120-specific CTL cytolysis, as delineated by Siliciano et al (1988). This group isolated CD4+ T-lymphocyte clones from seronegative donors cytolytic for autologous CD4 bearing, MHC class II bearing T-lymphocytes. Cytolysis was dependent upon target cell CD4-mediated uptake and internalisation of free gp120 and subsequent presentation of a processed gp120 peptide in conjunction with MHC class II. By this mechanism uninfected CD4+ cells, if MHC class II positive, become targets for elimination by these CTL. Moreover, the presence of CD4+

gp120-specific CTL may account for the early loss of recall memory responses: since most circulating T-cells are unactivated and therefore MHC class II negative, this response may focus on previously activated MHC class II positive T-lymphocytes or those T-cells which rapidly regain MHC class II during the restimulation process (reviewed by Germain, 1988). Miedema et al (1990) have recently postulated that selective loss of memory T-lymphocytes early in infection plays a fundamental role in the pathogenesis of HIV-1.

Lyerly et al (1987) have shown, in vitro, that when uninfected CD4+ cells bind free gp120 they are also rendered targets for ADCC immune responses, via anti-gp120 antibodies. Several other groups have demonstrated that HIV-1-infected cell lines are eliminated when exposed to small amounts of sera obtained from HIV-1 infected subjects and normal control peripheral blood mononuclear cells by ADCC in vitro (Blumberg et al., 1987; Ljunggren et al., 1987; Ojo-Amaize et al., 1987; Rook et al., 1987). In addition, anti-HIV-1 antibodies may damage uninfected cells by cross-reacting with normal cell-surface constituents and thereby instigate ADCC. This phenomenon of molecular mimicry may constitute the background for the generation of humoral autoreactivity observed in HIV-1 infected subjects (see section 1.3.4.c). In fact, non-polymorphic determinants of MHC class II products share some degree of homology with HIV-1 proteins such as gp41 and gp120 and anti-DR antibody has been found in the serum of HIV-1 infected individuals (Golding et al., 1988; Golding et al., 1989).

If these laboratory findings outlined above can be extrapolated to the in vivo situation, host CTL and ADCC immune responses to HIV-1 may be important mechanisms of CD4+ T-lymphocyte depletion.

1.3.3.c Failure of CD4+ T-lymphocyte regeneration

In order to delineate the long-term in vivo mechanisms of HIV-1-induced CD4+ T-lymphocyte depletion,

consideration must also be given to the inherent ability of the host to regenerate these CD4+ cells. Although the dynamics of CD4+ lymphocyte regeneration either from stem cells or by clonal expansion and proliferation of mature cells is unclear, clinical studies with ZDV suggest that in advanced HIV-1 disease immune reconstitution is limited (see section 1.4.1.b).

Several studies have indicated that certain bone marrow progenitor cells are targets of HIV-1 infection; this may directly impair CD4+ lymphocyte production or indirectly cause insufficient proliferation of CD4+ precursor cells (Donahue et al., 1987; Zuckler-Franklin and Cao, 1989). Furthermore, T-cell colony forming activity and interleukin-2 (Il-2) receptor expression is suppressed in HIV-1 infection (Winkelstein et al., 1988). Finally, several studies have found evidence of productive HIV-1 infection in thymic tissue (Namikawa et al., 1988; Numazaki et al., 1989) and substantial thymic damage, similar to that incurred by congenital immunodeficiency and allogenic bone marrow transplantation (Schuurman et al., 1989). Therefore it is possible that once thymic damage occurs, any potential regeneration of CD4+ T-lymphocytes is limited, with important consequences for the treatment of HIV-1 disease (see section 1.4.8.).

1.3.3.d Summary of HIV-1 immunopathogenesis

It seems improbable that any one of the pathogenetic mechanisms outlined above provides adequate explanation of HIV-1 mediated CD4+ T-lymphocyte depletion in vivo. It is much more plausible that the overall effect of HIV-1 infection on the CD4+ T-lymphocyte population is a consequence of a mixture of these mechanisms, the extent of each perhaps varying in different individuals. Moreover, the defective function of the CD4+ T-cells (see section 1.3.4.a,b) provides a further basis for the biological effects of HIV-1 disease, and may in part account for the imperfect association between peripheral CD4+ T-lymphocyte

number and disease (see section 1.5.1.a). Temporal progression to severe immunodeficiency will further depend on the virulence of the effector HIV-1 variant since a high rate of HIV-1 replication is associated with rapid development of AIDS (Cheng-Mayer et al., 1988; Miedema et al., 1990; Tersmette et al., 1989a,b) and on the host's immunogenetically determined anti-viral response (Pinching, 1990).

1.3.4. Immune dysfunction and HIV-1 infection

Progressive HIV-1 disease ultimately causes the selective depletion of both circulating and tissue CD4+ cells, putatively by the concert of mechanisms outlined by section 1.3.3. Given that CD4+ T-lymphocytes play a central regulatory role in the immune system through cognate interaction or cytokine release (see section 1.3.1.), loss from this T-lymphocyte subset consequently compromises the function of other cell types. Functional casualties attributed to HIV-1-specific CD4+ lymphocyte cytopathicity include: failure of antigen presentation in neoantigen response; failure of cytotoxic response induction; defective macrophage activation; impaired NK cell function; and defective B-lymphocyte differentiation and maturation (Pinching, 1990; Seligmann et al., 1987).

However, substantial data now indicate that T-cell, B-cell and monocyte/macrophage qualitative defects occur independently of quantitative CD4+ T-lymphocyte change in both symptomatic and asymptomatic HIV-1 disease. By implication, the HIV-1 retrovirus mediates these functional defects.

1.3.4.a T-cell dysfunction

A number of studies using various in vitro parameters of T-lymphocyte functional immunocompetence, have reported mitogen and recall antigen response defects in T-lymphocytes isolated from patients with asymptomatic and symptomatic HIV-1 disease (see section 1.5.3.a,b,d). In

symptomatic disease, Lane et al (1985) using enriched CD4+ T-lymphocytes from an AIDS patient, demonstrated a defect in the proliferative response to tetanus toxoid but not to the T-cell mitogen phytohaemagglutinin (PHA). Similarly, T-lymphocytes obtained from an AIDS (Smolen et al., 1985) and ARC (Gabrecht et al., 1987) patients exhibited marked reduction of proliferative response in autologous mixed lymphocyte reaction, but a normal proliferative response to human lymphocyte associated (HLA) alloantigens. Evidence of T-lymphocyte qualitative defects is also plentiful in HIV-1 asymptomatic disease, before CD4+ T-lymphocyte depletion occurs. Murray et al (1988), in an on-going study, reported normal numbers of circulating CD4+ T-lymphocytes and essentially normal peripheral blood mononuclear cell gamma-interferon (gIFN; see section 1.5.3.c for a full discussion of gIFN) production in response to Concanavalin A (Con A), whereas nominal antigen responses were reduced in 40 to 46 percent of 23 asymptomatic subjects. In each of these studies, defective Il-2 mediated intercellular communication was suggested as explanation of the observations. However, Miedema et al (1988) showed that in asymptomatic HIV-1 disease decreased CD4+ T-helper activity for pokeweed mitogen (PWM) driven B-cell differentiation (perceived by decreased immunoglobulin, Ig, M and G production) was not corrected by exogenous Il-2.

Shearer and colleagues (1984, 1985, 1986) provided further support of a T-lymphocyte functional defect by their investigations of CTL. Symptomatic, asymptomatic and high-risk individuals of unknown HIV-1 serological status demonstrated a selective in vitro defect in CTL responses to influenza-infected autologous cells (flu) but not to alloantigens. Furthermore, in peripheral blood lymphocytes obtained from three different HIV-1 seropositive donors, the defect in flu-specific CTL response could be corrected by recombinant Il-2 or by costimulation with alloantigen (Shearer et al., 1986). This suggested flu-specific CD8+ CTL were present and functional but that there was an underlying

qualitative deficiency in CD4+ T-cell help. Rook et al (1983) reported a similar Il-2-correctable defect in the CTL obtained from AIDS patients. Clerici et al (1989) expanded on this work and showed, in 74 asymptomatic patients, time-dependent sequential loss of peripheral blood lymphocyte Il-2 response to nominal antigen, followed by allogenic cells and lastly to PHA. Clerici interpreted these observations by the work of Via et al (1990b) to indicate the early loss of Il-2 response to antigen as a selective defect in CD4+ MHC self-restricted T-helper function; while the later loss of allogenic and PHA responses suggested more severe T-helper dysfunction involving both CD4+ and CD8+ T-lymphocytes.

Clearly several lines of evidence indicate that HIV-1 infection is typically associated with suppressed CD4+ cellular function, as distinct from the effects of viral CD4+ cellular depletion. A number of explanations have been proposed to account for this dysfunction, the aetiology of which is probably multifactorial.

With respect to HIV-1 asymptomatic disease, Via et al (1990a) recently advanced that host suppressor cells specific for CD4+ helper T-cell function are triggered, most likely by HIV-1 induced B-cell hyperactivity (see section 1.3.4.c), and thereby contribute to the in vitro CD4+ T-cell defect. However, this hypothesis is based on murine models of autoimmunity and immunodeficiency and is experimentally difficult to test in the human system. Interestingly, such a model questions whether CD4+ T-cell dysfunction is detrimental or beneficial to the HIV-1-infected host.

It has been found that banded HIV-1 proteins (Pahwa et al., 1985), gp120 (Weinhold et al., 1989) or tat encoded protein (Viscidi et al., 1989) suppress normal CD4+ T-lymphocyte mitogenic or antigenic response in vitro and that HIV-1 antigen concomitantly enhances the expression of the T-cell activation antigen (Tac; CD25), and suppresses PHA-induced Tac expression (Diamond et al., 1988). In view of these observations, several theories of T-lymphocyte dysfunction encompass the direct effects of HIV-1 or its

proteins.

Rosenberg and Fauci (1990) recently proposed that soluble gp120 bound to T-cell surface CD4 may directly disrupt CD4-MHC class II interaction (see section 1.3.1.), thereby physically interfering with macrophage T-lymphocyte collaboration and antigen presentation. Similarly, internal image anti-idiotypic antibodies to the gp120 CD4 binding site could interfere with these critical events of immune response. It has also been suggested that such antibodies may cause cellular calcium influx and thus signal functional modification (Kornfeld et al., 1988). However, evidence for the existence of these anti-idiotypic antibodies is weak (Chams et al., 1988; Thiriart et al., 1988).

Such signalling events may be mediated more effectively by anti-gp120 antibodies targeted to CD4 through bound soluble gp120. The resultant cross-linking of contiguous CD4 molecules would thereby mimick the anti-CD4 antibody effect (see section 1.3.1.). Recent findings demonstrated that normal CD4+ cells incubated with gp120 and anti-gp120 antibodies were consequently unable to respond to anti-CD3 antibody stimulation and down-regulated CD4 expression (Mittler and Hoffmann, 1989). Anti-CD3 antibody normally activates CD4+ T-lymphocytes to express Tac and proliferate (Imboden and Weiss, 1988). Peripheral blood lymphocytes obtained from AIDS patients also show evidence of CD4 down-regulation and an antigen receptor signalling defect in their CD4+ T-lymphocytes (Gerald et al., 1990).

In essence, it seems that HIV-1 or its proteins, particularly gp120, induce chronic activation of CD4+ T-lymphocytes such that these cells show impaired responses to further stimulation in vitro; these observations are interpreted as evidence of suppression (Pinching and Nye, 1990). It is apparent from the previous discussion that signalling events are involved in this manifestation of suppression. A similar argument is pertinent to B-cells and cells of monocyte/macrophage lineage obtained from HIV-1 infected individuals.

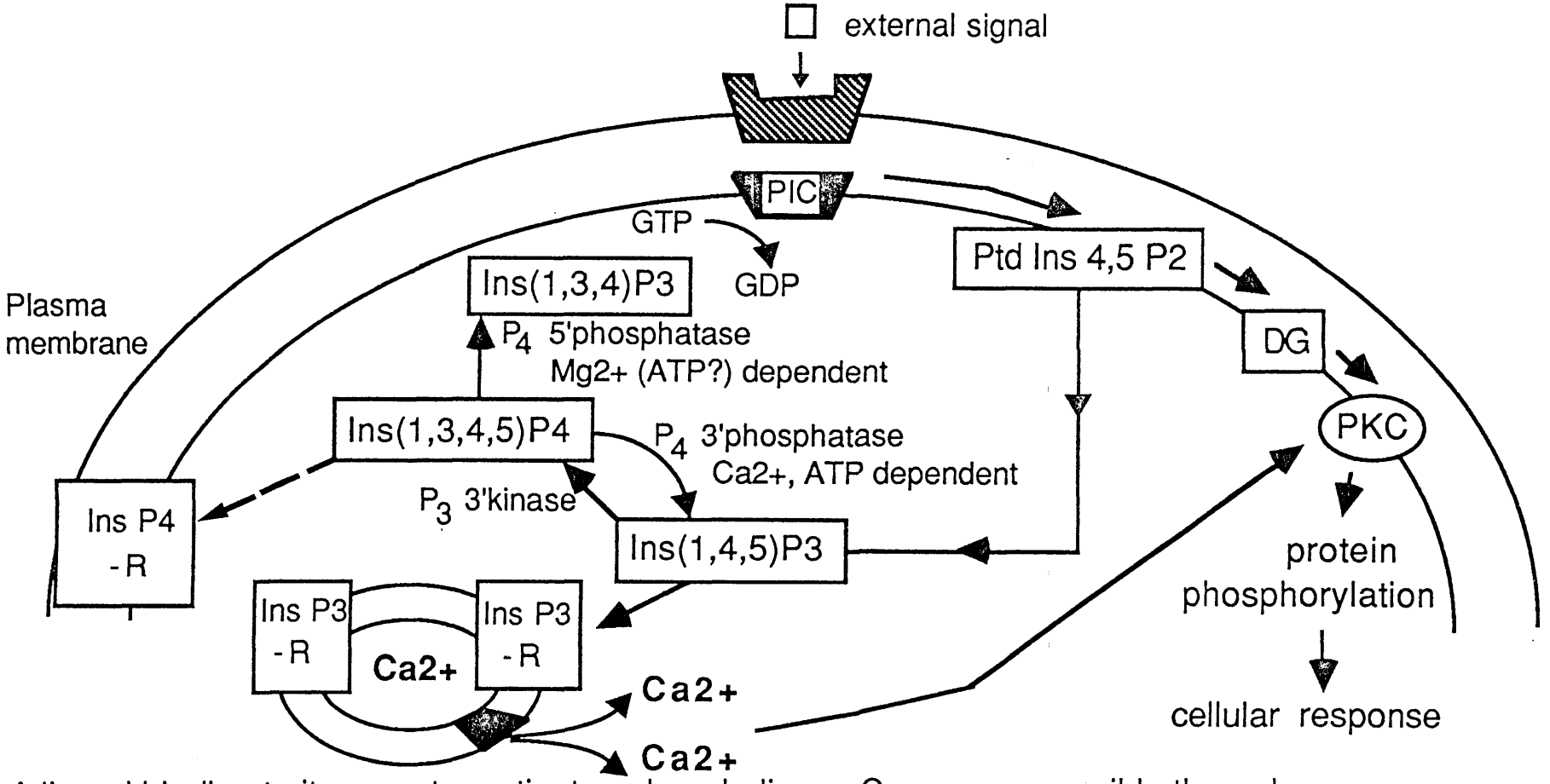
1.3.4.b T-cell dysfunction: HIV-1 and signal transduction by the inositol polyphosphate pathway

The T-lymphocyte TCR complex mediates signal transduction by the inositol polyphosphate pathway, which represents a major signal transduction pathway not only in T-cells but in B-cells, macrophages and in other cell types (cited by Berridge and Irvine, 1989).

As summarised in Figure 01, interaction of ligands with their receptors initiates rapid hydrolysis of membrane-bound inositol lipid, particularly phosphatidylinositol 4,5-bisphosphate ($PtdIns(4,5)P_2$), by phosphoinositidase C to 1,2-diacylglycerol (DG) and inositol 1,4,5-trisphosphate ($Ins(1,4,5)P_3$): two key second messengers. DG activates protein kinase C. $Ins(1,4,5)P_3$ releases calcium from intracellular stores, but is rapidly metabolised either through dephosphorylation by a 5'-phosphatase to $Ins(1,4)P_2$ or by phosphorylation via calcium/calmodulin dependent 3'-kinase to $Ins(1,3,4,5)P_4$ (Shears, 1989). $Ins(1,3,4,5)P_4$ may co-operate with $Ins(1,4,5)P_3$ in overall cellular calcium regulation (Irvine, 1990). It is well established that $Ins(1,3,4,5)P_4$ can be dephosphorylated to $Ins(1,3,4)P_3$ by 5'-phosphatase and sequentially thereafter to inositol (Batty et al., 1985; Shears et al., 1988) but it is now also recognised that dephosphorylation can also occur via an $Ins(1,3,4,5)P_4$ 3'-phosphatase giving the potential to maintain cellular $Ins(1,4,5)P_3$ levels (Cullen et al., 1989). Cellular activation occurs as a consequence of elevated intracellular calcium ($[Ca^{2+}]_i$) and protein kinase C protein phosphorylation by acting in concert (cited by Berridge and Irvine, 1989).

Several lines of evidence are indicative of HIV-1-associated defective signal transduction: resting $[Ca^{2+}]_i$ levels are raised in chronically infected H9 T-lymphoblastoid cells with impaired $[Ca^{2+}]_i$ responses to anti-CD3 antibody and PHA (Gupta and Vayuvegula, 1987; Linette et al., 1988). PHA may also act through CD3

Figure 01: Summary of inositol phospholipid signal transduction.



A ligand binding to its receptor activates phospholipase C enzyme, possibly through a transducing G-protein. Phosphoinositides, especially Ptd Ins 4,5-P_2, are hydrolysed to DG and Ins(1,4,5)P_3. DG is an activator of PKC, which phosphorylates and activates a number of intracellular proteins. Ins(1,4,5)P_3 liberates Ca2+ from endoplasmic reticulum (Guy et al., 1989).

(Valentine et al., 1985). Moreover, Nye and Pinching (1990) have delineated the inositol polyphosphate pathway of chronically HIV-1 infected H9 cells at rest or in response to PHA or anti-CD3 antibody. At rest these cells showed chronically elevated $[Ca^{2+}]_i$, $InsP_3$ and $InsP_4$ levels; following stimulation with anti-CD3 or PHA, $InsP_3$ fell and $InsP_4$ levels were further elevated. It is unclear if these altered responses were consequent or causal of the observed attenuated $[Ca^{2+}]_i$ response. Since the majority of H9 cells are infected under these experimental conditions, baseline increases of second messengers may be attributed to cellular HIV-1 infection. However, cells obtained from HIV-1 infected patients in whom a minority of viable circulating CD4+ T-lymphocytes are infected (Harper et al., 1986; see section 1.3.3.) show similar changes suggesting that cellular infection is not essential (Nye et al., 1991; see results section 5.1.).

In support Kornfeld et al (1988) found that soluble gp120 bound cellular CD4 activates the $InsP_3$ and calcium transduction pathways of resting uninfected T-lymphocytes, as manifested by increased Il-2 receptor (including Tac) expression and a chemotactic motile response. In contrast, Mittler and Hoffmann (1989) could not show an inhibitory effect of gp120 on anti-TCR antibody T-cell activation; however, when anti-gp120 antibody cross-linked soluble gp120 bound cellular CD4, T-cell activation was suppressed with respect to $[Ca^{2+}]_i$ changes (see section 1.3.4.a).

In summary, direct HIV-1 infection or HIV-1 proteins, in particular gp120, cause chronic activation of CD4+ T-lymphocytes as manifested by defects in the inositol polyphosphate signal transduction pathway: $[Ca^{2+}]_i$, $InsP_3$ and $InsP_4$ levels are elevated while responses to anti-CD3 antibody and PHA are reduced. Therefore, these in vitro observations may provide explanation of CD4+ T-lymphocyte dysfunction in vivo, especially if these changes are related to CD4-gp120 binding as Kornfeld et al (1988)

proposed.

The mechanics of inositol polyphosphate changes are unclear, especially since CD3 and not CD4 is linked to the inositol polyphosphate pathway (Imboden and Stobo, 1985); although CD4 has been shown to have tyrosine kinase activity (Veillette et al., 1989). It is likely that physical and functional alterations of the CD4 CD3 relationship within the TCR play a role in this scenario, and such mechanistic explanations are discussed by Pinching and Nye (1990). Alternatively, gp120 may directly affect part of the pathway for second messenger generation, for example at the G protein or a regulatory enzyme. Current work in our laboratory provides some support for the latter (Nye et al., 1991; see results section 5.2.), implicating defective function of the 5'-phosphatase.

The observed polyphosphate defects of CD4+ T-lymphocytes may also provide explanation of B-cell and monocyte/macrophage dysfunction (see section 1.3.4.c and 1.3.4.d), as these cell types show similar profiles of activation and stimulant suppression (Pinching and Nye, 1990).

1.3.4.c B-cell dysfunction

Abnormal B-cell function is as ubiquitous a feature of HIV-1 infection as the T-lymphocyte qualitative defects described previously (see section 1.3.4.a,b). These functional defects are characterised by hypergammaglobulinaemia; high numbers of circulating activated B-cells; raised titres of antibodies against other pathogens; and the presence of autoantibodies (cited by Amadori and Chieco-Bianchi, 1990).

HIV-1 associated B-cell activation is perceived by elevated serum levels and increased in vitro production of IgG1, IgG3, IgA and most notably in children IgM; IgG2 levels are often reduced and are relevant to the development of infection with capsulated bacteria (Parkin et al., 1989; Rogers, 1987; Rogers et al., 1989; Seligmann et al., 1987).

IgE levels are also increased in atopic subjects and may lead to recrudescence of atopic disease as immunodeficiency becomes manifest (Parkin et al., 1987). In addition, raised serum IgD and spontaneous in vitro IgD production is a prominent feature of HIV-1 infection, although unusual in other post-infectious causes of hypergammaglobulinaemia (Mizuma et al., 1987; Rogers, 1987; Rogers et al., 1989). B-cell activation is most pronounced in HIV-1 seropositive subjects with no or few symptoms (CDC group III) and in patients with early symptomatic disease (CDC group IVD; Mizuma et al., 1988; Rogers, 1987; Rogers et al., 1989). This intense B-cell activation was initially considered to be polyclonal in nature, however data now suggest that a strong response to HIV-1 antigens may underlie this phenomenon (Amadori and Chieco-Bianchi, 1990).

The mechanisms responsible for the steady state activation of B-cells in HIV-1 infection are unclear, especially with respect to the first steps. A number of workers have reported that HIV-1 banded viral preparations and specific viral antigens induce B-cells, obtained from HIV-1 seronegative donors, to proliferate and secrete immunoglobulin (Martinez-Maza et al., 1987; Nair et al., 1988; Pahwa et al., 1985; Pahwa et al., 1986; Schnittman et al., 1986). Moreover, the source of T-lymphocyte help to B-cells is unclear. Mizuma et al (1988) failed to correlate in vitro spontaneous IgD secretion, overproduction of which can perturb T-lymphocyte immunoregulatory circuits (Xue et al., 1984), by B-cells from HIV-1 infected subjects with either percentage or absolute numbers of circulating CD4+ or CD8+ T-cells; indicating a T-lymphocyte independent mechanism of B-cell dysfunction. This observation is corroborated by T-cell independent spontaneous production of anti-HIV-1 antibodies, mainly to viral envelope proteins (Weber and Jeffries, 1989; see section 1.5.5.a). However, Mizuma et al (1988) correlated IgM and IgG levels to T-lymphocyte percentage but not to absolute counts; indicating the critical importance of T-lymphocyte proportional

representation to spontaneous B-cell synthesis and secretion of these two Igs. Indeed, it has been advanced that there is excessive T-cell help for B-lymphocyte differentiation to Ig secreting cells (Maggi et al., 1987) and that HIV-1 induced loss of T-cell regulatory function then allows uncontrolled B-cell activity (Birx et al., 1986). Therefore, several mechanisms of B-cell activation are possible and are not necessarily exclusive.

Paradoxically while spontaneously producing elevated levels of Ig, B-cells, particularly those obtained from AIDS patients, are refractory to further in vitro stimulation by T-cell independent mitogens (for example, Staphlococcus aureus) and T-cell dependent mitogen (for example, PWM), which target resting and partially activated B-cells respectively (Lane et al., 1983; Rogers, 1987; Rogers et al., 1989).

A clue to the mechanism of B-cell response dysfunction is provided by the recent work of Edelman and Zolla-Pazner (1990). Seronegative mixed-lymphocyte culture supernatants increased the proliferative response of peripheral blood mononuclear cells, obtained from 76 HIV-1 seropositive patients, to Staphlococcus aureus Cowan 1 strain, by a similar magnitude as 39 laboratory controls. This observation suggests that there is no intrinsic defect in the ability of B-cells from HIV-1 infected patients to respond to T-cell factors. However, the mixed-lymphocyte supernatants failed to reconstitute the patient responses fully. These data indicate that at least part of HIV-1-associated B-cell dysfunction is intrinsic and is likely to be mediated by the virus itself or by its proteins. It would be interesting to extend this work to Ig production responses. A plausible role of dysfunction may be assigned to the inositol polyphosphate pathway, since a similar failure in response to anti-IgM antibody was noted: surface Ig is the major B-lymphocyte inositol polyphosphate pathway receptor. The mechanism of HIV-1 or HIV-1 derived protein interaction with the B-cell is unclear as these cells are neither

infected in vivo nor constitutively express CD4 (Pinching and Nye, 1990).

Peripheral B-lymphocytes also show evidence of immaturity in HIV-1 infection. In evidence IgD production, which is a marker of B-cell immaturity, is raised both in vivo and in vitro as mentioned previously (Mizuma et al., 1987; Rogers, 1987; Rogers et al., 1989). Phenotypic analysis of B-lymphocytes has shown an increase in the expression of CD10, another marker of immaturity (Martinez-Maza et al., 1987). Thus in addition to B-cell activation, two lines of evidence indicate that there is an increase in the proportion of immature B-cells; these data suggest that HIV-1 infection is associated with the recruitment of immature B-lymphocytes into the circulation as well as B-cell activation.

Irrespective of aetiology, the resulting inability of HIV-1 infected subjects to produce the antibodies required for a protective response to many bacterial pathogens plays an important part in their predisposition to bacterial sepsis and pneumonia. In addition, it has been suggested that B-cell hyperactivity manifested as humoral autoreactivity, such as rheumatoid factor, anti-nuclear antibody and antibody to cellular elements, participate in the pathogenesis of HIV-1 infection (Amadori and Chieco-Bianchi, 1990; Via et al., 1990a).

1.3.4.d Monocyte/macrophage dysfunction

The functional, numerical and phenotypic alterations in macrophage subpopulations during HIV-1 infection are not well defined, although investigative attention is now focusing on this area. However, virtually all studies to date suffer two major interpretative problems. First patient selection has been limited to those individuals with HIV-1 symptomatic disease. Second the macrophage subpopulation most frequently analysed in vitro has been the precursor cell to all tissue macrophages, the blood monocyte (Meltzer, 1990a; Pinching, 1990).

Most studies have reported normal numbers of blood

monocytes even in advanced HIV-1 disease (Enk et al., 1986; Poli et al., 1985; Siegal et al., 1986a). Similarly, most studies have documented normal phenotypic expression of plasma membrane antigens by flow cytometric analysis with monoclonal antibodies; for example, expression of MHC class II determinants DP, DQ, and DR, by monocytes obtained from HIV-1 seropositive individuals is indistinguishable from that of cells obtained from seronegative donors (Haas et al., 1987).

Analysis of macrophage function has been approached both in vivo and in vitro. Bender et al (1988) showed impaired clearance from the circulation of particles that express Fc (spleen macrophage-mediated clearance) or C3 (hepatic Kupffer cell-mediated clearance) determinants in most patients with late stage HIV-1 infection. Interestingly, defective Fc-mediated particle clearance was observed in early HIV-1 disease only in those patients with immune thrombocytopenic purpura. By in vitro assay, monocyte microbicidal activity (Eales et al., 1987; Murray et al., 1983; Nielsen et al., 1986), phagocytosis (Estevez et al., 1986; Murray et al., 1983; Poli et al., 1985), and release of hydrogen peroxide, Il-1 or tumour necrosis factor-alpha (Haas et al., 1987; Murray et al., 1983; Murray et al., 1987a; Nielsen et al., 1986) are normal in steady state and gIFN treated cells obtained from HIV-1 infected donors. However, monocytes also demonstrate defects in functions that are dependent upon cytokine signals from CD4+ T-lymphocytes, such as chemotactic response (Poli et al., 1985; Smith et al., 1984).

Other cells of monocyte/macrophage lineage show HIV-1 associated changes in phenotype, number and function. For example, Langerhans' cells and peripheral dendritic cells express reduced levels of MHC class II antigen (Belsito et al., 1984; Eales et al., 1988). Macatonia et al (1990) have demonstrated reduced numbers of peripheral dendritic cells in HIV-1 infected subjects with asymptomatic infection (CDC group II) and AIDS (CDC group IV). However, these cells also

show reduced function in antigen presentation and responsiveness to stimulation throughout HIV-1 infection (Eales et al., 1988; Macatonia et al., 1990). In contrast, monocyte/macrophages show evidence of apparent activation, for example by increased responsiveness to macrophage activating cytokines (Eales et al., 1987) and production of neopterin (Fahey et al., 1990; Fuchs et al., 1988).

Therefore monocyte/macrophage function may appear normal, suppressed in some respects and activated in others. Explanation of this puzzling phenomenon may lie in signal transduction events (Pinching, 1990). In evidence, it has been shown that purified gp120 or gp41 proteins can cause activation of monocytes obtained from HIV-1 seronegative donors, while suppressing their response to chemotactic factors (Tas et al., 1988; Wahl et al., 1989). The chemotactic factors fMetLeuPhe and leukotriene B_4 cause a rise in $InsP_3$ in macrophages (cited by Pinching and Nye, 1990) and activation of macrophage signal transduction by HIV-1, similar to that seen in CD4+ T-cells has been reported (Gupta and Vayuvegula, 1989).

1.3.4.e Summary of HIV-1 associated immune dysfunction

Thus, HIV-1 infection is associated with dysfunction of diverse cell types of the immune system including T-lymphocytes, B-lymphocytes and cells of monocyte/macrophage lineage.

1.4. Anti-HIV-1 therapy

Following the recognition in 1983 by Barre-Sinoussi et al, that AIDS was caused by a human retrovirus HIV-1, optimism concerning a safe effective vaccine prevailed. The development of an AIDS vaccine, however, has proved more difficult than many anticipated and is not yet an immediate prospect. Consequently, much attention is currently devoted to the development and evaluation of anti-HIV-1 therapeutic agents.

This section will discuss new and promising

approaches to HIV-1 therapy, together with ZDV which remains the main-stay of treatment, emphasising various points of attack in the virus replicative cycle (Figure 02).

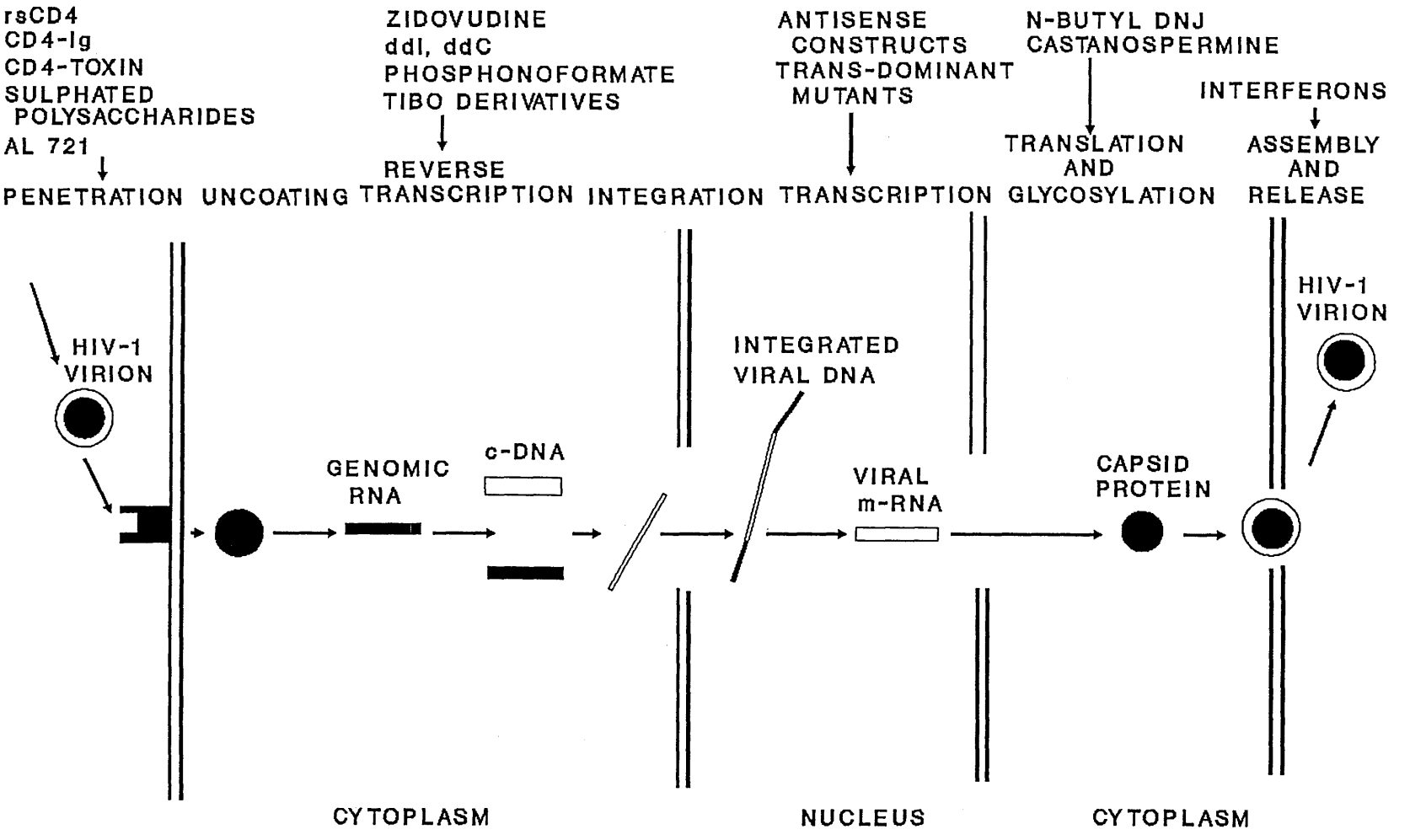
1.4.1. Inhibitors of HIV-1 reverse transcriptase: the dideoxynucleosides

The capacity to cultivate large quantities of HIV-1 *in vitro* has allowed the identification, study and practical development of antiretroviral agents. Initially, this work was an outgrowth of early research on animal retroviral systems (Yarchoan *et al.*, 1989a). The HIV-1 enzyme reverse transcriptase is essential to viral replication and has been the major target for antiretrovirals developed to date (Mitsuya and Broder, 1987; Yarchoan *et al.*, 1989a).

The HIV-1 RNA-dependent DNA polymerase, like any other retroviral reverse transcriptase, catalyses the formation of a DNA double helical copy of the viral genomic RNA. First, reverse transcriptase binds a lysine transfer RNA primer; inhibitors of this step may affect subsequent events. Thereafter, deoxynucleotides are added to the 3' end of the growing minus DNA copy which anneals to the complementary RNA, resulting in a DNA-RNA hybrid. Concomitantly with minus DNA strand elongation the RNA template is degraded to mono- and dinucleotides by ribonuclease H cleavage towards the 5'end of the RNA (Schatz *et al.*, 1990). Mutants defective in ribonuclease H have shown defective viral synthesis and therefore this enzyme provides a possible therapeutic target. A second positive DNA strand is synthesised and the resultant HIV-1 DNA double helix circularises and integrates into a host cell chromosome (Hirsch, 1990; Mitsuya and Broder, 1987).

Zidovudine (3'-azido-3'-deoxythymidine, 3'-azido-2',3'-dideoxythymidine, azidothymidine, AZT or ZDV) was found to be a potent inhibitor of HIV-1 infectivity and cytopathogenicity in H9 and peripheral blood T-lymphocytes at levels of 1 to 5 uM (Mitsuya *et al.*, 1985). Several other members of the dideoxynucleoside family were subsequently

Figure 02: The HIV-1 replicative cycle and sites of therapy



(HIRSCH, 1990
YARCHOAN, et al., 1990a)

found to inhibit HIV-1 replication in vitro (Mitsuya and Broder 1986; Mitsuya et al., 1987). Dideoxynucleosides in fact exhibit activity against a broad spectrum of retroviruses including HIV-2, human T-lymphotropic virus type 1 (HTLV-1), animal lentiviruses and murine retroviruses (cited by Yarchoan et al., 1989a).

The dideoxynucleoside compounds differ from physiological deoxynucleotides in that the ribose 3'hydroxyl group (-OH) is replaced by an azido group ($-N_3$), hydrogen (-H) or another group that cannot form phosphodiester linkages. As well as ZDV, these include 2',3'-dideoxycytidine (ddC), 2',3'-dideoxyinosine (ddI) and 2',3'-didehydro-2',3'-dideoxythymidine (D4T). Although substantial differences exist among the dideoxynucleosides, several general comments on their mechanism of action are possible.

In a process known as anabolic phosphorylation each dideoxynucleoside is phosphorylated to a 5'-triphosphate by a series of cellular cytoplasmic kinases. These triphosphates are thought to selectively inhibit reverse transcriptase and hence HIV-1 replication (Furman et al., 1986; Mitsuya et al., 1987; Waqar et al., 1984). The basis of subsequent dideoxynucleoside antiretroviral activity may be explained by two mechanisms acting in concert.

Firstly, as triphosphates these analogues compete with the deoxynucleoside-5'-triphosphates that are the natural substrates of cellular DNA polymerases and reverse transcriptase. Inhibition constants of the dideoxynucleoside-5'-triphosphates are of the range 0.005 to 0.2 uM and these concentrations can be attained in cells. The apparent preference of reverse transcriptase for dideoxynucleoside-5'-triphosphate over the corresponding deoxynucleotide partly explains their selective antiretroviral activity (Furman et al., 1986; Yarchoan et al., 1989a). A further basis of selectivity relies on the fact that mammalian DNA polymerase alpha, which is of major importance to DNA synthesis and repair, is relatively resistant to the effects of dideoxynucleoside-5'-triphosphates, expressing an inhibition

constant of 100 to 230 uM. However, mitochondrial DNA polymerase gamma and DNA polymerase beta are both sensitive to these 5'-triphosphates, having inhibition constants 0.016 to 0.4 uM and 2.6 to 70 uM respectively. This may provide a rationale of dideoxynucleoside toxicity (Furman et al., 1986; Waqar et al., 1984; Yarchoan et al., 1989a). In the second mechanism of dideoxynucleoside action, viral DNA strand elongation is terminated on addition of a dideoxynucleoside-5'-triphosphate as the 3' modification does not permit 5'-3'phosphodiester linkage with additional nucleotides (Mitsuya et al., 1987; Yarchoan et al., 1989a).

Evidence suggests that the kinases which activate dideoxynucleosides, and are essential to their antiretroviral activity, are cell cycle-dependent. Non-proliferating cells such as monocytes or macrophages have low levels of kinases relative to dividing lymphocytes (Yarchoan et al., 1990a). Therefore, cells of the monocyte/macrophage line and those derived from it, such as the brain microglia, while permissive of infection (Clapham et al., 1989; Gartner et al., 1986; Koenig et al., 1986) may not be protected by dideoxynucleosides. One study indicated that ZDV is poorly phosphorylated in peripheral blood monocytes and macrophages and does not protect these cells from infection by a T-lymphocyte tropic strain of HIV-1 (Richman et al., 1987b). However, Perno et al (1988) and Skinner et al (1988) demonstrated that low concentrations of ZDV and other dideoxynucleosides inhibited the replication of a monocytotropic strain of HIV-1 in fresh and cultured monocytes and macrophages. Explanation of the anti-HIV-1 activity of dideoxynucleosides relies on the fact that monocytes and macrophages have very low levels of physiological deoxynucleoside-5'-triphosphate. Therefore the ratio of physiological to analogue 5'-triphosphates may favour the latter on competition for reverse transcriptase (Perno et al., 1988; Yarchoan et al., 1989a).

1.4.1.a The pharmacokinetics of zidovudine

Initial pharmacokinetic and bioavailability data were obtained from a phase I, open-label, dose-escalating, study of intravenously and orally administered ZDV (Klecker et al., 1987; Yarchoan et al., 1986). On oral dosing, ZDV was rapidly absorbed from the gastrointestinal tract, with a bioavailability of approximately 65 percent caused by first-pass metabolism rather than incomplete absorption. Peak ZDV plasma levels of 3 to 4 uM were attained 30 to 90 minutes following a 200 mg oral dose and concentrations were compatible with in vitro anti-HIV-1 activity (Mitsuya et al., 1987). However, the plasma half-life of ZDV was only 1.1 hours (Klecker et al., 1987) while that of its active intracellular moiety was almost 3 hours (Furman et el., 1986). ZDV effectively penetrated the blood-brain barrier and subsequently demonstrated a salutory effect on HIV-1-related neurological abnormalities (Yarchoan et al., 1987) and inhibited HIV-1 expression in the cerebrospinal fluid (De Gans et al., 1988). ZDV's major route of elimination was by hepatic glucuronidation, after which its 5'-glucuronide conjugate was rapidly excreted in the urine. Consequently, factors affecting liver and to a lesser extent kidney function may alter ZDV clearance.

Based on these studies, a 250 mg four-hourly oral dosing regimen was adopted to maintain ZDV plasma levels above the minimum concentration required for an in vitro antiretroviral effect. Considering that the active 5'-triphosphate has a relatively extended half-life, recent studies hoped to improve ZDV's therapeutic index by employing lower doses.

Fischl et al (1990a) conducted a comparative study of the ZDV standard dose (250 mg, 4 hourly) and a lower dose (200 mg every 4 hours for 4 weeks, followed by 100 mg, 4 hourly) in 524 subjects with first episode of Pneumocystis carinii pneumonia. Over a 25.6 month median follow-up, survival was significantly better in the lower dose group, while serious anaemia and neutropenia was more common in the

standard group. However frequency of infection, CD4+ T-lymphocyte counts and p24 antigen levels were equivalent. Two studies further report that oral dosing regimens of 200 mg (Dominique et al., 1990) and 300 mg (Michel et al., 1990) every 8 hours were as efficacious as the reference dose of 200 mg, 4 hourly. Michel et al (1990) additionally record improved tolerance for the lower dose cohort, while Dominique et al (1990) found no difference in adverse effects between the two schedules.

These studies indicate that lower daily doses of ZDV may be as effective as the standard regimen of treatment adopted at present.

1.4.1.b Clinical results with zidovudine

Phase I study of ZDV found it to be well tolerated and associated with clinical improvement over 6 weeks in 19 ARC and AIDS subjects (Yarchoan et al., 1986; Yarchoan et al., 1987). Consequently a multicentre, placebo-controlled study of 282 HIV-1 infected individuals was initiated in February 1987. Entry criteria restricted subjects to those with AIDS, manifested by Pneumocystis carinii pneumonia alone, or with severe ARC (Fischl et al., 1987; Richman et al., 1987a). Nineteen placebo and one ZDV recipient had died by September 1987 and the placebo-arm was discontinued in that same month. The reduced mortality of treated subjects was attributed to decreased frequency and severity of opportunistic infection evident from 6 to 8 weeks of therapy. Further benefits included sustained improvement of cognitive function indicating that underlying encephalopathy may be caused, at least partly, by neuronal dysfunction rather than loss (Fischl et al., 1987; Schmitt et al., 1988). CD4+ T-lymphocyte counts transiently increased in the first 2 months of therapy, suggesting incomplete immunoreconstitution. Immunological parameters were further assessed by skin-test anergy which was partially reversed, however the significance of this observation remains unclear. Finally, subjects receiving ZDV had a decreased viral load,

interpreted from suppressed although not abrogated serum HIV-1 p24 levels (Fischl et al., 1987; Jackson et al., 1988). Although ZDV treatment of subjects with Kaposi's sarcoma has proven similar antiretroviral effects, a variable tumour response has been observed (de Wit et al., 1988; Lane et al., 1989a; Pinching et al., 1989; Stambuk et al., 1989a,b).

The therapeutic benefits of ZDV therapy must be considered in context of its substantial toxicity. During phase II trials anaemia and latterly neutropenia, caused by ZDV bone marrow suppression particularly of the erythroid and myeloid series, was dose-limiting and more commonly seen in subjects with low CD4+ lymphocyte counts and those with AIDS. The initial rise of platelet numbers exhibited a tendency to fall off in all subjects, but was rarely severe. Headache, nausea, myalgia, and insomnia were also reported, although these were reversible and generally tolerable (Richman et al., 1987a).

Based on data collected from these original trials, most countries approved ZDV for the treatment of severe HIV-1 infection.

Since ZDV's licensure, placebo-controlled trials in subjects with AIDS, diagnosed as the result of Pneumocystis carinii, or those with CD4+ T-lymphocyte counts of less than 400 per cubic millimetre (mm^3) have not been possible. However, a number of open-label studies have since confirmed and further defined the efficacy and toxicity of ZDV therapy in such individuals (Pinching et al., 1989; Stambuk et al., 1989a,b). Although several authors have compared results of ZDV treated subjects with those of a natural history cohort, interpretation has proved limited. The availability of ZDV since 1986 and improvements in health care, implies that diagnosis of AIDS or severe ARC is now likely to be made earlier in the course of disease than previously (Fischl et al., 1989; Stambuk et al., 1989a,b).

Data indicate that ZDV therapy together with efficient prevention and treatment of infectious complications, has substantially improved the survival of

subjects with advanced HIV-1 disease. However progressive disease does eventually occur (Creagh-Kirk et al., 1988; Fischl et al., 1989; Pinching et al., 1989; Stambuk et al., 1989a,b).

The benefits of ZDV treatment among subjects with less advanced disease include weight gain, improved neuropsychological status, transient increase of CD4+ T-lymphocyte count and decreased serum p24 antigen levels (Fischl et al., 1989; Pinching et al., 1989; Stambuk et al., 1989a,b). Significantly, a decrease in the number of new AIDS cases is postulated as secondary to ZDV prevention of progressive disease (Fischl, 1989). In evidence Fischl et al (1989) recorded a 36 percent progression rate of ZDV treated ARC subjects to AIDS, during an 18 month median follow-up and referenced to a historical group. Furthermore, Pinching et al (1989) reported a less than expected progression and Stambuk et al (1989a,b) saw no opportunistic infection in ARC subjects. Both of the latter observations were made over approximately 6 months. Such cumulative evidence, coupled with speculation that ZDV may prevent or slow HIV-1 damage to the immune and central nervous systems, has prompted studies of ZDV treatment in asymptomatic infection.

Although the Anglo-French Concorde study is still in progress, the results of an American double-blind trial involving 1338 asymptomatic subjects with CD4+ counts of fewer than 500 per cubic mm^3 and followed for a median period of 55 weeks were recently published (Volberding et el., 1990). The rate of progression to AIDS or advanced ARC of subjects randomised to placebo, 500 or 1500 mg ZDV daily were 7.6, 3.6 and 4.3 percent respectively. Improvement of standard immunological and virological parameters was observed in the treated groups. Anaemia and neutropenia were prevalent toxicities of the high-dose ZDV group only. In addition, preliminary data suggest that ZDV may decrease the development frequency of neuropsychological abnormalities in asymptomatic subjects (McArthur et al., 1990).

As Volberding et al (1990) point out, conclusions

from these studies, involving relatively small numbers of subjects, must be drawn with caution. Consideration must be given to the long-term toxicity of ZDV therapy. It is already known that prolonged ZDV treatment is associated with necrotising myopathy (Gertner et al., 1989; Helbert et al., 1988; Panegyres et al., 1988) and with an increasing incidence of non-Hodgkins lymphoma, presumably as the result of failed immunosurveillance (Yarchoan et al., 1990a). The potential long-term survival benefits, as well as the cost not only of ZDV therapy, but of counselling and monitoring must also be weighed against the overall beneficial observations of these studies. Mounting opinion recommends that HIV-1 seropositive adults with or without symptoms, who have less than 500 peripheral blood CD4+ lymphocytes per mm^3 be treated with ZDV and it is now likely that ZDV will be prescribed earlier in infection. Although it would be more rational to target ZDV therapy to those individuals at high risk of progression to symptomatic disease, the validity of CD4+ numbers as a criteria of treatment remains uncertain - clearly such a strategy would require comprehensive and as yet incomplete information on laboratory markers of HIV-1 disease progression (see section 1.5.).

A similar and likely scenario involves extended ZDV treatment of infants and children. Although the incidence of children and infants infected, 80 percent by transplacental or perinatal transmission of HIV-1, is rising ZDV treatment of these subjects is only now being investigated.

The clinical manifestations of HIV-1 in children appears to differ from those in adults. For example, they seem more susceptible to recurrent bacterial infection and neuropsychological deterioration. Pizzo et al (1988) found that on intravenous ZDV treatment of 21 children, neurodevelopmental abnormalities improved and intelligence quotients rose in the 13 who had presented with encephalopathy. Toxicity included anaemia-requiring transfusion and dose-limiting neutropenia. A number of

reports to the San Francisco conference (1990) noted clinical benefit, transient increase of CD4+ lymphocyte numbers and decreased serum and cerebrospinal p24 concentrations on oral as well as intravenous ZDV administration to children (De Jose et al; Frenkel et al; McKinney et al; Mitchell et al). Although ZDV treatment in early pregnancy is not recommended because this antiretroviral is reported to be mutagenic and can induce chromosomal abnormality (Yarchoan et al., 1989a), it may be utilised to render perinatal protection from HIV-1 infection. A recent phase I study of ZDV administration to 10 pregnant HIV-1 infected women in their last trimester, revealed that ZDV freely crossed the placenta and a 200 mg oral dose given before delivery resulted in similar plasma levels in infant and mother. It remains unresolved whether ZDV given during labour will prevent HIV-1 transmission during parturition (Schuman et al., 1990).

Clinical benefits of ZDV therapy have been observed as early as 6 weeks after the initiation of treatment and appear to persist at least through the first 6 months of therapy (Fischl et al., 1987; Pinching et al., 1989; Stambuk et al., 1989a,b). Still it is evident that immunological recovery is partial at best; HIV-1 disease progresses sometime during treatment and a substantial decline in the survival of subjects with AIDS or ARC has been observed from 1 year of therapy onwards. Whether the increased fatality rate represents the diminished efficacy of ZDV with time, the emergence of viral resistance or the natural outcome of treatment in advanced HIV-1 disease is unclear (Fischl et al., 1989).

Consistent with the in vitro observation that ZDV does not suppress chronic HIV-1 infection (Ho et al., 1985) the virus is not eradicated on treatment in vivo (Ho et al., 1989a). Even low grade HIV-1 replication may exert T-lymphocyte destruction on cell-to-cell infection and immunological suppression by continued production of potentially immunosuppressive proteins (Siliciano et al., 1988; Tyler et al., 1989; Yarchoan et al., 1990a).

Furthermore, as outlined in section 1.3.3.c, HIV-1 has been found to infect certain bone marrow progenitor cells (Donahue et al., 1987; Zuckler-Franklin and Cao, 1989), thymic epithelial cells (Numazaki et al., 1989) and cause thymic tissue damage (Schuurman et al., 1989). Therefore, HIV-1 damage of the host immune system is likely to be exacerbated by ZDV-associated bone marrow suppression.

ZDV efficacy may be compromised in vivo for several reasons. It is possible that plasma and cerebrospinal concentrations become suboptimal as a result of dose attenuation (Fischl et al., 1989; Richman et al., 1987a; Pinching et al., 1989; Stambuk et al., 1989a,b). Furthermore, HIV-1 strains with reduced sensitivity to ZDV have been isolated from subjects receiving therapy for 6 months or more (Larder et al., 1989; Rooke et al., 1989). The molecular mechanisms of HIV-1 resistance to ZDV are unclear, although the full resistant phenotype is conferred by multiple mutations at specific amino acids of the reverse transcriptase gene (Larder and Kemp, 1989). Possibly resistance occurs more frequently with advanced disease where a higher rate of viral replication increases the probability of mutagenesis (Hirsch, 1990). However, resistant HIV-1 isolates retain sensitivity to the other dideoxynucleosides (Larder et al., 1989; Rooke et al., 1989).

In summary, ZDV is currently the only widely available antiretroviral drug demonstrated to be effective for the treatment of symptomatic HIV-1 disease, at least in Western society. However, this therapy has been associated with severe toxicity, especially in subjects with advanced disease. In addition, the beneficial effects of ZDV in individuals with AIDS or severe ARC appears to wane after 12 months of treatment and HIV-1 strains resistant to ZDV have been observed with prolonged therapy. Therefore, with increasing numbers of adults and children requiring long-term treatment of HIV-1 infection, it is evident that alternative or a combination of approaches to therapy are required.

1.4.1.c The pharmacokinetics of other dideoxynucleosides

In addition to ZDV, the 2',3'-dideoxynucleoside analogues of cytidine, adenosine and inosine have demonstrated anti-HIV-1 activity in freshly isolated and cellular lines of human T-lymphocytes, monocytes and macrophages (Mitsuya and Broder, 1986; Mitsuya et al., 1987; Perno et al., 1988).

Of these, 2',3'-dideoxycytidine (ddC) has proved most active, suppressing HIV-1 at concentrations of 0.01 to 0.5 uM for more than 30 days in vitro (Mitsuya and Broder, 1986; Mitsuya et al., 1987). Although in vivo, following a 2 mg oral dose, ddC has a bioavailabilty of 87 percent and attains peak plasma levels of 0.1 to 0.2 uM, its terminal half-life is only 1.2 hours (Klecker et al., 1988; Starnes et al., 1987; Yarchoan et al., 1988). The basis of ddC's potent activity seems dependent on its resistance, unlike other cytidine analogues, to the ubiquitous enzyme cytidine deaminase (Cooney et al., 1986; Starnes et al., 1987) and that dephosphorylation of its active intracellular moiety slows when accumulation of the 5'-triphosphate nears maximal concentration for this process (Starnes et al., 1987). It has also been found that ddC does not affect levels of deoxycytidine-5'-triphosphate, whereas ZDV, at high concentrations in certain T-lymphoblastoid lines, acts to decrease thymidine-5'-triphosphate (Furman et al., 1986; Yarchoan et al., 1989a). Further, ddC penetrates at least partially into the cerebrospinal fluid. The major metabolites of ddC, 5'-diphosphate-choline and related compounds, are produced in lymphocytes and other cells. Any relation to the peripheral neuropathy associated with in vivo ddC administration is unknown. Dideoxycytidine and its metabolites are cleared chiefly by the kidney (Klecker et al., 1988; Merigan et al., 1989; Starnes et al., 1987; Yarchoan et al., 1988).

After entry to a cell, the purine analogue dideoxyadenosine (ddA) is phosphorylated to its 5'-triphosphate and converted, by adenosine deaminase, to

2',3'-dideoxyinosine (ddI). This deamination has been found to be almost instantaneous in human plasma and cell extracts (Cooney et al., 1987). Therefore, for the purpose of this discussion these drugs can be considered as alternate forms. Dideoxyinosine also undergoes anabolic phosphorylation to ddA-5'-triphosphate (Ahluwali et al., 1987). Unlike the active intracellular moieties of the pyrimidine dideoxynucleosides, ZDV and ddC, ddA-5'-triphosphate has a prolonged half-life of 12 hours or more. Though ddI has a plasma half-life of approximately 90 minutes, it seems clinically effective to administer ddA and ddI infrequently (Cooley et al., 1990; Lambert et al., 1990; Yarchoan et al., 1988). In fact, ddI has a relatively high therapeutic index in vitro relative to other dideoxynucleosides (Mitsuya and Broder, 1986) and including human bone marrow progenitor cells (Bhalla et al., 1989). Both ddA and ddI are cleaved to their purine base and deoxyribose under acidic conditions (Cooney et al., 1987) and this may reduce their oral absorption; ddI has an average bioavailability of 35 percent given on an empty stomach after ingestion of antacids (Yarchoan et al., 1988). High concentrations of ddA's free base, adenine, have been reported to cause renal damage in dogs whereas, hypoxanthine, the free base of ddI, does not have similar toxicity. El-hawari et al (1989) reported a favourable toxicity profile for ddI in animal studies. Therefore, ddI may be preferable for oral administration (Yarchoan et al., 1989a). Certain analogues of ddA, for example 2',3'-dideoxy-2'-fluoro-ara-adenosine, are resistant to acid hydrolysis (Marquez et al., 1987), although the issue of ddA and ddI acid lability could be addressed by buffering gastric secretion.

Finally, a number of analogues of 2',3'-dideoxythymidine, like ZDV, have demonstrated virustatic activity against HIV-1 in vitro. One, an unsaturated form of dideoxythymidine, 2',3'-didehydro-2',3'-dideoxythymidine (D4T) is as about as active as ZDV on a molar basis (Balzarini et al., 1987; Hamamoto et al., 1987; Ho et al.,

1989b; Lin et al., 1987). Also 3'-azido-2',3'-deoxyuridine has shown some anti-HIV-1 activity in vitro, although HIV-1 ZDV resistant isolates have shown cross-resistance with this analogue (Larder et al., 1989).

1.4.1.d Clinical studies with dideoxycytidine and dideoxyinosine

On the basis of promising results collected from initial clinical studies in subjects with AIDS and severe ARC, both ddC and ddI are currently being tested for their effects on survival and HIV-1 disease progression in large-scale phase II/III trials (Yarchoan et al., 1990a).

Dideoxycytidine was the first antiretroviral drug to be studied in a clinical scenario after ZDV. Two open-label, dose-escalating phase I trials of intravenously and orally administered ddC were conducted in a total of 81 subjects (Merigan et al., 1989; Yarchoan et al., 1988). The results of these studies are discussed in combination. At doses of 0.03 to 0.09 mg/kg every 4 hours, a transient increase of CD4+ T-lymphocyte numbers and decreases in serum p24 concentrations were reported in most subjects. These benefits were noted as early as week 2 and were maximal at weeks 8 to 12 of therapy. Toxicity was noted at doses of greater than 0.01 mg/kg every 4 hours and included skin rashes, fever and mouth sores. A reversible painful peripheral neuropathy, occurring at 6 to 14 weeks of therapy, was dose-limiting, however different administration schedules or lower doses may reduce its frequency and severity.

The initial uncontrolled, dose-escalating studies of ddI carried out to date have achieved similar results. The drug was administered intravenously for two weeks and then orally once or twice daily (Cooley et al., 1990; Lambert et al., 1990; Yarchoan et al., 1989b). All groups noted significant decreases in serum p24 levels and increases in circulating CD4+ lymphocytes at minimum daily doses of 0.8 mg/kg intravenously followed by 1.6 mg/kg given orally. These observations were made as early as 2 weeks of therapy

and persisted for 20 to 24 weeks. Adverse effects included asymptomatic elevation of hepatic and serum aminotransferase and serum urate. A reversible painful peripheral neuropathy, with clinical manifestations similar to those seen in ddC treatment, and pancreatitis were dose-limiting. The pathogenesis of both the neuropathy and pancreatitis are unknown, although in the latter case ddI may exacerbate underlying conditions associated with advanced HIV-1 infection (Schwartz and Brandt, 1989) and aerosolised pentamidine prophylaxis (Hart, 1989).

Although information is not yet available on the long-term benefits or toxicities of ddC monotherapy, Yarchoan et al (1990c) have recently published the results collected from their extended phase I study of ddI (Yarchoan et al., 1990b). The data indicate that individuals with AIDS or ARC tolerated ddI doses of up to 9.6 mg/kg per day for up to 12 to 21 months and that virological and immunological improvement were sustained for at least 9 months. Both ddC and ddI seem to offer a promising treatment for subjects with AIDS or severe ARC. Since both exhibit non-overlapping toxicities with ZDV and in particular are not associated with bone marrow suppression, ddI and ddC may be useful for the treatment of ZDV intolerant subjects. In addition, alternating ddC or ddI administration with ZDV may limit the toxicities of each drug and provide the means to counteract the emergence of HIV-1 resistant strains in prolonged therapy.

1.4.1.e Other inhibitors of HIV-1 reverse transcriptase

Pauwels et al (1990) recently reported that members of a novel series of tetrahydro-imidazo [4,5,1,jk] [1,4]-benzodiazepin-2(1h)-one and -thione (TIBO) inhibited the replication of HIV-1 in four cell lines and in freshly isolated peripheral blood lymphocytes. The concentrations of TIBO-derivatives required were nanomolar, and 10^4 to 10^5 lower than their cytotoxic concentrations in these sytems. These derivatives appear to inhibit HIV-1 reverse

transcriptase activity and are extremely specific, failing to affect HIV-2, HTLV-1 and SIV replication, as well as several other animal retroviruses. TIBO derivatives may be clinically useful because of their specificity; cross reaction with cellular DNA polymerases seems minimal and therefore toxicity may be limited. However, HIV-2 is a major cause of AIDS in the Third World and may become so in the West. Also, with compounds so specific, it is more than likely that HIV-1 would rapidly mutate to a resistant form.

1.4.2. Inhibitors of HIV-1 binding to its target cell: soluble recombinant CD4 and related approaches

The CD4 glycoprotein has been subverted as a virus receptor by the human and simian immunodeficiency viruses (HIV-1, HIV-2 and SIV) in most permissive cell types (Dalgleish et al., 1984; Klatzmann et al., 1984b; Sattentau and Weiss, 1988). Specifically, the virus envelope glycoprotein, gp120, binds the cellular CD4 external NH_2-terminal domain. The high affinity (Kd: 10^{-9} M) of interaction suggests binding is crucial to viral entry.

Recombinant soluble CD4 (rsCD4) is likely to inhibit, to some degree, all forms of HIV that use CD4 as a receptor. Recombinant CD4 has been produced by DNA transfection of the four CD4 extracellular domains but not transmembrane or intracellular regions. These preparations are non-cytotoxic and inhibit HIV-1 binding, replication and syncytium formation in CD4+ T-lymphoblastoid lines at concentrations of 2 to 20 ug/ml (Fisher et al., 1988; Hussey et al., 1988; Smith et al., 1987). In addition, rsCD4 has demonstrated a virustatic effect against HIV-1 in myelomonocytic cell lines (Clapham et al., 1989). Furthermore, rsCD4 does not interfere with MHC-class II specific T-lymphocyte interactions (Hussey et al., 1988) and this is substantiated by a report that class II and gp120 binding functions of CD4 are distinct (Lamarre et al., 1989). Limited studies of rsCD4 in rhesus monkeys infected with SIV of macaque suggested reduced levels of recoverable virus and

improved bone marrow cell growth during treatment (Watanabe et al., 1989). The conserved nature of CD4 and gp120 interaction ensures rsCD4 blocks cellular infection of diverse HIV and SIV strains provided that infection is mediated by the fluid phase; rsCD4 inhibition of infection by cellular contact appears relatively inefficient (Clapham et al., 1989). Moreover, rsCD4 binds free gp120 (Kd: 10^{-9} M) and therefore presumably reduces its absorption by CD4+ T-lymphocytes. Consequently, syncytium formation and ADCC or T-lymphocyte cytotoxicity may be avoided (Germain, 1988; Siliciano et al., 1988; see sections 1.3.3.a,b). Unfortunately clinical studies have not yet reflected these promising in vitro or animal results.

Administration of rsCD4 to ARC and AIDS subjects in pilot trials was not associated with toxicity (Kahn et al., 1990; Schooley et al., 1990; Yarchoan et al., 1989c). However, no significant changes of CD4+ T-lymphocyte number or function (in vitro by mitogenic or antigenic proliferative response) were reported. Kahn et al (1990) and Yarchoan et al (1989c) found no evidence of antiretroviral activity, while Schooley et al (1990) reported a significant decrease of serum p24 levels at the highest rsCD4 administered dose only.

Initial trials may have foundered because average serum rsCD4 levels were too low or because rsCD4 has a serum half-life of only 45 minutes. Moreover, Daar et al (1990) found that HIV-1 freshly isolated from infected subjects was substantially less sensitive to the inhibitory effects of rsCD4 than the laboratory strains initially used to define its activity. Antibodies to rsCD4 may interfere with its function, however this seems unlikely as one subject only developed these antibodies during clinical trial (Kahn et al., 1990; Schooley et al., 1990). The role of antibodies to gp120 is however uncertain. Several possible approaches may improve current rsCD4 serum half-life and concentrations.

Increasing steady-state serum rsCD4 concentrations is possible as clinical studies indicated that the maximum tolerated rsCD4 dose has not yet been reached (Kahn et al.,

1990; Schooley et al., 1990; Yarchoan et al., 1989c). Truncated preparations (Lifson et al., 1988) may allow delivery of more concentrated doses and additionally increase organ penetration, especially of the central nervous system.

Improvements on the serum half-life of rsCD4 utilise CD4's membership of the immunoglobulin gene superfamily and its resemblance to an immunoglobulin light chain (Capon et al., 1989). As such CD4 and the Fc region of immunoglobulin are structurally compatible. Consequently workers fused the first two domains of CD4, including the gp120 binding region, to the immunoglobulin Fc effector domain creating a hybrid protein or immunoadhesin (Capon et al., 1989; Traunecker et al., 1989). The Fc moiety bestows a prolonged half-life to the hybrids, as confirmed in rabbits (Capon et al., 1989), which selectively block HIV-1 infection of T-lymphoblastoid and myelomonocytic cell lines in vitro (Capon et al., 1989; Traunecker et al., 1989). Phase I studies of immunoadhesins are now underway in the National Cancer Institute and elsewhere (Hirsch, 1990; Yarchoan et al., 1990a). Variations of fusion include conjugation of Pseudomonas exotoxin A (Chaudhary et al., 1989) or ricin (Till et al., 1988) active subunits with the CD4 gp120 binding domain. Such hybrids may elicit problems of increased immunogenicity or non-specific toxicity but could be useful against cells resistant to the cytopathic effects of HIV-1, such as macrophages (Hirsch, 1990).

Although CD4 is the major HIV-1 receptor, CD4-negative muscle, brain (Clapham et al., 1989) and fibroblast cell lines (Tatento et al., 1989) are susceptible to infection. Furthermore low levels of anti-HIV-1 antibodies enhance infection of certain cells, possibly via CD4-independent mechanisms (Homsy et al., 1989; McKeating et al., 1990; see section 1.3.2.). The reference of these in vitro systems to the in vivo pathogenesis of AIDS is unclear (Yarchoan et al., 1990a). Therefore, recombinant soluble and hybrid CD4 may be used most effectively in combination with other drug regimens (see section 1.4.8.d).

1.4.2.a AL 721

Active lipids 721 (AL 721) is a lipid compound composed of neutral glycerides, phosphatidylcholine and phosphatidylethanolamine at a ratio of 7:2:1. In globular droplet form, AL 721 inserts phosphatidylcholine into and extracts cholesterol from cellular membrane, thus increasing membrane fluidity, both in vitro and in vivo (Lyte and Shinitzky, 1985; Shinitzky et al., 1983). Therefore, AL 721 may alter the lipid content of viruses that undergo membrane budding. Employing AL 721 as an anti-HIV-1 agent presumes that such lipid changes may modify viral gp120 and cellular CD4 receptor complementarity, causing reduced attachment of HIV-1 to its target cell membrane. A preliminary study demonstrated that AL 721 inhibited HIV-1 replication in peripheral blood lymphocytes or H9 cells in vitro. AL 721 was coadministered with HIV-1 to the host cells and therefore no conclusions as to the mechanism of antiviral activity were possible (Sarin et al., 1985). It is not clear if AL 721 is absorbed intact from the gastrointestinal tract and then circulates in droplet form in vivo.

An open study of AL 721 treatment of HIV-1 infected subjects with generalised lymphadenopathy syndrome (CDC group III) was reported by Grieco et al in 1988. No consistent or substantial trend was noted in CD4+ T-lymphocyte numbers nor in the in vitro proliferative response to PHA, while the Con A and PWM response was augmented in 4 of 8 subjects studied. Reverse transcriptase activity decreased in 5 subjects. No antiviral or immunorestorative effect of AL 721 was indicated in an open-study of 40 PGL and ARC subjects (Mildvan et al., 1989). Skorruk et al (1988) reported similarly inconclusive results. We (Peters et al., 1990) conducted a pilot study of 6 AIDS and 1 ARC p24 antigenaemic subjects treated with AL 721. Laboratory evidence (assessed by p24 levels and in vitro PHA response; see results sections 3.3.4. and 4.5.4.) indicated no benefit from AL 721. Furthermore, 4 of the AIDS subjects, still receiving AL 721, died within 12 months of commencing

treatment. The study of 6 HIV-1-antigen negative subjects was terminated early.

Subnormal *in vitro* lymphocyte PHA responses are restored on AL 721 treatment of elderly subjects. The previously diminished response is accounted for by increased membrane microviscosity, mainly due to increased cholesterol and sphingomyelin levels. Therefore, the immunorestorative effects of AL 721 are thought to be caused by its membrane fludisation properties (Shinitzky *et al.*, 1983). Apostolov *et al* (1989) have proposed that HIV-1 causes a rise in cellular oleic acid and consequently increases membrane fluidity. The original premise of AL 721 treatment of HIV-1 infected subjects does not consider the effect of further increasing already abnormally heightened levels of membrane fluidity. This observation may partly explain the ineffectiveness of AL 721 therapy *in vivo*. AL 721 treatment has now largely been abandoned.

1.4.2.b Anionic polysaccharides and antibodies to gp120

Additional agents that inhibit HIV-1 binding include low molecular weight dextran sulphate (MW: 7 to 8 kDa). This polyanionic polysaccharide comprises 17 to 20 percent sulphur. Dextran sulphate was active against HIV-1 and synergistc with ZDV *in vitro* (Ueno and Kuno, 1987) by preventing viral binding (Mitsuya *et al.*, 1988). An initial, dose-escalating 8-week study of orally administered dextran sulphate suggested that it had little toxicity but also little effect on circulating CD4+ T-lymphocyte numbers or serum p24 antigenaemia. However as there was no evidence of systemic absorption, the relevance of these findings is uncertain (Abrams *et al.*, 1989).

Vaccination regimens, based on host humoral and cellular responses to HIV-1 envelope and core antigens, may provide protection against infection and in addition, the means of reducing or preventing viral damage in seropositive individuals. In this respect, anti-gp120 antibodies may inhibit the HIV-1 binding step and, with the effects

described for rsCD4 (see section 1.4.2.), absorb free serum gp120. A possible beneficial effect of high titre antibodies is being investigated; however, HIV-1 disease progression occurs even in the presence of these antibodies (Yarchoan et al., 1990a).

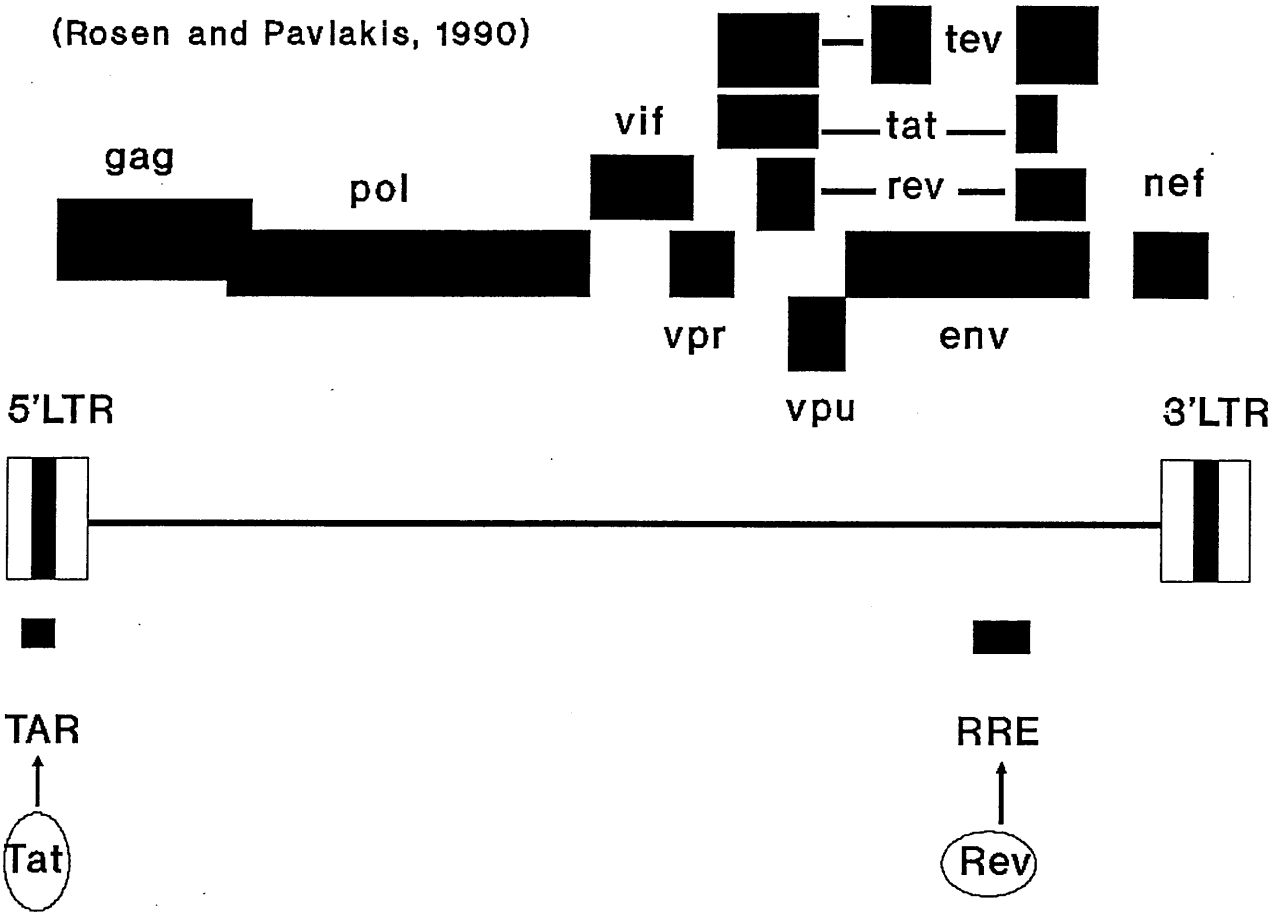
1.4.3. Polyprotein cleavage: protease (proteinase)

The HIV-1 gag and pol genes (Figure 03) are translated as $Pr55^{gag}$ and, by a ribosomal frame shift, $Pr160^{gag-pol}$ polyproteins (Jacks et al., 1988; Ratner et al., 1985). These precursors are subsequently processed by the HIV-1 specific protease to mature core proteins (p17, p24, p7 and p6) and essential viral enzymes (protease, implying autoproteolysis, reverse transcriptase, ribonuclease H and endonuclease). Kramer et al (1986) first suggested the HIV-1 protease as a potential therapeutic target since its inhibition putatively limits virus maturation at two or more replication sites (Hirsch, 1990).

Navia et al (1989) determined the crystal stucture of HIV-1 protease to 3 angstrom (Å) resolution. Corrections were made in a 2.8 Å X-ray study with synthetic protease (Weber et al., 1989) and confirmed with cloned material (Lapatto et al., 1989). Large regions of the protease homodimer, including the conserved Asp-Thr-Gly active site sequence, are homologous to the family of microbial aspartyl proteases. For a review of retroviral protease structure see Skalka (1989).

Pepstatin A, a modified oligopeptide inhibitor, has been reported to inhibit HIV-1 protease and retroviral replication in H9 cells (Grinde et al., 1989). Recent studies focus on peptides synthesised according to the natural substrate ($Pr55^{gag}$) cleavage site, but where the scissile bond is replaced with a bond stable to aspartic proteolysis. Meek et al (1990) demonstrated that peptide hydroxyethylene isosteres prevent HIV-1 infection of T-lymphocytes in vitro. However, the most potent inhibitors contain hydroxyethylamine isostere and effectively block proteolytic processing in

Figure 03: The HIV-1 genome



The three major genes of the HIV-1 genome, gag, pol and env, are common to all retroviruses and encode virion proteins. tat, rev and nef encode nonstructural regulatory proteins. vif and vpu are required for virion morphogenesis and maturation. vpr has no known function. vif, vpu and vpr will not be discussed further. Benko et al (1990) have recently characterised tev and postulate a regulatory role. The positions of the Tat-responsive element (TAR) and of the Rev-responsive element (RRE) are indicated.

cell-free assays (Dreyer et al., 1989) and suppress HIV-1 replication in vitro (Roberts et al., 1990). These workers also found differential inhibition of viral and mammalian aspartyl proteases, indicating a high therapeutic index. Furthermore, Moelling et al (1990) suggest mimetic modelling of cerulenin, a naturally occurring antibiotic, for rational design of protease inhibitors.

Although no clinical data have been reported as yet, the protease inhibitors are clearly promising antiretroviral agents.

1.4.4. Integration of HIV-1 DNA into host chromosome: endonuclease (integrase)

Integration of proviral DNA into host cell chromosome is common to the retroviral life cycle, including HIV-1 (see Figure 02), and is mediated by virally encoded endonucleases. Although Stevenson et al (1990) have reported that unintegrated HIV-1 DNA serves as a template for HIV-1 protein production, both this group and Haseltine (1989) found that integration was required for the production of infectious HIV-1.

Retroviral endonucleases appear to act on linear forms of the provirus (Brown et al., 1989). The initial step is probably a cleavage that removes the terminal 2 bases from the 3'end of the viral DNA exposing the 3'-OH group to be joined to the target host DNA. The target DNA is then cut and viral 3'-OH ends are coupled with target 5'-P DNA ends. The resulting gapped intermediate is then repaired.

HIV-1 endonuclease inhibitors have not yet been described (Hirsch, 1990).

1.4.5. Early HIV-1 gene expression: tat and nef

Cellular transcription factors, such as NF-kappaβ and Sp1, present in the activated T-lymphocyte are sufficient to stimulate low but significant transcription of the HIV-1 provirus (Cullen and Greene, 1989). The initial population of cytoplasmic genomic length viral mRNAs encodes the HIV-1

regulatory gene products tat, nef and rev (Feinberg et al., 1986; Malim et al., 1989a).

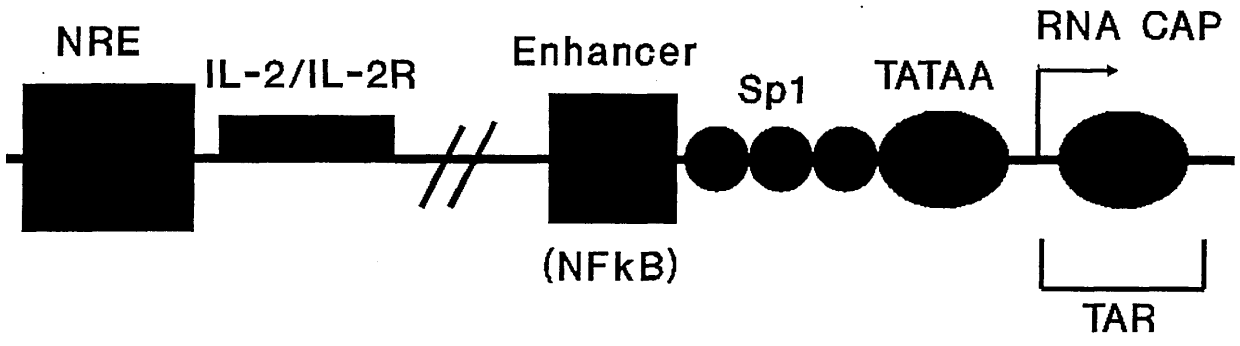
The 15.5 kDa tat protein is a potent trans-activator of viral long terminal repeat (LTR; Figure 04) directed gene expression, including the tat gene itself (Sodroski et al., 1985b). Functional mapping of cDNA clones (Arya et al., 1985) and partial genomes (Sodroski et al., 1985a) identified tat as a tripartite gene encoding an 86 amino acid nuclear protein. Site directed mutagenesis revealed that the first 57 amino acids only are essential for full tat function (Wright et al., 1986; Siegal et al., 1986b). The tat gene is essential to HIV-1 replication (Dayton et al., 1986; Fisher et al., 1986a; Sadaie et al., 1988).

TAR, the cis-acting tat-responsive sequence, maps within the R region of HIV-1 LTR (Figure 04; Hauber and Cullen, 1988; Rosen et al., 1985). The function of TAR is position and orientation dependent (Rosen et al., 1985) and evidence favours TAR to function as RNA (Muesing et al., 1987).

The precise mechanism of tat trans-activation remains unresolved. The increased steady state LTR-directed RNA commonly observed to accompany trans-activation (Sadaie et al., 1988; Wright et al., 1986) may be explained by transcription initiation, post-transcriptional or even translational events. Based on cotransfection experiments Cullen (1986) suggested a bimodal mechanism of tat action. Rosen and Pavlakis (1990) have recently reviewed tat regulation of gene expression.

Unlike tat, the 27 kDa nef gene product is not essential to HIV-1 replication and cytopathogenicity in vitro (Terwilliger et al., 1986). Nef is reported to downregulate transcription from the HIV-1 LTR (Ahmad and Venkatesan, 1988; Niederman et al., 1989) and as such may facilitate HIV-1 latency in vivo (Cheng-Mayer et al., 1989; Niederman et al 1989). However, Hammes et al (1989) and Kim et al (1989) have been unable to detect any inhibitory effects of nef on

Figure 04: The LTR of HIV-1



(Wong-Staal, 1989)

The HIV-1 LTR consisits of a 453 base pair U3 region, a 98 base pair R region and a 83 base pair U5 region. The region upstream of the transcription initiation site contains a TATA box and binding sites for cellular proteins that activate transcription. The best studied are NF-kB (Nable and Baltimore, 1987) and Sp1 (Jones et al., 1986). The Tat-responsive element, TAR, and the Negative regulating element, NRE, regulate LTR-directed transcription.

transcriptional activity of the HIV-1 LTR. Hammes *et al* ascribe this difference with Niederman *et al*, in part, to the proportion of 3' untranslated U3 and R sequences incorporated into the latter's transfect.

Guy *et al* (1987) have reported that purified bacterial nef has GTPase, autophosphorylation and GTP-binding activities reported for the *ras* oncogene product and is largely associated with cytoplasmic membrane structures. Furthermore, nef down-regulates CD4 antigen expression in the CEM CD4+ cell line. Therefore, it is possible that nef affects intracellular signal transduction.

The *nef* open reading frame appears conserved in most strains of HIV-1, HIV-2 and SIV-1 (Allan *et al*., 1985). Therefore, nef may subserve an important, although as yet undefined, function.

Clearly the tat and nef proteins provide a good target for inhibition and modulators are under development (Hirsch, 1990). Short segments or modified DNA in an antisense configuration (complementary to sequences of HIV-1 RNA) may hybridise viral RNA and arrest viral protein translation. Several forms of modified oligodeoxynucleotides, for example methylphosphonates and phosphorthioates, have demonstrated anti-HIV-1 activity *in vitro* (Agrawal *et al*., 1988; Sarin *et al*., 1988). Phosphorthioates were found to be particularly effective *in vitro*, being partly resistant to nuclease degradation. These oligodeoxynucleotides also exhibit sequence non-specific inhibitory effects (Matsukura *et al*., 1987). Allowing that problems of stability, nuclease resistance, solubility and specificity are addressed, these agents may become useful clinical drugs (Hirsch, 1990).

1.4.6. Transition to HIV-1 structural gene expression: rev

Early HIV-1 gene expression is characterised by cytoplasmic RNA encoding tat, nef and rev (see section 1.4.5.). The *rev* gene induces transition from early to late phase HIV-1 expression of structural proteins (Cullen and

Greene, 1989).

Discovered during investigation of tat, rev was also found to be critical for virus expression. HIV-1 mutants with deletions within or near the two tat coding exons expressed low or undetectable levels of viral proteins and were not complemented by functional tat cDNA clones (Feinberg et al., 1986; Sodroski et al., 1986b). A second open reading frame overlapping with tat was identified and found to encode a 19 kDa phosphorylated protein which is primarily accumulated in the nucleoli (Cullen et al., 1988; Schwartz et al., 1990). Rev function is mediated through a cis-acting element, rev-responsive element (RRE), found in the env region of HIV-1 (Rosen et al., 1988). In vitro binding experiments have presented evidence of direct rev and RNA containing RRE interaction (Cochrane et al., 1990; Daly et al., 1989; Zapp and Green, 1989).

Analysis of the subcellular localisation of the mRNAs in the absence or presence of rev demonstrated that this protein regulates the export of viral structural mRNAs from the nucleus to the cytoplasm (Felber et al., 1989; Malim et al., 1989a). Arrigo et al (1989) provided further evidence for this data employing a modified polymerase chain reaction. Rosen and Pavlakis (1990) have reviewed the major hypothesis of rev function mechanism.

The approach to inhibition adopted for tat and nef (see section 1.4.5.) is applicable to rev. Additionally, Malim et al (1989a) have characterised several rev mutants that inhibit wild-type rev function (trans-dominant rev mutants) in cotransfection experiments. Suppression of HIV-1 replication has yet to be verified in human lymphocytes. It is likely that inhibition of viral production by rev analogues is specific and may lead to novel therapeutic approaches.

1.4.7. Translation: glucosidase enzymes

The successful interaction of HIV-1 envelope glycoprotein gp120 and the T-lymphocyte subset antigen CD4 is

critical to viral life cycle initiation.

HIV-1 gp120 glycosylation (31 to 36 N-linked glycans per molecule) occurs late in viral replication and depends on host cell glucosidases. These enzymes remove three glucose residues while the protein is in the rough endoplasmic reticulum. The inhibition of glucosidase processing results in high molecular weight immature viral gp120 which is functionally disabled (Hirsch, 1990).

Castanospermine (CAS) and N-butyl deoxynojirimycin (N-Butyl DNJ) inhibit glucosidases and interfere with HIV-1 replication in vitro (Gruters et al., 1987; Karpas et al., 1988; Walker et al., 1987b). These inhibitors target host cellular enzymes and therefore a therapeutic window between antiretroviral effects and host toxicity needs to be demonstrated. Hirsch (1990) has reported that phase I clinical trials of N-Butyl DNJ are in progress following animal toxicology tests.

1.4.8. Immunomodulation and combination therapy

To date, research has focused on antiretroviral monotherapy of HIV-1 infection. However, it has become evident from detailed work with ZDV (see section 1.4.1.b) that, although viral suppression has short-term clinical benefits, it does not reverse the underlying immunological deficit caused by HIV-1 disease. Therefore, the optimal management of HIV-1 may require immunorestorative therapy administered with a combination or alternation of drug regimens.

The availability of effective treatments for HIV-1 infection has caused a resurgence of interest in putative immunorestorative or immunostimulating therapies, although the former are thought most appropriate.

1.4.8.a The class I interferons

In 1985, Ho et al observed recombinant interferon-alpha (rIFNα) expressed a dose related inhibitory effect on HIV-1 replication in vitro. Interferon-beta also

proved anti-HIV-1 activity (Hartshorn et al., 1987a).

The mechanics of interferon (IFN) inhibition of HIV-1 have yet to be clearly established. In vitro studies of murine leukaemia virus have indicated that the mechanism of IFN inhibition differs between acute and chronic infections. In acute infections, IFN predominantly suppresses early events of viral replication such as transcription or translation, while virion release from the plasma membrane is largely affected during chronic episode (Pitha et al., 1979). However, recombinant IFNα has been shown to inhibit HIV-1 replication of both acute and chronically infected cell lines (Ho et al., 1985; Poli et al., 1989). In addition to its antiviral activity, IFNα has a direct antineoplastic effect against cutaneous Kaposi's sarcoma.

AIDS-related Kaposi's sarcoma or epidemic Kaposi's sarcoma is seen in one third of all AIDS subjects. However, major response rates of approximately 30 percent are observed on high-dose rIFNα treatment (Krown, 1988). While low dose IFNα avoids characteristic toxicity (fever, fatigue, influenza-like syndromes, leucopenia and thrombocytopenia) treatment was generally ineffective.

The high dose mechanism of response to rIFN is unclear. Anti-HIV-1 activity, reflected by reductions of serum p24 antigen, negative blood cultures and increases in circulating CD4+ T-lymphocytes have been observed in responders to therapy (de Wit et al., 1988; Lane et al., 1988). However, the role of HIV-1 in Kaposi's sarcoma remains obscure. Vogel et al (1988) reported a tat gene introduced in transgenic mice produced a syndrome resembling Kaposi's. However, a direct effect of HIV-1 seems unlikely as ZDV had no significant effect on the extent of the sarcoma (de Wit et al., 1989; Lane et al., 1989b). Response to rIFNα does not correlate with tumour burden or the presence of visceral disease but with higher CD4+ T-lymphocyte numbers. Antiviral activity is most pronounced with the highest CD4+ counts. Therefore, IFNα may be more beneficial to subjects at earlier stages of HIV-1 infection where immune function is relatively

preserved (Hirsch, 1990; Krown, 1988). In fact, Ellis et al (1989) reported results from an open study of rIFNα in HIV-1-antibody positive men, excluded for PGL, ARC, and AIDS. Although therapy was not associated with clinical toxicity, no conclusions of beneficial effects were drawn. However, p24 antigen levels implied a lower viral load and CD4+ T-lymphocyte counts were relatively normal.

1.4.8.b The class II interferons

Gamma-interferon (gIFN) is a class II IFN which, in contrast to the class I IFNs, is derived from activated T-lymphocytes (see section 1.5.3.c). gIFN has demonstrated very little in vitro activity against HIV-1 and does not enhance the NK cell or T-lymphocyte mediated cytotoxicity of peripheral blood mononuclear cells obtained from HIV-1 infected subjects.

Phase I studies of gIFN have identified a maximum tolerated dose in the range of 0.1 mg per m^2 (Lane et al., 1989b). During these trials, performed predominantly in patients with AIDS and Kaposi's sarcoma, no antitumour effects or other obvious clinical benefits were observed. In one trial (Murray et al., 1987b), patients with AIDS, diagnosed by opportunistic infections, received 4 once-weekly 24-hour continuous infusions of 30 or 500 ug/m^2 recombinant gIFN. Blood monocytes obtained from patients following both low- and high-dose infusion, remained persistently activated for up to 5 days in vitro and showed increased ability to act against intracellular Toxoplasma gondii or Leishmania donovani. Although deficient T-lymphocyte function (see section 1.3.4.a) clearly disposes an HIV-1 infected subject to Pneumocystis carinii or CMV infection, it is not yet known if gIFN or the activated macrophage play important host defense roles against these particular pathogens.

1.4.8.c Granulocyte-macrophage colony stimulating factor and immunorestoration

Groopman et al (1987) has previously demonstrated

that administration of granulocyte-macrophage colony stimulating factor (GM-CSF) to AIDS subjects increased their circulating leucocyte numbers in a dose-dependent fashion. Although these individuals were not receiving ZDV, this result offers a precedent for the treatment of ZDV-induced bone marrow suppression with GM-CSF and similar factors. Clinical studies of GM-CSF, granulocyte colony stimulating factor and erythropoietin are now underway (Yarchoan et al., 1990a). However, the use of such agents must be carefully considered in evidence of their potential ability, demonstrated of GM-CSF, to augment HIV-1 replication in vitro (Koyanagi et al., 1988; Perno et al., 1989). Initial clinical studies with Il-2 were abandoned for this reason. Therefore, the value of immunostimulation remains unclear.

Effective immunorestoration is now feasible by bone marrow transplantation, given the recent results of Holland et al (1989). This group reported that HIV-1 could not be detected, even by polymerase chain reaction, in a subject with non-Hodgkin lymphoma who expired 47 days after receiving allogenic bone marrow transplantation and high-dose ZDV therapy. The limiting factor seems to be control of HIV-1 replication in vivo, as marrow transplantation earlier in the AIDS epidemic achieved partial immune reconstitution but was hampered by the lack of effective antiretroviral therapy (Lane et al., 1984). A similar approach may employ thymic hormone or thymus transplantation therapy (Hong, 1986). Therefore, prospective treatment of HIV-1 disease could combine antiretroviral treatment with immunorestorative therapy.

1.4.8.d Combination therpay

Combined antiviral therapy offers several potential advantages over monotherapy. These include inhibition of different sites of the viral replicative cycle, targeting of different cell populations and tissues, and a means to combat the emergence of drug-resistant HIV-1 strains. In addition, such a strategy allows reduction of the

doses of component synergistic drugs, thereby diminishing toxicity. Synergism is proved when combined drug effects are greater than the additive activities of the constitutents and is objectively calculated by the mathematical techniques of median-effect principle, isobologram analysis and fraction product method (Hirsch, 1990). Several *in vitro* systems have shown ZDV to act synergistically against HIV-1 with rIFNα (Berman *et al.*, 1989; Hartshorn *et al.*, 1987b); rsCD4 (Johnson *et al.*, 1989a) or the glucosidase inhibitors, CAS and N-butyl DNJ (Johnson *et al.*, 1989b; Hirsch, 1990) without enhanced toxicity. Promising *in vitro* studies with ZDV and rIFNα have been corroborated by a phase I *in vivo* trial (Kovacs *et al.*, 1989). Johnson *et al* (1990) have further demonstrated synergism using a three drug regimens of ZDV, rsCD4 and rIFNα *in vitro*.

1.4.9. Summary of anti-HIV-1 therapy

Research is currently devoted to the development of antiretroviral therapy for HIV-1 infection, both in symptomatic disease where ZDV has already shown clear, though limited benefit, and in asymptomatic infection, where preliminary evidence is encouraging for those with low peripheral CD4+ T-lymphocyte counts. Alternative antiretrovirals to ZDV which also target reverse transcriptase, for example ddI, have proved useful therapeutic agents in their own right, and may be efficacious in combination with ZDV. It is now evident that optimum therapeutic strategy must target a number of sites in the HIV-1 replicative cycle, such as the HIV-1 protease and regulatory gene products and viral binding to target cell. However, it is clear that in AIDS and ARC, suppression of HIV-1 replication does not reverse the immunological deficit, rather slowing its progression. Therefore there is a need to evaluate combined antiviral and immunorestorative therapy, for example with cytokine combinations.

In the future dramatic breakthroughs, such as ZDV development, may be less common than incremental advances.

These advances may be difficult to evaluate, since small differences among drugs requires a large number of patients to demonstrate an effect. With respect to this difficulty, it is anticipated that laboratory markers may replace clinical end-points in trials of new agents, thereby allowing more rapid evaluation.

1.5. Laboratory markers of antiretroviral efficacy

HIV-1 causes a chronic and progressive decline in cell-mediated immunity ultimately resulting in a lethal immunodeficiency. Yet this retroviral infection is typically asymptomatic for prolonged periods, until punctuated by episodes of opportunistic infections or by tumours (CDC, 1986). Thus, a great need exists for accurate indicators of the progression of asymptomatic HIV-1 infection to symptomatic disease that do not rely upon clinical symptoms yet predict their development.

The laboratory parameters or 'surrogate markers' of HIV-1 disease progression can be considered in three categories: 1) direct viral markers of HIV-1 activity, such as serum p24 antigen concentration; 2) markers of the status of the immune system, such as CD4+ T-lymphocyte count; 3) markers of immune activation, such as serum β_2-microglobulin or neopterin concentration. Data has been accumulated as to the utility of each of these types of markers in predicting progression of clinical symptoms in studies of the natural history of HIV-1 infection (Detels et al., 1988; Fahey et al., 1990; Lang et al., 1989; Moss et al., 1988; Munoz et al., 1988; Pedersen et al., 1990; Taylor et al., 1989). However, information is more limited concerning the use of laboratory markers in predicting the efficacy of antiretroviral treatment of HIV-1 disease.

The standard method of evaluating the efficacy of new drugs has been to conduct controlled, prospective trials using clinically relevant end-points as the main outcome of measure. In the case of HIV-1 disease the standard end-points of death or development of new AIDS defining opportunistic

infections or neoplasms can take months or years to reach (Fischl et al., 1987; Fischl et al., 1990b). Furthermore, because prophylactic medications can successfully prevent or greatly delay the onset of opportunistic infections, future studies of anti-HIV-1 agents will be even more difficult to perform using clinical end-points. Thus the development of laboratory markers that can serve as surrogate end-points for quickly evaluating new antiretroviral treatment has become a high priority for investigation. Indeed, a symposium convened by the National Academy of Sciences Institute of Medicine in September 1989 emphasised the urgent need for defining and validating such end-points for antiretroviral efficacy in patients with HIV-1 disease (Weiss and Mazade, 1990).

Laboratory markers identified in epidemiological studies as predictors of progression of HIV-1 asymptomatic infection to AIDS, have been primarily investigated as markers of antiretroviral efficacy. However, it is possible that a given marker may be an excellent predictor in a study of natural history but not prove as useful in a clinical trial of therapy.

An ideal ideal surrogate marker for clinical efficacy in the evaluation of anti-HIV-1 agents should be detectable in the majority of HIV-1 infected individuals at all stages of the disease, should change towards normal soon after the initiation of an effective agent and most importantly, changes in the marker should accurately predict the ultimate clinical response in individuals receiving therapy. Ideally, changes in the marker should discriminate between treated subjects who will improve clinically and those who will not improve clinically. If the ability of a marker to predict is validated for a trial also using clinical end-points, then this validated marker could be used as a true surrogate marker for clinical efficacy in subsequent studies of drugs of the same class, and investigators need not wait for clinical end-points (Valentine and Jacobson, 1990).

At present the evaluation of anti-HIV-1 agents

should be monitored by a direct viral marker, such as serum p24 concentration. However, the symptoms of HIV-1 disease are usually a result of the underlying immunodeficiency; therefore it is possible that the prevention of progressive clinical disease correlates more directly with an improvement in immune status than with a decrease in HIV-1 replicative activity following therapy (Valentine and Jacobson, 1990). Immunological status is now routinely assessed by CD4+ T-lymphocyte enumeration. However, a strong case exists for lymphocyte functional assays as laboratory markers of therapeutic efficacy; it could be argued that lymphocyte functional capacity is less a surrogate marker than a direct measure of the relevant variables underlying changes in immunological status.

The relationship between host immunological status and HIV-1 activity following treatment is unknown, and indeed may differ between individuals and between therapeutic agents. It is also likely that the administration of higher doses of some drugs may cause a drug-related inhibition of a potential marker of improvement, for example ZDV's associated bone-marrow toxicity may inhibit CD4+ T-lymphocyte number increase. Therefore, some laboratory markers may be more suitable for some treatments than others. For these reasons, a range of virological and immunological markers should be measured and validated in clinical trials of therapy.

1.5.1.a Peripheral CD4+ T-lymphocyte enumeration: immunological marker of natural history

Peripheral CD4+ T-lymphocyte counts are now routinely enumerated in patients at risk for and with AIDS and are widely utilised as a prognostic immunological marker on the basis of well established patterns of decline in natural history cohorts.

Acute HIV-1 infection is typically associated with a rapid decline of peripheral CD4+ T-lymphocytes (Cooper et al., 1985), after which asymptomatic subjects may exhibit a

prolonged period of stability (Detels et al., 1988) or a gradual decline (Lang et al., 1989). Finally during the 18 months prior to the development of AIDS, CD4+ levels exhibit further fall (Detels et al., 1988; Lang et al., 1989; Taylor et al., 1989). Subsequently, the level of circulating CD4+ T-lymphocytes has prognostic value for predicting the development of AIDS in HIV-1 infected subjects.

In evidence, Goedert et al (1987) documented a prospective study of 2 cohorts comprising 86 homosexual men from Washington and New York and followed for 3 years. AIDS developed in at least 43 percent of subjects with absolute CD4+ counts less than 0.3×10^9 cells per litre (cells/l) as compared with at least 5 percent of those with CD4+ T-lymphocyte counts greater than 0.55×10^9 cells/l at entry. Similarly in a cohort of 288 homosexual men from San Francisco, Moss et al (1988) reported that a 3-year acturial progression rate to AIDS was 87 percent for men with less than 0.2×10^9 CD4+ cells/l; 46 percent for those with 0.2 to 0.4×10^9 CD4+ cells/l; and 16 percent where CD4+ T-lymphocytes numbered greater than 0.4×10^9 cells/l. However in these Washington, New York and San Francisco cohorts, subjects were eligible with HIV-1 related symptoms although not with AIDS (Goedert et al., 1987; Moss et al., 1988).

De Wolf et al (1989) recently reported on an Amsterdam cohort of 199 asymptomatic HIV-1-core antibody seropositive and 76 seroconverted homosexual men; only subjects classified as CDC II or II asymptomatics were enrolled. The AIDS attack rate after 30 months follow-up was 51.9 percent for subjects with CD4+ cell counts less than 0.5×10^9 cells/l and 6.1 percent for those with CD4+ lymphocyte counts greater than 0.5×10^9 cells/l. Similar results have been reported from haemophiliac (Eyster et al., 1987) and intravenous drug user (Fernandez-Cruz et al., 1988) cohorts.

While selective loss of peripheral CD4+ T-lymphocytes generally reflects HIV-1 disease progression,

it is evident from the cohort studies described previously that a significant proportion of individuals fail to comply with this trend. In evidence of this observation, Weber et al (1986) found that CD4+ T-lymphocyte count at entry to a 3-year study of 170 symptom-free homosexual men, was not of value in predicting progression to AIDS. Furthermore Lau et al (submitted for publication) subsequently demonstrated in an 8-year prospective study of 350 homosexual men, that peripheral CD4+ T-lymphocyte counts alone failed to distinguish a clinically prevalent asymptomatic group (N=108) from a progressor group (N=62).

It is likely that considerable naturally occurring individual variability accounts for these described limitations of the CD4+ T-lymphocyte system. The wide range of patient CD4+ levels at a given clinical stage and individual rate of fall may contribute to such variability. Furthermore if the virulence of HIV-1 changes significantly over a period of months or years, changes in individual pattern of CD4+ cell decline and time to AIDS might be expected (Taylor et al., 1989; Valentine and Jacobson, 1990). However, individual variability may also be due to measurement error.

Taylor et al (1989) sought to minimise such measurement error by assessing the prognostic value of CD4+ T-lymphocyte percent rather than absolute CD4+ T-lymphocyte count. Since an absolute CD4+ T-lymphocyte count is derived by multiplication of the percent CD4+ T-lymphocytes, the percent lymphocytes, as determined on differential count, and the white blood cell count, using the percent figure alone eliminates any error arising from these procedures.

Data from a 3-year longitudinal study of 813 HIV-1 seropositive men, demonstrated that absolute CD4+ lymphocyte number and CD4+ lymphocyte percent were strongly correlated and similar in their ability to predict the development of AIDS. However, the CD4+ percent had slightly greater prognostic significance and showed slightly less variability on repeated measurements than absolute CD4+ number (Taylor et

al., 1989). Consequently, Taylor et al (1989) advocate the use of CD4+ T-lymphocyte percent rather than CD4+ absolute number to assess prognosis and also in the stratification and entry requirements for clinical trials. However, such a change may be difficult to implement since the absolute CD4+ number has already worked its way into common practice.

Finally the utility of the peripheral CD4+ T-lymphocyte level, in the form of an absolute number or percentage, may be improved as an immunological marker of prognosis by measuring certain subsets of this lymphocyte population. De Paoli et al (1988) observed selective loss of CD45RA- CD29+ (CD4.4B4, helper/inducer) memory T-lymphocytes in asymptomatic HIV-1 infected drug abusers. In contrast less-mature CD45RA+ CD29- (CD4.2H4, helper/suppressor) memory population numbers were initially normal and declined with progressive HIV-1 disease (De Paoli et al., 1988). In view of such observations, Miedema et al (1990) postulated that differential loss of these memory T-lymphocytes is intrinsic to HIV-1 pathogenesis in asymptomatic infection. Therefore it may be of more effect to measure these CD45RA- and CD45RA+ memory T-lymphocyte subsets and to monitor their relationship with each other, rather than enumerating total CD4+ T-lymphocyte levels, as an immunological marker of progression to AIDS in HIV-1 asymptomatic infection.

1.5.1.b Peripheral CD4+ T-lymphocyte enumeration: immunological marker of antiretroviral efficacy

It is evident from the natural history cohorts previously discussed, that peripheral CD4+ T-lymphocyte levels are a useful predictor of progression to AIDS in HIV-1 infected subjects. Therefore, CD4+ T-lymphocyte level was an obvious choice as an immunological marker of efficacy in clinical trials of ZDV and subsequently tested anti-HIV-1 agents.

The initial placebo-controlled trial of ZDV documented a transient rise of absolute CD4+ T-lymphocyte number in 145 patients with advanced ARC or AIDS receiving

1200 mg of the antiretroviral daily (Fischl et al., 1987; see section 1.4.1.b). The patients receiving ZDV showed a peak increase in CD4+ count at 4 weeks treatment which declined thereafter, although an increase relative to the pretreatment level was maintained until 20 weeks administration. The increase in CD4+ cell level was better sustained in ARC than AIDS patients. In comparison, those patients receiving the placebo most frequently showed a decline in absolute CD4+ T-lymphocyte number.

Although this study demonstrated that ZDV increased peripheral CD4+ T-lymphocyte number towards normal, the transient trend established did not reflect ZDVs associated clinical benefit. Clinical benefit was evident from 4 to 8 weeks of administration and persisted until termination of the study at 24 weeks. This was assessed by body weight, Karnofsky performance and development of opportunistic infection. However it may be argued that in patients with such advanced HIV-1 disease, the ZDV induced clinical response may have been sufficiently poor and the CD4+ T-lymphocyte count so low that these parameters may not have correlated with one another as well they might in less advanced disease (Valentine and Jacobson, 1990).

This discussion must now be considered in context of 2 placebo-controlled trials recently conducted in HIV-1 infected patients with asymptomatic or mildly symptomatic disease (Fischl et al., 1990b; Volberding et al., 1990).

Fischl et al (1990b) reported that ZDV treatment of patients with mildly symptomatic ARC was associated with a median rise of 25 cells per cubic millimetre ($cells/mm^3$; 0.025×10^9 cells/l) above entry level; this increase was apparent from week 4 through week 16 and subsequently declined to pretreatment level at week 40. The absolute number of peripheral CD4+ cells was always higher in patients receiving ZDV than in those receiving placebo, whose CD4+ cells declined steadily. The median increase of CD4+ T-lymphocytes in the ZDV arm was 2.4 $cells/mm^3$ per month and the median decline in the placebo arm was 4.9 $cells/mm^3$

per month (Fischl et al., 1990b). In a comparable study of asymptomatic subjects, the median annual changes for individual subjects were 39 and 26 CD4+ cell/mm^3 increases per year for recipients of 500 mg and 1500 mg ZDV daily and a decrease of 16 cells/mm^3 per year for recipients of placebo (Volberding et al., 1990).

Several points of discussion are raised by these studies of ZDV therapy in asymptomatic and symptomatic HIV-1 disease. First it is clear that since ZDV associated clinical benefit is usually evident from 6 weeks to at least 6 months of administration (Fischl et al., 1987; Pinching et al., 1989; Stambuk et al., 189a,b), these observations question whether CD4+ T-lymphocyte counts parallel the temporal clinical benefits of ZDV treatment. Furthermore, it is difficult to explain the profound clinical benefits of ZDV by the relatively small ZDV induced increases in CD4+ T-lymphocyte number. Indeed on the basis of such observations, Valentine and Jacobson (1990) have suggested that improvements of immune function may better account for ZDV's clinical benefit.

Similar observations to those previously outlined for ZDV have been reported in phase I trial of other clinically effective reverse transcriptase inhibitors, namely 2',3'-dideoxycytidine (Merigan et al., 1989; Yarchoan et al., 1988; see section 1.4.1.d) and 2',3'-dideoxyinosine (Cooley et al., 1990; Lambert et al., 1990; Yarchoan et al., 1989b; Yarchoan et al., 1990a; see section 1.4.1.d).

Thus, several trials have shown the clinical efficacy of the dideoxynucleosides ZDV, 2'3'-dideoxycytidine and 2',3,-dideoxyinosine in patients with symptomatic HIV-1 disease. However, it is clear from work with ZDV that this treatment does not result in improved long-term survival for all patients (Creagh-Kirk et al., 1988). The studies of CD4+ T-lymphocyte count previously discussed indicate only that ZDV for example, increases absolute CD4+ T-lymphocyte counts during 8 to 12 weeks administration (Fischl et al., 1987; Fischl et al., 1990b). These investigations have not

established if those subjects with improved CD4+ T-lymphocyte counts were the long-term survivors.

Jacobson et al (1991) sought to determine whether the early effects of ZDV treatment on CD4+ T-lymphocyte levels correlated with long-term clinical outcome in a study of 90 AIDS or ARC patients followed for a median of 2 years or until death. After 8 to 12 weeks ZDV treatment the most powerful of five potential predictors of long term survival, when controlled for pretreatment characteristics and conditional survival over 12 weeks, was the logarithm of CD4+ T-lymphocyte count. The raw CD4+ T-lymphocyte count itself was a less powerful predictor. However, change in the CD4+ absolute T-lymphocyte count during treatment was not a marker for survival. Thus patients maintaining a particular CD4+ lymphocyte count at 8 to 12 weeks of ZDV administration had the same prognosis as those subjects whose count rose and fell to a similar level during this treatment period.

In summary, these data suggest that CD4+ T-lymphocyte count at 8 to 12 weeks could act as a surrogate end-point for clinical outcome in trials of antiretroviral drugs for patients with HIV-1 disease.

It is noteworthy that when antiretroviral treatment of HIV-1 disease is clinically ineffective, CD4+ counts are accordingly unresponsive. This has been demonstrated with compounds which inhibit HIV-1 attachment to its target cell, for example: Active Lipids 721 (Grieco et al., 1988; Peters et al., 1990; see section 1.4.2.a); recombinant soluble CD4 (Kahn et al., 1990; Schooley et al., 1990; Yarchoan et al ., 1989c; see section 1.4.2.) or dextran sulphate treatment (Abrams et al., 1989; see section 1.4.2.b).

1.5.2. Immunological markers: peripheral CD8+ T-lymphocyte enumeration

Whereas circulating CD4+ T-lymphocyte numbers typically decline during HIV-1 asymptomatic infection (Lang et al., 1989), CD8+ T-cell counts remain raised following

seroconversion; thereby causing persistent inversion of the CD4:CD8 ratio (Gaines et al., 1990; Pedersen et al., 1990). Peripheral CD8+ cell levels are then relatively stable until progression to AIDS, where their numbers fall shortly before the onset of severe clinical deterioration (Lang et al., 1989; Lau et al., submitted for publication).

The marked CD8+ lymphocytosis observed shortly after seroconversion is reminiscent of Epstein Barr virus (EBV) associated mononucleosis (de Waele et al., 1981) and CMV infection (Carney et al., 1981). However, Gaines et al (1990) found no serological evidence of primary or activated EBV or CMV in 20 such HIV-1 infected homosexual men and concluded that the activated CD8+ T-lymphocytes were HIV-1-specific CTL. In support, large numbers of circulating CTL and CTL-precursor cells have been found in asymptomatic HIV-1 infected individuals (Hoffenbach et al., 1989; Plata et al., 1987; Walker et al., 1987a). Furthermore, Hoffenbach et al (1989) reported that circulating CTL and CTL-precursor numbers decreased gradually with time, such that HIV-1-specific CTL were undetectable prior to major secondary infection. These observations are consistent with the quantitative alterations of circulating CD8+ T-cells seen in HIV-1 infection.

The physiological role of the CD8+ T-lymphocyte in HIV-1 disease is unclear since the relative importance of CD8+ T-lymphocyte inhibition of HIV-1 infection or contribution to host damage remains unresolved. While these views of CD8+ cell role are not necessarily mutually exclusive, the nature of their relationship has yet to be clarified. Confusion is additionally generated by a number of conflicting observations concerning CD8+ T-lymphocyte level association with HIV-1 disease progression to AIDS.

CD8+ T-lymphocytes are thought to be inhibitors of HIV-1 infection as delineated by Via et al (1990b) and supported by the ability of such cells to suppress HIV-1 infection in vitro (Walker et al., 1986). In evidence, Fernandez-Cruz et al (1990) has recently found, in a

prospective 33-month follow-up study of 76 HIV-1 seropositive intravenous drug users, that actuarial progression was associated with suppressed numbers of peripheral CD8+ cells.

In contrast several lines of evidence now indicate that CD8+ cells may participate in certain immunopathological manifestations of HIV-1 infection, such as local inflammatory reaction in the lungs and central nervous system (cited by Plata et al., 1989). Moss et al (1988) found by actuarial study of 288 homosexual men, that increased CD8+ cell numbers were associated with the development of AIDS. CD8+ T-lymphocytes have also been implicated in CD4+ T-lymphocyte destruction or functional modification (see section 1.3.3.b) and indeed in the Multicentre AIDS Cohort Study, high CD8+ cell counts were found to be a significant predictor of a steeper decline of CD4+ cell number (Munoz et al., 1988).

These disparate findings are difficult to reconcile, not in least because of differences in subject cohorts and absolute cell number cut-off points. However from the evidence outlined, it seems unlikely that absolute peripheral CD8+ T-lymphocyte numbers will provide a reliable laboratory parameter of the course of HIV-1 infection. Indeed Fahey et al (1990) recently found that the absolute level of peripheral CD8+ T-lymphocytes had little value in predicting progression to AIDS in a 3-year prospective study of 395 HIV-1 seropositive homosexual men.

Following this line of argument, although no evidence is yet available, it also seems unlikely that peripheral CD8+ counts would provide a reliable marker of antiretroviral efficacy. However, HIV-1-specific CTL enumeration and functional ability may provide useful information on strategies for immune reconstitution (see section 1.4.8.).

1.5.3. Parameters of immune dysfunction: potential immunological markers of the natural history of HIV-1 infection and antiretroviral efficacy

A number of qualitative immunological parameters

have been studied throughout the course of HIV-1 infection and the observed trends of dysfunction correlated with HIV-1 disease progression. The more established of these markers include decreased mitogen and antigen stimulated T-lymphocyte proliferative response and gIFN generation; elevated spontaneous Ig production *in vivo* and *in vitro*; and decreased B-cell mitogen induced proliferation and Ig secretion. These changes become more pronounced as symptomatic disease emerges and are thus candidate laboratory markers of HIV-1 disease progression and of antiretroviral efficacy.

1.5.3.a Parameters of T-lymphocyte dysfunction: the proliferative response

Both the mitogen and antigen induced lymphocyte proliferative response, assessed by tritiated thymidine incorporation into newly synthesised cellular DNA, decrease in parallel with HIV-1 disease progression. The patterns of responsiveness to several antigens, for example to tetanus toxoid or *Candida* are closely interrelated, revealing similar trends of decline. Similar observations have been noted of T-cell specific mitogens, such as PHA or Con A. PWM, a B-cell T-cell dependent mitogen (see section 1.3.4.c), has also been employed in the delineation of these trends.

Pedersen *et al* (1990) recently reported that lymphocytes obtained from 6 homosexual men, participating in longitudinal natural history cohort studies and with severe clinical manifestations of HIV-1 seroconversion, were profoundly hyporesponsive to mitogens (PHA and PWM) and to soluble recall antigen (*Candida*). Moreover, the proliferative responses of these subjects remained depressed for 52 weeks from the onset of seroconversion illness; during this time 5 of the original 6 were classified as asymptomatic, with or without PGL.

Several cross-sectional studies have previously demonstrated depressed mitogen or antigen induced lymphocyte proliferation in similarly classified asymptomatic HIV-1 infected individuals (Lane *et al*., 1985; Rogers, 1987; Rogers

et al., 1989). These investigators further reported that lymphocytes from healthy homosexual men were also hyporesponsive to mitogen and antigen stimulation, in comparison with heterosexual controls. The cause of these abnormalities and of decreased antigen stimulated gIFN production (Murray et al., 1988) and of altered T-lymphocyte subsets (Weber et al., 1986) in HIV-1 seronegative homosexual men is unknown. It is unclear if they reflect biologically significant alterations in the immune responsiveness of homosexual men, although this is often inferred (Rogers et al., 1989).

The mitogen and antigen driven lymphocyte proliferative response defect becomes more pronounced as symptomatic disease emerges (Lane et al., 1985; Rogers 1987; Rogers et al., 1989). The severe hyporesponsiveness observed with the onset of AIDS is accounted for by the synergism of CD4+ T-lymphocyte depletion (see section 1.3.3.) and the intrinsic dysfunction of those CD4+ cells remaining (see section 1.3.4.a,b; Hofmann et al., 1985; Lane et al., 1985) and is associated with defective Il-2 production and Il-2 receptor expression (Murray et al., 1984; Prince et al., 1984).

Lane et al (1985) delineated a uniform decrease in PHA and PWM proliferation, from patients with chronic lymphadenopathy syndrome to those with AIDS manifested as Kaposi's sarcoma and finally to AIDS patients classified by opportunistic infection. The trend of response to soluble recall antigen revealed a similar decline, though failed to distinguish the AIDS patient subgroups. Gluckman et al (1985) and Rogers et al (1989), utilising the PHA proliferative response as a laboratory marker, were also able to distinguish asymptomatic patients from those with AIDS in cross-sectional studies.

The evidence available does not formally correlate the antigen or mitogen induced lymphocyte proliferative response level with HIV-1 disease progression, as performed for CD4+ T-lymphocyte phenotypic marker (Goedert et al.,

1987; Moss et al., 1988 see section 1.5.1.a); however a trend for declining response with HIV-1 disease progression is clearly evident. As such, the mitogen and antigen lymphocyte proliferative response may prove a useful laboratory marker of HIV-1 disease progression in natural history studies. Although data in this area are limited, Coates et al (1989) have recently found that the PHA, Con A and PWM proliferative response together with CD4:CD8 ratio were significantly associated with progression to AIDS in an asymptomatic cohort comprising 143 members. However, their final model revealed that only the PHA proliferative response and the CD4:CD8 ratio remained independently and significantly associated with the risk of disease progression.

No reported study has yet evaluated mitogen or antigen driven proliferation as possible in vitro markers of antiretroviral treatment in HIV-1 disease, although the present project was designed with this aim (see result sections 3.5. and 3.6.2 and discussion sections 8.2.2. and 8.2.6.). However the demonstrated ability of these immunological functional parameters to distinguish clinically defined states of disease, together with preliminary evidence of their value as predictors of HIV-1 disease progression in natural history cohorts is promising, particularly with respect to the PHA induced proliferative response. Furthermore such data may better explain the striking clinical benefits seen with ZDV therapy than the observed small changes in CD4+ lymphocyte number (see section 1.5.1.b).

1.5.3.b Parameters of T-lymphocyte dysfunction: the gamma-interferon (gIFN) response

Among the numerous T-lymphocyte qualitative defects associated with HIV-1 disease (see section 1.3.4.a, b), failure of gIFN in vitro generation (Lane et al., 1985; Murray et al., 1984; Murray, 1988; Murray et al., 1988) is central to the pathogenesis of characteristic opportunistic infection in vivo; gIFN potentiates macrophage antimicrobial

activity and exerts a pivotal regulatory role in a variety of other cellular immune reactions including T-cell activation and B-cell differentiation and maturation (Murray, 1988; Trinchieri and Perussia, 1985).

1.5.3.c gIFN

Native type II or immune (gamma)-interferon is a pH 2-labile, highly basic heterogeneous glycoprotein (estimated weight: 40 to 50 kDa) which can be resolved into 3 monomeric forms (25 kDa; 20k Da; 15.5 kDa). However, there is evidence of only one gIFN gene and resolution to component form is due to post-translational processing of a 17 kDa protein (Murray, 1988; Trinchieri and Perussia, 1985).

Mitogens, alloantigens and Il-2 induce human CD4+, CD8+ and NK cells to secrete gIFN in vitro (Chang et al., 1982; Epstein et al., 1981; O'Malley et al., 1982; Trinchieri et al., 1984), whereas only CD4+ T-lymphocytes produce gIFN after exposure to soluble microbial antigen in an anamnestic immune response (Cunningham and Merigan, 1984; Kelly et al., 1987). The molecular mechanisms of mitogen or antigen stimulated gIFN production are complex and are at best only partially defined; antigen induced secretion is dependent on Il-1, Il-2 and Il-2 receptor expression (Murray, 1988; Trinchieri and Perussia, 1985).

A series of collaborative studies demonstrated lymphokine-induced macrophage activation and enhanced microbicidal activity to be both gIFN dependent and inducible by gIFN alone (Murray et al., 1983; Nathan et al., 1983; Rothermel et al., 1983). Although the gIFN stimulated macrophage is broadly reactive, killing or inhibiting by oxygen-dependent or oxygen-independent mechanisms such diverse human intracellular organisms as Toxoplasma gondii, Leishmania donovani and Chlamydia psittaci (Murray et al., 1983; Nathan et al., 1983; Rothermel et al., 1983), its efficacy against certain extracellular pathogens such as Pneumocystis carinii are unclear (Murray, 1988). Evidence from murine studies shows that administered gIFN enhances in

vivo host mechanisms against systemic intracellular infection (Murray et al., 1985). Monocyte activation has also been observed on recombinant gIFN therapy of AIDS patients (Murray et al., 1987b; see section 1.4.8.b).

Accumulating data from clinical trials of recombinant gIFN has also indicated that this lymphokine has in vivo immunoregulatory activities that had previously only been documented in vitro. These effects, demonstrated in patients with cancer, leprosy, multiple sclerosis or AIDS include enhanced or increased MHC class II antigen expression; NK cell number and cytotoxicity; CD4:CD8 ratio and monocyte Ig receptor expression. Moreover, recombinant gIFN exerts other in vitro immunoregulatory activites relevant to the pathogenesis of HIV-1 disease such as, inhibition of monocyte migration; stimulation of complement activation and of monocyte antibody dependent cellular cytotoxicity; gIFN also potentiates Ig secretion by mature B-cells and differentiation and maturation of resting B-cells. These in vitro activities of gIFN have yet to be demonstrated in vivo (cited by Murray, 1988; Trinchieri and Perussia, 1985).

1.5.3.d gIFN: potential immunological marker of natural history and antiretroviral efficacy

Natural history cohort studies have revealed that mitogen or antigen induced lymphocyte synthesis of gIFN declines with HIV-1 disease progression (Lane et al., 1985; Murray et al., 1984; Murray et al., 1988). This trend is similar to that of the proliferative response decrease (see section 1.5.3.a).

Whereas mitogen induced gIFN production has been observed to decline noticeably with the onset of symptomatic HIV-1 disease, a decreased antigen response is apparent in lymphocytes obtained from both HIV-1 seropositive and seronegative homosexual men (Lane et al., 1985; Murray et al., 1984; Murray et al., 1988). In evidence, Murray et al (1988) found peripheral mononuclear cell production of gIFN

stimulated by mitogen (Con A) was similar to healthy heterosexual controls, while the antigen (Toxoplasma gondii lysate or CMV antigen) induced response was decreased in 40 to 46 percent of 23 healthy homosexual men, 10 of whom were HIV-1 seropositive. This observation implied that modest changes in the antigen induced gIFN response of healthy homosexual men was independent of the HIV-1 retrovirus (Murray et al., 1988); this concept has been discussed previously (see section 1.5.3.a).

In the same study, Murray et al (1988) observed that 40 patients with clinical symptoms of advanced ARC displayed appreciable defects in both mitogen (43 percent) and antigen induced gIFN production (92 percent). In fact the antigen induced lymphocyte gIFN response was undetectable, by bioassay, in 40 percent of these patients. An undetectable response to antigen readily discriminated asymptomatic (0 percent) from symptomatic ARC (40 percent) and AIDS (63 percent) patients. However, abnormal antigen gIFN production failed to distinguish the symptomatic patient groups, whereas abnormal mitogen induced production of gIFN was considerably more pronounced in those subjects with AIDS than those with ARC; moreover the mitogen driven response differentiated AIDS patients subgrouped as those with Kaposi's sarcoma alone (46 percent) from those with opportunistic infection alone (73 percent) or clinical evidence of both of these (77 percent). In comparison with subjects with opportunistic infection or Kaposi's sarcoma plus opportunistic infection, those individuals with Kaposi's sarcoma alone were considerably less deficient in both antigen (46 percent) and mitogen (76 percent) function.

It is evident from Murray's study that an undetectable antigen gIFN response readily distinguished the asymptomatic, ARC and AIDS patient cohorts; undetectable antigen driven gIFN synthesis may prove to be a useful surrogate marker of HIV-1 disease progression (Murray et al., 1988). In evidence, undetectable lymphocyte gIFN production to specific microbial antigen successfully identified those

patients who clinically progressed during a 3.5 year programme (Murray et al., 1984; Murray et al., 1989).

Although an undetectable antigen gIFN response has proved a useful predictor of HIV-1 disease progression, it is unlikely to be an ideal laboratory marker of therapeutic efficacy. Ideally, a laboratory marker for clinical efficacy in the evaluation of antiretrovirals against HIV-1 should be detectable in the majority of individuals at all stages of the disease (Valentine and Jacobson, 1990); it is advantageous to have a pretreatment baseline to which even small changes of benefit or detriment may be compared. The deficient, although not abrogated, mitogen induced gIFN response observed in symptomatic HIV-1 patients (Murray et al., 1988) provides a better potential marker of therapy than the antigen driven response. Data accumulated by the present project provide evidence in favour of this assumption (see result sections 4.7. and 4.8.2. and discussion sections 8.2.3. and 8.2.6.).

1.5.3.e Parameters of B-cell dysfunction

The main changes observed in B-cell function across the spectrum of HIV-1 infection have been discussed previously and from which it is evident that HIV-1 associated B-cell dysfunction provides numerous potential parameters of antiretroviral efficacy (see section 1.3.4.c). Such measurable in vitro parameters include spontaneous and mitogen induced B-cell production of Ig. Although no published evidence is available as to the utility of these laboratory measurements as markers of antiretroviral efficacy, this project suggests that B-cell spontaneous production of IgM, IgG and IgD and production of these Igs in response to the T-cell dependent mitogen, PWM, are not promising markers, in this context (see result sections 6.4.1. to 6.4.4. and discussion section 8.2.7.).

The in vivo antibody response to HIV-1, as perceived by serum levels of antibody to retroviral proteins such as p24, is discussed with respect to HIV-1 disease

progression and therapeutic efficacy by sections 1.5.5.a and 1.5.5.b respectively.

1.5.4. β_2-microglobulin and neopterin: immunological markers of natural history and antiretroviral efficacy

The serum immunological markers β_2-microglobulin and neopterin have been identified in natural history cohorts as predictors of disease progression to AIDS in HIV-1 infected subjects. These markers also show promise as laboratory parameters of antiretroviral efficacy since, during clinical trials, they exhibit a tendency to revert to normal in response to ZDV treatment (Crocchiolo et al., 1988; Fahey et al., 1990; Jacobson et al., 1989; Jacobson et al., 1991; Moss et al., 1988; Reddy et al., 1989).

1.5.4.a β_2-microglobulin

β_2-microglobulin is a low molecular weight compound comprising the constant subunit of the MHC class I antigen, which is present on the surface of most somatic cells, including T-cells, B-cells and macrophages (Cresswell et al., 1974). Since endogenous β_2-microglobulin is produced mainly by cells of the immune system, its serum level reflects immunological activation during HIV-1 infection (Lang et al., 1989; Sonnerborg et al., 1989; Valentine and Jacobson et al., 1990).

Although elevated β_2-microglobulin is not a specific marker of HIV-1 infection, increased serum concentrations appear to be associated with HIV-1 infection, levels being highest during acute infection and again after development of symptomatic disease (Fuchs et al., 1988; Melmed et al., 1989; Moss et al., 1988; Murray et al., 1989). Indeed in a natural history cohort of 288 HIV-1 seropositive men, serum β_2-microglobulin concentration was elevated in 40 percent of subjects and was found to be a better singular marker of prognosis than peripheral CD4+ T-lymphocyte counts (see section 1.5.1.a) and p24 antigenaemia (see section

1.5.6.a). The 3-year actuarial progression rate to AIDS in this study was 69 percent for subjects with serum β_2-microglobulin levels greater than 5.0 mg/l; 33 percent for levels 3.1 to 5.0 mg/l; and 12 percent for levels less than 3.0 mg/l. The normal value of β_2-microglobulin found in HIV-1 seronegative blood donors and homosexual men was 1.7 mg/l (Moss et al., 1988). Interestingly, high concentrations of β_2-microglobulin are found in the cerebrospinal fluid of HIV-1 infected patients with severe neurological symptomatology (Sonnerborg et al., 1989) and therefore β_2-microglobulin may be a useful predictor of this HIV-1 associated condition.

Data concerning the effect of antiretroviral treatment on serum β_2-microglobulin level is limited and that available pertains to ZDV therapy. Reddy et al (1989) recently reported that ZDV treatment (1500 mg or 1200 mg daily) of 9 ARC and 11 AIDS patients caused a decrease of serum β_2-microglobulin concentration 12 weeks post administration; however, these levels increased towards pretreatment values by 20 weeks. Jacobson et al (1989) also found that mean serum β_2-microglobulin concentration decreased, from 4.1 mg/l before treatment to 3.55 mg/l at 12 weeks of ZDV administration, in a prospective study of 41 asymptomatic HIV-1 infected patients. This study also showed that individual changes in serum β_2-microglobulin level correlated highly to individual changes in serum p24 antigen level; however neither changes in serum β_2-microglobulin nor changes in p24 antigen correlated to changes in CD4+ T-lymphocyte number during treatment (Jacobson et al., 1989). Jacobson et al (1991) further reported, in a retrospective study of 90 patients with ARC or AIDS, that patients who had higher CD4+ T-lymphocyte counts and whose serum β_2-microglobulin concentration did not increase after the first 8 to 12 weeks of ZDV tretment had better long-term survival. However, neither absolute nor relative measures of p24 antigen concentration during ZDV treatment correlated with survival (see section 1.5.6.b; Jacobson et al., 1991).

The evidence obtained by these studies suggest that the immunological marker, β_2-microglobulin, is a slightly better parameter of antiretroviral efficacy than the direct virological marker, serum p24 antigenaemia. With respect to this observation, it is possible that the prevention of progressive clinical disease correlates more directly with an improvement in immune status than with a decrease in HIV-1 replicative activity following therapy. Another potential advantage of β_2-microglobulin over p24 antigen is that β_2-microglobulin level can be accurately quantified in the serum of all subjects. Also a higher proportion of asymptomatic HIV-1 infected individuals have elevated serum β_2-microglobulin than have detectable serum p24 antigen (Moss et al., 1988).

1.5.4.b Neopterin

Neopterin is an endogenous metabolic product of guanosine trisphosphate, produced by macrophages when they are stimulated by gIFN derived from activated T-lymphocytes. This serum factor, like β_2-microglobulin, is another potential immunological marker for in vivo HIV-1 activity (Fuchs et al., 1988).

Although serum neopterin level is noticeably increased in HIV-1 seronegative homosexual men, intravenous drug abusers and haemophiliacs (Heering et al., 1987; Sandstrom et al., 1989), elevated serum neopterin has been found to be a strong predictor of progression to AIDS in asymptomatic HIV-1 infected individuals (Fahey et al., 1990; Kramer et al., 1989; Melmed et al., 1989). For example in a cohort of 395 HIV-1 seropositive homosexual men, initially free of AIDS and who were followed prospectively for 4 years, serum neopterin, β_2-microglobulin and CD4+ T-lymphocyte number each had a similarly high predictive value for subsequent progression to AIDS and substantially more predictive value than p24 antigen (Fahey et al., 1990).

Like β_2-microglobulin, data is limited concerning the effect of antiretroviral treatment on serum

neopterin levels. However, Crocchiolo et al (1988) have documented a decrease in serum neopterin concentration during ZDV therapy (1200 mg/day) in 12 symptomatic HIV-1 infected individuals and which was associated with an increase in CD4+ T-lymphocyte number. Similarly, Jacobson et al (1991) reported a significant decrease of serum neopterin concentration during 8 to 12 weeks of ZDV treatment in a retrospective study of 90 patients with ARC or AIDS. However, this decrease did not correlate with survival when controlled for baseline predictors. Perhaps if Jacobson et al (1991) had assayed at a different time during treatment, changes in neopterin concentration may have correlated with clinical outcome. Alternatively, if increased serum neopterin concentration results from direct HIV-1 activation of chronically infected monocytes or macrophages, ZDV, at plasma achievable concentrations, may not effectively inhibit virus replication in these long lived cells. This hypothesis may be tested only when drugs targeted against HIV-1 infected monocytes or macrophages are available. Such drugs might produce greater decreases in serum neopterin during treatment that could correlate with clinical outcome (Jacobson et al., 1991).

1.5.5.a Parameters of the humoral immune response to HIV-1 as markers of natural history

Early after the identification of HIV-1 as the aetiological agent of AIDS (Barre-Sinoussi et al., 1983; Popovic et al., 1984), it became apparent that patterns of humoral immune reactivity differed markedly between the retroviral glycosylated envelope (env) proteins and non-glycosylated core (gag) proteins (Barin et al., 1985; Casey et al., 1985; Montagnier et al., 1985). Investigators found that after HIV-1 infection, antibodies to the env gene products gp160/gp120 and gp41 were produced early followed shortly by antibodies to the gag gene product p24 (Allain et al., 1986; Gaines et al., 1987). Moreover, a number of workers demonstrated that serum antibodies to the envelope

proteins were present throughout the course of HIV-1 infection, but that antibodies to the gag protein, p24, were undetectable in the majority of individuals with advanced HIV-1 disease (Barin et al., 1985; Casey et al., 1985; Montagnier et al., 1985; Weber et al., 1987). Consequently, there is some controversy as to the cause of anti-p24 antibody decline with HIV-1 disease progression.

It has been repeatedly shown that 50 to 70 percent of patients with AIDS have detectable titres of p24 antigen, whereas only rarely is p24 antigen detectable in asymptomatic subjects (Moss et al., 1988; Pedersen et al., 1987; Simmonds et al., 1988). The loss of anti-p24 antibody precedes p24 antigenaemia by 12 to 18 months (Forster et al., 1987) and this has been interpreted as being due to the formation of immune complexes of anti-p24 antibodies with rising concentrations of p24 antigen (Amadori and Chieco-Bianchi, 1990; Lange et al., 1987; Morrow et al., 1986; see section 1.3.4.c). However, undetectable titres of anti-p24 antibodies in antigenaemic patients do not rise after the reduction of p24 antigen with successful ZDV treatment; this suggests that the mechanism for the decline in titre of anti-p24 antibodies is unlikely to be the simple formation of immune complexes (Weber and Jeffries, 1989). Nevertheless, the differential antibody response observed suggests serum anti-p24 antibody level as a candidate laboratory marker of HIV-1 disease progression.

In evidence Weber et al (1987) showed that in an asymptomatic cohort, a lower or falling titre of anti-p24 antibody was associated significantly with disease progression up to 27 months before the development of ARC or AIDS. Similar results were also reported from a number of other cohort or cross-sectional studies (for example, Mayer et al., 1987; Pan et al., 1987). However subsequent cohort data has shown that, over time, increasing numbers of patients with preserved high titres of anti-p24 antibody and without detectable titres of p24 antigen develop AIDS, and the association of loss of anti-p24 antibodies with prognosis

has become less significant (Weber and Jeffries, 1989). In support, Simmonds et al (1988) have previously demonstrated that not all subjects at high risk of AIDS lose anti-p24 antibodies and that not all asymptomatic subjects mount a high titre anti-p24 antibody response. In view of these interpretative problems, Cheingsong-Popov et al (1991) studied the role of the initial humoral response to HIV-1 gag and env proteins at seroconversion in relation to disease outcome in an 84-month retrospective longitudinal study of 88 haemophiliac patients infected with HIV-1.

This study showed highly significant associations between anti-p24 and anti-gp120 antibody titres at seroconversion and the time to HIV-1 symptomatic disease. Twenty-two patients with low anti-p24 (median titre of 200) and high anti-gp120 (median titre of 51 200) antibody titres developed stage IV disease (ARC or AIDS) relatively more rapidly than 66 patients with high anti-p24 (median titre 1600) and low anti-gp120 (median titre 12 800) antibody titres. It remains unclear whether the association between a strong initial anti-p24 antibody response and slower progression of HIV-1 disease is due to host or viral factors (Cheingsong-Popov et al., 1991).

1.5.5.b Parameters of the humoral immune response to HIV-1 as markers of antiretroviral efficacy

Little evidence is available concerning the effect of antiretroviral treatment on the humoral response to HIV-1.

Weber and Jeffries (1989) initially reported that successful ZDV treatment was not associated with the reappearance of formerly undetectable anti-p24 antibody in patients with symptomatic HIV-1 disease. The recent retrospective work of Jacobson et al (1991) on 90 ZDV treated patients with ARC or AIDS is compatible with this observation. Although Jacobson et al (1991) reported a marginal increase in anti-p24 antibody level at 8 to 12 weeks ZDV administration, when controlled for AIDS, ARC, age and baseline neopterin concentration, neither absolute values nor

changes anti-p24 antibody concentration during ZDV treatment correlated with survival.

These results indicate that the anti-p24 antibody response is not a suitable marker of ZDV efficacy and most likely of other nucleoside analogue treatments. This may reflect the inability of the immune sytem to reconstitute itself and as such the anti-p24 antibody level may be a useful marker of treatment employing immune reconstitution regimens (see section 1.4.8.).

1.5.6.a Serum p24 antigen: virological marker of natural history

The HIV-1 gene gag (see Figure 02) encodes a core protein antigen (molecular weight: 24 kDa), measurable by enzyme linked immonosorbent assay (ELISA) in the serum of about half to two-thirds of individuals with ARC or AIDS (Cao et al., 1987) and in 10 to 15 percent of individuals with asymptomatic infection (Moss et al., 1988). P24 antigen is also found in the culture supernatant of HIV-1-infected cells actively replicating HIV-1. Hence the presence of p24 antigen in the serum is thought to correlate with active HIV-1 replication in vivo (Valentine and Jacobson, 1990).

Circulating p24 antigen has been detected prior to antibody seroconversion (Allain et al., 1986; Lange et al., 1987) becoming rapidly undetectable with the emergence of anti-p24 antibody (von Sydow et al., 1988; see section 1.5.5.a). The return of detectable p24 antigenaemia following the decline of anti-p24 antibody is strongly associated with disease progression (Allain et al., 1986; Moss et al., 1988; Murray et al., 1989; Pedersen et al., 1987). In evidence in a 3-year actuarial analysis of 288 HIV-1 seropositive men, Moss et al (1988) estimated that the progression to AIDS was 59 percent in p24 antigenaemic individuals as compared to 15 percent in those who remained antigen negative.

1.5.5.b Serum p24 antigen: virological marker of antiretroviral efficacy

It is clear from natural history cohorts, that serum p24 antigen level has proven predictive value as a virological marker of HIV-1 disease progression. This, together with the simplicity, low cost and reproducibility of the p24 antigen assay and the reliability of results obtained from frozen stored samples, makes this virological marker particularly attractive for application in large multicentre trials of therapy for HIV-1 disease.

Chaisson et al (1988) reported that 59 percent of AIDS patients and 37 percent of ARC patients participating in the original phase II trial of ZDV had detectable serum p24 levels at entry. Median serum p24 antigen levels decreased from 111 pg/ml at entry to 40 pg/ml at 8 weeks in those ZDV treated patients, whereas no change (median 85 pg/ml at entry and 86 pg/ml at 8 weeks) was observed in placebo-treated subjects. The significant decrease of p24 antigen in the treated group was paralleled by a doubling of peripheral CD4+ T-lymphocytes, while the lack of change in p24 in the placebo group was accompanied by a 20 percent decrease in CD4+ cells. However, the correlation of change in p24 antigen over time with clinical outcome was not examined.

Jackson et al (1988) studied a subset of 16 AIDS or ARC patients entered into the American phase II study. A direct relationship was observed between decrease in serum p24 antigen concentrations and a decrease in symptoms of fever, sweats, weakness and fatigue post ZDV treatment. The clinical benefits of ZDV therapy have since been correlated with decreasing serum p24 antigen concentrations in several open-label studies of patients with ARC or AIDS (Pinching et al., 1989; Stambuk et al., 1989a,b). For example Stambuk et al (1989a,b), in a study of 87 patients with symptomatic HIV-1 disease, found that of 61 percent of patients who were p24 antigenaemic prior to treatment one half had a 50 percent or greater decrease in serum p24 antigen concentration during ZDV therapy. However, this subgroup had a substantially lower

mortality (4 percent) than the subgroup whose p24 level did not decrease during the treatment period (27 percent). Surprisingly, those patients who were antigen negative at the beginning of the study and remained so during the treatment also had a higher mortality (28 percent). Thus, the relationship between low or decreased p24 antigenaemia and positive clinical outcome was not consistent. In support of this observation, Jacobson et al (1991) found that neither absolute nor relative measures of p24 antigen concentration at 8 to 12 weeks ZDV treatment correlated with survival, when controlled for baseline predictors, in a retrospective study of 90 patients with ARC or AIDS.

Although a majority of individuals with early HIV-1 disease do not have detectable p24 antigen, levels do decrease during ZDV therapy in those that do (Valentine and Jacobson, 1990). De Wolf et al (1988) first reported that 13 (72 percent) of 18 asymptomatic, p24 antigenaemic individuals treated with 1 to 2 gm ZDV daily had substantial decreases in serum antigen levels during treatment. Subsequent large, multicentre placebo-controlled trials of ZDV therapy in populations with asymptomatic HIV-1 infection or early HIV-1 disease symptoms have confirmed a significant decrease in serum p24 antigen among ZDV treated subjects compared with untreated controls (Fischl et al., 1990b; Volberding et al., 1990). However, patients have not been followed for long enough in any of these studies to correlate this data with HIV-1 disease progression.

Serum p24 antigen concentration has been used widely to evaluate the in vivo antiretroviral efficacy of drugs, other than ZDV, for HIV-1 infection. Recent trials of nucleoside analogues 2'3'-dideoxycytidine (Merigan et al., 1989; Yarchcoan et al., 1988; see section 1.4.1.d) and 2'3'-dideoxyinosine (Cooley et al., 1990; Lambert et al., 1990; Yarchoan et al., 1989; see section 1.4.1.d) showed that suppression of circulating p24 antigen correlated with clinical benefit. Moreover, lack of clinical response to, for example, Active Lipids 721 (Peters et al., 1990; see section

1.4.2.a) and recombinant soluble CD4 (Schooley et al., 1990; see section 1.4.2.), which inhibit HIV-1 binding to its target cell, was reflected by unchanged levels of serum p24.

However as a surrogate end-point of antiretroviral efficacy, serum p24 antigen has the disadvantage of being detectable in only two-thirds of patients with advanced HIV-1 disease (Chaisson et al., 1988; Jacobson et al., 1989). It may be that other direct viral markers will be of use in this respect.

Recently, quantifiable measures of viraemia in plasma have been reported to be sensitive markers of clinical disease, and plasma virus titres have been reported to decrease during ZDV treatment (Coombs et al., 1989; Ho et al., 1989a). However the true sensitivity and reproducibility of these techniques remains to be established, and their cost is likely to exceed that of other serum markers. This last disadvantage may be particularly important in future definitive trials of efficacy for new antiretroviral drugs; since control patients will necessarily receive some active standard antiretroviral treatment, such as ZDV, hundreds to thousands of patients will be required to demonstrate an effect.

1.5.7. Summary of the utility of laboratory markers in the evaluation of anti-HIV-1 therapy

The need to evaluate new anti-HIV-1 agents as quickly as possible, together with limitations in the enormous resources required to perform large clinical trials, has compelled investigators to rely increasingly on immunological and virological markers of therapeutic efficacy. From the data published to date we can conclude that several of these laboratory parameters, which were predominantly identified in natural history studies as predictors of HIV-1 disease progression to AIDS, tend to revert towards more normal values in response to ZDV and other nucleoside analogues, such as 2',3'-dideoxycytidine and 2',3'-dideoxyinosine. Although two studies have recently been

published correlating changes in immunological and virological markers with eventual clinical outcome following ZDV administration (Cheingsong-Popov et al., 1991; Jacobson et al., 1991), larger and longer follow-up studies are needed to define and validate the change in a marker, or combination of markers, that best predict subsequent clinical benefit following administration in a range of experimental therapeutic regimens.

At the present time, laboratory markers are being used to evaluate the potential efficacy of new drugs during phase I studies, where the primary goals are to determine the safety and pharmacological properties of different doses of the agent (Kahn et al., 1990; Krown et al., 1990; Lambert et al., 1990; Merigan et al., 1990). Even though these markers may not have yet been shown to predict clinical benefit in individual recipients, a new compound that at low doses causes a rise in CD4+ T-lymphocyte count and a fall in p24 and plasma viraemia should be evaluated in larger trials to prove clinical efficacy. Yet, should new agents that showed promise in vitro but that failed to induce these changes in laboratory markers during phase I trial in paients be abandoned without a larger trial of clinical efficacy ? (Valentine and Jacobson, 1990).

Finally, if changes in laboratory markers are to be used to assess the efficacy of anti-HIV-1 agents, great attention must be paid to controlling the quality of measurements; small differences in laboratory values may be associated with substantial differences in clinical progression. Intralaboratory and interlaboratory variations can be enormous, and participation in a quality assurance programmme is essential.

1.6. Aims of this study

As our understanding of the aetiology, pathogenesis, natural history and clinical profile of AIDS, its associated disorders and HIV-1 infection have increased, the investigation of therapeutic strategies has accordingly

progressed. In recent years antiretroviral agents have been the main focus of attention, as illustrated by the rapid licensure of ZDV in 1987 for routine use in the UK. However ZDV therapy is not associated with improved long term survival in all treated subjects, yet it remains the only widely available agent shown to be clinically effective against progressive HIV-1 disease. Clearly there is a great need to evaluate new anti-HIV-1 agents alone, or in combination with ZDV. It is now evident that present and future trials of anti-HIV-1 agents must be evaluated by laboratory markers of efficacy rather than by clinically determined end-points in order to expedite their outcome.

The immunodeficiency observed in patients with HIV-1 infection is due to the selective depletion of CD4+ T-lymphocytes and the intrinsic dysfunction of those CD4+ cells remaining, together with the dysfunction of other cells of the immune system. Therefore, it is likely that prevention of progressive clinical disease correlates with an improvement of immunological status following treatment. Therefore, the present study was designed to assess laboratory markers of immunological change as correlates of clinical efficacy following ZDV treatment of symptomatic HIV-1 disease.

Previous workers have mainly utilised absolute peripheral CD4+ T-lymphocyte counts as a guide to immunological status; however change in CD4+ T-cell level does not correlate well with the prolonged temporal benefits of ZDV therapy. Moreover the small transient increase in circulating CD4+ T-lymphocyte number observed with ZDV therapy, fails to provide satisfactory explanation of the profound clinical effects of this drug. In contrast, it could be argued that lymphocyte functional capacity is less a marker than a direct measure of the relevant variables underlying changes in immunological status. Therefore, this study aimed to evaluate immunological functional markers, in parallel with phenotypic studies, following ZDV treatment. It was thought this would also better clarify the relationship

between quantitative and qualitative immunological defects shown in HIV-1 infected subjects.

The effect of ZDV on the following laboratory parameters was assessed by cross-sectional and longitudinal study of HIV-1 infected subjects:-

1) lymphocyte proliferative response to mitogen and antigen
2) lymphocyte gIFN synthesis in response to mitogen and antigen
3) lymphocyte inositol polyphosphate metabolism
4) spontaneous and mitogen induced B-cell production of IgM, IgG and IgD
5) absolute CD4+, CD8+ and CD3+ T-lymphocyte counts.

Immunological markers of proved utility in this system may be of use in trials of other antiretrovirals, particularly nuclesoide analogues. These results may also be useful to clinicians in counselling ZDV treated patients as to the potential efficacy of their treatment.

CHAPTER 2

MATERIALS AND METHODS

2.1. Patient subject cohort

All HIV-1 seropositive subject samples were obtained from homosexual men attending specialist HIV/AIDS clinics at St. Mary's Hospital. While some of these individuals were participants in formal trials, others were studied during their clinical treatment. Classification was according to Centers for Disease Control (CDC) grouping: asymptomatic or persistent generalised lymphadenopathy (CDC II and III), ARC (CDC IVC2) and AIDS (CDC IVC1 and IVD) (CDC, 1986). Functional and phenotypic studies were performed on the day of venesection, unless specified otherwise.

2.1.1. Longitudinal study: zidovudine

Eight ARC (CDC IVC2) and 6 AIDS (CDC IVC1/D) subjects regularly attending clinics were studied serially. Testing was pre or day zero zidovudine (ZDV) administration and then throughout 1 to 16 months (median: 3 months) for ARC and 1 to 12 months (median: 3 months) for AIDS subjects. The standard treatment during this period was 250 mg ZDV 4 times daily.

2.1.2. Longitudinal study: AL 721

Subjects participating in an open pilot study of AL 721 (Peters *et al.*, 1990) were included. AL 721 is a lipid compound with apparent anti-HIV-1 activity *in vitro* (Sarin *et al.*, 1985; see section 1.4.2.a). Subjects received 15 mg AL 721 (Ethigen) twice daily for at least 3 months. The serial results of 1 ARC (CDC IVC2) and 2 AIDS (CDC IVC1) p24 antigenaemic subjects are reported here. One subject was followed through ZDV treatment given after he discontinued AL 721.

2.1.3.a Cross-sectional study: AIDS patients

The majority of AIDS subject samples obtained for this study, were taken from HIV-1 infected individuals primarily involved in an investigation of peripheral neuropathy.

The neuropathy study sought to determine whether this disorder had an immunological basis. It became evident that prior ZDV treatment of the symptomatic cohort influenced the incidence of neuropathy and was the main variable of note in both studies (Table 02; Knox et al., 1991). Limited numbers prevented interpretation of the asymptomatic group data (Table 03).

The cross-sectional cohort of untreated AIDS subjects consisted of 14 neuropathy study participants and an additional 10 subjects recruited pre AL 721 or ZDV treatment.

Eleven peripheral neuropathy study AIDS patients had been treated with the standard regimen of ZDV for a median of 1 to 30 months (median: 4 months). These individuals, together with 2 patients attending regular clinics, in receipt of ZDV for 1 and 14 months, formed the cross-sectional ZDV treated AIDS group.

2.1.3.b Cross-sectional study: ARC patients

The cross-sectional cohort of non-ZDV treated ARC subjects consisted of eight subjects who were pre AL 721 or ZDV treatment.

Nine subjects made up the ZDV treated cross-sectional group. One of these was from the peripheral neuropathy study and had received therapy for 18 months. The other 8 were part of the longitudinal studies. Where these subjects have been tested more than once post ZDV therapy, the median result is given. Therefore, the length of ZDV treatment is quoted with the appropriate assay.

2.1.3.c Cross-sectional study: asymptomatic patients

The non-ZDV treated asymptomatic cohort consisted of 7 patients. Five subjects classified as CDC II or III were from the peripheral neuropathy study and an additional 2 (CDC II) were subjects admitted to hospital.

Four ZDV treated patients (CDC III) were participants in the neuropathy study and had received ZDV for a range of 1 to 23 months (median: 3.5 months).

Table 02: Symptomatic subjects
Zidovudine and peripheral neuropathy

	Number of subjects Receiving zidovudine	No zidovudine treatment
Peripheral neuropathy diagnosed	1	9
Peripheral neuropathy excluded	10 (1)	4

Twenty-four subjects all classified as CDC IVC1 were categorised with respect to peripheral neuropathy diagnosis and zidovudine treatment.
(1): this subject was CDC IVC2

Table 03: Asymptomatic subjects
Zidovudine and peripheral neuropathy

	Number of subjects Receiving zidovudine	No zidovudine treatment
Peripheral neuropathy diagnosed	2	3
Peripheral neuropathy excluded	2	2

Nine subjects classified CDC II/III were categorised with respect to peripheral neuropathy diagnosis and zidovudine treatment.
Three Concorde subjects (CDC III) had no peripheral neuropathy.

In addition, 3 asymptomatics were part of the Medical Research Council (MRC) Concorde trial and therefore their ZDV status was unknown.

2.2. Laboratory control subjects

The control subjects used were heterosexual laboratory or hospital workers at low risk of contracting HIV-1.

Longitudinal study: A study of serial determinations was performed for several controls. Unfortunately it proved impossible to match one control for each HIV-1 infected subject.

Cross sectional study: The median result is reported where more than one assay was performed for a particular individual.

2.3. Isolation of peripheral blood mononuclear cells (PBMC)

Peripheral blood was collected into 10 ml sterile preservative free Lithium heparin coated evacuated tubes using the Labco system (Labco Ltd, UK).

The following cell culturing techniques were carried out within the class 3 containment laboratory (see Safety, Appendix V.2.).

The plasma was separated by centrifugation at 2000 rpm (580g) for 10 min, aliquoted and stored at -80°C. The buffy coat was removed into 110 x 16 mm conical tubes (Sterilin Ltd, Hounslow, UK) and diluted to 10 ml with RPMI 1640 (Flow Laboratories, Irvine, Scotland / Gibco Ltd, Paisley, Scotland) containing 2 g/l Sodium bicarbonate, 2 mM L-Glutamine and supplemented with Benzylpenicillin (Glaxo Laboratories Ltd, Greenford, England) at 100 units/ml and Streptomycin sulphate (Evans Medical Ltd, Hoesham, England) at 100 ug/ml (RPMI pen/strep).

Peripheral blood mononuclear cells (PBMC) were

then prepared by density gradient sedimentation (Boyum, 1968). 6 ml of the diluted blood was carefully layered onto 4 ml of Lymphoprep™ (Density 1.077 g/ml; Nycomed AS, Oslo, Norway) and the gradient centrifuged at 2000 rpm (580g) for 30 min at room temperature. The resulting interface of PBMC was harvested and washed three times by centrifugation at 1800 rpm (470g) for 10 min in 10 ml RPMI pen/strep.

The PBMC were adjusted to the appropriate concentration with RPMI pen/strep supplemented with 5 % heat inactivated Fetal Calf Serum (hiFCS) (GIBCO Ltd, UK), (RPMI/C).

Human PBMC are known to be activated in a non-specific manner by Fetal Calf Serum (FCS). The level of proliferation observed varies between batches of FCS and according to the concentration of FCS used in the culture medium. Preliminary experiments ascertained the optimum concentration and identified a batch of FCS which caused low levels of background proliferation whilst providing enough supplements to support optimal proliferative levels in response to polyclonal activators. Aliquots of the same batch of FCS stored at -20°C and thawed prior to use were subsequently employed. Autologous plasma has been used by some workers as an alternative to FCS, and consistently lower levels of background proliferation have been observed (Rogers, 1987). Autologous plasma/serum could not be used in these functional assays however, since a variety of serum abnormalities have been reported in HIV-1 infected individuals. These abnormalities include the presence of circulating antibodies which bind to CD4+ and CD8+ lymphocytes (Tomar et al., 1985) as well as other unidentified factors which inhibit lymphocyte responses to mitogens and alloantigens in normal subjects (Cunningham-Rundles et al., 1983; Laurence et al., 1983; Siegal et al., 1985).

2.4. Cell counts and viability

All cell counts were performed using a 0.1 mm

Improved Neaubauer Haemocytometer and white cell counting fluid (Appendix VI.1.). Cell viability was assessed by the exclusion of 0.1 % trypan blue dye (BDH Chemicals Ltd, Poole, England) in Phosphate buffered saline (PBS) (OXOID, England). Cell populations with viabilities greater than 95 % were regularly obtained and all figures quoted in the text refer to viable cells unless specified otherwise. The yield of PBMC from control donors was consistently around 1.0×10^6 PBMC per ml of blood, whereas for HIV-1 seropositive subjects this figure was invariably lower.

LYMPHOCYTE FUNCTION STUDIES

2.5. PBMC Proliferative response

The T-lymphocyte proliferative response following culture with mitogen or antigen was determined by tritiated thymidine ([^{3}H]TdR) incorporation into cellular dideoxynucleic acid (DNA).

2.5.1. Determination of PBMC proliferative response to mitogen

190 ul aliquots of the PBMC suspension at 1.0×10^6 cells/ml were plated out in 96 flat bottom well microtitre tissue culture plates (F96LC, Sterilin Ltd, UK), and cultured with one of the following:- 10 ul RPMI/C; 10 ul Phytohaemagglutinin (PHA) (HA16, Wellcome, Dartford, England); or 10 ul Concanavalin A (Con A) (C5275, Sigma, Poole, England). The cells were then incubated at 37^{o}C in a humidified atmosphere with 5 % CO_2 for 72 h.

2.5.2. Determination of PBMC proliferative response to antigen

PBMC were isolated as previously described (see sections 2.3. and 2.4.) and adjusted to 2.0×10^6 cells/ml. 190 ul aliquots were then plated in 96 flat bottom well tissue culture plates and cultured with either 10 ul RPMI/C

or 10 ul Streptokinase/Streptodornase (SK/SD) 400/100 (originally supplied by D. Grennan, Royal Postgraduate Medical School, London). The cultures were incubated at 37 °C in a humidified atmosphere with 5 % CO_2 for 144 h.

The SK/SD soluble antigen preparation was kindly supplied by L.A. Rogers, and is identical to the preparation used *in vivo* (Pinching *et al.*, 1983).

2.5.3. Mitogen and antigen storage

The mitogen preparations were reconstituted as recommended by the manufacturers. Both mitogen and antigen aliquots were stored at -80 °C and discarded after a single use.

2.5.4. Measurement of tritiated thymidine [^{3}H]TdR incorporation

PHA, Con A and SK/SD induced PBMC proliferation was determined by tritiated thymidine, [^{3}H]Tdr (Amersham International, Amersham, England), incorporation into the cellular DNA of triplicate wells 4 h prior to culture termination.

100 ul of supernatant was carefully removed from triplicate wells with a Finn pipette. The cultures were then pulsed with 1.0 uCi [^{3}H]Tdr in 100 ul RPMI/C, [^{3}H]TdR,RPMI/C. The separated supernatant was used to measure interferon-gamma (see section 2.6.). After incubation for 4 h at 37 °C in 5 % CO_2 the culture plates were frozen at -20 °C.

Frozen culture plates were thawed and harvested by batch weekly. The cultures were harvested with a Skatron cell harvester, where the cells were lysed with distilled water and the intracellular DNA deposited on glassfibre filter paper (Skatron AS, Norway). The filter papers were allowed to dry overnight, the discs transferred to plastic insert vials (Sterilin Ltd, Hounslow, UK) and saturated with 70 % ethanol (BDH Chemicals Ltd, Poole, England) which was left to evaporate. This entire procedure was carried out within a

class I microbiological laminar flow cabinet in the class 3 laboratory (see Safety, Appendix V.3.).

2 ml of Ecoscint (National Diagnostics, New Jersey, USA) was added to each vial and the number of β-emissions, counts per minute (cpm), determined per vial in a liquid scintillation spectrometer (B2450, Canberra Packard Ltd, Berks, England).

Inter-batch variation of [^{3}H]Tdr in RPMI/C was checked weekly. The cpm per 10 ul of [^{3}H]TdR,RPMI/C in 2 ml Ecoscint was ascertained in triplicate and also provided an indication of B-counter precision.

2.6. Quantitative assay of human gamma-interferon (hu gIFN)

Human gamma-interferon (hu gIFN) was determined quantitatively in cell culture supernatants by immunoradiometric assay (IRMA), bought as the SUCROSEP™ kit from Novo Bio Ltd, (Cambridge, UK) formerly Celltech Diagnostics Ltd, (Berkshire, UK). However, when production of this kit ceased on the 30th March 1990, supernatants collected subsequently were tested using the immunoradiometric kit produced by Medgenix Diagnostics (Brussels, Belgium). Comparison of these systems is discussed fully (see section 4.1.6.).

2.6.1.a Sample collection and preparation: plasma

A limited number of plasma samples were assayed for gIFN using the Celltech Diagnostics IRMA. These were separated from peripheral blood collected in Lithium heparin coated tubes (Labco Ltd, UK) and subsequently stored at -80^{o}C. Plasma samples were centrifuged at 2000 rpm (580g) for 5 min before assay. No dilution was required.

Preliminary investigations failed to detect hu gIFN (data not shown) and this line of enquiry was abandoned.

2.6.1.b Sample collection and preparation: cell supernatants

PHA, Con A and SK/SD PBMC cultures were established as described in sections 2.5.1. and 2.5.2. 100 ul of supernatant was collected with a Finn pipette and pooled minimally from five identical wells, centrifuged at 2000 rpm (580g) for 10 min, aliquoted into labelled Sarstedt vials (Sarstedt, W. Germany) and stored at -80°C. Harvesting was at 72 h for mitogen and 168 h for antigen cultures. This coincided with optimal proliferation and as such the proliferative and gIFN assays ran simultaneously (see sections 3.1.1. and 4.3.1.).

Supernatant samples were thawed, allowed to equilibrate to room temperature and vortexed gently before assay.

Where dilution of culture supernatant was appropriate, the dilutent (RPMI/C) was included in the assay as a control sample. The gIFN concentration value obtained for the dilutent was subtracted from diluted sample values.

2.6.2.a Determination of hu gIFN using the Celltech SUCROSEP™ kit: principle of the assay procedure

The Celltech gIFN kit was a two-site IRMA, where the concentration of analyte (gIFN) was quantified by direct incubation with ^{125}Iodine (^{125}I)-labelled monoclonal antibody (Mab) to gIFN. The analyte-^{125}I-labelled antibody complex was then immobilised by incubation with a sheep anti-gIFN Mab coupled to a solid phase and directed to an epitope distinct from the tracer antibody. Separation of bound from unbound Mab was effected by a semi-automated method which relied on gravity rather than centrifugation. After separation the radioactivity in the assay tubes reflected the analyte concentration.

2.6.2.b Specificity of the SUCROSEP™ gIFN IRMA

Celltech Diagnostics reported the SUCROSEP™ gIFN kit to have no detectable cross-reactivity with alpha-

or beta-IFN.

2.6.2.c Materials provided and preparation of standards

The assay standard was prepared from purified natural hu gIFN (Finnish Red Cross Blood Transfusion Service, Helsinki, Finland). This was calibrated against the National Institute of Health (NIH, Bethesda, USA) hu gIFN reference preparation Gg 23-901-530.

The gIFN standard was reconstituted as directed with 3.0 ml of the serum dilutent provided. This stock solution had a nominal value of 1024 U/ml. It was allowed to stand at room temperature for 15 min and then used with the serum dilutent to prepare a series of gIFN standards covering the concentration range 0-1024 U/ml (Table 04). These standards were prepared on the day of assay, and a standard curve was included with each assay.

Each assay included RPMI/C as a negative control and several positive controls. Initially, the positive controls were provided by Celltech Diagnostics Ltd, and latterly prepared from excess kit reagents (gIFN stock standard and serum dilutent) and batch stored at -80°C (Table 05). These were additionally used to check the precision (inter-assay variation; see section 4.1.1.) and accuracy (determined by dilution-test; see section 4.1.2.) of the kits, as well as their reliability of detection across the range of the standard curve.

2.6.2.d Materials provided and preparation of reagents: antibodies and buffers

The tracer (^{125}I-monoclonal) and solid phase antibody preparations were supplied ready for use and were stored at 2-4°C.

The concentrated pre-wash buffer and SUCROSEP™ separation reagents were diluted to 300 ml and 600 ml with distilled water (dH_2O) respectively and stored at 2-4°C.

Table 04: Preparation of SUCROSEP-TM human gIFN standards

gIFN standard U/ml	volume of standard ul	volume of dilutent ul	nominal value of gIFN standard U/ml
1024	stock	0	1024
1024	200	600	256
1024	100	1500	64
64	200	600	16
64	100	1500	4
4	200	600	1
-	0	1000	0

Table 05: Preparation of human gIFN positive controls

control	volume of stock standard ul	volume of dilutent ul	nominal value of gIFN control U/ml
A	25	2475	10
B	100	1900	51
C	500	2500	171
D	500	500	512
E	stock	0	1024

2.6.2.e Assay protocol: standard, sample and Mab application

200 ul of standard or unknown sample was pipetted in duplicate into previously labelled assay tubes (Celltech Diagnostics Ltd) arranged in racks of 20. Each kit allowed manipulation of 120 tubes.

50 ul of ^{125}I-anti-gIFN Mab was then added to all tubes. The racks were covered with plastic film, vortexed and incubated for 2 hours at room temperature. Two tubes containing 50 ul of radiolabelled antibody only served as a control for total counts, and were kept separate throughout the assay.

Ensuring that the solid phase monoclonal antibody was well mixed by repeated inversion during the dispensing process, 50 ul was dispensed into each tube. The racks were covered with plastic film and agitated on an orbital agitator (BC127, Celltech Diagnostics Ltd) at mark 9 (300-350 rpm) for 2 hours at room temperature.

2.6.2.f Assay protocol: separation of bound from unbound Mab

1 ml of the provided Pre-wash Buffer, previously diluted to 300 ml with dH_2O, was dispensed into all assay tubes.

2 ml of SUCROSEP™ Separation Reagent, previously diluted to 600 ml with dH_2O, was then layered below the incubate in each tube using a dispenser (SUCRODISPENSER™, BC 123, Celltech Diagnostics Ltd).

Following a 15 min incubation at room temperature, during which the solid phase settled, the tubes were carefully aspirated leaving approximately 0.3 ml. The aspiration step was carried out with a multi-aspirator (SUCROASPIRATOR™, BC 129, Celltech Diagnostics Ltd) attached to the vacuum pump and reservoir used in cell harvesting. This procedure was repeated once more.

200 ul 10 % Formol saline was added to each tube (see Safety, Appendix V.4.).

The radioactivity was determined per tube in a gamma scintillation spectrometer (5266, Caberra Packard Ltd) for a time sufficient to accumulate 50 000 counts in the total counts tubes.

2.6.2.g Calculation of gIFN concentration, U/ml

Intially, gIFN concentration was calculated from the standard curve prepared for each assay of ^{125}I counts versus standard concentration on a log/log plot. This time-consuming method was superseded on the introduction of the Sucrosep IRMA Data Reduction Package (Celltech Diagnostics Ltd). All gIFN concentrations obtained by this assay and quoted in the text were calculated using the computer programme.

2.6.3.a Detection of hu gIFN using the Medgenix kit: principle of the assay procedure

The Medgenix IRMA was based on coated-tube separation and on the oligoclonal system R, which utilised several Mabs directed against a distinct epitope of gIFN. Capture antibodies were attached to the lower and inner surface of plastic tubes. On incubation gIFN bound from the standards or samples were detected with ^{125}I-labelled signal antibodies. The radioactivity bound to the tubes after washing reflected gIFN concentration.

2.6.3.b Specificity of the Medgenix gIFN kit

No significant interference with the apparent gIFN response was reported by Medgenix up to: IL-1, 1000 ng/ml; IL-2, 5000 U/ml; IFNα, 1000 ng/ml; IFNβ, 1000 U/ml; TNFα, 100 ng/ml; TNFβ, 100 ng/ml.

2.6.3.c Materials provided and preparation of standards

The assay standard was calibrated against the NIH (Bethesda, USA) hu gIFN reference preparation Gg 23-901-530 such that 1 IU/ml of Medgenix standard was equivalent to 1 IU/ml of NIH Gg 23-901-530.

The gIFN standards provided represented 0, 1, 2, 5, 10, 30, and 90 IU/ml. These were reconstituted as directed with dH_2O. Standards were prepared on the day of assay and a standard curve was included in each assay.

Three additional positive controls were provided, 2 in human serum and 1 in cell culture medium. These provided the means to check both intra and inter-assay precision.

2.6.3.d Materials provided

96 tubes coated with Mabs to gIFN in 2 racks of 48 and 1 vial of ^{125}I labelled Mabs to gIFN in 5.5 ml of phosphate buffer with bovine serum albumin and azide were supplied ready to use.

One vial was provided containing 10 ml of washing solution Tween 20, 20 %. This was diluted to 400 ml with dH_2O.

All kit reagents were stored at 2-8 ^{o}C until the expiry date and any residual solution up to 5 days after reconstitution.

2.6.3.e Assay protocol: standard, sample and antibody application

The coated tubes were labelled in duplicate. 100 ul of each standard, control and sample were dispensed appropriately and 50 ul of signal antibody added. The racks of tubes were shaken gently by hand and incubated for 16 $\pm$ 1 h at room temperature.

Two uncoated tubes were utilised for total counts and kept separately.

2.6.3.f Assay protocol: separation of bound from unbound antibody

The tubes were carefully aspirated with a multi-aspirator (SUCROASPIRATOR^{TM}, BC 129, Celltech Diagnostics Ltd) as described previously (see section 2.6.2.f).

2 ml of the washing solution provided, previously

diluted to 400 ml with dH_2O, was added to each tube and aspirated as before. 200 ul of 10 % Formol saline was dispensed per tube (see Safety, Appendix V.4.).

The tubes were counted in a gamma counter (5266, Canberra Packard Ltd) for 1 min.

2.6.3.g Calculation of gIFN concentration, IU/ml

The standard curve for each assay was prepared by plotting cpm or bound radioactivity/total radioactivity, expressed as a percentage (B/T %), against the standard concentration using semi-log graph paper and a best-fit curve drawn. gIFN concentration was determined by interpolation of cpm or B/T (%) values.

2.7. Changes in the phosphatidyl inositol hydrolysis pathway of HIV-1 infected subjects receiving zidovudine therapy

Several studies have demonstrated HIV-1 associated defects of T-lymphoblastoid cellular signal transduction (Gupta and Vayavegula, 1987; Nye and Pinching, 1990) and in lymphocytes obtained from HIV-1 infected individuals (Nye et al., 1991). This study examined inositol polyphosphate metabolism in lymphocytes obtained from HIV-1 infected patients pre and post ZDV administration.

2.7.1. Sample preparation

10 ml of peripheral blood was taken into a universal (Sterilin Ltd, UK) containing 75 ul preservative free heparin (5 units/ml of blood) (Paines & Byrne Ltd, Greenford, England). PBMC were then isolated over Lymphoprep (Nycomed AS, Oslo, Norway) as described previously (see section 2.3.).

Isolated PBMC were incubated at 2.0×10^6/ml with 4.0 uCi/ml of myo-[^{3}H] inositol with PTS-271 (Amersham International, Amersham, England) in RPMI (Gibco Ltd, UK) supplemented with 5 % dialysed hiFCS (Gibco Ltd, UK). Dialysis prevented contamination by constitutive

inositol. This procedure was necessary to prevent unlabelled and radiolabelled inositol competition (K. Nye, personal communication).

Incubation was at 37^{o}C in a humidified atmosphere with 5 % CO_2 for at least 16 h to achieve isotopic equilibrium. This occurs when the specific activities of all inositol containing compartments are equal. It has been proposed that there are metabolically distinct pools of inositol lipid (K. Nye, personal communication). It is therefore impossible to know how the label is distributed between agonist-sensitive and agonist-insensitive pools.

Following incubation, Lithium chloride (L-0505, Sigma) was added at a final concentration of 10 mM and incubation continued for 10 min. By inhibiting conversion of InsP to inositol and Ins(1,4)P_2 to InsP, lithium greatly amplifies the accumulation of inositol phosphates during agonist stimulation both *in vitro* and *in vivo* (Berridge *et al.*, 1982). This decreases metabolism of Ins(1,4,5)P_3 to inositol and causes a build up of InsP and $InsP_2$, which is also a good marker of PtdIns(1,4)P_2 hydrolysis.

The cells were then stimulated with PHA (HA16, Wellcome) at 3.0 ug/ml for two min and the reaction terminated with 10 % iced Trichloroacetic acid, TCA (BDH Chemicals Ltd). The cell debris was removed by centrifugation and the supernatants washed with Diethylether (BDH Chemicals Ltd) and neutralised with NH_4OH.

2.7.2. Sample analysis by high performance liquid chromatography (HPLC)

The aqueous phase was then run on High Performance Liquid Chromatography (HPLC; Gilson, Villiers le Bel, France) using a 200 ul sample loop, a 5 cm x 4.6 mm ID guard column and a 25 cm x 4.6 mm analytical column both packed with Partisil SAX (N-propyl-N-N-trimethyl-ammonium resin). The water soluble inositol phosphates were then eluted using a linear gradient from deionised distilled water to 2.0 M Ammonium formate/0.5 M Orthophosphoric acid (Sigma) at

pH 3.7. InsP was therefore displaced according to the degree of phosphorylation: IP, IP_2, IP_3 and finally IP_4. The flow rate was 1.25 ml/min. Free inositol was eluted during the water elution and the column was washed with water for 12 min following each run.

The column elutate was mixed automatically with scintillant (Flo-SCINT IV, Packard Radiometric, UK) in a ratio of 1:3 and β-emissions counted by an in-line beta counter (Radiomatic flow detector A250, Radiometric Chemical Co, Florida). The results were analysed on the Flo-One Data programme.

2.7.3. Inositol (1,3,4,5)P_4 metabolism

Lymphocytes were washed and resuspended in buffer containing 70 mM potassium glutamate, 30 mM potassium chloride, 10 mM sodium chloride, 24 mM magnesium sulphate (4 mM free Mg^{2+}), 0.33 mM calcium chloride (0.1 uM free Ca^{2+}), 1 mM EGTA, 10 mM HEPES and 2 mM ATP. It has been reported that ATP inhibits Ins(1,4,5)P_3/Ins(1,3,4,5)P_4-5'-phosphatase activity; however, 2 mM ATP gives optimum metabolism of Ins(1,3,4,5)P_4, although ATP at 5 mM or greater does inhibit 5'-phosphatase activity (K. Nye, personal communication). Cells were then freeze-thawed 3 times in this buffer and the lysate incubated at 37°C for 10 min with [^{3}H]-inositol (1,3,4,5) tetrakisphosphate. The rection was then terminated with iced TCA, washed and neutralised and metabolites analysed by HPLC.

2.8. Enzyme linked immunosorbent assay (ELISA) for human immunoglobulin classes IgM, IgG, and IgD.

The concentration of IgM, IgG, and IgD present in culture supernatants of PBMC cultured with or without PWM was ascertained by an enzyme linked immunosorbent assay (ELISA). This assay was previously detailed by Rogers (1987) and Rogers et al (1989), and is a modification of the assay described by Farrant et al (1985).

2.8.1. Culture supernatant harvest

Triplicate PWM culture supernatants, harvested with a Finn pipette, were pooled and centrifuged at 2600 rpm (800g) for 10 min. The pooled supernatants were transferred into coded Sarstedt vials (Sarstedt, W. Germany) and stored at -80°C.

2.8.2. General procedures

All incubations were carried out in a humidified sandwich box, and all plate washings were by hand using a wash bottle into Asep (Galen, Craigavon). This procedure was carried out in a Class I microbiologcal cabinet (see Safety, Appendix V.4.).

2.8.3. Coating of microtitre plates with anti-hu Ig subclass antibody

A goat anti-human IgG (1-9881 Sigma, Poole, England) was used when measuring IgG. Rabbit anti-human IgM (A091, Dakopatts Ltd, UK) was used when measuring IgM and rabbit anti-human IgD (A093, Dakopatts Ltd, UK) was used to measure IgD.

96 flat bottomed well microtitre plates (M29AR, Sterilin, Feltham, England) were coated with 50 ul per well anti-human Ig subclass antibody, dissolved in Coating Buffer E014 (Don Whitely Scientific Ltd, Yorkshire, England). The IgG, IgM, and IgD preparations were diluted 1/500, 1/800, and 1/1000 respectively.

Two columns per plate were coated with buffer alone, serving as a control for non-specific binding.

Following overnight incubation at room temperature, the plates were washed 3 times with Phosphate Buffered Saline (PBS) containing 0.05% polyoxyetheylene (20)-sorbitan monolaurate (Tween 20) (BDH Chemicals Ltd, Poole, England) (PBS/T).

2.8.4. Addition of blocking solution (BS)

200 ul of blocking solution (BS) (Appendix VI.3.)

was added to each well and the microtitre plates incubated for 1 hour at 37°C. The Gelatin (BDH Chemicals Ltd, Poole, England) and Bovine serum albumin (A7030 Sigma, St. Louis, USA) in the BS prevented Ig from adhering non-specifically to the plastic wells of the microtitre plates.

The plates were washed once with PBS/T.

2.8.5. Addition of reference standard hu Ig and test samples

Sigma Calibrated Human Serum Normal Concentration (S7011, Sigma Diagnostics, St. Louis, USA) was used as the reference standard for both IgG and IgM. The undiluted standard contained 9.64 mg/ml IgG and 0.806 mg/ml IgM as determined against the College of American Pathologists Reference Preparation for Serum Proteins.

The IgD standard was supplied by Behring (OTRD 02/03, Behring, W. Germany) and was calibrated with reference to the British Research Standard No. 67/37. The concentration was batch-dependent and given in units of IgD per ml (Rowe et al., 1970).

The relevant standard was included in duplicate on each microtitre plate. 50 ul aliquots of reference standard human Ig diluted in BS (1/40 000 for IgG, 1/1000 for IgM, and 1/20 000 for IgD) were added per well and six doubling dilutions made using a multichannel pipette.

Culture supernatants were introduced by the same protocol as the standards, except initial dilutions in BS were 1/10 for IgG and IgM, and 1/2 for IgD PWM driven production. The supernatants collected from spontaneous cultures were diluted 1/2.

The microtitre plates were then incubated for 1 hour at 37°C and subsequently washed 3 times with PBS.

2.8.6. Addition of antibody-enzyme conjugate

IgM, IgG and IgD antibodies bound in the wells were determined using goat anti-human IgG (A3150 Sigma), goat anti-human IgM (A3275 Sigma), or goat anti-human IgD (A6406)

conjugated to alkaline phosphatase. These were diluted 1/1000 for both IgM and IgD and 1/800 for IgG in BS. 50 ul of the appropriate conjugate was added to each well.

Following incubation for 1 hour at 37°C, the microtitre plates were washed 3 times with PBS.

2.8.7. Addition of substrate

50 ul aliquots of alkaline phosphatase substrate solution, p-Nitrophenyl Phosphate, Disodium (104-105 Sigma) dissolved in Substrate Buffer E016 (Don Whiteley Scientific Ltd, Yorkshire, England) at 1 mg/ml were added to each well.

The microtitre plates were incubated at 37°C for 30 min when measuring IgG, 20 min at room temperature for IgM and overnight at room temperature for IgD. The substrate reaction was then stopped in the linear phase of the reaction curve by the addition of 50 ul 3 M NaOH (BDH Chemicals Ltd, Poole, England; see Safety, Appendix V.4) to each well.

The absorbance at 405 nm was measured on a Flow Titrek Multiscan MCC ELISA plate reader blanked against an empty column of wells on each ELISA plate.

2.8.8. Calculation of Ig concentration

Mean Optical Density (OD) values were corrected for background levels obtained in the absence of coating anti-Ig (OD < 0.15). The culture supernatant Ig concentration was calculated from the linear relationship of OD against log standard concentration.

PHENOTYPIC STUDIES

2.9. Lymphocyte phenotypic studies: enumeration of T-lymphocyte subsets

Both immunocytochemical and flow cytometry methodologies were employed to enumerate total T-lymphocyte, T-helper/inducer and T-cytotoxic/suppressor populations. These subsets are defined by surface expression of the

protein receptor molecules CD3, CD4, and CD8 respectively .

Immunocytochemical labelling proved to be time-consuming and temperamental and was superseded when a suitable flow cytometry system became available in the department. Comparison of these techniques is discussed fully (see section 7.3.).

2.9.1. T-lymphocyte subset enumeration by immunocytochemical labelling

Immunocytochemical enumeration of T-lymphocyte subsets was adapted from the method of Erber et al (1984). Briefly, Mab labelling of the CD3, 4 and 8 markers was visualised by a alkaline phosphatase anti-alkaline phosphatase/Napthol AS-MX phosphate/Fast red system. The protocol followed is described in detail by Hutchinson (1991).

2.9.1.a Slide preparation

Cytopreps were made by spinning 100 ul PBMCs, resuspended at 1.0×10^5 cells/ml in RPMI/C, for 2 min at 1500 rpm onto methanol (BDH Chemicals Ltd, Poole, England) cleaned slides (BS 3836, Arnold R. Horwell Ltd, London, UK) using a cytospin.

The cytopreps were then air-dried overnight at room temperature, wrapped in aluminium foil, and stored maximally for 6 months at -20^{o}C.

At least 8 slides were prepared for each individual.

2.9.1.b Fixation of slides

Frozen slides were allowed to equilibrate at room temperature for 60 mins before removing the aluminium foil. Subsequently, the slides were fixed in acetone (BDH Chemicals Ltd, Poole, England) at 4^{o}C for 10 min, rinsed three times in distilled water and washed in 0.5 M Tris-buffered saline (TBS) pH 7.6 (Appendix VI.4.) for 3-5 min.

2.9.1.c Staining protocol

Sections 2.9.1.d to 2.9.1.i describe the staining protocol. All incubations were at room temperature in a humidified sandwich box, and all washings between incubations in TBS pH 7.6 for 3-5 min.

2.9.1.d Application of Mab

Four cytopreps per subject were incubated with the appropriate Mab diluted in TBS pH 7.6, or with TBS pH 7.6 alone, as a negative control (Table 06) for 45-60 min. The preliminary experiments deciding Mab and optimum dilution were performed in this department and are precisely detailed by Hutchinson (1991).

2.9.1.e Application of rabbit anti-mouse immunoglobulins (RAMIg)

40 ul of rabbit anti-mouse immunoglobulins (RAMIg) (Dakopatts, Glostrup, Denmark) diluted 1:10 with TBS pH 7.6, supplemented with 5 % normal human serum (NHS) was applied to each slide and incubated for 30 min. The NHS reduces cross-reactivity of RAMIg with other cell surface proteins.

2.9.1.f Application of alkaline phosphatase anti-alkaline phosphatase (APAAP)

40 ul of APAAP (Dakopatts, Glostrup, Denmark) diluted 1:10 with TBS pH 7.6 (5 % NHS) was applied per slide and incubated for 30 min.

2.9.1.g Repetition of steps (e) and (f)

Steps (e) and (f) were repeated once for negative control and CD3 and twice more for CD4 and CD8 slides. The final incubation time was 15 min. The low density T-lymphocyte expression of CD4 in particular necessitates this amplification.

Table 06: T-lymphocyte subset enumeration by immunocytochemical labelling: Mab application

Mab to	Mab	dilution in TBS, pH 7.6	volume applied to cytoprep, ul
-	TBS, pH 7.6	neat	35
CD3	UCHT 1	1/20	35
CD4	Leu3a3b	neat	35
CD8	Dako T8	1/3	35

2.9.1.h Application of napthol AS-MX phosphate/fast red mixture

The Napthol AS-MX phosphate/fast red (Sigma, St. Louis, USA) mixture (Appendix VI.6.) was filtered onto the slides and incubated for 20 min.

2.9.1.i Haematoxylin counterstain

Following washing in tap water, the leucocyte nuclei were counterstained blue with haematoxylin (Appendix VI.7.) for 1 min.

2.9.1.j Enumeration of cells

By light microscopy positively stained cells exhibit a purple/blue cytoplasm and blue nucleus, whereas negative cells show a blue nucleus only.

Approximately 200 lymphocytes were counted per slide, the number of positives expressed as a percentage, and on receipt of the haematology differential count as an absolute figure.

2.9.2. T-lymphocyte subset enumeration by flow cytometry (FCM)

Flow cytometry (FCM) is a technique of automated cell analysis. The physical or chemical characteristics of individual cells, or other biological particles, are measured as they pass at high speed suspended in a liquid medium through a sensing area. As each cell passes through this area, electrical or optical signals are generated. These signals which may represent fluorescence, light scatter, light absorbance, or electrical resistance of cells can be accurately measured and the information stored (Braylan, 1983; Shapiro, 1985).

Salzman et al (1975), observed that human lymphocytes, monocytes and granulocytes have differing light scattering properties and suggested FCM to distinguish lymphocytes from other peripheral leucocyte types. Focusing on the lymphocyte population by this means Hoffman et al

(1980) demonstrated that lymphocytes labelled with fluorochrome conjugated Mabs could be assigned to subclass by cellular immunofluorescence.

2.9.2.a Principle of the technique

Whole peripheral blood samples were stained with the appropriate fluorochrome-conjugated Mabs and enriched for leucocytes by erythrocyte lysis (see section 2.9.2.b).

T-lymphocyte subset enumeration of the prepared samples by FCM employed Becton Dickinson's FACScan System coupled with their SimulSET software (Becton Dickinson Immuncytometry Systems, San Jose, USA). The basic mechanics of this system are outlined below (Carter, 1990; Landay et al., 1990).

The single cell suspension is taken up through a biohazard nozzle (see Safety, Appendix V.5.) into a flowing sheath fluid which hydrodynamically focused the cells to the centre of the stream. The cells are then passed individually through a focused light beam generated by a laser. Each cell traversing the beam scatters light and any associated fluorochrome becomes excited and emits a signal. These optical signals are then collected through appropriately arranged filters and photodetectors (photodiodes and photomultiplier tubes), digitised and analysed by the computer software as the sample is run.

These optical signals are two light-scatter parameters (forward or low angle scatter [FSC] and side or 90^{o} scatter [SSC]) together with two fluorescence signals (fluorescein isothiocyanate [FITC] and phycoerythrin [PE]). A fifth parameter, a duochrome or tandem parameter is also available. The light-scatter signals provide information about cell size (FSC) as well as cytoplasmic and nuclear characteristics (SSC). The fluorescent signals of fluorochrome-conjugated Mabs can be utilised to characterise a wide range of cellular parameters, such as the density and distribution of cell surface and cytoplasmic determinants and receptors.

2.9.2.b Sample preparation

Venous blood was collected into EDTA (ethylenediaminetetra-acetic acid) tubes (LIP Ltd).

100 ul of whole blood was aliquoted into 12 x 75 mm test tubes (2054 Falcon, Becton Dickinson Labware, New Jersey, USA), mixed gently, and fluorescently labelled with 20 ul of the appropriate Simulset reagent (Table 07). The samples were then incubated in the dark for 15 min at room temperature. The erythrocyte population was lysed on addition of 2.0 ml FACS lysing solution (Appendix VI.8.). Once gently vortexed the tubes were incubated for 10 min at room temperature in the dark, and then centrifuged at 1200 rpm (200g) for 5 min.

Following aspiration, 1.0 ml of PBS (Oxoid, England) containing 0.1 % Sodium azide (BDH Chemicals Ltd, Poole, England) was added and following gentle vortex mixing centrifugation was as before.

The cell pellets were gently resuspended in 1.0 ml 1.0 % Paraformaldehyde solution (Appendix VI.9.; see Safety, Appendix V.5.) and stored in the dark at 4 ^{o}C, maximally for 24 h, until analysis.

2.9.2.c. Sample analysis: **See FACScan Analysis Panel (Pocket)**

An example of a typical laboratory control print-out is delineated by Figures 106 and 107 (Pocket) and is used to describe sample analysis.

The leucogate stained sample (Table 07) was initially used to differentiate the lymphocyte (L) population from monocyte (M) and granulocyte (G) clusters and from debris (d) utilising FSC and SSC. A scatter cytogram of these populations is shown by panel (1) of Figure 106 (Pocket). The lymphocyte population was then selected by demarkating the lymphocyte cluster (panel (1); Figure 106, Pocket) and using this as a gate.

The fluorescence cytogram of the leucogate sample was subsequently analysed to quantify the accuracy of selective scatter gating; only the fluorescence intensity of

Table 07: T-lymphocyte subset enumeration by flow cytometry: simulset Mab reagents

Reagent	Mab	specificity	description
leucogate	anti-Hle-1-FITC	leucocytes	select lymphocyte population
	anti-Leu-M3-PE	moncytes & macrophages	
control	IgG1-FITC	keyhole limpet haemocyanin	quantify non-specific fluorescence
	IgG2a-PE		
T- & B-cell test	anti-Leu4 (CD3)-FITC	T-cells	simultaneous analysis of T-cells, B-cells & activated T-cells
	anti-HLA-DR-PE	B-cells, acvtivated T-cells & monocytes	
T-helper/ suppressor test	anti-Leu3a (CD4)-FITC	T-helper cells	analysis of T-helper & T-cytotoxic/ suppressor subsets
	anti-Leu2a (CD8)-PE	T-cytotoxic/ suppressor cells	

FITC: Fluorescein isothiocyanate PE: Phycoerythrin

cells falling within this gate are displayed by immunofluorescence profile (panel (2); Figure 106, Pocket). The FITC-conjugated Mab to the common leucocyte antigen, Hle (FL1), provided a positive control of lymphocyte population staining and by means of weaker staining a measure of granulocyte contamination. The PE-conjugated Mab to M3 (FL2) indicated contamination by monocytes or macrophages. In addition, any negative cells within this gate were, by definition, neither lymphocytes, monocyte/macrophages nor granulocytes and usually represented erythrocyte or nucleated red blood cell contamination (Carter, 1990).

The negative control was then utilised to set the threshold on the immunofluorescence histogram above which cells were considered positive for antibody binding (panel (3); Figure 106, Pocket). The negative control consisted of FITC- and PE-conjugated Mabs to keyhole limpet haemocyanin; an irrelevant antibody of the same immunoglobulin subclasses as the test antibodies.

Immunofluorescence measurements were calibrated regularly with conjugated microspheres in order to discriminate background optical and electrical noise from signal.

Finally, T-lymphocyte subsets were quantified by staining for the CD3, CD4, CD8 and HLA-DR antigens (panels (1) and (2); Figure 107, Pocket).

2.10.1. Cross-sectional data analysis

Data were analysed and stored using the CSS computer programme devised by StatSoft 1988. Parametric (Student t-test) and non-parametric (Mann-Whitney U test) tests were applied.

2.10.2. Longitudinal data analysis

Longitudinal studies were made of individual laboratory control and HIV-1 infected subjects' lymphocyte phenotype and function *in vitro*. The patient cohort was categorised by therapy to AL 721 and ZDV treated groups. The

ZDV treated patient group was further subdivided by CDC classification to ARC (CDC IVC2) or AIDS (CDC IVC1 or IVD).

These serial studies were designed to describe contrasts between the different control and patient groups and the nature of subject response time trends. As is common with clinical studies, the data collected were not rigorous enough to employ time series analytical techniques (J. Wadsworth, personal communication).

However, comparison of control and patient coefficients of variation (CV; Armitage and Berry, 1987),

$$CV(\bar{x}) = \frac{SD}{\bar{x}} \times 100\ \%$$

SD: standard deviation

$\bar{x}$: mean

provides a useful descriptive tool of temporal individual and experimental variation. Therefore, by informal comparison of individual patient CV and laboratory control response CVs, it is possible to ascertain the influence of therapy on the patient's <u>in vitro</u> response (J. Wadsworth, personal communication).

2.11. Graphics

Graphics were by Harvard Graphics, version 2.12 programme (Software Publishing Corporation, 1988).

CHAPTER 3: RESULTS

LYMPHOCYTE FUNCTION STUDIES: PROLIFERATIVE RESPONSE

3. Lymphocyte Function Studies: Mitogenic Proliferative Response

Two mitogens, phytohaemagglutinin (PHA) and concanavalin A (Con A) were utilised specifically to assess T-lymphocyte proliferative capacity. Each activates T-lymphocytes by different ligands and second messenger pathways (Beverley et al., 1987) and were employed for this reason. PHA was utilised primarily and Con A cultures established only when PBMC numbers permitted.

3.1. Optimal culture conditions for mitogen induced proliferation

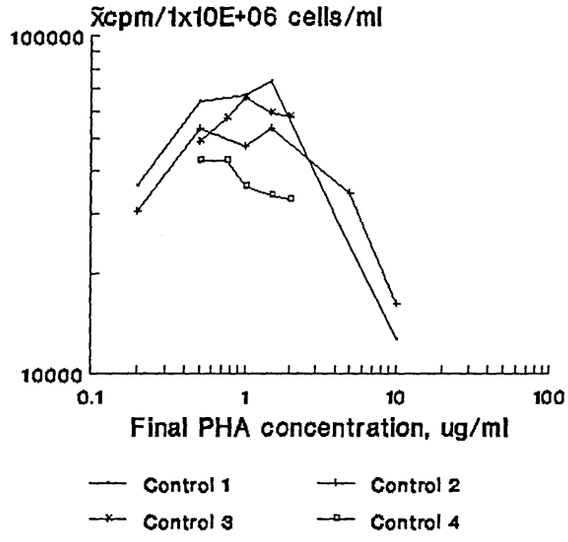
In view of the low numbers of PBMC isolated from HIV-1 infected individuals, proliferative and gIFN cultures were studied concomitantly (see sections 3.1.1. and 4.3.1.). Response measurement was also necessarily limited to one mitogen concentration, as other polyclonal activators and antigen were tested. Ideally, individual response should be assessed over a concentration range as the optimal response may vary individually.

Preliminary experiments were performed to assess relevant variables for proliferative assay, in conjunction with optimal gIFN synthesis (see section 4.3.). These included mitogen concentration, culture time and PBMC concentration. The optimal hiFCS concentration was also briefly investigated in view of previously reported results (Rogers, 1987; see section 2.3.).

3.1.1. Optimal mitogen concentration

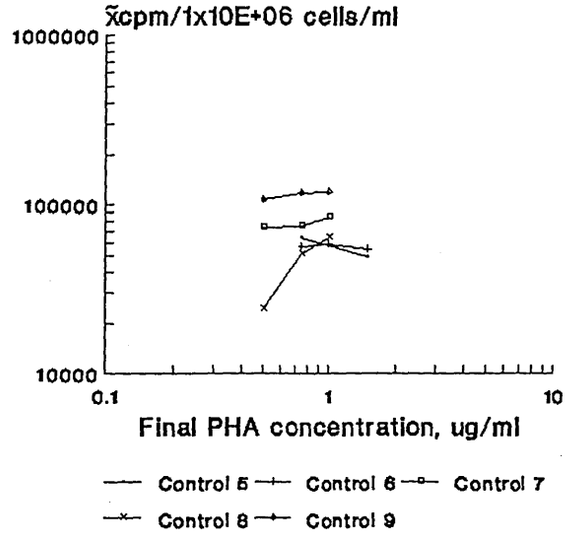
PBMC from several laboratory controls were cultured with an extensive PHA or Con A concentration range in quintuplieate at $1x10^6$ cells/ml for 72 h as previously described (see section 2.5.). The peak response range was then evaluated in further controls. Figures 05, 06 (a) and (b) illustrate the net proliferative response in each case. The net response was calculated by subtracting the mean cpm from cultures without mitogen from the mean cpm of PBMC

Figure 05a
The effect of PHA concentration on PBMC net proliferative response



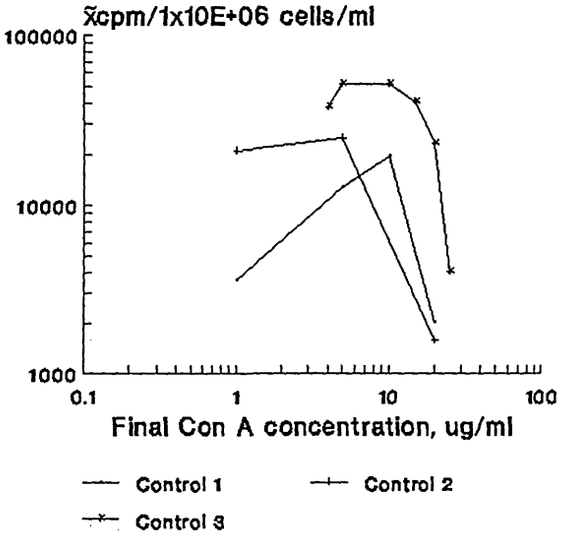
PBMC from four controls were cultured with a PHA concentration range of 0.2ug/ml to 10ug/ml.

Figure 05b
Further effect of PHA concentration on PBMC net proliferative response



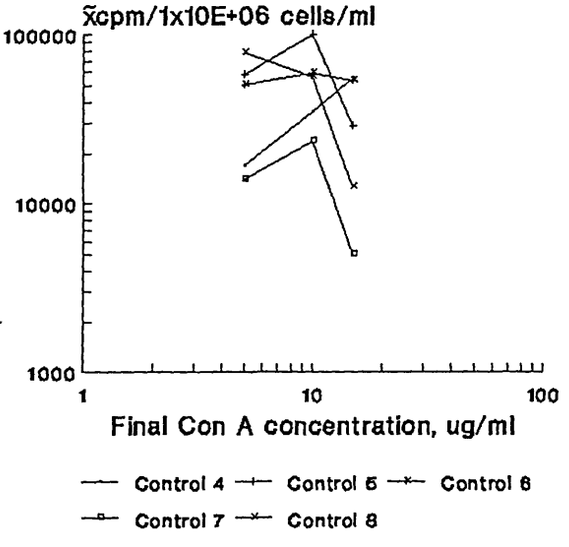
PBMC from a further five controls were cultured with a PHA concentration range of 0.5ug/ml to 1.5ug/ml.

Figure 06a
The effect of Con A concentration
on PBMC net proliferative response



PBMC from three controls were cultured with a Con A concentration range of 1ug/ml to 20ug/ml.

Figure 06b
Further effect of Con A concentration
on PBMC net proliferative response



PBMC from a further five controls were cultured with a Con A concentration range of 5ug/ml to 15ug/ml.

cultured with mitogen for each subject.

The final PHA concentration adopted throughout, in agreement with that producing optimal gIFN synthesis (see section 4.3.1.), was 1.0 ug/ml and caused significant PBMC proliferation ($p < 0.01$) in control and patient groups.

Con A induced maximal proliferative response at final concentrations of around 10 ug/ml and this was adopted as the working concentration. Optimal Con A concentration caused significant PBMC proliferation ($p < 0.01$) in control and subject groups. Although Con A induced maximal gIFN synthesis at approximately 5 ug/ml, 10 ug/ml caused significant production (see section 4.3.1.).

3.1.2. Optimal culture time

Extensive documentation suggests 72 h as optimal for mitogen induced proliferative response (for example, Eales et al., 1987). A time course study of net PHA and Con A induced proliferation confirming this in our hands is shown in Figures 07 and 08. This was in accordance with induced gIFN production and so these mitogen cultures were for 72 h throughout.

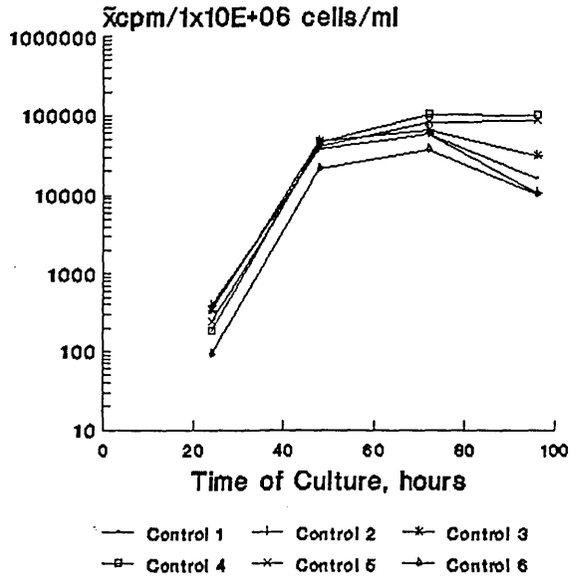
3.1.3. Optimal PBMC concentration

Figure 09 demonstrates the effect of PBMC concentration on PHA and Con A proliferative response in four laboratory controls. Two of three PHA cultures showed little difference between $1x10^6$ cells/ml and $2x10^6$ cells/ml, while the remaining PHA and Con A cultures suggested optimal proliferative response at $1x10^6$ cells/ml. PBMC for proliferative and gIFN cultures were therefore adjusted to $1x10^6$ cells/ml.

3.1.4. The effect of hiFCS concentration

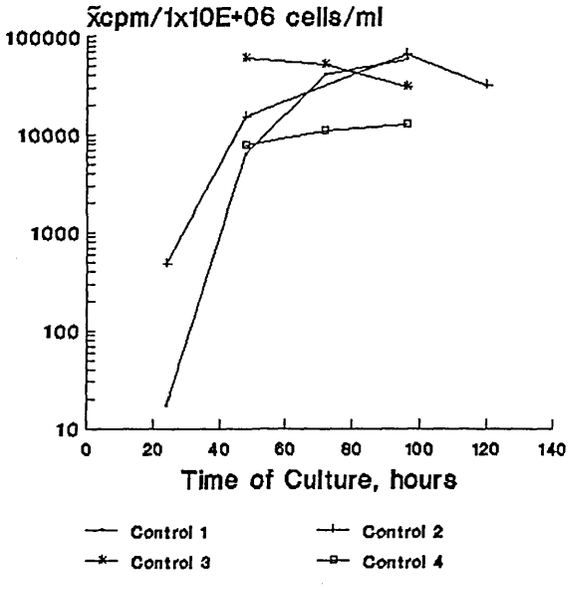
The results of two PHA proliferation cultures (Figure 10) agree with those of Rogers (1987). Concentration of hiFCS at less then 5 % does not support optimal proliferation, while higher levels increase spontaneous

Figure 07
Time course : net PHA driven
PBMC proliferative response



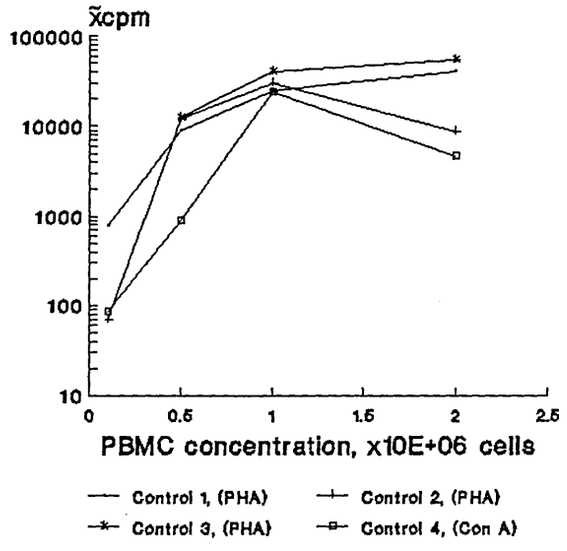
PBMC from six controls were cultured
with PHA (1ug/ml) or without.

Figure 08
Time course : net Con A driven
PBMC proliferative response



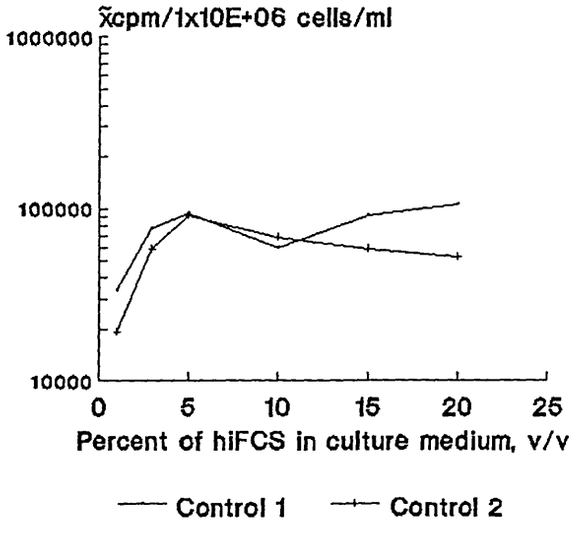
PBMC from four controls were cultured
with Con A (10ug/ml) or without.

Figure 09
The effect of PBMC concentration on mitogen-driven net proliferative response



PBMC from three controls were cultured with PHA (1ug/ml), Con A (10 ug/ml) or without. PBMC concentration as variable.

Figure 10
The effect of hiFCS concentration on the PHA-driven net proliferative response



PBMC from two laboratory controls were cultured with PHA (1ug/ml) or without for 72h.

measurement, detracting from net response. Culture medium was therefore made up with 5 % hiFCS throughout.

3.2. Mitogenic proliferative response: cross-sectional study

Tables 41 to 56 (Appendices I and II) record the spontaneous and total PHA or Con A induced proliferative response of PBMC obtained from laboratory controls and HIV-1 infected subjects. HIV-1 seropositive individuals were categorised by their CDC grouping to asymptomatic or persistent generalised lymphadenopathy (CDC II and III); ARC (CDC IVC2) and AIDS (CDC IVC1 and IVD). These subjects were further subdivided by zidovudine (ZDV) therapy. Tables 08 and 09 summarise the spontaneous, total and net mitogenic response for the control and subject groups. Formal analysis of the asymptomatic group was limited to non-ZDV treated subjects as few of these individuals were receiving ZDV.

The net mitogenic proliferative response is composed of two elements: spontaneous PBMC proliferation and total mitogen induced proliferation. Analysis of the spontaneous proliferation revealed no significant difference between the control and HIV-1 infected subject groups nor between the latter categories. Therefore, the changes in mitogenic response observed were caused solely by changes in mitogen induced proliferation.

3.2.1. Net proliferative response to PHA and Con A by non-zidovudine treated HIV-1 infected individuals: natural history

The net PBMC proliferative response to PHA and Con A was significantly depressed in all HIV-1 seropositive groups as compared with heterosexual controls at low risk of contracting HIV-1 ($p < 0.01$; Tables 10 and 11). Although formal analysis of the ARC patients' Con A response was prevented by limited subject numbers (N=4), their results were consistent with this observation. Thus, the decrease in both PHA and Con A proliferation occurred early in the

spectrum of HIV-1 infection before the onset of symptomatic disease and has been observed in previous studies of PHA proliferative response (Rogers, 1987; Rogers et al., 1989).

A decrease in net mitogenic response follows disease progression. The graphical representation of results (Figures 11 and 12) suggests that the ARC response was depressed relative to the symptomless cohort, with or without persistent generalised lymphadenopathy, although statistical significance was not noted (Tables 10 and 11). Similar to the observations of Rogers (1987) and Rogers et al (1989), a significant reduction of PHA proliferative response was observed for AIDS individuals relative to asymptomatic ($p < 0.01$; Table 10) and ARC ($p < 0.05$; Table 10) subjects. This pattern was reflected by the Con A net response (Table 11).

3.2.2. The effect of zidovudine therapy on the net mitogenic proliferative response of HIV-1 infected subjects

The net PHA proliferative response of ARC and AIDS patients was substantially augmented by ZDV treatment ($p < 0.05$ and $p < 0.01$ respectively; Table 10) over median treatment times of 3 months (range: 1 to 18 months) and 4 months (range: 1 to 30 months) respectively. The Con A assay further indicated that ZDV elevated the proliferative response of AIDS subjects ($p < 0.01$; Table 11) and a similar, although non-significant, increase was observed in individuals classified ARC. The Con A response was ascertained over ZDV median treatment periods of 3 months for both ARC and AIDS patients (range: 1 to 16 months for ARC subjects and 1 to 30 months in the case of AIDS subjects).

No clear trend was obvious on informal analysis of the ZDV treated asymptomatic nor of MRC Concorde groups because of limited subject numbers.

Table 08: PHA proliferative response: summary of control and patient subject response

	[3H]TdR incorporation x̃cpm per 1x10E+06 cells/ml ± SEM			
	without PHA	with PHA	net response	
CONTROL	622 ± 155 (467 - 777)	66105 ± 4509 (61596 - 70614)	65047 ± 4329 (60718 - 69376)	N=23
As, no ZDV	269 ± 72 (197 - 341)	21002 ± 4871 (16131 - 25873)	20733 ± 4871 (15862 - 25604)	N=7
ARC, no ZDV	418 ± 154 (264 - 572)	16157 ± 5445 (10712 - 21602)	15739 ± 5460 (10279 - 21199)	N=8
ARC, ZDV	404 ± 96 (308 - 500)	28746 ± 5697 (23049 - 34443)	28342 ± 5671 (22671 - 34013)	N=9
AIDS, no ZDV	503 ± 119 (384 - 622)	5750 ± 1646 (4104 - 7396)	5247 ± 1574 (3673 - 6821)	N=24
AIDS, ZDV	395 ± 106 (289 - 501)	12522 ± 1825 (10727 - 14377)	12156 ± 1847 (10309 - 14003)	N=13

The mean cpm ± SEM is given for the spontaneous, total and net PHA proliferative response of each subject group. The SEM range is in parenthesis.
ZDV: zidovudine

Table 09: Con A proliferative response: summary of control and patient subject response

	[3H]TdR incorporation $\tilde{x}$cpm per 1x10E+06 cells/ml ± SEM			
	without Con A	with Con A	net response	
CONTROL	291 ± 29 (262 - 320)	25071 ± 2148 (22923 - 27219)	24780 ± 2147 (22633 - 26927)	N=16
As, no ZDV	286 ± 83 (203 - 369)	10145 ± 2553 (7592 - 12698)	9859 ± 2516 (7343 - 12375)	N=6
ARC, no ZDV	**** (184 - 548)	**** (1937 - 12069)	**** (1753 - 11644)	N=4
ARC, ZDV	415 ± 114 (301 - 529)	16831 ± 6934 (9897 - 23764)	16416 ± 6885 (9531 - 23301)	N=7
AIDS, no ZDV	467 ± 199 (268 - 666)	3301 ± 1051 (2250 - 4352)	2834 ± 949 (1885 - 3783)	N=12
AIDS, ZDV	340 ± 70 (270 - 410)	13313 ± 3474 (9839 - 16787)	12973 ± 3474 (9499 - 16447)	N=9

The mean cpm ± SEM is given for the spontaneous, total and net Con A proliferative response of each subject group with, the range^{SEM} in parenthesis. The range of the four untreated ARC subjects is given.
ZDV: zidovudine

Table 10
Net PHA proliferative response

	AS no ZDV	ARC no ZDV	ARC on ZDV	AIDS no ZDV	AIDS on ZDV
CONTROL	0.01	0.01	0.01	0.01	0.01
AS, no ZDV	-	ns	ns	0.01	ns
ARC, no ZDV	-	-	0.05	0.05	ns
ARC, on ZDV	-	-	-	0.01	0.01
AIDS, no ZDV	-	-	-	-	0.01

Comparison of PHA net PBMC proliferative response, the independent variables being CDC classification groups and zidovudine (ZDV) therapy.
Significant results are quoted as figures and represent probability less than 0.01 or 0.05 ($p < 0.01$ or 0.05).
Non significant results are represented as ns.

N.B. Statistical analysis was by Student t-test.
Although we must be aware that the greater the number of separate t-tests the greater the risk of mistakenly rejecting the null hypothesis, the data shown here is unlikely to present this problem (J. Wadsworth, personal communication).

Table 11
Net Con A proliferative response

	AS no ZDV	ARC no ZDV	ARC on ZDV	AIDS no ZDV	AIDS on ZDV
CONTROL	0.01	na	0.05	0.01	0.01
AS, no ZDV	-	na	ns	0.05	ns
ARC, no ZDV	-	-	na	na	na
ARC, on ZDV	-	-	-	0.01	ns
AIDS, no ZDV	-	-	-	-	0.01

Comparison of Con A net PBMC proliferative response, the independent variables being CDC classification groups and zidovudine (ZDV) therapy.
Significant results are quoted as figures and represent probability less than 0.01 or 0.05 ($p < 0.01$ or 0.05).
Non significant results are represented as ns.
Formal analysis of ARC, no zidovudine (ZDV) was not applicable (na) because of limited numbers (N=4).

N.B. Statistical analysis was by Student t-test. Although we must be aware that the greater the number of separate t-tests the greater the risk of mistakenly rejecting the null hypothesis, the data shown here is unlikely to present this problem (J. Wadsworth, personal communication).

Figure 11: Cross-sectional summary of the net PHA proliferative response

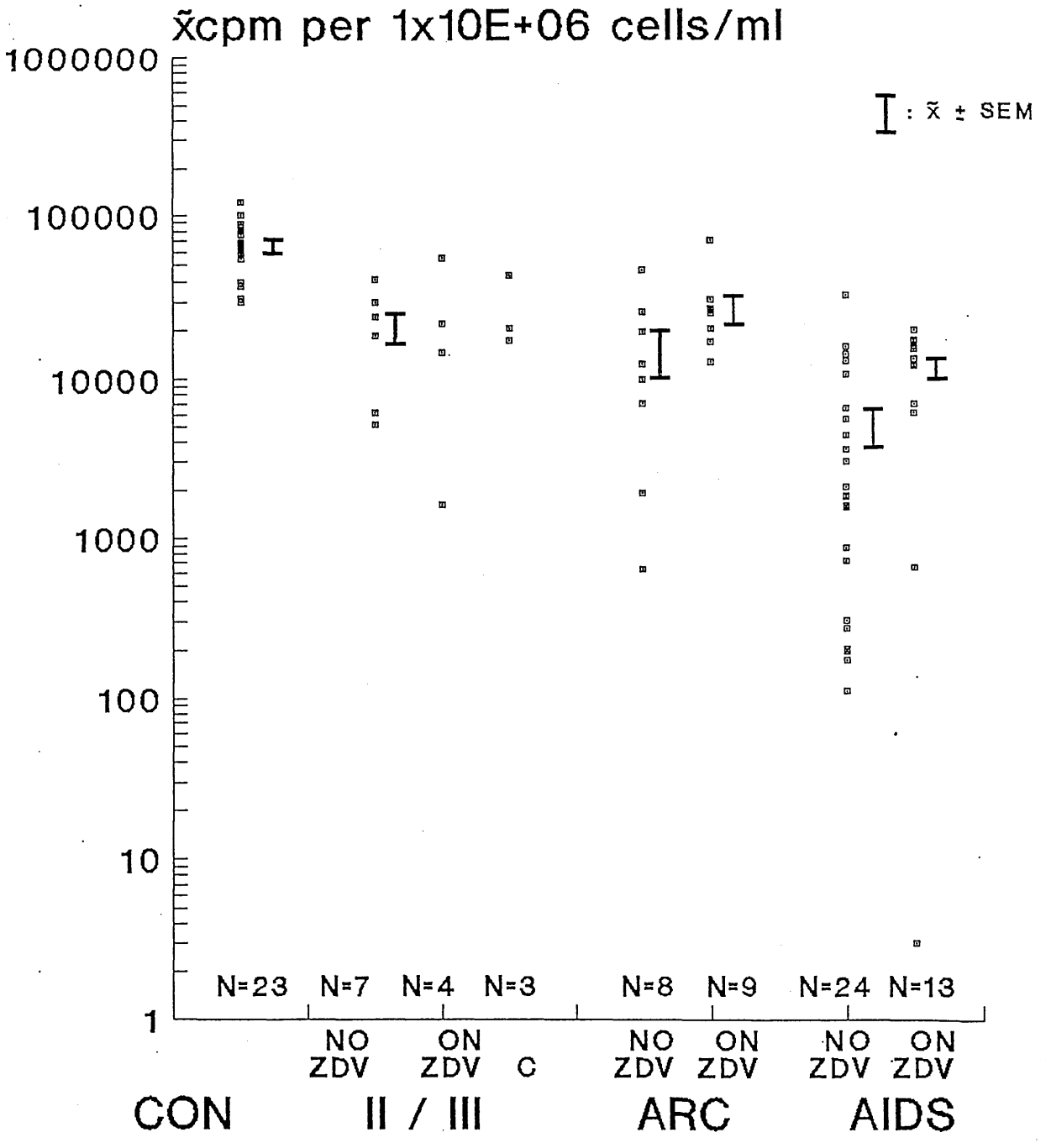
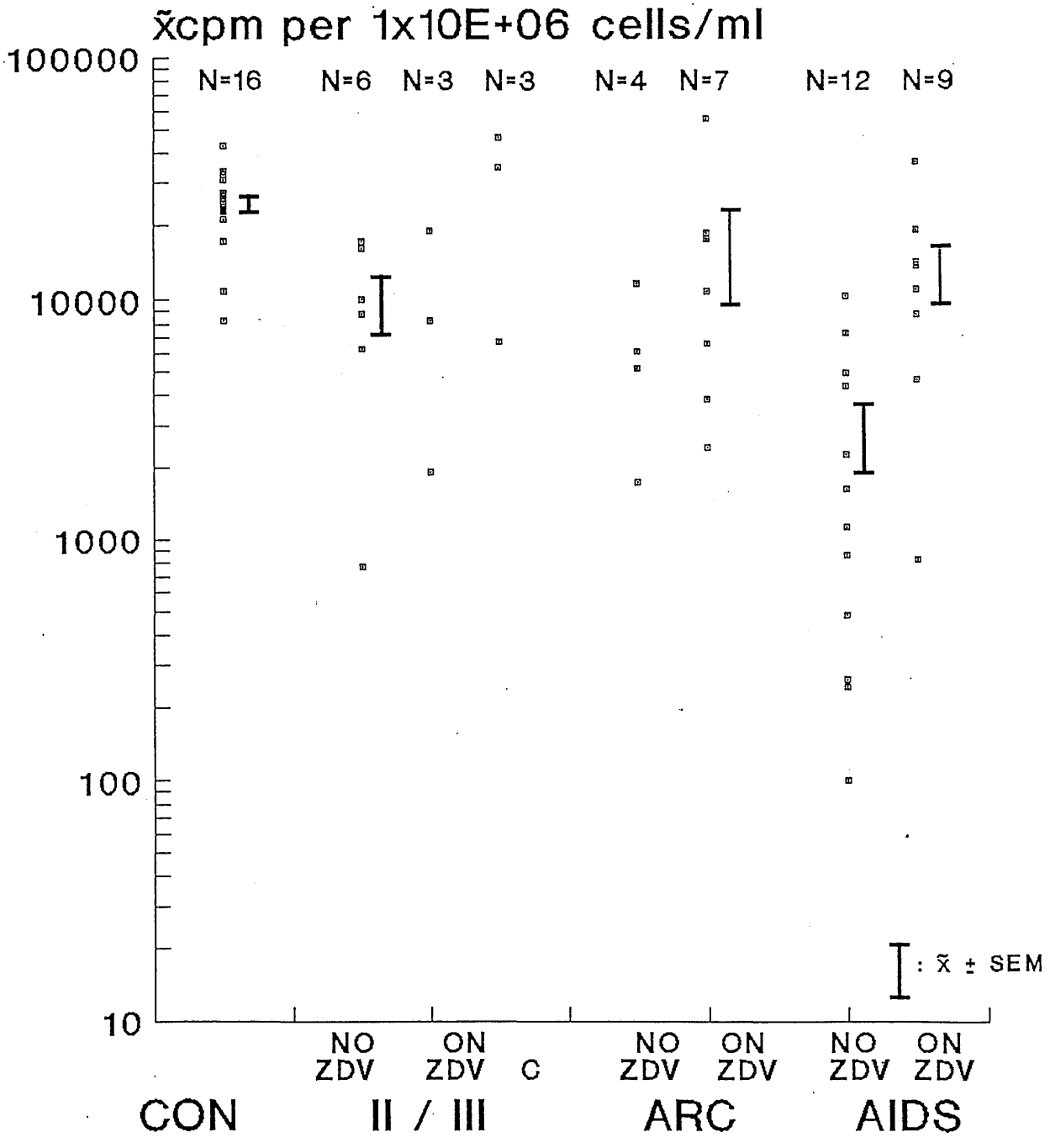


Figure 12: Cross-sectional summary of the net Con A proliferative response



3.2.3. The mitogenic response level of zidovudine treated ARC and AIDS patients

Both assays employed suggest that the mitogen induced proliferative response of AIDS patients was elevated to or towards the untreated asymptomatic response level by ZDV therapy (Tables 10 and 11). Furthermore, the PHA assay indicated that ZDV augments the response of ARC subjects to a slightly higher level than the response of asymptomatics. A similar although less obvious trend was observed of the Con A ARC response and may indicate the Con A system to be less sensitive than the corresponding PHA proliferative assay.

3.3. Mitogenic proliferative response: longitudinal study of the PHA induced response

The serial spontaneous, total and net PHA induced PBMC proliferative response is tabulated for each laboratory control (Figures 13 and 14) and HIV-1 infected subject.

The longitudinal patient cohort was categorised by therapy to ZDV and AL 721 treated groups. Patients receiving ZDV were further subdivided by their CDC grouping to ARC (Figures 16 and 17) or AIDS (Figures 21 and 22). The serial data of 3 AL 721 treated individuals is recorded (Figure 25), as is the response of one of these subjects followed through ZDV therapy after he discontinued AL 721 (Table 12).

Restricted data prevented formal analysis of the laboratory control and patient groups' net PHA proliferative response using time series analytical techniques. However, comparison of control and patient coefficients of variation (CV) provides an appropriate and useful gauge of patient response trends with therapy; since CV is a measure of temporal individual and experimental variation (J. Wadsworth, personal communication; see section 2.10.2.). In addition, the laboratory control group mean net response plus or minus 2 standard deviations ($\bar{x} \pm 2SD$ cpm per $1x10^6$ cells/ml) is indicated on ZDV treated patient graphs (J. Wadsworth, personal communication). These methods of comparison were applied to all longitudinal studies conducted for this

report.

3.3.1. Laboratory control subjects

The longitudinal net PBMC proliferative response to PHA is shown graphically for 5 laboratory controls (Figure 15). The magnitude of response ($\bar{x} \pm$ SD: 58847 $\pm$ 18493 $\bar{x}$cpm/1x10^6 cells/ml) conforms with cross-sectional data ($\bar{x} \pm$ SEM: 65047 $\pm$ 4329 $\bar{x}$cpm/1x10^6 cells/ml; see Table 08)

These heterosexual controls were monitored over a median time of 7 months (range: 3 to 8.5 months) and calculated to have net response CVs of 26.3 %; 26.3 %; 33.5 %; 12.2 % and 7.1 % respectively (median: 26.3 %; range: 7.1 to 33.5 %).

3.3.2. Zidovudine treated ARC subjects

Figures 18 to 20 graph the serial net proliferative response to PHA by PBMC obtained from 8 ARC (CDC IVC2) subjects. Testing was from before or at day zero of ZDV administration in 6 cases, and from 1 month of therapy in the remaining 2, and then throughout treatment (median: 3 months; range 1 to 16 months of ZDV).

The net PHA induced proliferative response of 5 subjects (individuals 1 and 4-7) before therapy or on day zero ($\bar{x} \pm$ SD: 13457 $\pm$ 8244 $\bar{x}$cpm/1x10^6 cells/ml) is decreased relative to the laboratory control group and conforms with the data collected from the cross-sectional study ($\bar{x} \pm$ SEM: 15739 $\pm$ 5460 $\bar{x}$cpm/1x10^6 cells/ml; see Table 08).

Serial determination revealed a clear improvement of net proliferative response to PHA during ZDV therapy for patients numbered 1, 5 and 6. This increase is reflected by their CVs of 50.9 %, 96.7 % and 64.6 % respectively. A trend of response improvement was observed in subjects 4 and 7, although these patients CVs were similar to those of the control group (30.5 % and 35.4 % respectively).
Therefore, in the latter 2 cases, individual or experimental variation cannot be dismissed as causal of this trend.

Figure 13: PHA proliferative response
Longitudinal study of control subjects

CONTROL 1: PHA PROLIFERATIVE RESPONSE

	[3H]TdR incorporation x̄cpm per 1x10E+06 cells/ml at								
month	0	2	3	3.5	4	5	6	7	7.5
with PHA	41757	61714	36017	67860	69530	76581	57337	89830	68364
no PHA	334	757	255	460	162	894	188	206	543
net	41423	60957	35910	67400	69368	75687	57149	89624	67821

CONTROL 2: PHA PROLIFERATIVE RESPONSE

	[3H]TdR incorporation x̄cpm per 1x10E+06 cells/ml at								
month	0	2	4	5	5.5	6	7	8	8.5
with PHA	63157	51124	57446	65927	89192	40721	63136	39756	74167
no PHA	360	213	114	182	149	65	412	1002	257
net	62797	50911	57332	65745	89043	40656	62724	38754	73910

FIGURE 14: PHA proliferative response
Longitudinal study of control subjects

CONTROL 3: PHA PROLIFERATIVE RESPONSE

	[3H]TdR incorporation x̃cpm per 1x10E+06 cells/ml at					
month	0	2	3	4	5	7
with PHA	49514	46218	25857	20109	30040	41359
no PHA	281	438	169	142	314	235
net	49233	45780	25688	19967	29726	41124

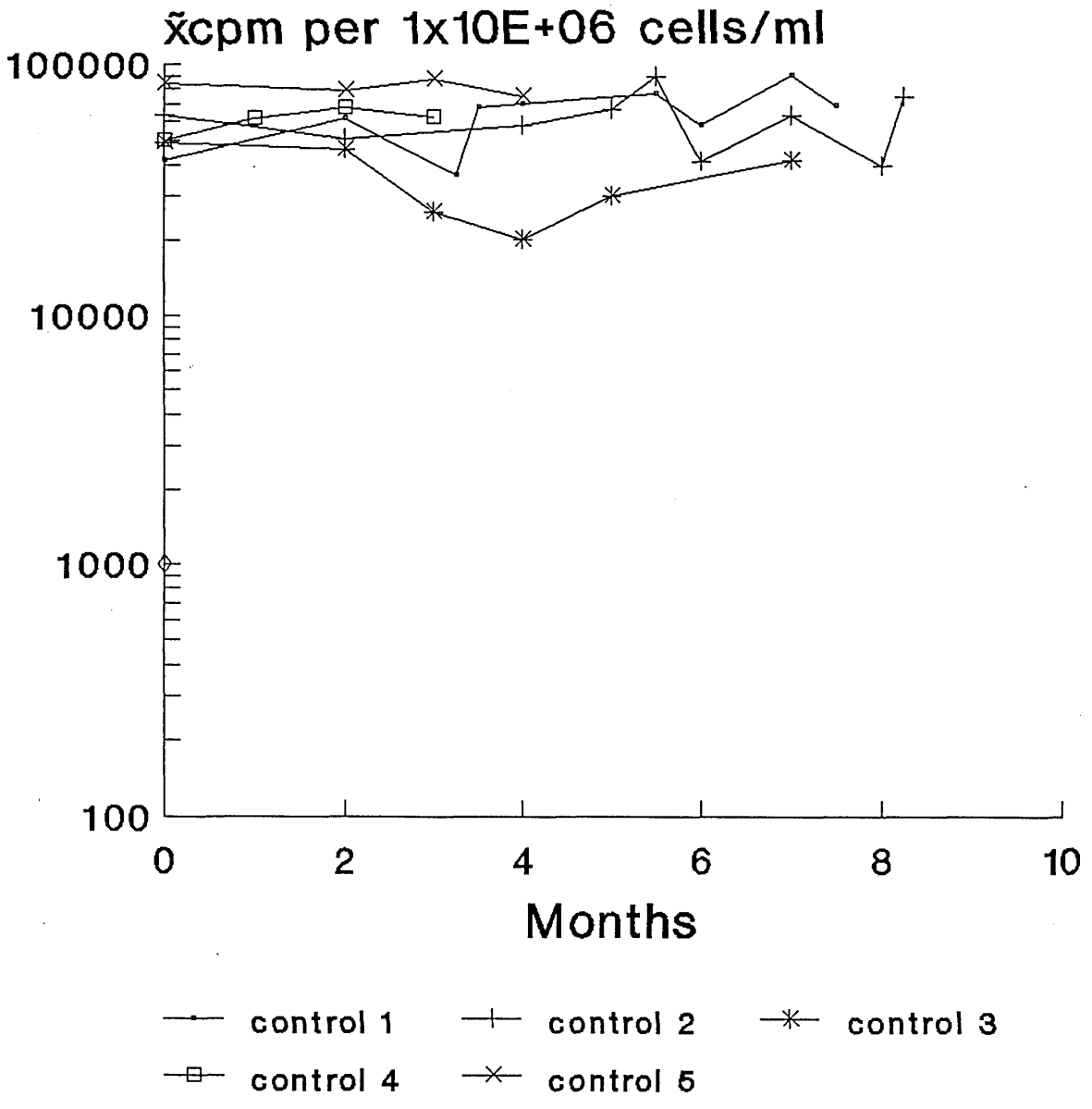
CONTROL 4: PHA PROLIFERATIVE RESPONSE

	[3H]TdR incorporation x̃cpm per 1x10E+06 cells/ml at			
month	0	1	2	3
with PHA	50224	61521	58008	61972
no PHA	128	250	458	254
net	50096	61271	67550	61718

CONTROL 5: PHA PROLIFERATIVE RESPONSE

	[3H]TdR incorporation x̃cpm per 1x10E+06 cells/ml at			
month	0	2	3	4
with PHA	84188	78623	87632	74415
no PHA	317	196	470	142
net	83871	78427	87162	74273

Figure 15: Longitudinal net PHA proliferative response of five laboratory controls



In contrast, subject 8's response was observed to decrease from day zero of therapy to month 1 of ZDV administration, although little difference was noted between months 1 and 2. Over the 3 serial determinations of response, CV was calculated to be 34.8 % and is similar to laboratory control values. It is noteworthy that the net PHA proliferative response pattern of this patient was not paralleled by his corresponding Con A proliferative response (see Figure 29; section 3.4.2.), PHA induced gIFN response (see Figures 49 and 51; section 4.5.2.) nor by the Con A induced gIFN response (see Figure 60; section 4.6.2.), where a trend of improvement was observed in each instance. Taken together, these observations indicate a technical error on measurement of subject 8's net PHA proliferative response.

Pre ZDV or day zero administration data were not available for subjects 2 and 3, and no clear trend was obvious from testing throughout treatment.

Of the 8 ARC subjects studied longitudinally, the net PHA proliferative response of 5 was augmented with ZDV administration over a range of 1 to 16 months (median: 3 months). These findings are consistent with cross-sectional data for similar periods of ZDV treatment (median: 3 months; range: 1 to 18 months: see section 3.2.2.). In each of the 5 subject cases, the PHA induced proliferative response was elevated to or towards the control range (described as $\bar{x} \pm 2SD$ cpm per $1x10^6$ cells/ml) by ZDV administration.

These findings of ZDV associated immunological improvement, using lymphocyte functional response to mitogen as a means of assessment, parallel the general clinical improvements observed in these and other treated patients seen at this centre (B. Peters, personal communication) and with previous studies delineating clinical benefit in severe ARC (Fischl et al., 1987; Pinching et al., 1989; Stambuk et al., 1989a,b).

SUBJECT 1: PHA proliferative response of ARC subject receiving zidovudine

	[3H]TdR incorporation x̃cpm/1x10E+06 cells/ml at				
month	-1	4	11	15	16
with PHA	8520	21020	14116	26477	10082
no PHA	1440	369	327	117	232
net	7080	20651	13789	26360	9850

SUBJECT 2: PHA proliferative response of ARC subject receiving zidovudine

	[3H]TdR incorporation x̃cpm/1x10E+06 cells/ml at			
month	1	2	3	4
with PHA	25867	50004	20413	36722
no PHA	46	3259	166	1193
net	25821	43745	20247	35529

SUBJECT 3: PHA proliferative response of ARC subject receiving zidovudine

	[3H]TdR incorporation x̃cpm/1x10E+06 cells/ml at		
month	1	2	7
with PHA	33519	13977	28129
no PHA	150	195	597
net	33369	13782	27532

SUBJECT 4: PHA proliferative response of ARC subject receiving zidovudine

	[3H]TdR incorporation x̃cpm/1x10E+06 cells/ml at		
month	-1	0	3
with PHA	22611	17229	31678
no PHA	139	132	293
net	22472	17097	31385

Figure 16
PHA proliferative response
Longitudinal study
ARC subjects receiving zidovudine

SUBJECT 5: PHA proliferative response
of ARC subject receiving zidovudine

	[3H]TdR incorporation x̄cpm/1x10E+06 cells/ml at			
month	0	1	2	3
with PHA	2118	12924	13198	40701
no PHA	184	138	278	203
net	1934	12786	12920	40498

SUBJECT 6: PHA proliferative response
of ARC subject receiving zidovudine

	[3H]TdR incorporation x̄cpm/1x10E+06 cells/ml at	
month	0	1
with PHA	26692	71624
no PHA	201	542
net	26491	71082

SUBJECT 7: PHA proliferative response
of ARC subject receiving zidovudine

	[3H]TdR incorporation x̄cpm/1x10E+06 cells/ml at	
month	0	1
with PHA	12799	21180
no PHA	234	237
net	12565	20943

SUBJECT 8: PHA proliferative response
of ARC subject receiving zidovudine

	[3H]TdR incorporation x̄cpm/1x10E+06 cells/ml at		
month	0	1	2
with PHA	47810	25503	31040
no PHA	425	249	1826
net	47385	25254	29214

Figure 17
PHA proliferative response
Longitudinal study
ARC subjects receiving zidovudine

Figure 18: Longitudinal net PHA proliferative response of ARC subject, CDC IVC2, receiving zidovudine

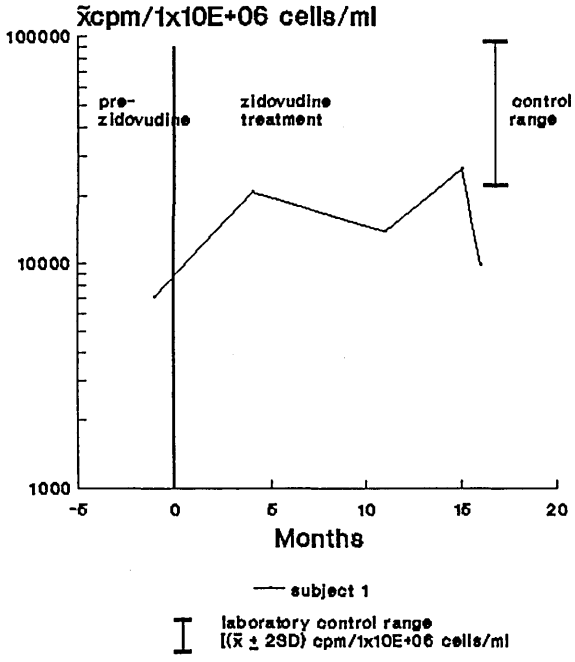


Figure 19: Longitudinal net PHA proliferative response of two ARC subjects, CDC IVC2, receiving zidovudine

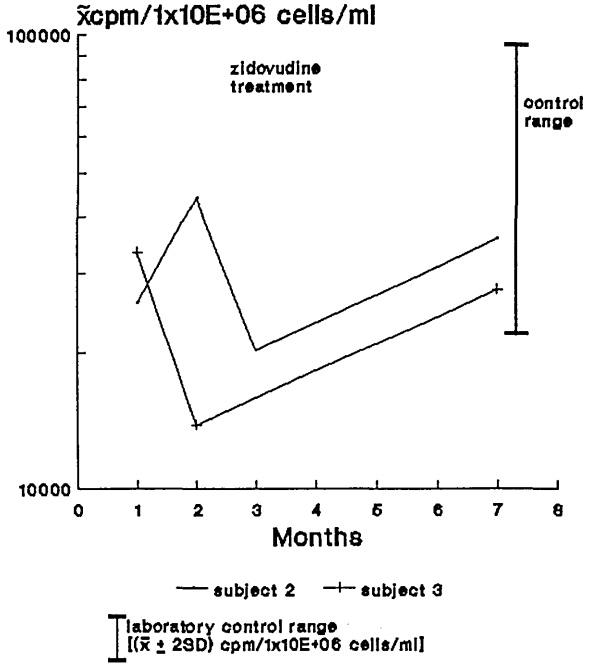
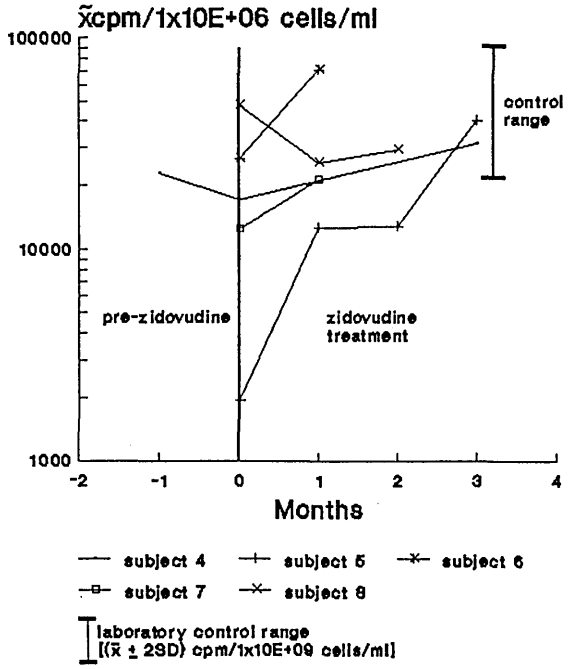


Figure 20: Longitudinal net PHA proliferative response of five ARC subjects, CDC IVC2, receiving zidovudine



LONGITUDINAL STUDY: PHA PROLIFERATIVE RESPONSE OF EIGHT ARC (CDC IVC2) SUBJECTS RECEIVING ZIDOVUDINE TREATMENT

3.3.3. Zidovudine treated AIDS subjects

Figures 23 and 24 illustrate the serial net PBMC proliferative responses to PHA for 6 AIDS patients, receiving ZDV for a median of 3 months (range: 1 to 12 months). Subjects 3 and 5 were classified as CDC IVD and the remainder as CDC IVCI.

Subjects 2 to 6 were tested before ZDV or at day zero administration, and similar to the cross-sectional study ($\bar{x} \pm$ SEM: 5247 $\pm$ 1574 $\bar{x}$cpm/1x10^6 cells/ml; see Table 08), the net response of these patients ($\bar{x} \pm$ SD: 3110 $\pm$ 5568 $\bar{x}$cpm/1x10^6 cells/ml) was profoundly depressed in comparison with the heterosexual controls. However, the subsequent use of ZDV was associated with a striking improvement of net PHA induced proliferation towards the control range, over 1 to 12 months (median: 2 months) of antiretroviral administration. This response improvement is unlikely to be caused by individual or experimental variation as the CVs of subjects 2 to 6 were calculated as 96.8 %; 92.0 %; 73.9 %; 43.5 %; 104 % and 75.4 % respectively. ZDV was also observed to augment the net PHA proliferative response of AIDS patients by cross-sectional study over 1 to 30 months (median: 4 months). These patients generally showed ZDV associated clinical benefit or at least maintenance of clinical status; the most marked clinical improvement is observed with those receiving ZDV soon after diagnosis of AIDS (B. Peters, personal communication).

However ZDV treatment of subject 1, an afro-carribean male, was associated with profound and progressive deterioration of net PHA proliferative response (CV: 197 %) from months 1 to 11 of administration. This patient presented with pulmonary Kaposi's sarcoma and was started on ZDV in the autumn of 1988. His Kaposi's repeated and became severe with progressive pyogenic infection particularly in the last months before his death in 1990. His general clinical status deteriorated rapidly during this time (B. Peters, personal communication). The marked decline of his *in vitro* mitogenic proliferative response

SUBJECT 1: PHA proliferative response
of AIDS subject receiving zidovudine

	[3H]TdR incorporation x̄cpm/1x10E+06 cells/ml at						
month	1	2	5	8	9	10	11
with PHA	20858	5535	856	318	245	272	217
no PHA	576	402	te	251	138	191	96
net	20282	5133	856	67	107	81	121

te: technical error

SUBJECT 2: PHA proliferative response
of AIDS subject receiving zidovudine

	[3H]TdR incorporation x̄cpm/1x10E+06 cells/ml at							
month	-1	0	3	4	8	10	11	12
with PHA	380	8334	123	1368	4731	1440	8897	3337
no PHA	354	306	267	103	175	118	161	58
net	26	5273	856	1265	4556	1322	8736	3279

SUBJECT 3: PHA proliferative response
of AIDS subject receiving zidovudine

	[3H]TdR incorporation x̄cpm/1x10E+06 cells/ml at	
month	0	1
with PHA	2094	9131
no PHA	222	292
net	1872	8839

FIGURE 21
PHA PROLIFERATIVE RESPONSE
LONGITUDINAL STUDY
AIDS SUBJECTS RECEIVING ZIDOVUDINE

SUBJECT 4: PHA proliferative response of AIDS subject receiving zidovudine

	[3H]TdR incorporation x̄cpm/1x10E+06 cells/ml at		
month	-4	0	2
with PHA	1887	10227	14370
no PHA	231	481	429
net	1656	9746	13941

SUBJECT 5: PHA proliferative response of AIDS subject receiving zidovudine

	[3H]TdR incorporation x̄cpm/1x10E+06 cells/ml at		
month	0	2	4
with PHA	14640	te	28481
no PHA	271	243	1360
net	14369	na	27121

te: technical error
na: not applicable

SUBJECT 6: PHA proliferative response of AIDS subject receiving zidovudine

	[3H]TdR incorporation x̄cpm/1x10E+06 cells/ml at		
month	0	1	2
with PHA	427	14859	33513
no PHA	221	396	442
net	206	14463	33071

FIGURE 22
PHA PROLIFERATIVE RESPONSE
LONGITUDINAL STUDY
AIDS SUBJECTS RECEIVING ZIDOVUDINE

Figure 23: Longitudinal net PHA proliferative response of two AIDS subjects, CDC IVC1, receiving zidovudine

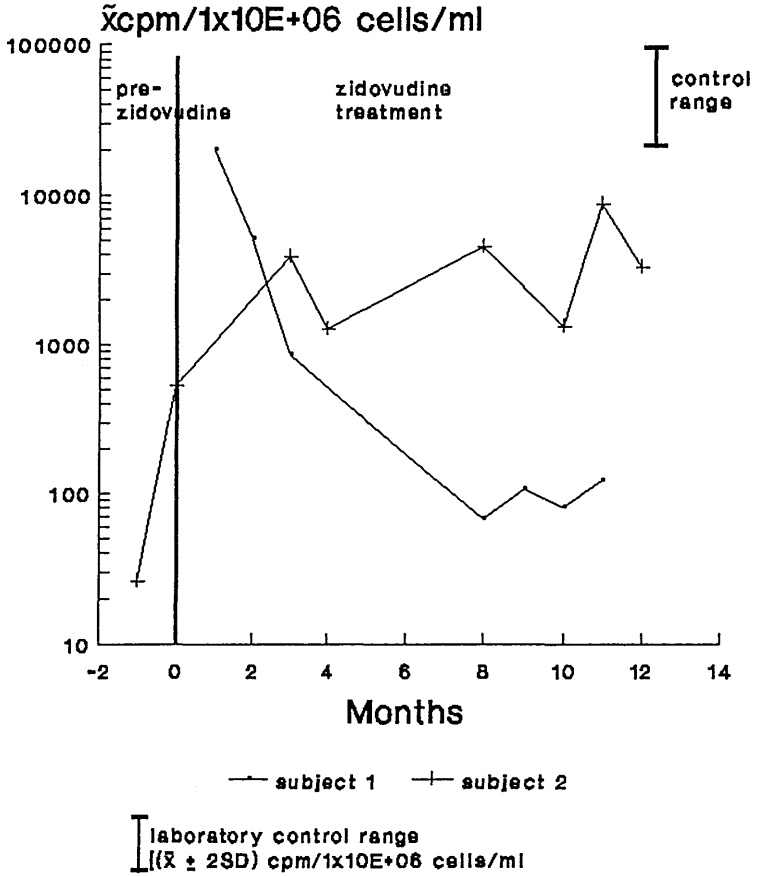
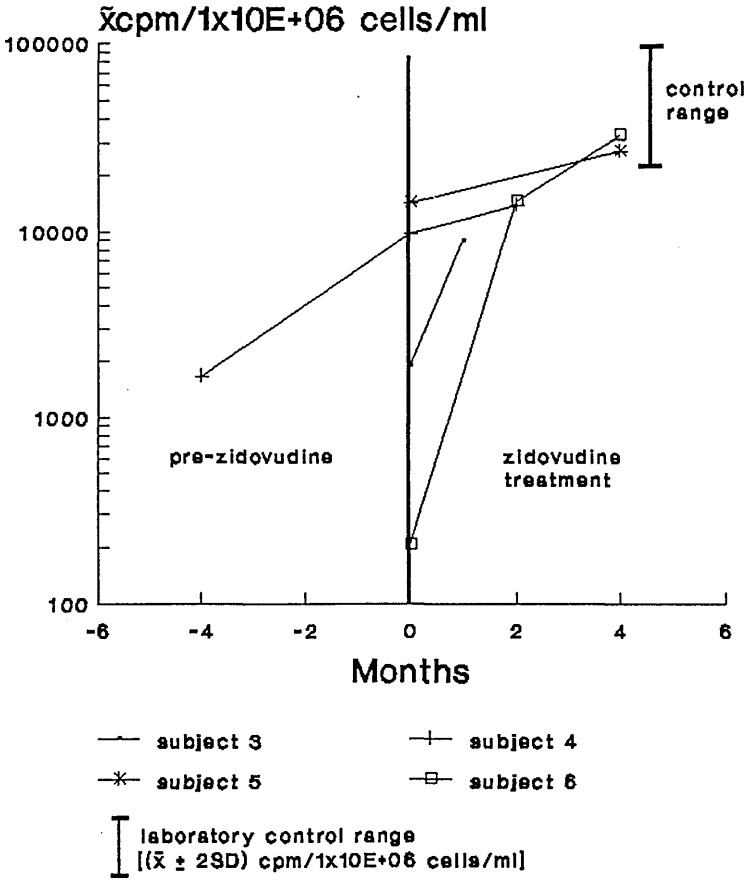


Figure 24: Longitudinal net PHA proliferative response of four AIDS subjects receiving zidovudine



certainly reflected this underlying clinical status throughout these latter months, and falling as it did before severe clinical deterioration may have acted as a predictor.

In summary, these data on AIDS subjects indicate that serial net PHA proliferative determination parallel temporal trends of clinical efficacy following ZDV therapy.

3.3.4. AL 721 treated HIV-1 infected subjects

An open pilot study of AL 721 treatment was conducted at St. Mary's (Peters et al., 1990) and provided an opportunity to conduct a comparative longitudinal study with the effects of ZDV treatment. Clinical, virological and neuropsychological data on these patients showed no evidence of benefit from this treatment and patients fared significantly worse than comparable patients on ZDV. The serial results on 1 ARC (CDC IVC2) and 2 AIDS (CDC IVC1) subjects are reported here.

The patients' net PHA response before therapy or at day zero of treatment (Figure 25) was noticeably depressed relative to the laboratory controls and concurs with the cross-sectional results (see Table 08).

The serial data (Figures 25 and 26) reveal a progressive decrease in net PHA proliferative response during 4 to 11 months of AL 721 treatment. The calculated CV of response for subjects 1 to 3 was 131 %, 116 % and 85.8 % respectively. Comparison of these CVs with control values (see section 2.10.2.) suggests the change in response with AL 721 therapy is not due to individual or experimental variation.

Therefore AL 721 treatment of these subjects is associated with a decline in net PHA proliferative response: this could reflect the failure of AL 721 to stem the progression of HIV-1 induced immunosuppression or even a detrimental effect of the drug. These findings provide a useful contrast to the studies with ZDV therapy as it was not possible to study patients with AIDS or severe ARC receiving no ZDV treatment (see section 1.4.1.b).

SUBJECT 1: PHA proliferative response of an AIDS subject receiving AL 721

	[3H]TdR incorporation x̃cpm/1x10E+06 cells/ml at			
month	-1	1	3	4
with PHA	2390	740	194	878
no PHA	813	608	200	356
net	1577	132	-6	522

SUBJECT 2: PHA proliferative response of an AIDS subject receiving AL 721

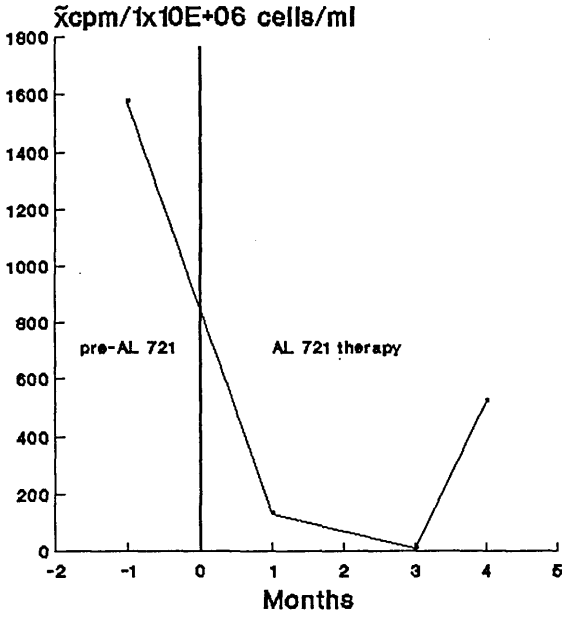
	[3H]TdR incorporation x̃cpm/1x10E+06 cells/ml at					
month	0	3	4	7	8	11
with PHA	3340	331	3755	571	756	638
no PHA	256	322	347	351	232	103
net	3084	9	3408	220	756	406

SUBJECT 3: PHA proliferative response of an ARC subject receiving AL 721

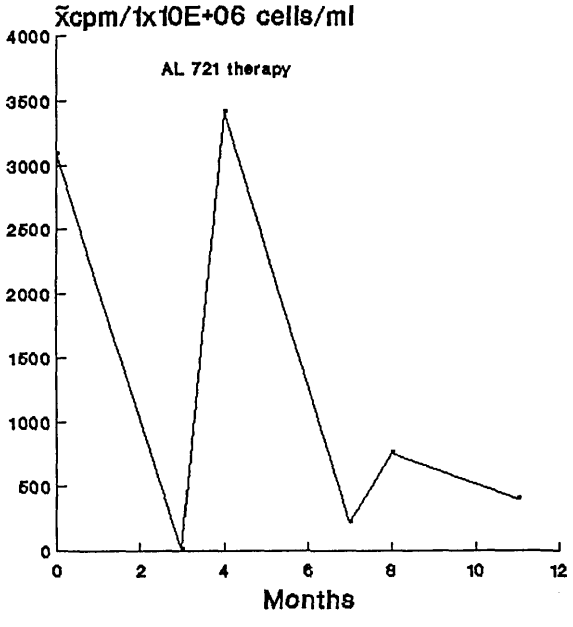
	[3H]TdR incorporation x̃cpm/1x10E+06 cells/ml at					
month	0	1	4	7	8	10
with PHA	8194	1143	971	3439	3611	2757
no PHA	174	490	154	439	298	287
net	8020	653	817	3000	3313	2470

FIGURE 25
PHA PROLIFERATIVE RESPONSE
LONGITUDINAL STUDY: AL 721

SUBJECT 1: Longitudinal net PHA proliferative response of AIDS subject, CDC IVC1, receiving AL 721



SUBJECT 2: Longitudinal net PHA proliferative response of AIDS subject, CDC IVC1, receiving AL 721



SUBJECT 3: Longitudinal net PHA proliferative response of ARC subject, CDC IVC2, receiving AL 721

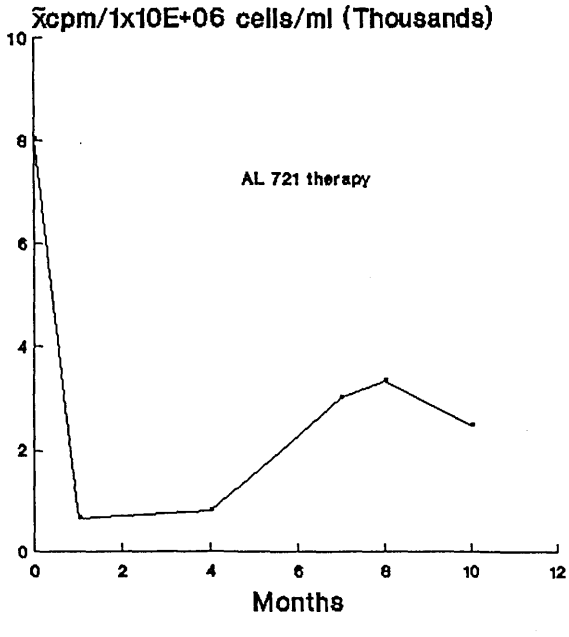


FIGURE 26
PHA PROLIFERATIVE RESPONSE
LONGITUDINAL STUDY: AL 721

3.3.5. Sequential treatment of a patient with AIDS using AL 721 and zidovudine

One AL 721 treated subject (patient 1), classified as CDC IVC1, received ZDV therapy after he discontinued AL 721. The serial, spontaneous, total and net PHA induced PBMC proliferative response was monitored throughout both treatments (Table 12) and the net response is shown graphically (Figure 27).

Four monthly tests during ZDV therapy show a clear improvement (CV: 75.4 %) of net PHA proliferation, contrasting with the deteriorating trend (CV: 131 %) during AL 721 treatment. However, the magnitude of the proliferative response with ZDV remains lower than the laboratory control group. The three month break from AL 721 was associated with a slight improvement in net PHA proliferative response.

Thus, in this case, there was progressive deterioration in PHA proliferative response during AL 721 treatment which improved slightly on its discontinuation. The subsequent use of ZDV was followed by a striking improvement indicating that this antiretroviral drug can reverse previous deterioration and implies that the deterioration on AL 721 was not an irreversible consequence of HIV-1 disease progression.

3.4. Mitogenic proliferative response: longitudinal study of the Con A induced response

Figures 28 to 30 record the serial PBMC spontaneous, total and net Con A response of laboratory control and ZDV treated subjects. HIV-1 infected patients were subdivided by their CDC groupings to ARC or AIDS. Data are limited since Con A cultures were established secondary to PHA and only when PBMC numbers permitted.

3.4.1. Laboratory control subjects

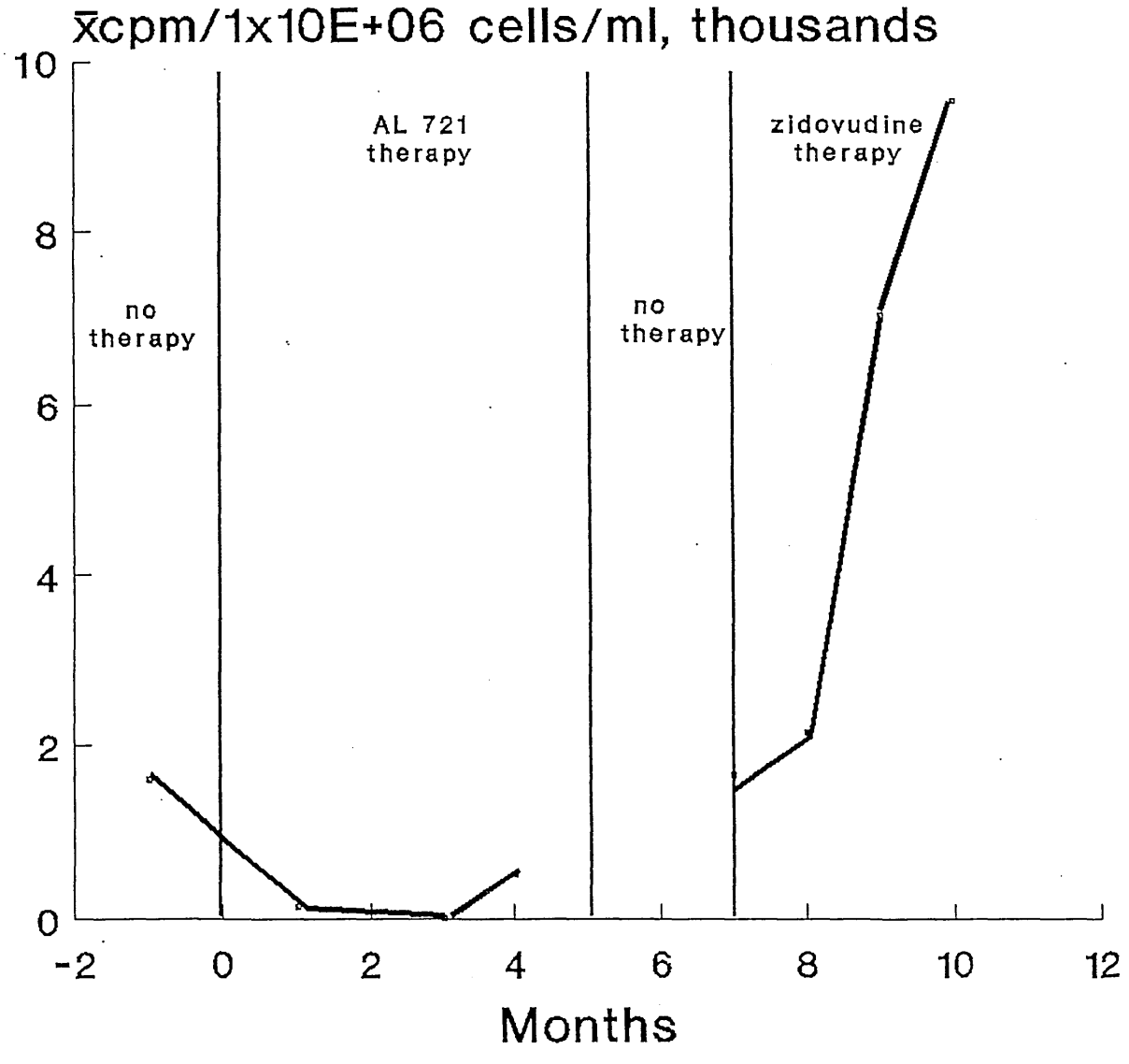
Two laboratory controls were tested over 4 and 7 months (Figure 28), and the CVs of their net Con A proliferative responses were calculated to be 21.0 % and

Table 12: PHA proliferative response of a subject receiving AL 721 and then zidovudine

	[3H]TdR incorporation x̄cpm/1x10E+06 cells/ml at						
month	-1	1	3	4	7	8	9	10
with PHA	2390	740	194	878	1773	2517	7174	9717
no PHA	813	608	200	356	134	376	146	186
net	1577	132	-6	522	1639	2141	7028	9531

Longitudinal PHA proliferative response of a subject classified CDC IVC1. Testing was at pre-AL 721 (month -1), during AL 721 treatment (months 1, 3, and 4), and throughout zidovudine treatment (months 7 to 10). There was a three month break between AL 721 and zidovudine therapy. Net PHA response is defined as total PHA minus spontaneous response.

Figure 27: Net PHA proliferative response of AIDS subject receiving AL 721 followed by zidovudine



Net PHA-driven proliferative response of subject, CDC IVC1, receiving AL 721 followed by zidovudine therapy

33.0 %. The magnitude of response ($\bar{x}$ ± SD: 17698 ± 7060 $\bar{x}$cpm/1x10^6 cells/ml) was in line with cross-sectional data ($\bar{x}$ ± SEM: 24780 ± 2147 $\bar{x}$cpm/1x10^6 cells/ml; see Table 09).

3.4.2. Zidovudine treated ARC subjects

Serial Con A proliferative determination was performed for 2 ARC (CDC IVC2) patients treated with ZDV for 3 and 2 months (Figure 29). The subjects correspond with patients 5 and 8 of the PHA proliferative longitudinal study.

Although the net Con A proliferative response of both subjects is depressed relative to controls at day zero of treatment, both of the patients' net response improve with ZDV administration, in parallel with observed clinical benefit, and this is confirmed by CVs of 92.3 % and 76.3 % respectively. In emphasis, the PHA proliferative (Figures 17 and 20), gIFN (Figures 48 and 51) and Con A gIFN (Figure 60) response of subject 5 also improved as did the PHA and Con A gIFN synthesis of patient 8. Subject 8's response has been discussed elsewhere (see section 3.3.2.).

3.4.3. Zidovudine treated AIDS subjects

The serial spontaneous, total and net Con A proliferative response was ascertained for 3 AIDS subjects receiving ZDV for 1 or 2 months (Figure 30). These individuals were patients 3 (CDC IVD), 4 (CDC IVC1) and 6 (CDC IVC1) of the longitudinal PHA proliferative study.

In cases 3 (CV: 53.4 %) and 4 (CV: 60.8 %), ZDV augmented the net Con A proliferative response and at least maintained the response of subject 6. These observations were paralleled by the serial PHA and clinical data on these patients (see section 3.3.2.).

3.5. Summary of the serial PHA and Con A proliferative responses of PBMC obtained from AL 721 and zidovudine treated ARC and AIDS patients

Both cross-sectional and serial studies demonstrated that ZDV augmented the previously decreased net

CONTROL 1: Con A proliferative response of a laboratory control subject

	[3H]TdR incorporation x̄cpm per 1x10E+06 cells/ml at			
month	0	1	2	4
with Con A	22343	31019	20336	20208
no Con A	404	894	188	355
net	21939	30125	20148	19853

CONTROL 2: Con A proliferative response of a laboratory control subject

	[3H]TdR incorporation x̄cpm per 1x10E+06 cells/ml at			
month	0	2	3	7
with Con A	8671	15427	9349	17122
no Con A	206	438	169	235
net	8465	14989	9180	16887

The longitudinal net Con A proliferative response of two laboratory controls

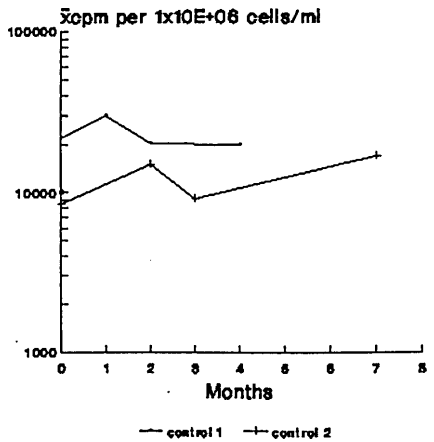


Figure 28
Con A Proliferative Response
Longitudinal Study
Laboratory Control Subjects

SUBJECT 6: Con A proliferative response of ARC subject receiving zidovudine

	[3H]TdR incorporation x̄cpm/1x10E+06 cells/ml at		
month	0	2	3
with Con A	1937	6493	15559
no Con A	184	288	203
net	1753	6215	15356

SUBJECT 8: Con A proliferative response of ARC subject 8 receiving zidovudine

	[3H]TdR incorporation x̄cpm/1x10E+06 cells/ml at		
month	0	1	2
with Con A	12069	8327	30629
no Con A	425	249	1826
net	11644	8088	28803

Longitudinal net Con A proliferativ response of two ARC subjects, CDC IVC2, receiving zidovudine

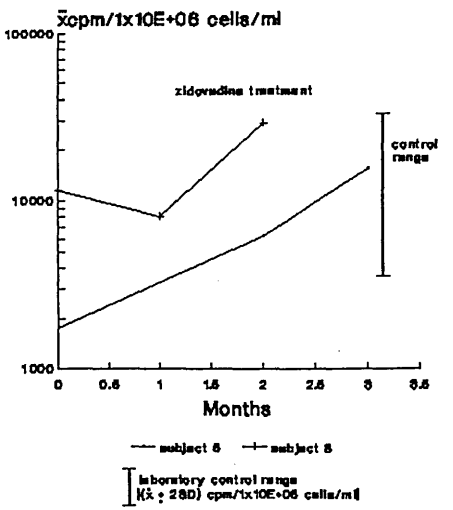


Figure 29
Con A proliferative response
Longitudinal study
ARC subjects receiving zidovudine

SUBJECT 3: Con A proliferative response of AIDS subject receiving zidovudine

	[3H]TdR incorporation x̃cpm/1x10E+06 cells/ml at	
month	0	1
with Con A	1853	6731
no Con A	222	292
net	1631	6439

SUBJECT 4: Con A proliferative response of AIDS subject receiving zidovudine

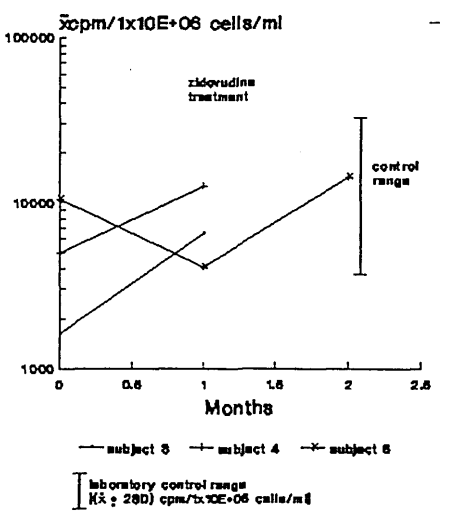
	[3H]TdR incorporation x̃cpm/1x10E+06 cells/ml at	
month	0	2
with Con A	5464	13369
no Con A	481	429
net	4983	12490

Figure 30
Con A proliferative response
Longitudinal study
AIDS subjects receiving zidovudine

SUBJECT 6: Con A proliferative response of AIDS subject receiving zidovudine

	[3H]TdR incorporation x̃cpm/1x10E+06 cells/ml at		
month	0	1	2
with Con A	10595	4463	14567
no Con A	221	396	442
net	10374	4067	14125

Longitudinal net Con A proliferative response of three AIDS subjects receiving zidovudine



mitogenic proliferative response of PBMC obtained from patients with ARC and AIDS. These findings suggest that ZDV can stem and even reverse HIV-1 induced immunosuppression. In contrast to the studies with ZDV, 3 subjects receiving AL 721 exhibited progressive deterioration of net PHA proliferative response. Furthermore, one of these patients showed significant response improvement when treated with ZDV after discontinuing AL 721.

These lymphocyte proliferative responses followed temporal trends in clinical status. Serial improvement of mitogenic proliferative response paralleled ZDV associated clinical benefit, even in those patients whose net PHA responses were studied for extended times (for example, subject 1 of ARC and 2 of AIDS PHA cohort who were tested over 16 and 12 months of therapy respectively). The cross-sectional PHA and Con A responses were consistent with these observations over similar ranges of ZDV treatment. In addition, the serial net PHA proliferative response trend also paralleled AIDS subject 1's rapid clinical deterioration. Finally, AL 721 showed no clinical, virological or neuropsychological benefit and this was reflected by deterioration of longitudinal PHA proliferative response. Therefore, mitogenic lymphocyte proliferation may act as a guide to immunological change and parallel clinical status following therapy.

3.6. Lymphocyte Function Studies: Proliferative Response to the Soluble Recall Antigen Streptokinase/Streptodornase (SK/SD)

Streptokinase/Streptodornase (SK/SD) was employed specifically to assess the antigen induced T-lymphocyte proliferative capacity of PBMC obtained from laboratory controls and HIV-1 infected subjects.

3.6.1. Optimal culture conditions for SK/SD induced PBMC proliferation

PBMC obtained from 4 SK/SD skin-test positive (as

defined by Pinching et al., 1983) laboratory controls were cultured in triplicate at $2x10^6$ cells/ml for 3, 7 and 9 days without or with SK/SD. The SK/SD skin-test preparation was used at final dilutions of 1/200, 1/2000 and 1/20000 in vitro. Figures 31, 32 and 33 illustrate the net SK/SD induced proliferative response, calculated by subtracting the mean cpm obtained without SK/SD from the mean cpm obtained with SK/SD, in each instance.

In accordance with previous studies conducted in this department (Rogers, 1987), the optimal net SK/SD induced proliferative response was observed at 7 days culture. These results are similar to those of D'Andrea et al (1986), where optimal blastogenic response to cytomegalovirus antigen was at 6 days.

Three of four controls showed optimal PBMC net proliferative response at a final dilution of 1/2000 of the SK/SD preparation, whereas Rogers (1987) found 1/20000 to be optimal. This discrepancy may illustrate the need to assess individual response over a stimulant concentration range. Unfortunately, response measurement was necessarily limited to one antigen concentration (see section 3.1.).

The SK/SD induced proliferative response was additionally tested at PBMC concentrations of $0.5x10^6$ and $1x10^6$ cells/ml. These cultures were established with SK/SD (final dilution 1/2000) or without for 7 days. However such conditions failed to elicit a detectable or consistent net proliferative response (data not shown) and possibly reflect the smaller numbers of SK/SD reactive or potentially reactive clones present at these concentrations than at $2x10^6$ cells/ml.

Therefore, PBMC were adjusted to $2x10^6$ cells/ml and cultured in triplicate with SK/SD at a final dilution of 1/2000 or without for 7 days. Employing these culture parameters caused a significant proliferative response to SK/SD in laboratory control subjects ($p < 0.05$; Figure 34). The SK/SD induced proliferative response did not attain statistical significance in the AIDS groups (Figure 34).

Figure 31: The effect of SK/SD concentration on PBMC net proliferative response after 3 days culture

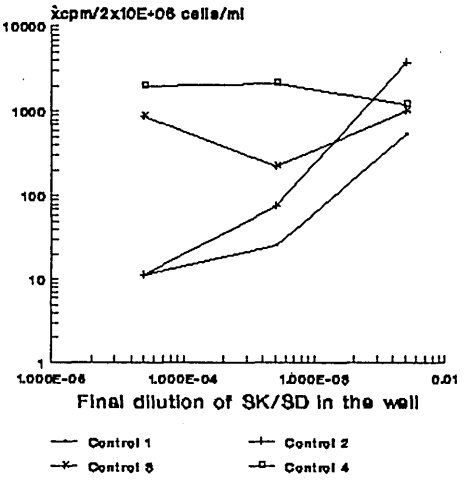


Figure 32: The effect of SK/SD concentration on PBMC net proliferative response after 9 days culture

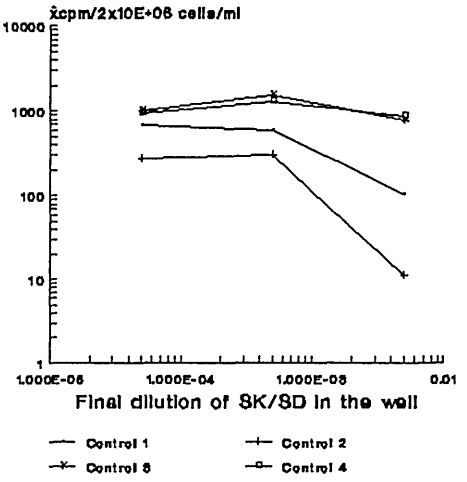
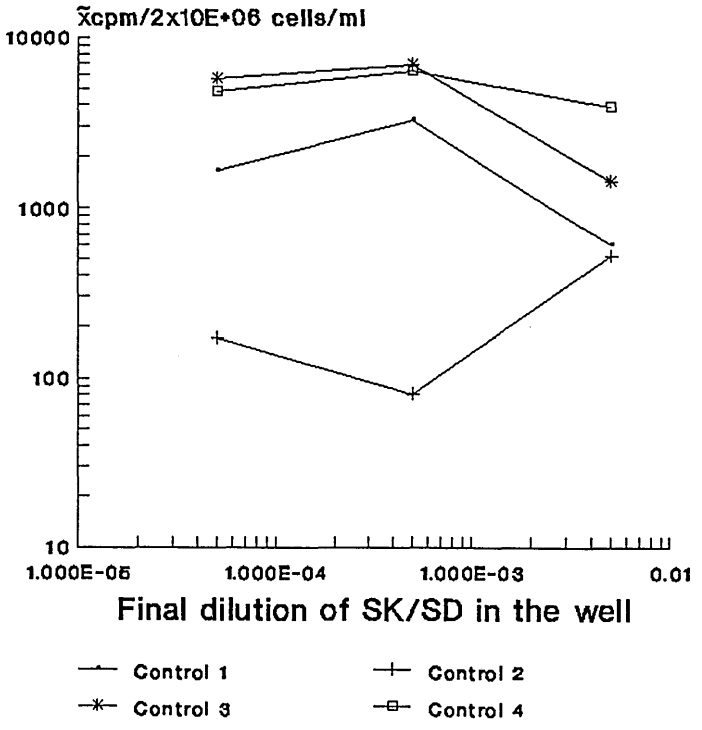


Figure 33: The effect of SK/SD concentration on PBMC net proliferative response after 7 days culture



3.6.2. The SK/SD induced net proliferative response pattern: cross-sectional study

Figure 34 tabulates the spontaneous, total and net SK/SD induced proliferative response of PBMC obtained from laboratory controls and ZDV untreated or treated AIDS patients. ZDV treatment was for a median of 2.5 months (range: 4 months). In addition, the net SK/SD proliferative response is shown graphically by Figure 35. The spontaneous PBMC proliferative levels did not differ significantly between the control and AIDS groups nor between the AIDS patient subgroups. Therefore, changes in the net proliferative response were caused only by changes in SK/SD induced PBMC proliferation.

A range of net proliferative responses was observed within the laboratory control group of 4 skin-test positive and 2 skin-test negative subjects. It was not possible to ascertain the skin-test status with respect to SK/SD for the AIDS subjects tested, but previous studies suggest cutaneous anergy would have been present in all even if they had previously been reacting (Pinching *et al.*, 1983). Less than 10 % of lymphocytes become transformed *in vitro* even in sensitised individuals challenged with the sensitising antigen; as such lymphocyte transformation *in vitro* may correlate poorly with *in vivo* delayed hypersensitivity responses (reviewed by Turk, 1980).

Similar to the observations of Rogers (1987), the net SK/SD PBMC proliferative response of ZDV untreated AIDS patients was significantly depressed in comparison with heterosexual controls ($p < 0.05$). The net SK/SD induced proliferative response of ZDV treated AIDS patients was also, although non-significantly, depressed relative to laboratory controls (Figure 34 and 35).

In contrast to the mitogen induced PBMC proliferative response (see sections 3.2.2. and 3.2.3), ZDV had no apparent effect on the net SK/SD proliferative response of PBMC obtained from AIDS subjects. Since SK/SD induced PBMC proliferation, like that of any recall antigen,

is determined by a small number of T-lymphocyte reactive clones; this system may be too insensitive to detect changes in PBMC functional capacity. Indeed SK/SD failed to induce PBMC proliferation significantly above spontaneous levels in both AIDS patient groups. This may be compounded by the selective loss of memory T-lymphocytes early in HIV-1 infection (Germain, 1988; Miedema et al., 1990), and which may be unaffected by ZDV therapy.

PBMC proliferative response to SK/SD
Laboratory control subjects

Control	[3H]TdR incorporation x̄cpm per 2x10E+06 cells/ml (± SD)		
	without SK/SD	with SK/SD	net response
1	639 ± 147	3908 ± 226	3269
2	1022 ± 13	1102 ± 85	80
3	398 ± 73	7379 ± 766	6981
4	143	6369 ± 785	6226
5	103 ± 3	728 ± 125	625
6	79 ± 30	3906 ± 668	3827

PBMC from 6 control subjects were cultured for 7 days without or with SK/SD at a final dilution of 1/2000 in the well.

PBMC proliferative response to SK/SD
AIDS, receiving zidovudine therapy

Patient	[3H]TdR incorporation x̄cpm per 2x10E+06 cells/ml (± SD)		
	without SK/SD	with SK/SD	net response
1	346 ± 138	488 ± 14	142
2	138	152 ± 35	117
3	150 ± 53	651 ± 53	501
4	123 ± 21	251 ± 114	128
5	132 ± 2	287 ± 46	155

PBMC from 5 AIDS subjects were cultured for 7 days without or with SK/SD at a final dilution 1/2000 in the well. All patients were from the cross-sectional cohort and all were classified as CDC IVC1, except patient 3 who was CDC IVD.

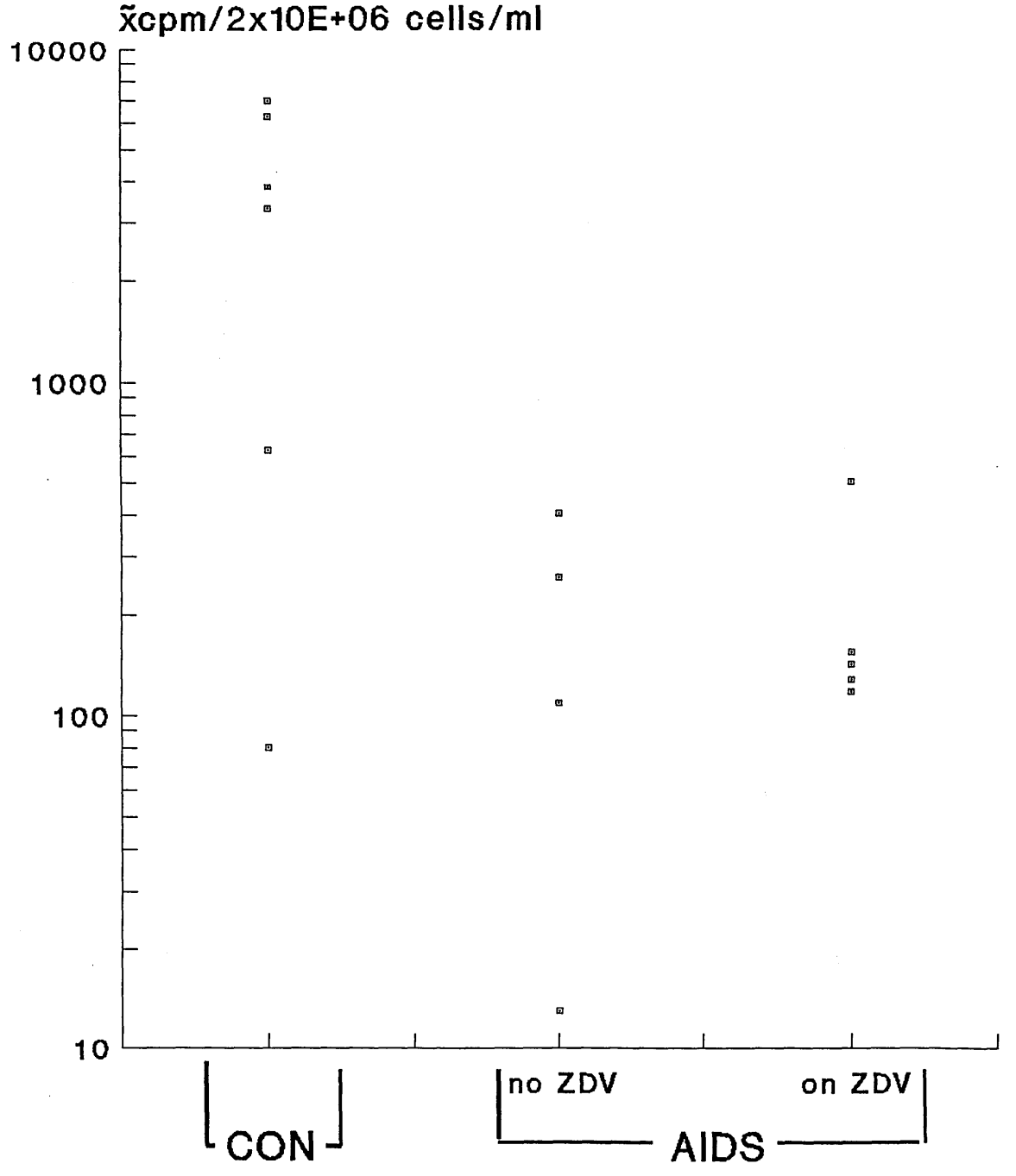
PBMC proliferative response to SK/SD
AIDS, no zidovudine therapy

Patient	[3H]TdR incorporation x̄cpm per 2x10E+06 cells/ml (± SD)		
	without SK/SD	with SK/SD	net response
1	119 ± 20	132 ± 32	13
2	194 ± 63	597 ± 150	403
3	66 + 38	325 ± 206	259
4	te	154 ± 40	NA
5	265 ± 55	374 ± 68	109

PBMC from 5 AIDS subjects, all classified as CDC IVC1, were cultured for 7 days without or with SK/SD at a final dilution of 1/2000 in the well. All patients were from the cross-sectional cohort.

Figure 34: The SK/SD proliferative response of PBMC obtained from laboratory controls and AIDS patients. HIV-1 infected individuals were subdivided by zidovudine therapy.

Figure 35: Summary of the net PBMC proliferative response to SK/SD



CHAPTER 4: RESULTS

LYMPHOCYTE FUNCTION STUDIES: gIFN RESPONSE

4. Lymphocyte Function Studies: Mitogenic gIFN Response

The majority of cell culture supernatants were assayed quantitatively for human gamma-interferon (hu gIFN) by the Celltech Diagnostic (CD) SUCROSEP™ system (see section 2.6.2.). However, when production of this kit ceased (March 1990) remaining samples and those subsequently collected were necessarily assayed using two Medgenix Diagnostics (MD) kits (see section 2.6.3.). Both are immunoradiometric assays (IRMA).

4.1. Performance characteristics of the Celltech Diagnostic gIFN kit

Discussion of the SUCROSEP™ IRMA considers precision, accuracy, sensitivity, and individual kit performance. Where data is available, characteristics of the MD system are defined. A comparative study of the two is also discussed.

4.1.1. Precision

Inter-assay variation: Precision with respect to inter-assay variation was of essential importance. Eighteen CD kits were used over a period of 9 months, all within the shelf-life of 11 weeks. It proved impossible to assay all longitudinal samples for any subject within the same kit. This was also true of cross-sectional groupings.

To control for inter-assay variation five controls were prepared at different concentrations from excess CD gIFN stock (1024 U/ml) and diluent. At least three controls and a culture medium (RPMI/C) sample were included with each run.

In summary, Table 13 suggests inter-assay variation to be acceptable except for that part of the range less than 50 U/ml. This indicates that the gIFN values obtained on assay of antigen or spontaneous supernatants may be unreliable as results were consistently less than this value.

CD quote coefficient of variance (CV) for three

Table 13: gIFN performance characteristics
Inter-assay precision (CD)

	Nominal gIFN value, U/ml	Number of assays	gIFN $\bar{x}$U/ml	SD	%CV
A	10	14	9	3	33
B	51	9	49	13	27
C	171	15	181	17	9
D	512	5	489	20	4
E	1024	5	939	66	7

Controls A to E were prepared by dilution of excess CD stock standard gIFN, 1024 U/ml. Nominal gIFN values were assigned with respect to dilution. These controls were stored in aliquots at -80°C.

mean concentrations of 6.66, 27.42, and 98.5 U/ml as 16.8 %, 12.6 % and 8.9 % respectively. Results were from twenty-two assays run over 9 periods of 6 months. Clearly CD found less inter-assay variation at the lower end of the range than this study. The number of kits assessed or the shelf-life age at assay may provide an explanation for this.

MD for their part provided three controls of mean concentrations with standard deviation of 2.5 $\pm$ 0.5, 6.5 $\pm$ 1.0 and 17.1 $\pm$ 3.0 IU/ml. For the two assays, only one control concentration average fell outside the appropriate range and that by 0.2 IU/ml.

Intra-assay variation: Intra-assay variation may also be considered as a measure of precision. CD found the intra-assay CV typically less than 10 percent over the range 6.3 - 1000 U/ml during the first 8 weeks of the products shelf-life. Over the range 3.4 - 46.7 IU/ml, MD found the CV to be less than 3.0 %.

4.1.2. Accuracy

Accuracy can be assessed by recovery or dilution test. A dilution test only was employed for both CD and MD kits. Financial constraints limited these studies to assay at neat, and dilutions 1/5 and 1/10 for the CD system and at neat, 1/10 and 1/50 for MD. This test also provided the means to ascertain optimal dilution of cell culture supernatant for assay. From the data collected (Tables 14 and 15), supernatants were processed neat by the CD system and if necessary repetition at dilution of 1/5. All mitogen stimulated supernatants were assayed at 1/50 and antigen stimulated and spontaneous neat by the MD system.

On addition of exogenous hu gIFN at three concentrations (3, 30, 300 U/ml) to 20 individual sera, CD quote recovery greater than 90 % for all. MD utilised both recovery and dilution test to assess accuracy. The former test details recovery of seven gIFN concentrations (0.5 - 36.6 IU/ml) as greater than 95 %, while serial

Table 14: gIFN performance characteristics
Dilution test of accuracy (CD)

Sample	$\bar{x}$gIFN, U/ml at Dilution 1/1	1/5	1/10
Standard	0	nd	nd
	1	nd	nd
	4	12	nd
	16	24	nd
	64	66	36
	256	243	209
	1024	985	839
Cell culture supernatant	50	71	58
	66	77	115
	386	469	449
	412	556	484
	878	1135	1160
	1760	1777	2198
	1048	1259	-

The CD standards and seven cell culture supernatants spanning the range of the Celltech Diagnostic kit were diluted 1/5 and 1/10 with culture medium. Cell culture medium was assayed alone as control. The results shown are corrected for the dilution factor.
nd: not detected

Table 15: gIFN performance characteristics
Dilution test of accuracy (MD)

Sample	$\bar{x}$gIFN, IU/ml at dilution 1/1	1/10	1/50
Standard (CD)	2.7	1.6	30
	8.0	12.0	85
	30.0	30.0	85
	*	117.0	120
	*	*	585
Cell culture supernatant	*	*	1235

CD standards 4 to 1024 and cell culture supernatant were assayed by the Medgenix system. Dilutions of 1/10 and 1/50 were made in culture medium. Cell culture medium was assayed alone as control. The results shown are corrected for the dilution factor.
nd: not detected
* : greater than upper limit of detection

dilution of culture supernatant to 1/64 showed a maximum of 1.5 IU/ml difference between theoretical and measurable concentration. However the number of tests in each case was not disclosed.

From the data collected and that quoted, it seems that greater accuracy for CD exists within the higher concentration ranges and for MD the lower. This is consistent with their intended purpose: CD for culture supernatant, and MD for sera assay.

4.1.3. Sensitivity

CD found the minimum detection limit (defined as the gIFN concentration equivalent 2.5 times the intra-assay standard deviation at zero gIFN concentration) as typically less than one U/ml. This figure is quoted for the first eight weeks of the product's shelf-life only. Included in the SUCROSEP™ IRMA data reduction package for an individual assay was the sensitivity calculation. The mean minimum detection limit for sixteen assays was found to be 2.83 U/ml, with standard error of the mean 0.41. Of the two remaining assays, one result was clearly spurious and data is not available for the other. The explanation of difference may lie again in numbers and age of kit at assay.

On compilation of gIFN results, a net concentration of less than 3 U/ml was interpreted as not detectable and all net figures were corrected to whole numbers.

MD defined sensitivity by measuring the zero dose standard 20 times and assigned a gIFN concentration corresponding to the mean cpm plus two standard deviations, 0.2 IU/ml. Values obtained by the MD system found to be less than 0.2 IU/ml were considered not detectable and all values quoted to one decimal place.

4.1.4. Individual kit performance

The performance of any individual assay was assessed by minimal and maximal binding and by the variance

ratio of standard values with best fit line.

Minimal or non-specific binding (Bmin) and maximal binding (Bmax) are defined as the mean cpm of the zero or stock dose respectively, expressed as a percentage of the mean total count cpm. Mean values for the eighteen CD assays with SEM are 0.8 % $\pm$ 0.07 and 28.8 % $\pm$ 1.26. This is acceptable for IRMA (K. Nye, personal communication).

The two MD kits had average Bmin and Bmax values of 2.3 % and 46.8 %.

The F ratio or variance of points about the drawn line should be as close to unity as possible. For fifteen CD assays the mean F ratio was found to be 1.84 with SEM 0.37. Two assays produced spurious results and data is not available for the third.

By the criteria outlined, the assays performed were satisfactory.

4.1.5. Typical gIFN reference curve

Table 16 with Figure 36 demonstrate an example of a typical CD reference curve, while Table 17 with Figure 37 show one of the two reference curves obtained by the MD system.

4.1.6. Comparison of Celltech and Medgenix Diagnostics hu gIFN measurement

A comparative study of CD and MD hu gIFN IRMA became essential when production of CD kits ceased and a number of supernatant samples were processed by the MD system. It seemed unlikely that a linear relationship existed between the two kits given their difference of units (CD values quoted as U/ml whereas MD as IU/ml); standard range (0 - 1024 U/ml for CD and 0 - 90 IU/ml for MD) and designed assay sample population (culture supernatant as opposed to sera for CD and MD respectively). Eighteen samples were assayed by CD and MD IRMA simultaneously. Samples consisted of the MD standard range (neat); CD standards 4 to 1024 U/ml (neat all but standard 16 diluted 1/10 with cell culture

Table 16: Example of typical gIFN reference curve - Celltech Diagnostics

	x̄cpm	SD	%CV
Total Counts	52975	566	1.1
Standard, U/ml	537	40	7.4
1	614	38	6.2
4	987	256	26.0
16	1700	25	1.5
64	4493	67	1.5
256	10403	36	0.4
1024	20229	115	0.6

Bmin: 1.0%
Bmax: 38.2%

Table 17: Example of typical gIFN reference curve - Medgenix

	x̄cpm	SD	%CV
Total Counts	72328	291	0.4
Standard, IU/ml	1552	4	0.2
1	1831	192	10.5
2	2846	267	9.4
5	4269	203	4.8
10	6672	146	2.2
30	16153	85	0.5
90	35951	1076	3.0

Bmin : 2.2%
Bmax: 49.7%

Figure 36: Example of typical gIFN reference curve - Celltech Diagnostics

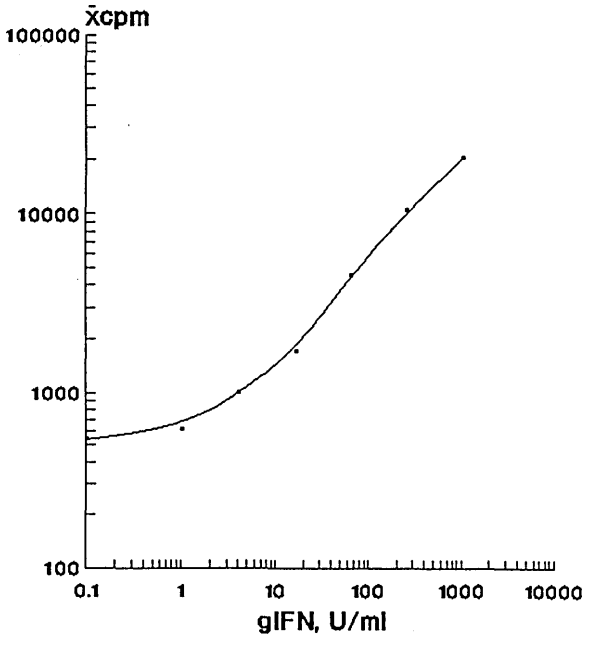
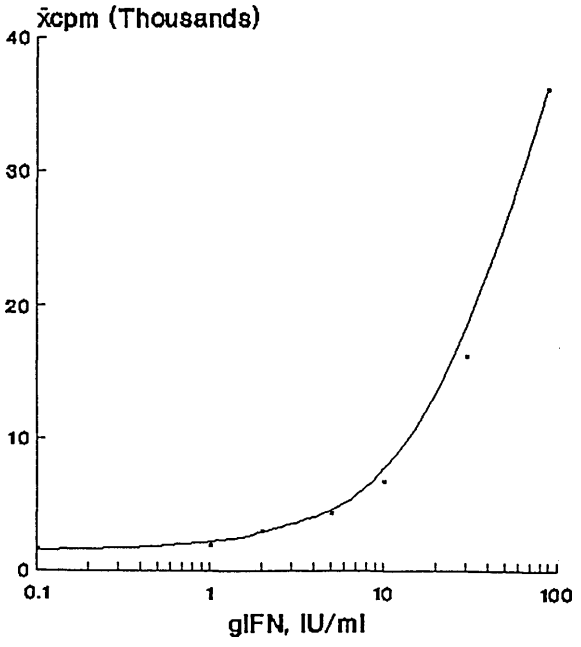


Figure 37: Example of typical gIFN reference curve - Medgenix



medium), CD controls A, B, C (neat), D (diluted 1/10); and a PHA stimulated supernatant diluted 1/50. The samples chosen ensured definition for the complete MD concentration range. A conversion factor was calculated and applied to MD obtained values correcting them for compatibility with the CD system.

4.1.6.a Calculation of conversion factor

Table 18 records the raw data collected from the above experiment and also the CD to MD ratio for each sample. The ratio mean and SEM was found to be 2.6 $\pm$ 0.14 for fifteen samples. Two ratios were excluded as spurious and zero dose ratio not applicable.

4.1.6.b Validity of the conversion factor

Each MD sample value (IU/ml) was converted to a CD compatible value (U/ml) by multiplication with the factor of 2.6 (Table 19).

The conversion factor was validated by comparing the CD and adjusted MD results as two alternative methodologies (J. Wadsworth, personal communication). By the suggested strategy of Altman and Bland (1983) it was shown that the latter results could replace the former with sufficient accuracy of measurement.

Figure 38 plots the adjusted MD values (method B) against those obtained by CD (method A) for each sample. For the purpose of comparison the line of identity (A=B) was drawn. The figure suggests a linear relationship approximating to unity over the concentration range tested. However, much of this plot is empty space and a large range of measurements would better the appearance of agreement.

In confirmation of the suggested relationship, the method difference (A-B; Table 20) was plotted against the average (A+B/2; Table 20) for each sample (Figure 39). The even scatter about X = 0 indicates size of measurement has little or no effect on the relationship. No further analysis was thought necessary (J. Wadsworth, personal communication).

Table 18 : Arithmetic ratio relating Celltech and Medgenix gIFN measurements

Sample	Nominal gIFN value by:- CD, U/ml	Medgenix, IU/ml	Arithmetic association CD / Medgenix
1	0	0	-
2	3	1	3.0
3	8	2	4.0
4	14	5	2.8
5	31	10	3.1
6	85	30	2.8
7	228	90	2.5
8	16	8	2.0
9	64	30	2.1
10	10	5	2.0
11	48	22	2.2
12	162	72	2.3
13	10	2	5.0*
14	69	30	2.3
15	320	117	2.7
16	989	517	1.9
17	72	17	4.2*
18	3380	1235	2.7
Mean ± SEM			2.8 ± 0.14

* : not included in the calculation of mean ± SEM as obviously spurious

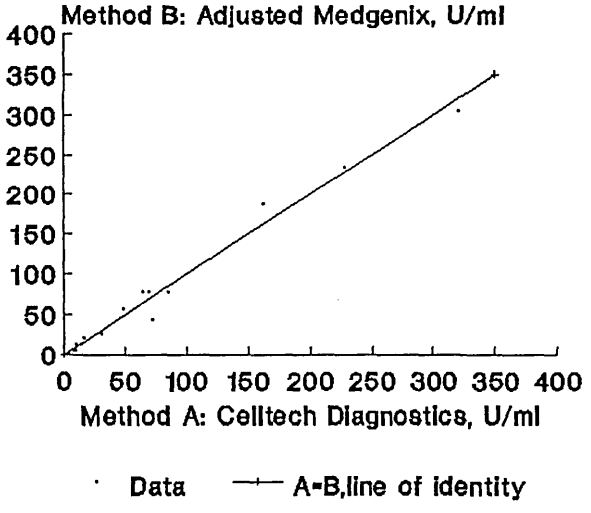
Table 19 : Medgenix gIFN values adjusted by the calculated constant

Sample	Nominal gIFN value by:- CD, U/ml	Medgenix, IU/ml	2.8 (Medgenix, IU/ml) U/ml
1	0	0	0
2	3	1	2.8
3	8	2	5.2
4	14	5	13.0
5	31	10	28.0
6	85	30	78.0
7	228	90	234.0
8	16	8	20.8
9	64	30	78.0
10	10	5	13.0
11	48	22	57.2
12	162	72	187.2
13	10	2	5.2
14	69	30	78.0
15	320	117	304.2
16	989	517	1344.2
17	72	17	44.2
18	3380	1235	3211.0

Table 20: Calculation of Method Difference (A-B) & Average (A+B/2) for CD & Adjusted Medgenix Values

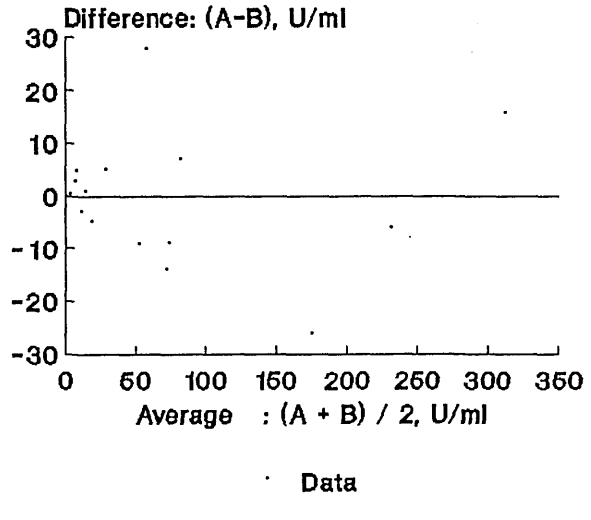
Sample	CD, U/ml A	Adjusted M. B	Difference A-B	Average (A+B)/2
1	0	0	0	0
2	3	2.8	0.4	2.8
3	8	5.2	2.8	6.6
4	14	13.0	1.0	13.5
5	31	26.0	5.0	28.5
6	85	78.0	7.0	81.5
7	228	234.0	- 6.0	231.0
8	16	20.8	- 4.8	18.4
9	64	78.0	- 14.0	71.0
10	10	13.0	- 3.0	11.5
11	48	57.2	- 9.2	52.6
12	162	187.2	- 25.2	174.6
13	10	5.2	4.8	7.6
14	69	78.0	- 9.0	73.5
15	320	304.2	15.8	312.1
16	989	1344.2	-355.2	1166.6
17	72	44.2	27.8	58.1
18	3380	3211.0	169.0	3295.5

Figure 38
Comparison of Celltech Diagnostic with adjusted Medgenix gIFN values



Samples 16 and 18 are not plotted because of scale. However both approximate to the line of identity.

Figure 39
Method diffrence (A-B) against average (A+B/2) for CD & adjusted Medgenix values



Samples 16 and 18 are not plotted because of scale.
Method A: CD Method B: Adjusted Medgenix

In summary, the conversion factor of 2.6 was found reliable over the MD concentration range and was applied to samples assayed by the two MD kits. gIFN values obtained by this method are however indicated in the text. This factor also provides the means to convert CD gIFN units to international units.

4.2. Addition of formol saline

gIFN assays were processed within the class 3 containment laboratory, however the gamma-counter was located in a communal departmental area. To inactivate any residual HIV-1 at the end of assay and comply with safety standards (see Safety, Appendix V.4.), 200 ul 10 % formol saline was added to each tube (see section 2.6.2.f). A set of standards was counted without and then with formol saline, thereby controlling for effect on counts. The results of Table 21 demonstrate no significant difference in counts by addition of formol saline.

4.3. Optimal culture conditions for gIFN production: mitogen induced gIFN synthesis

Two mitogens have proved particularly effective inducers of gIFN synthesis. These are PHA and Con A (Murray, 1988; Trinchieri and Perussia, 1985). Both specifically activate T-lymphocytes, although by different ligands and second messenger pathways (Beverley et al., 1987). In view of this difference, while PHA was utilised primarily, Con A cultures were established when PBMC numbers permitted. As polyclonal activators, both PHA and Con A reveal the gIFN synthesising potential of the entire repertoire of T-lymphocytes.

Parameters of mitogen culture for gIFN synthesis have been documented, listing variables as mitogen concentration, culture time and PBMC concentration (for example, Eales et al., 1987). Limited repetition served to establish agreement within this laboratory. Two further variables of hiFCS concentration and supernatant harvest

Table 21: The effect of formol saline on gIFN standard counts

Sample	mean counts before formol saline	mean counts after formol saline
Total counts	46381	46334
Standard 1024 U/ml	9777	9854
256 U/ml	4987	4750
64 U/ml	1757	1727
16 U/ml	732	743
4 U/ml	446	456
1 U/ml	366	335
0	354	339

A set CD standards were counted before and after the addition of 200 ul 10 % formol saline, thereby controlling for any effect on counts.

methodology were investigated. Figures 40 to 43 record these standardisation experiments.

Since HIV-1 seropositive subjects gave a low PBMC yield, results here were obtained using conditions which gave optimal proliferation, so that the proliferation and gIFN cultures could be combined.

4.3.1. Optimal mitogen concentration

PBMC obtained from two laboratory controls were cultured in quintupliate at 1.0×10^6 cells/ml for 72 h as previously described (see section 2.5.1.) with PHA or Con A at final concentration in the microtitre wells ranging from 0.1 to 10 ug/ml and 0.1 to 20 ug/ml respectively. PBMC were also cultured without mitogen. Figure 40 records the effect of mitogen concentration on net production of gIFN. Net gIFN production was calculated by subtracting the spontaneous from the total mitogen induced value for each subject.

gIFN synthesis was induced maximally at final PHA concentrations ranging from 1 to 10 ug/ml. The final PHA concentration adopted throughout was 1 ug/ml and caused significant, $p < 0.01$, gIFN production in control and all but one subject group where significance was $p < 0.05$. The PHA concentration was in agreement with optimal concentration for proliferative response (see section 3.1.1.).

Con A induced gIFN production most effectively at 5 ug/ml. Compromise was necessary as the proliferative response was maximal at 10 ug/ml (see section 3.1.1.). The final working concentration of Con A adopted was 10 ug/ml and caused significant gIFN production ($p < 0.01$) in control and subject groups.

4.3.2. Optimal culture time

Extensive documentation suggests 72 h as optimal for mitogen induced gIFN synthesis (for example Eales et al., 1987). A time course study of net PHA driven gIFN production for three laboratory controls is illustrated by Figure 41. Results indicate maximal net PHA induced gIFN synthesis

between 72 and 120 h. 72 h was in fact adopted as the PHA and Con A end-point and agrees with proliferative study.

4.3.3. Optimal PBMC concentration

The effect of PBMC concentration, donated by three laboratory controls, on PHA driven net production is demonstrated by Figure 42. For two controls little difference was observed between PBMC concentration of 1.0 and 2.0×10^6 cells/ml, while the third shows maximum gIFN production at 0.5×10^6 cells/ml. PBMC were adjusted to 1.0×10^6 cells/ml throughout for mitogen gIFN and proliferative cultures.

4.3.4. The effect of hiFCS concentration

Investigation of hiFCS concentration was prompted by its demonstrated effect on spontaneous and PHA induced total proliferation levels (Rogers, 1987). PBMC donated by three laboratory controls were cultured with or without PHA and hiFCS concentration was varied (Figure 43). No significant difference in spontaneous production of gIFN was observed over the hiFCS range. A slight increase in total PHA induced gIFN production was seen for two of the controls at 15 and 20 % hiFCS. Maximal gIFN production was not supported at less than 5 % hiFCS. The concentration of hiFCS adopted throughout was 5 %, in agreement with proliferative studies (see section 3.1.4.).

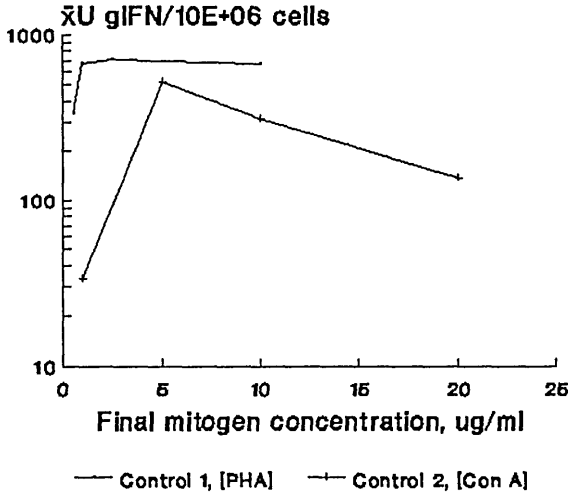
4.3.5. Supernatant harvest

Combination of proliferative and gIFN cultures was so far possible. However, a final experiment ensured that there was no significant difference between gIFN levels when the whole 200 ul supernatant was harvested as opposed to 100 ul from the culture (see section 2.5.4.).

Duplicate cultures of PBMC donated by three laboratory controls were established. These were cultured in quintuplet for 72 h with or without mitogen at 1.0×10^6 PBMC/ml. 200 ul or 100 ul of supernatant from duplicate cultures were harvested and assayed for gIFN (Table 22).

Figure 40

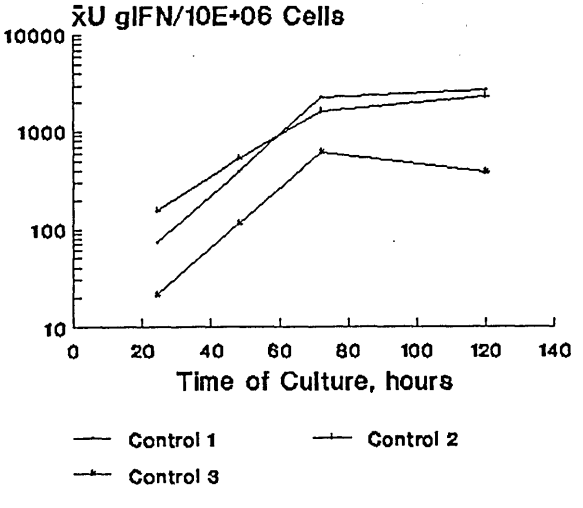
The effect of mitogen concentration on the net production of gIFN



PBMC from two controls were cultured with PHA or Con A concentration range Supernatants assayed for gIFN.

Figure 41

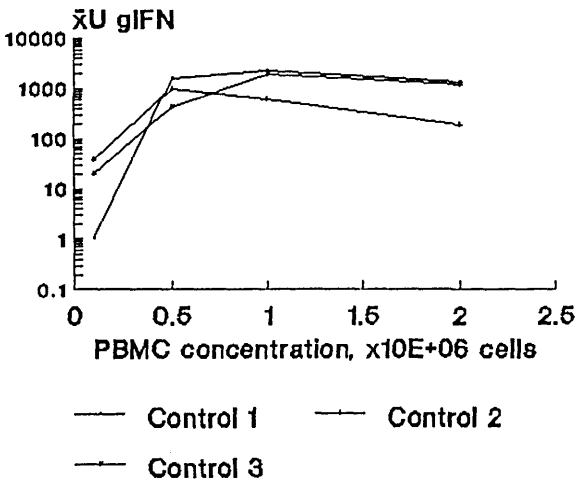
Time course: the net PHA driven production of gIFN against time



PBMC from three controls cultured with PHA (1ug/ml) or without. Supernatant harvested at 24, 48, 72 and 120 h.

Figure 42

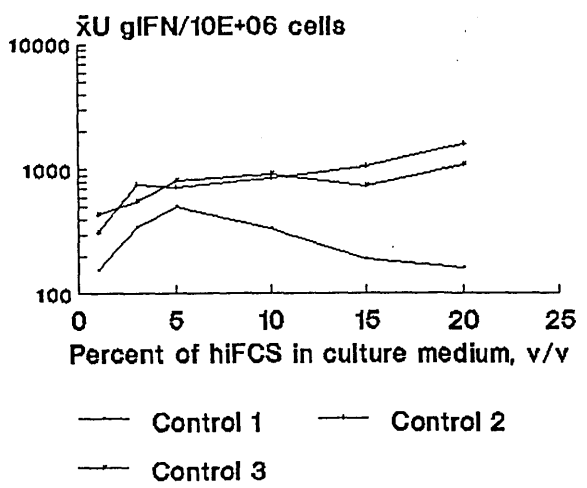
The effect of PBMC concentration on PHA driven net production of gIFN



PBMC from three controls were culture with PHA (1ug/ml) or without for 72h. PBMC concentration as variable.

Figure 43

The effect of hIFCS concentration on PHA net production of gIFN



PBMC from three laboratory controls cultured with PHA (1ug/ml) or without for 72h. Supernatant assayed for gIFN.

Table 22: Comparison of methodology for gIFN supernatant harvest

Subject	Net mitogen-driven gIFN, xU/ml	
	200ul supernatant	100ul supernatant
1	386	417
2	449	431
2*	229	208
3	3523	2280

Duplicate cultures were established for PBMC from three laboratory controls. PBMC were cultured for 72h with or without PHA (2* Con A) at 1.0x10E+06 cells/ml. 200ul or 100ul of supernatant were harvested from five identical wells and assayed for gIFN.

The results obtained showed no significant difference between methodologies for three of the four results recorded. The difference in subject 3 may be explained by the high concentration of gIFN. Proliferation and gIFN cultures were therefore run simultaneously.

4.4. Mitogenic gIFN response: cross-sectional study

The PBMC spontaneous and total PHA or Con A driven production of gIFN is recorded in Tables 57 to 72 (Appendices III and IV) for laboratory controls and HIV-1 infected subjects. Patients were categorised by CDC grouping and ZDV therapy, as for the proliferative cross-sectional study. A summary of spontaneous, total and net gIFN production for each group is presented by Tables 23 and 24. The spontaneous production of gIFN by PBMC did not influence net mitogenic synthesis in any subject group.

4.4.1. Net gIFN response to PHA and Con A by non-zidovudine treated HIV-1 infected individuals: natural history

Similar to earlier studies (Murray et al., 1984, 1985), the AIDS patients' net gIFN response to both PHA and Con A was profoundly decreased relative to laboratory controls ($p < 0.01$; Table 25 and 26), as was the PHA response of ARC subjects ($p < 0.01$; Table 25). Figure 45 indicates that the Con A response of ARC subjects and the mitogenic gIFN production by symptomless patients are likewise depressed, although these differences did not attain statistical significance (Table 26). Therefore, substantial depression of mitogenic gIFN response occurs with symptomatic HIV-1 disease.

There is an obvious decrease of net gIFN mitogenic response with HIV-1 disease progression and this observation reflects proliferative results (Figures 44 and 45). The mitogenic response of AIDS patients was significantly depressed in comparison with the asymptomatic ($p < 0.01$) and ARC subjects ($p < 0.05$). The PHA system demonstrated a

Table 23: PHA driven gIFN production: summary of control and patient subject response

	gIFN $\bar{x}$U per 1x10E+06 cells ± SEM			
	without PHA	with PHA	net response	
CONTROL	5 (0.2 - 13)	1340 ± 198 (1142 - 1538)	1337 ± 198 (1139 - 1535)	N=13
As, no ZDV	5 (2 - 9)	1007 ± 339 (674 - 1350)	1012 ± 338 (668 - 1346)	N=7
ARC, no ZDV	5 (0.2 - 6)	375 ± 165 (210 - 540)	374 ± 165 (209 - 539)	N=6
ARC, ZDV	2 (0.1 - 15)	835 ± 213 (622 - 1048)	831 ± 213 (618 - 1044)	N=8
AIDS, no ZDV	2 (0.1 - 23)	90 ± 32 (58 - 122)	88 ± 32 (56 - 120)	N=21
AIDS, ZDV	4 (0.1 - 45)	406 ± 113 (293 - 519)	399 ± 113 (286 - 512)	N=11

The mean value of gIFN is given for the total and net PHA response, with range^{SEM} in parenthesis, for each subject group. The median value of detectable spontaneous gIFN synthesis, with range, is quoted.
ZDV: zidovudine

Table 24: Con A driven gIFN production: summary of control and patient subject response

	gIFN $\bar{x}$U per 1x10E+06 cells ± SEM			
	without Con A	with Con A	net response	
CONTROL	1 (0.2 - 6)	670 ± 160 (510 - 830)	668 ± 160 (508 - 828)	N=12
As, no ZDV	7 (3 - 9)	504 ± 148 (356 - 652)	499 ± 149 (350 - 648)	N=6
ARC, no ZDV	2 (0.2 - 5)	269 ± 73 (196 - 342)	268 ± 72 (196 - 340)	N=6
ARC, ZDV	2 (1 - 15)	647 ± 196 (451 - 843)	644 ± 196 (448 - 840)	N=7
AIDS, no ZDV	0.2 (0.1 - 23)	96 ± 31 (65 - 127)	94 ± 31 (63 - 125)	N=13
AIDS, ZDV	3 (0.1 - 45)	484 ± 169 (315 - 653)	477 ± 171 (306 - 648)	N=9

The mean value of gIFN is given for the total and net Con A response, with range$_{SEM}$ in parenthesis, for each subject group. The median value of detectable spontaneous gIFN synthesis, with range, is quoted.
ZDV: zidovudine

Table 25
Net PHA driven gIFN response

	AS no ZDV	ARC no ZDV	ARC on ZDV	AIDS no ZDV	AIDS on ZDV
CONTROL	ns	0.01	ns	0.01	0.01
AS, no ZDV	-	0.05	ns	0.01	0.05
ARC, no ZDV	-	-	ns	0.05	ns
ARC, on ZDV	-	-	-	0.01	ns
AIDS, no ZDV	-	-	-	-	0.01

Comparison of PHA driven gIFN response, the independent variables being CDC classification groups and zidovudine (ZDV) therapy.
Significant results are quoted as figures and represent probability less than 0.01 or 0.05 ($p < 0.01$ or 0.05).
Non significant results are represented as ns.

N.B. Statistical analysis was by Student t-test.
Although we must be aware that the greater the number of separate t-tests the greater the risk of mistakenly rejecting the null hypothesis, the data shown here is unlikely to present this problem (J. Wadsworth, personal communication).

Table 26
Net Con A driven gIFN response

	AS no ZDV	ARC no ZDV	ARC on ZDV	AIDS no ZDV	AIDS on ZDV
CONTROL	ns	ns	ns	0.01	ns
AS, no ZDV	-	ns	ns	0.01	ns
ARC, no ZDV	-	-	ns	0.05	ns
ARC, on ZDV	-	-	-	0.01	ns
AIDS, no ZDV	-	-	-	-	0.01

Comparison of Con A driven gIFN response, the independent variables being CDC classification groups and zidovudine (ZDV) therapy.
Significant results are quoted as figures and represent probability less than 0.01 or 0.05 ($p < 0.01$ or 0.05).
Non significant results are represented as ns.

N.B. Statistical analysis was by Student t-test.
Although we must be aware that the greater the number of separate t-tests the greater the risk of mistakenly rejecting the null hypothesis, the data shown here is unlikely to present this problem (J. Wadsworth, personal communication).

Figure 44: Cross-sectional study Summary of the net PHA driven gIFN response

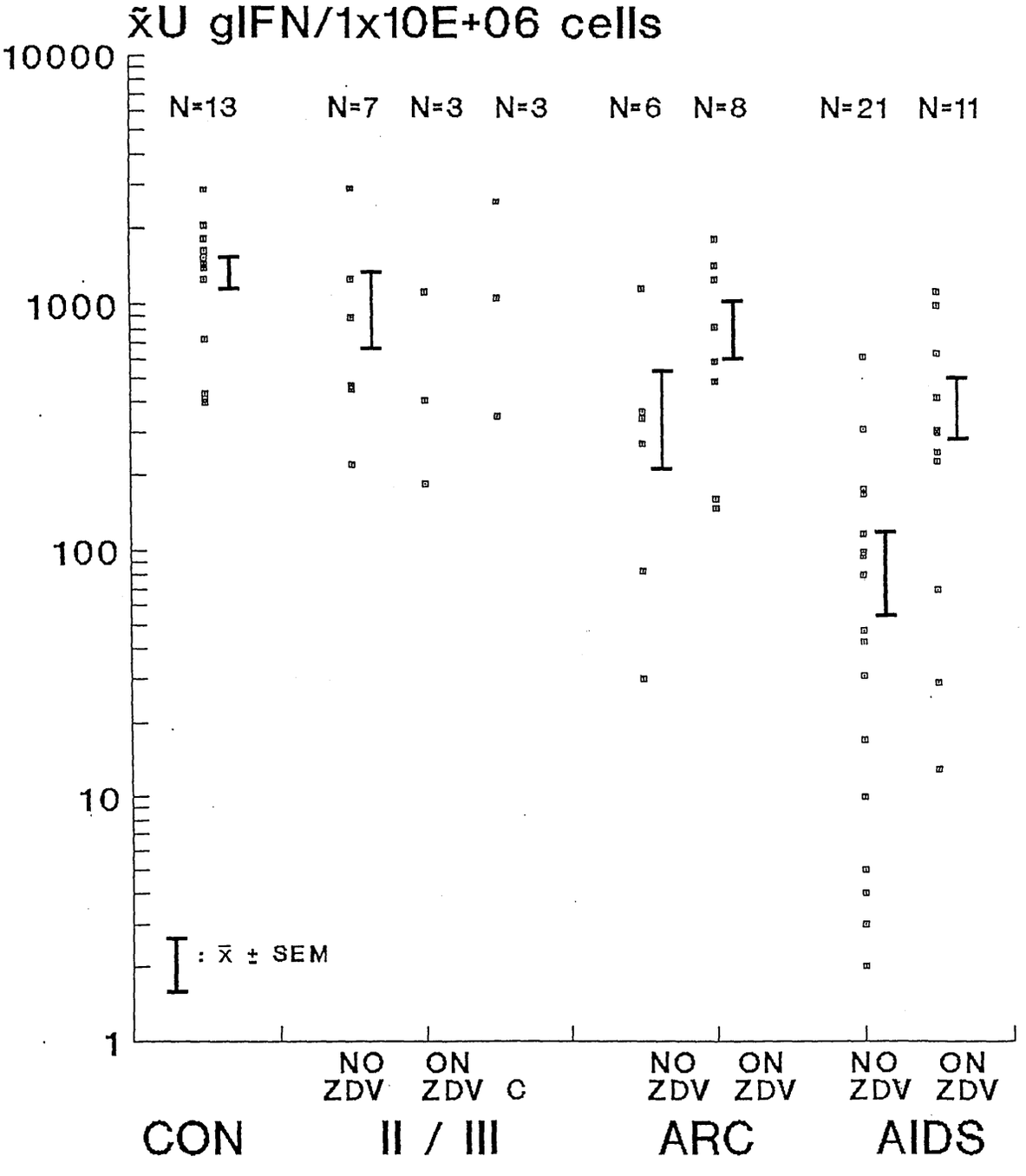
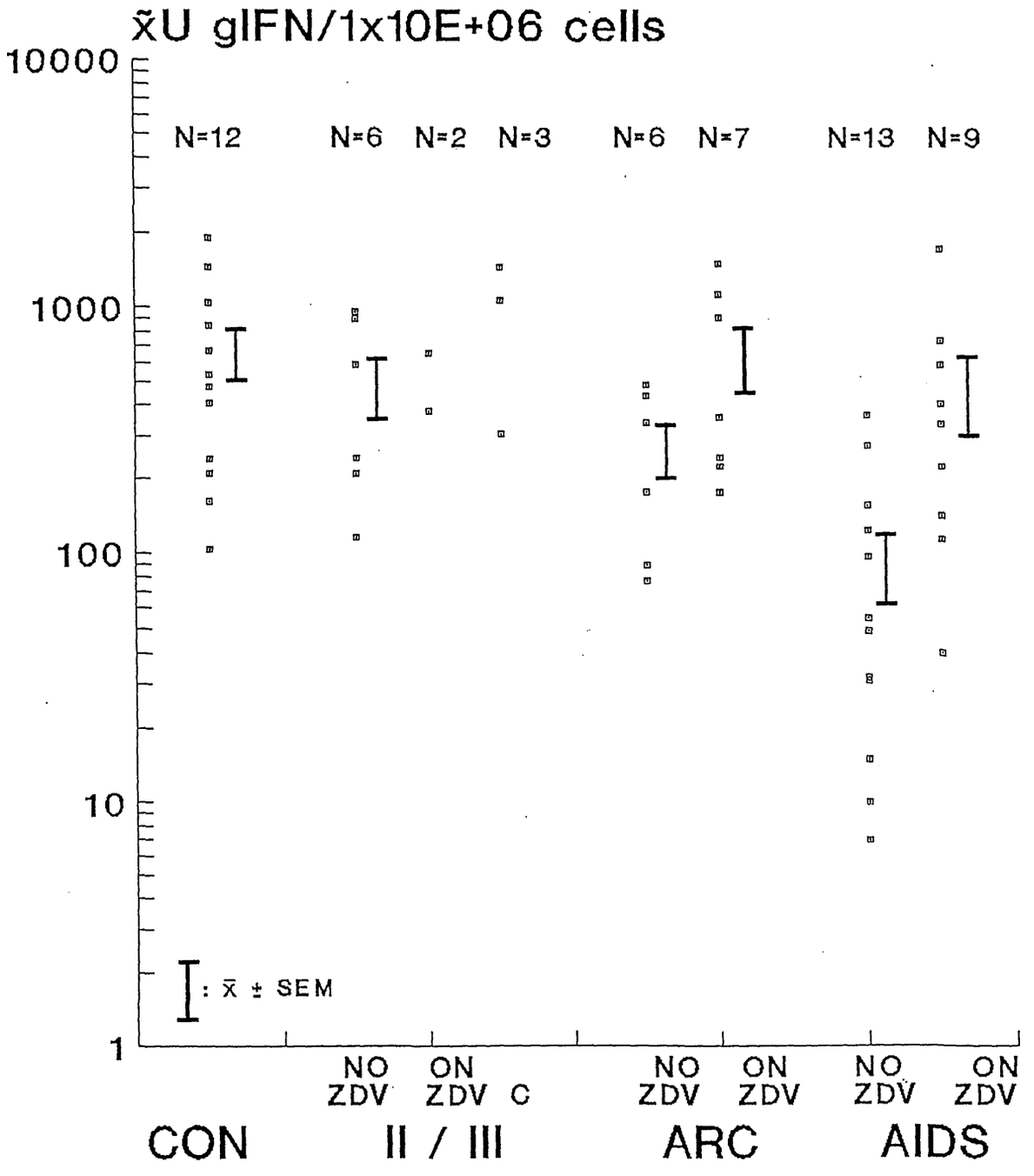


Figure 45: Cross-sectional study Summary of the net Con A driven gIFN response



profound depression of the ARC response relative to symptomless individuals ($p < 0.05$) and a similar trend was suggested by the Con A asssay.

4.4.2. The effect of zidovudine therapy on the net mitogenic gIFN response of HIV-1 infected individuals

ZDV treatment of AIDS patients caused a significant elevation of both their PHA and Con A gIFN responses ($p < 0.01$; Tables 25 and 26) over median treatment times of 3.5 and 4 months respectively (range: 1 to 30 months). An increase of ARC patients mitogenic response was also apparent with ZDV therapy, although this was not statistically significant. The ARC patients PHA response was ascertained at a median of 1.25 months (range: 1 to 18 months) ZDV administration and the Con A response at a median of 2.5 months (range: 1 to 4.5 months) treatment.

Subject numbers prevent comment on ZDV treatment of asymptomatic subjects and on the Concorde group.

4.4.3. The mitogenic response level of zidovudine treated ARC and AIDS subjects

The PHA assay indicated that ZDV therapy elevates AIDS patients' PBMC gIFN production to the response level of untreated ARC subjects, while the Con A system suggests that the increase is to laboratory control level. This discrepancy may indicate the Con A system to be less sensitive than the PHA system, and was also concluded from proliferative results (see section 3.2.3.). Both mitogens indicated that the ARC gIFN response is increased to control levels.

4.5. Mitogenic gIFN response: longitudinal study of the PHA induced response

The PBMC spontaneous, total and net PHA driven production of gIFN is recorded for each laboratory control (Figures 46 and 47) and HIV-1 infected subject. As for the longitudinal PHA proliferative response (see section 3.3.),

patients were categorised by therapy to ZDV and AL 721 treated groups. Subjects receiving ZDV therapy were subdivided by their CDC grouping to ARC (Figures 48 to 51) or AIDS (Figures 52 to 55). Three patients were followed through AL 721 therapy (Figure 56 and 57) and one of these subjects was monitored when he was subsequently treated with ZDV (Table 27, Figure 58).

Within the patient study groups, individuals are numerically labelled to correspond with the serial studies of their mitogenic proliferative response (see sections 3.3. and 3.4.).

Analysis is restricted to comparison of controls' and patients' coefficient of variation (see section 2.10.2.). The laboratory control range of net PHA response ($\bar{x} \pm 2SD$ U gIFN per $1x10^6$ cells) is indicated on ZDV treated patient graphs.

4.5.1. Laboratory control subjects

Four laboratory controls were followed for a median time of 4.75 months (range: 4 to 6 months). The magnitude of these controls net response ($\bar{x} \pm SD$: 2004 ± 750 $\bar{x}$U gIFN/$1x10^6$ cells) is consistent with cross-sectional data ($\bar{x} \pm SEM$: 1337 ± 198 $\bar{x}$U gIFN/$1x10^6$ cells; see Table 23). Individuals 1 to 4 were calculated to have net response CVs of 14.3 %; 35.7 %; 24.9 % and 36.1 % respectively (median 27.75 %; range: 14.3 % to 35.7 %).

4.5.2. Zidovudine treated ARC subjects

The PHA driven PBMC synthesis of gIFN was studied serially for 7 ZDV treated ARC (CDC IVC2) patients, and these correspond with individuals 2 to 8 of the longitudinal PHA proliferative study. These subjects were followed for a median of 3 months (range: 1 to 7 months) therapy from before ZDV or day zero administration in 6 instances and from 1 month of therapy in the remaining case. The net PHA induced gIFN response is shown graphically (Figures 50 and 51).

It was observed that the net PHA gIFN response

Figure 46: PHA gIFN response: longitudinal study of laboratory control subjects

CONTROL 1: PHA DRIVEN gIFN RESPONSE

	gIFN x̄U per 1x10E+06 cells at						
month	0	1	2	3	3.5	4	5	5.5
PHA	2276	2966	3370	2176	2967	2777	2619	2938
no PHA	nd	2	0.4	3	nd	nd	1	nd
net	2276	2964	3370	2173	2967	2777	2618	2938

nd: not detectable

CONTROL 2: PHA DRIVEN gIFN RESPONSE

	gIFN x̄U per 1x10E+06 cells at				
month	0	2	3	3.5	4
with PHA	2280	1236	2032	2475	1022
no PHA	0.4	nd	nd	0.2	nd
net	2280	1236	2032	2475	1022

nd: not detectable

CONTROL 3: PHA DRIVEN gIFN RESPONSE

	gIFN x̃U per 1x10E+06 cells at				
month	0	2	3	3.5	4
with PHA	1480	1760	1099	927	1272
no PHA	2	nd	nd	nd	6
net	1478	1760	1099	927	1266

nd: not detected

CONTROL 4: PHA DRIVEN gIFN RESPONSE

	gIFN x̃U per 1x10E+06 cells at				
month	0	2	3	4	6
with PHA	1418	2670	1457	1825	1071
no PHA	nd	4	nd	nd	nd
net	1418	2666	1457	1825	1071

nd: not detected

THE LONGITUDINAL NET PHA DRIVEN gIFN RESPONSE OF FOUR LABORATORY CONTROLS

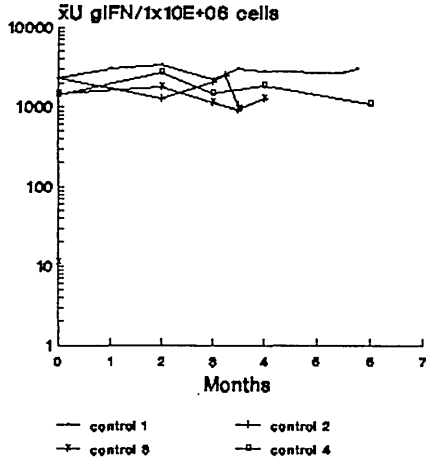


FIGURE 47
PHA gIFN RESPONSE
LONGITUDINAL STUDY
LABORATORY CONTROL SUBJECTS

SUBJECT 2: PHA driven gIFN response
of ARC subject receiving zidovudine

	gIFN x̃U per 1x10E+06 cells at					
month	0	1	2	3	4	7*
with PHA	1150	1188	2080	1105	1418	1749
no PHA	0.2	nd	1	0.4	1	nd
net	1150	1188	2079	1105	1417	1749

month 7* by Medgenix gIFN IRMA
nd: not detected

SUBJECT 3: PHA driven gIFN response
of ARC subject receiving zidovudine

	gIFN x̃U per 1x10E+06 cells at		
month	1	2	7*
with PHA	161	31	336
no PHA	1	nd	2
net	160	31	334

month 7* by Medgenix gIFN IRMA
nd: not detected

SUBJECT 4: PHA driven gIFN response
of ARC subject receiving zidovudine

	gIFN x̃U per 1x10E+06 cells at		
month	-1	0	3
with PHA	110	575	810
no PHA	nd	nd	nd
net	110	575	810

nd: not detected

SUBJECT 5: PHA driven gIFN response
of ARC subject receiving zidovudine

	gIFN x̃U per 1x10E+06 cells at			
month	0	1	2	3
with PHA	31	1265	2230	974
no PHA	0.5	8	nd	nd
net	31	1257	2230	974

nd: not detected

FIGURE 48
PHA DRIVEN gIFN RESPONSE
LONGITUDINAL STUDY
ARC SUBJECTS RECEIVING ZIDOVUDINE

SUBJECT 6: PHA driven gIFN response
of ARC subject receiving zidovudine

	gIFN x̃U per 1x10E+06 cells at	
month	0	1
with PHA	373	587
no PHA	5	2
net	368	585

SUBJECT 7: PHA driven gIFN response
of ARC subject receiving zidovudine

	gIFN x̃U per 1x10E+06 cells at	
month	0	1
with PHA	270	500
no PHA	nd	15
net	270	485

nd: not detected

SUBJECT 8: PHA driven gIFN response
of ARC subject receiving zidovudine

	gIFN x̃U per 1x10E+06 cells at		
month	0	1*	2*
with PHA	1000	1825	1749
no PHA	4	3	nd
net	996	1822	1749

month 1* and 2* : results by Medgenix gIFN IRMA
nd : not detected

FIGURE 49
PHA DRIVEN gIFN RESPONSE
LONGITUDINAL STUDY
ARC SUBJECTS RECEIVING ZIDOVUDINE

Figure 50: Longitudinal net PHA driven gIFN response of two ARC subjects, CDC IVC2, receiving zidovudine

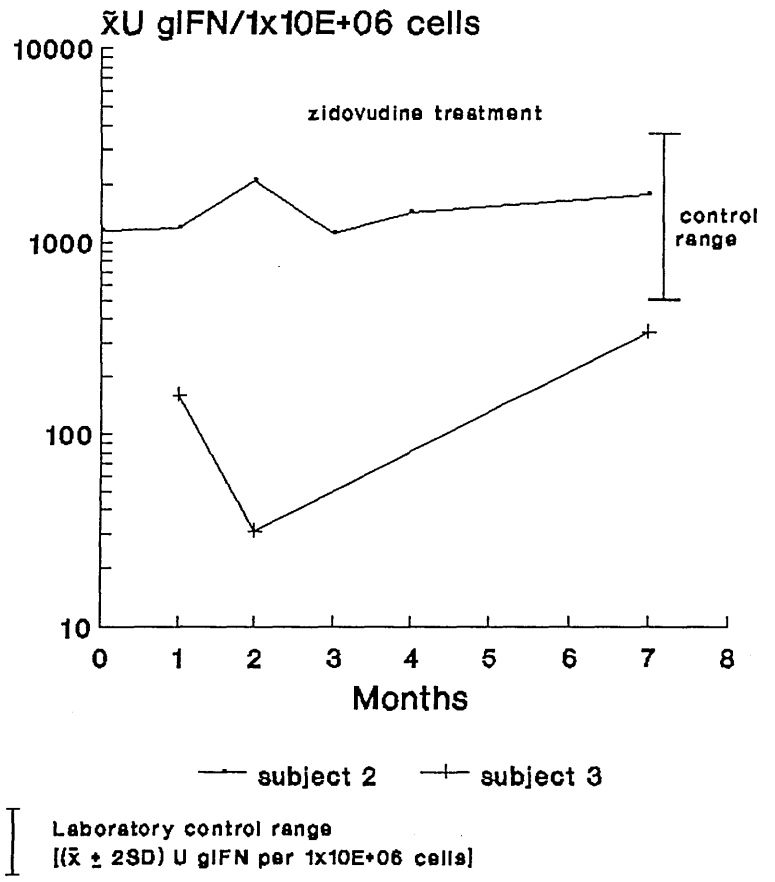
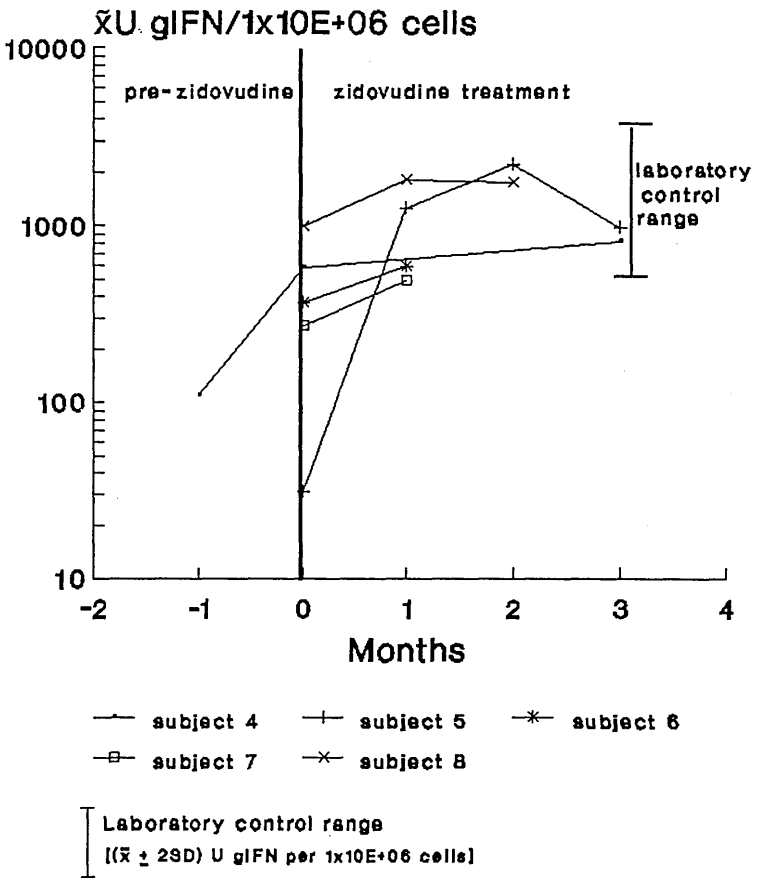


Figure 51: Longitudinal net PHA gIFN response of five ARC subjects, CDC IVC2, receiving zidovudine



values of 6 patients before ZDV therapy were decreased relative to the controls in 4 cases (patients 4 to 7), and approached control levels in 2 (patients 2 and 8).

Temporal monitoring revealed that ZDV caused improvement of net gIFN production in 6 subjects. These were individuals 2 (CV: 27.0 %); 4 (CV: 71.5 %); 5 (CV: 80.6 %); 6 (CV: 32.2 %); 7 (CV: 42.2 %) and 8 (CV: 30.0 %). However, since the CVs of subjects 2, 6 and 8 were similar to those of the controls (see section 2.10.2.), these results must be interpreted with respect to individual or experimental variation. The trend of improvement seen here was also observed by serial PHA proliferative assay for subjects 2 and 4 to 7. Subject 8 has been discussed elsewhere (see section 3.3.2.).

The net gIFN responses of individual 3, determined during ZDV treatment, was erratic.

Five of the seven ARC subjects showed improvement towards control levels, as determined by their net PBMC gIFN response to PHA, during 1 to 7 months (median: 2 months) of ZDV therapy and parallels ZDV associated clinical benefit (B. Peters, personal communication; see section 3.3.2.). This observation is consistent with the non-significant although apparent ZDV augmented increase of response made over a median of 1.25 months (range: 1 to 18 months) for the cross-sectional study (see section 3.2.2.).

4.5.3. Zidovudine treated AIDS subjects

The serial net PBMC gIFN response is shown graphically by Figures 54 and 55 for 6 ZDV treated AIDS subjects. These patients were followed for a median of 3 months (range: 1 to 12 months) of therapy and are numbered to correspond with the longitudinal PHA proliferative AIDS cohort (see section 3.3.3.).

ZDV treatment of 4 AIDS patients (subjects 3 to 6) was associated with a striking improvement of PHA gIFN response, and was confirmed by individual CVs of 131 %; 127 %; 135 % and 162 %. However, the gIFN response of subject

SUBJECT 1: PHA driven gIFN response
of AIDS subject receiving zidovudine

	gIFN x̃U per 1x10E+06 cells at			
month	5	9	10	11
with PHA	8	5	2	1
no PHA	nd	0.6	1	nd
net	8	4	1	1

nd : not detected

SUBJECT 2: PHA driven gIFN response
of AIDS subject receiving zidovudine

	gIFN x̃U per 1x10E+06 cells at							
month	-1	0	3	4	8	10	11	12
with PHA	2	59	27	21	40	5	88	6
no PHA	nd	nd	nd	7	nd	nd	nd	3
net	2	59	27	14	40	5	88	3

nd : not detected

SUBJECT 3: PHA driven gIFN response
of AIDS subject receiving zidovudine

	gIFN x̃U per 1x10E+06 cells at	
month	0	1
with PHA	5	207
no PHA	nd	0.2
net	5	207

nd : not detected

FIGURE 52
PHA DRIVEN gIFN RESPONSE
LONGITUDINAL STUDY
AIDS SUBJECTS RECEIVING ZIDOVUDINE

SUBJECT 4: PHA driven gIFN response
of AIDS subject receiving zidovudine

	gIFN x̃U per 1x10E+06 cells at		
month	-4	0	2*
with PHA	28	31	1259
no PHA	4	2	5
net	24	31	1254

month 2* : result by Medgenix gIFN IRMA
nd : not detected

SUBJECT 5: PHA driven gIFN response
of AIDS subject receiving zidovudine

	gIFN x̃U per 1x10E+06 cells at		
month	0	2	4*
with PHA	104	191	1463
no PHA	8	nd	2
net	96	191	1461

month 4* : result by Medgenix gIFN IRMA
nd : not detected

SUBJECT 6: PHA driven gIFN response
of AIDS subject receiving zidovudine

	gIFN x̃U per 1x10E+06 cells at		
month	0	1*	2*
with PHA	2	40	161
no PHA	0.1	5	nd
net	2	35	161

month 1*/2* : result by Medgenix gIFN IRMA
nd : not detected

FIGURE 53
PHA DRIVEN gIFN RESPONSE
LONGITUDINAL STUDY
AIDS SUBJECTS RECEIVING ZIDOVUDINE

Figure 54: Longitudinal net PHA driven gIFN response of two AIDS subjects, CDC IVC1, receiving zidovudine

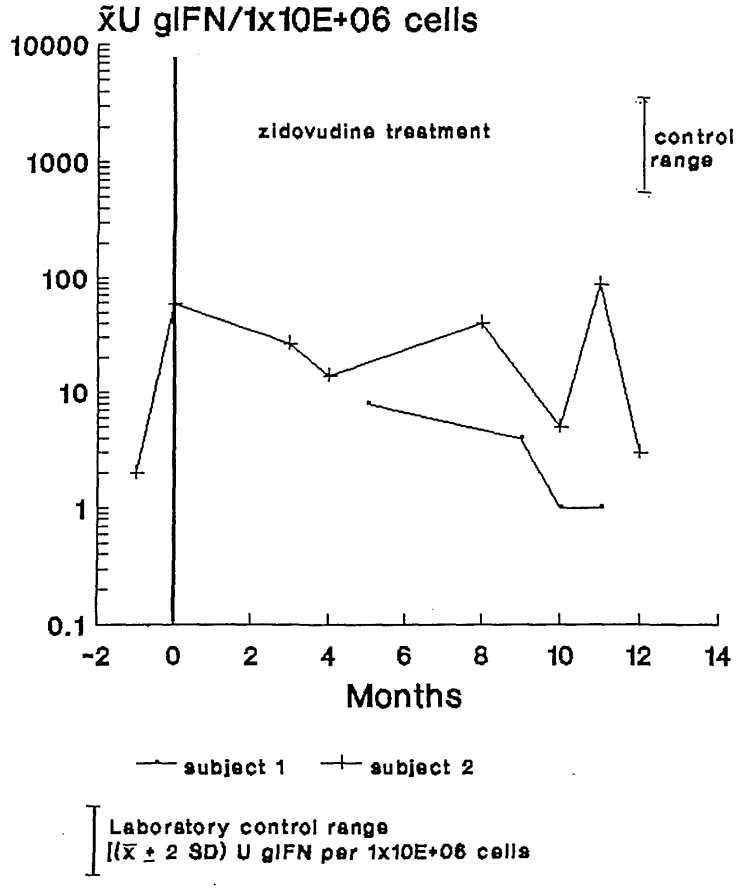
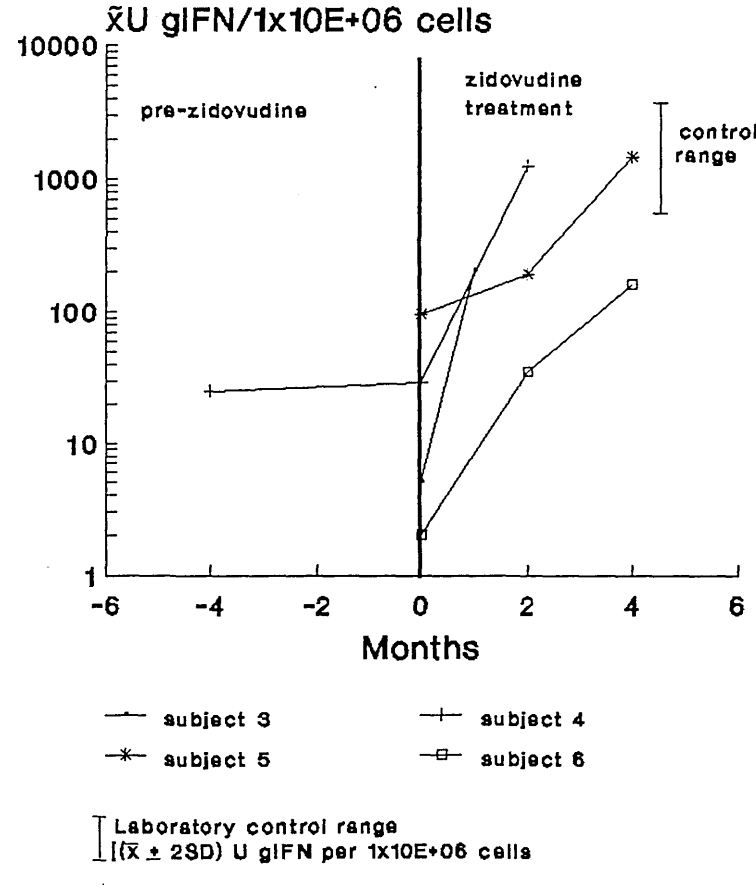


Figure 55: Longitudinal net PHA driven gIFN response of four AIDS subjects receiving zidovudine



1 was notably depressed throughout months 4 to 11 of treatment and deteriorated throughout that period. The trends of response delineated for these patients are consistent with proliferative results and clinical observations (see section 3.3.3.).

The net PHA induced production of gIFN by PBMC obtained from subject 2 was erratic and tended to decline from day zero of therapy; although the PHA proliferative response (see section 3.3.3.) of this patient was also erratic, a trend of improvement was observed. These results do not reflect well the observations that subject 2's clinical status was reasonable with ZDV, although therapy was discontinued earlier this year when ZDV associated bone marrow suppression manifested as low neutrophil count (B. Peters, personal communication). This patients results may be interpreted with regard to his unusual T-lymphocyte phenotype (see section 7.6.).

Serial testing showed marked ZDV associated improvement of the net PHA gIFN response in 4 of 6 AIDS patients. These 4 patients were monitored over 1 to 4 months (median: 2 months) of therapy. These results are consistent with cross-sectional observations (see section 3.2.2.) where ZDV treatment over 1 to 30 months (median: 3.5 months) caused a significant elevation of AIDS patients net gIFN response to PHA. The in vitro gIFN response to PHA also paralleled the clinical deterioration of subject 1.

4.5.4. AL 721 treated HIV-1 infected subjects

The serial net PHA induced gIFN response of PBMC obtained from 1 ARC and 2 AIDS AL 721 treated patients are shown graphically (Figure 57). The PHA proliferative responses of these patients have also been monitored, and details of the AL 721 pilot study, clinical, virological and neuropsychological data are discussed within that context (see section 3.3.4.).

The net gIFN response of all 3 study subjects, especially subject 1, were markedly decreased relative to

SUBJECT 1: PHA driven gIFN response
of AIDS subject receiving AL 721

	gIFN x̄U per 1x10E+06 cells at			
month	-1	1	3	4
with PHA	3	nd	nd	2
no PHA	nd	3	nd	2
net	3	nd	nd	nd

nd: not detected

SUBJECT 2: PHA driven gIFN response
of AIDS subject receiving AL 721

	gIFN x̄U per 1x10E+06 cells at					
month	0	2	3	7	8	11
with PHA	117	89	6	nd	8	12
no PHA	nd	nd	nd	nd	nd	7
net	117	89	6	nd	8	5

nd: not detected

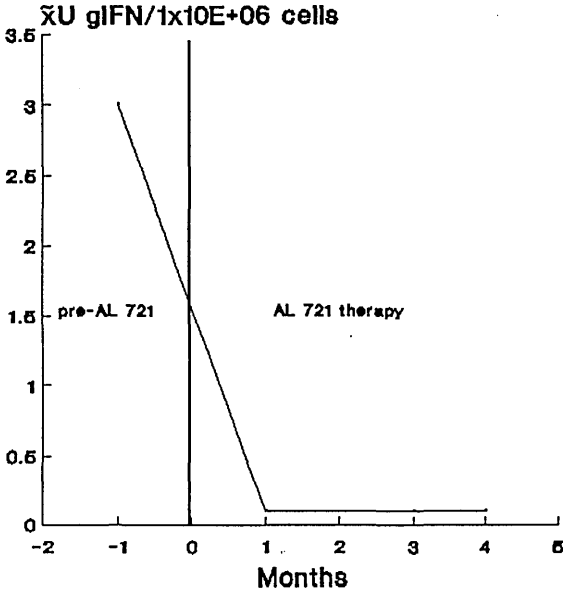
SUBJECT 3: PHA driven gIFN response
of ARC subject receiving AL 721

	gIFN x̄U per 1x10E+06 cells at					
month	0	2	4	7	8	10
with PHA	83	65	53	49	81	71
no PHA	nd	nd	2	1	1	3
net	83	65	51	48	80	68

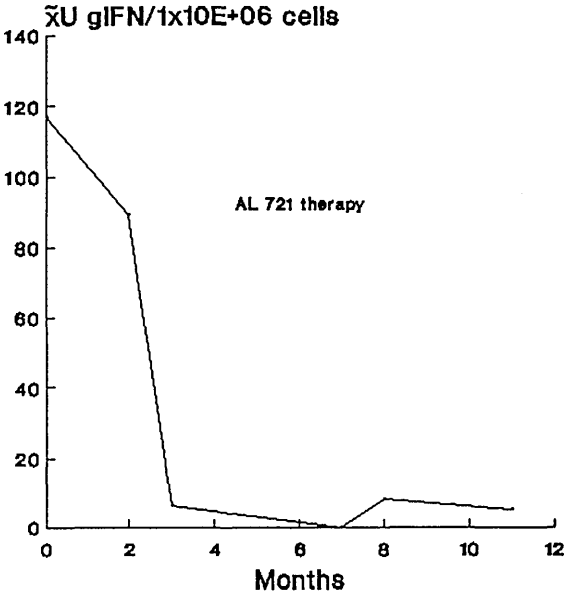
nd: not detected

FIGURE 56
PHA DRIVEN gIFN RESPONSE
LONGITUDINAL STUDY: AL 721

SUBJECT 1: Longitudinal net PHA gIFN response of AIDS subject, CDC IVC1, receiving AL 721



SUBJECT 2: Longitudinal net PHA gIFN response of AIDS subject, CDC IVC1, receiving AL 721



SUBJECT 3: Longitudinal net PHA gIFN response of ARC subject, CDC IVC2, receiving AL 721

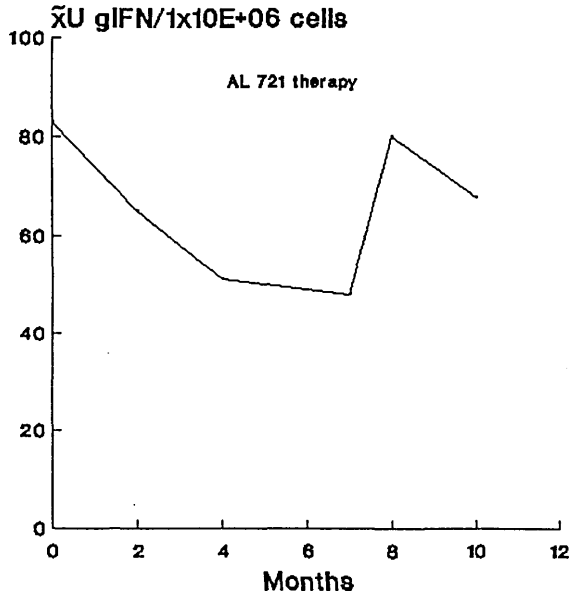


FIGURE 57
PHA DRIVEN gIFN RESPONSE
LONGITUDINAL STUDY: AL 721

laboratory control levels before therapy. This is consistent with cross-sectional observations (see Table 23).

AL 721 treatment is associated with a profound and progressive deterioration of net PHA response in subject 2. The serial gIFN levels for subject 1 remained at the lower limit of detection over 4 months of therapy. The third subect's response decreased to month 8 and then improved to almost pre treatment levels before dropping again; however, an overall trend of deterioration was apparent over 10 months of AL 721 therapy.

In summary AL 721 treatment of these subjects was associated with a decline of PBMC net gIFN production in response to PHA and reflect the observations of the proliferative serial study (see section 3.3.4.). Thus AL 721 appears to have no effect or even a detrimental effect on immunological competence, as determined by in vitro gIFN synthesis. Again, in emphasis, these studies of AL 721 provide a useful contrast to the studies with ZDV therapy.

4.5.5. Sequential treatment of a patient with AIDS using AL 721 and zidovudine

The serial net PHA gIFN response of PBMC obtained from this AIDS subject followed the pattern of his proliferative response (see section 3.3.5.); persistent depression duing AL 721 treatment was followed by a striking improvement with the use of ZDV (CV: 91.8 %; see Table 27 and Figure 58). However, the magnitude of response remained depressed in comparison with control levels throughout ZDV treatment. This observation reflects his clinical status during this time - he improved with ZDV, although starting therapy sometime after diagnosis of AIDS he did not do as well as comparable patients commenced shortly after diagnosis (B. Peters, personal communication). There was no detectable change of response in the 3 months between therapies.

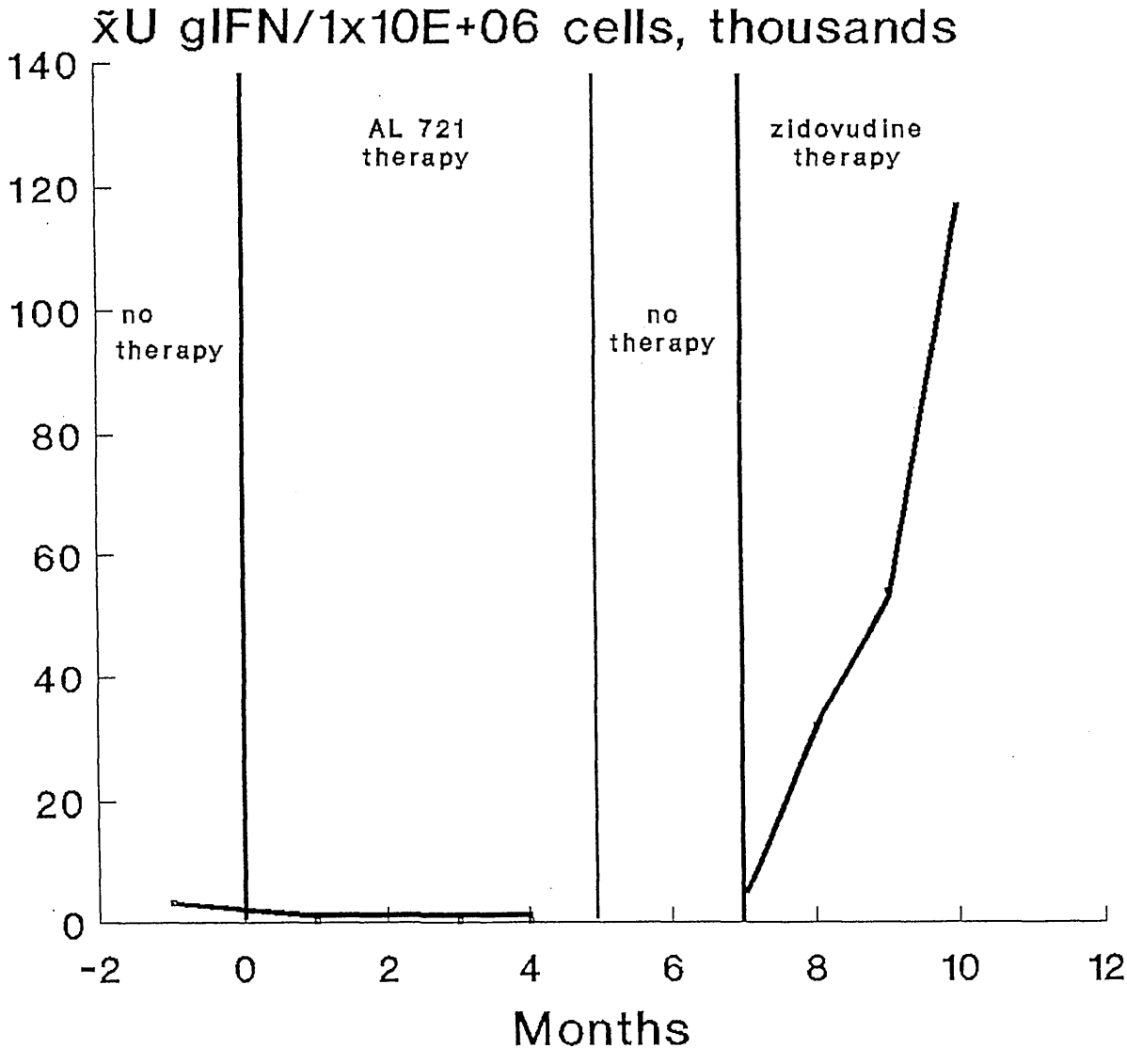
Similar to the proliferative results obtained for this patient (see section 3.3.5.), these data suggest that ZDV can reverse HIV-1-associated immunosuppression while

Table 27: PHA gIFN response of a subject receiving AL 721 and then zidovudine

	gIFN $\bar{x}$U per 1x10E+06 cells							
month	-1	1	3	4	7	8	9	10
with PHA	3	nd	nd	2	5	32	54	118
no PHA	nd	3	nd	2	nd	nd	nd	1
net	3	nd	nd	nd	5	32	54	117

Longitudinal PHA gIFN response of a subject classified CDC IVC1. Testing was at pre-AL 721 (month -1), during AL 721 treatment (months 1, 3, and 4), and throughout zidovudine treatment (months 7 to 10). There was a three month break between AL 721 and zidovudine therapy.
Net PHA response is defined as total PHA minus spontaneous response.
nd : not detected

Figure 58: Net PHA driven gIFN response of AIDS subject receiving AL 721 followed by zidovudine



Net PHA driven gIFN response of subject, CDC IVC1, receiving AL 721 followed by zidovudine therapy

AL 721 has no apparent effect.

4.6. Mitogenic gIFN response: longitudinal study of the Con A induced response

The serial spontaneous, total and net PBMC gIFN response to Con A is tabulated for each laboratory control and ZDV treated ARC or AIDS subject studied. HIV-1 infected individuals are labelled to correspond with previous longitudinal functional patient studies. Data are limited for identical reasons to those outlined in the Con A proliferative serial study (see section 3.4.).

4.6.1. Laboratory control subjects

A single laboratory control subject was tested regularly over a period of 4 months (Figure 59). His Con A induced PBMC net synthesis of gIFN had a CV of 27.3 % and the magnitude of response was consistent with cross-sectional data (see Table 24).

4.6.2. Zidovudine treated ARC subjects

Figure 60 delineates the serial Con A gIFN response of 2 ARC (CDC IVC2) patients, subjects 5 and 8 of previous studies, receiving ZDV for 3 and 2 months respectively.

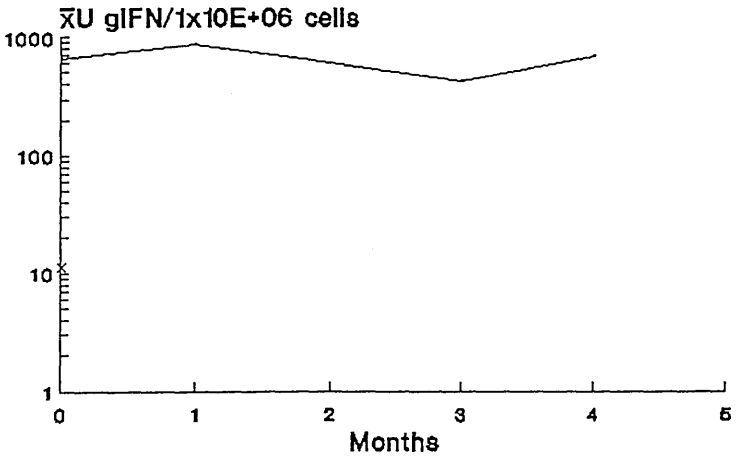
The net PBMC response to Con A of subject 5 was poor at day zero, while subject 8's synthesis of gIFN approached normal. Both subject responses were raised (CV: 93.5 % and 61.4 % respectively) above control levels during ZDV therapy. The data for subject 5 is supported by other serial functional studies (see Figures 17 and 20; 29; 48 and 51), while subject 8's results are in accordance with all but his serial PHA proliferative response and this has been discussed elsewhere (see section 3.3.2.). These observations are consistent with the cross-sectional study (see section 4.4.2.) where ZDV treatment was associated with an increase of ARC patient response over a median period of 2.5 months (range: 1 to 4.5 months) therapy.

Table 28: Con A driven gIFN response: longitudinal study of one laboratory control subject

	$\bar{x}$U gIFN per 1x10E+06 cells at			
month	0	1	3	4
with Con A	650	867	429	682
no Con A	nd	nd	nd	nd
net	650	867	429	682

nd: not detected

Figure 59: Longitudinal net Con A driven gIFN response of a laboratory control



SUBJECT 5: Con A driven gIFN response of ARC subject receiving zidovudine

	gIFN x̃U per 1x10E+06 cells at		
month	0	2	3
with Con A	89	1532	702
no Con A	0.5	nd	nd
net	89	1532	702

nd: not detected

SUBJECT 8: Con A driven gIFN response of ARC subject receiving zidovudine

	gIFN x̃U per 1x10E+06 cells at		
month	0	1*	2*
with Con A	440	1135	1825
no Con A	4	3	nd
net	436	1132	1825

month 1* and 2* : results by Medgenix gIFN IRMA
nd : not detected

The longitudinal net Con A gIF response of two ARC subjects CDC IVC2, receiving zidovudine

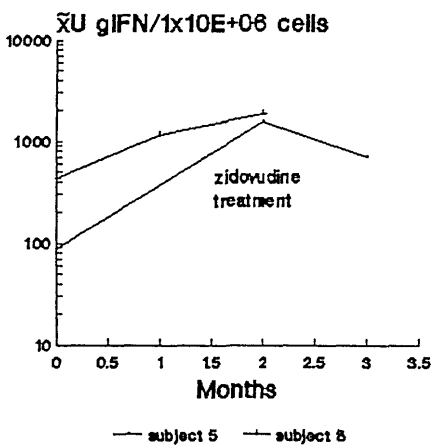


FIGURE 60: Con A driven gIFN response. The longitudinal study of two ARC subjects receiving zidovudine

SUBJECT 3: Con A driven gIFN response of AIDS subject receiving zidovudine

	gIFN ẍU per 1x10E+06 cells at	
month	0	1
with Con A	7	255
no Con A	nd	0.2
net	7	255

nd: not detected

SUBJECT 4: Con A driven gIFN response of AIDS subject receiving zidovudine

	gIFN ẍU per 1x10E+06 cells at	
month	0	2*
with Con A	5	49
no Con A	2	5
net	3	44

month 2* : result bu Medgenix gIFN IRMA
nd : not detected

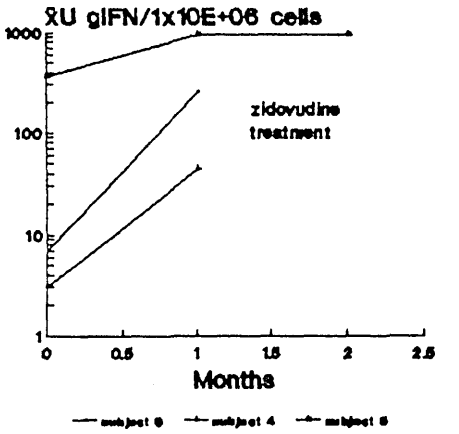
FIGURE 61: Con A driven gIFN response The longitudinal study of three AIDS subjects receiving zidovudine

SUBJECT 6: Con A driven gIFN response of AIDS subject receiving zidovudine

	gIFN ẍU per 1x10E+06 cells at		
month	0	1*	2*
with Con A	365	951	951
no Con A	0.1	5	nd
net	365	946	951

month 1* and 2* : result by Medgenix gIFN IRMA
nd : not detected

The longitudinal net Con A gIFN response of three AIDS subject receiving zidovudine



4.6.3. Zidovudine treated AIDS subjects

The serial Con A induced gIFN response of PBMC obtained from 3 AIDS patients - subjects 3 (CDC IVD); 4 (CDC IVC1); and 6 (CDC IVC1) - is shown by Figure 61. These patients were monitored from day zero of therapy through 1 or 2 months of ZDV treatment.

Day zero net Con A gIFN synthesis was markedly depressed in cases 3 and 4 and approached control levels for subject 6. ZDV therapy was observed to augment the net Con A response to or towards control levels in all 3 instances, and this is supported by CV values of 134 %, 123 % and 44.7 % calculated for subjects 3, 4 and 6 respectively. These results are confirmed by previous serial PHA (see sections 3.3.3. and 4.5.3.) and Con A (see section 3.4.3.) functional studies. In addition, cross-sectional analysis showed that ZDV treatment of AIDS patients caused significant elevation of their net Con A gIFN responses (see section 4.4.2.) over 1 to 30 months (median: 4 months) of therapy.

4.7. Summary of the serial PHA and Con A induced gIFN response by PBMC obtained from AL 721 and zidovudine treated ARC and AIDS patients

The results of mitogen driven gIFN production by PBMC obtained from ARC and AIDS subjects reflected the observations of mitogenic proliferative studies and supports the conclusions drawn from those findings (see section 3.5.). In summary, both cross-sectional and serial studies demonstrated that ZDV generally raised these patients' previously decreased net gIFN levels and emphasised that ZDV can stem and even reverse HIV-1 associated immunosuppression. However in contrast, AL 721 treatment of 3 subjects was associated with a general deterioration of net PHA induced gIFN response.

The mitogenic net gIFN response trends followed temporal changes of patient clinical status associated with each treatment group. Serial testing within 2 or 3 months of ZDV therapy revealed that improvement of response paralleled

ZDV associated clinical benefit. In the case of ARC subject 2, his PHA response level was maintained during 7 months of therapy and reflected his clinical well-being (B. Peters, personal communication). The cross-sectional PHA and Con A responses were consistent with these observations over similar times of ZDV treatment (see section 4.4.2.). The serial net PHA gIFN response trend also paralleled AIDS subject 1's rapid deterioration over months 5 to 11 of ZDV (see section 3.3.3.). The gIFN response of AL 721 treated subjects generally declined and this reflected this drug's clinical, virological and neuropsychological ineffectiveness (Peters et al., 1990).

Similar to mitogenic proliferative studies, serial testing of mitogen driven gIFN response may act as a guide to immunological change and parallel clinical status following antiretroviral therapy.

4.8.1. Lymphocyte Function Studies

The gIFN Response to the Soluble Recall Antigen SK/SD: optimal culture conditions

The soluble recall antigen SK/SD was utilised to assess antigen driven T-lymphocyte gIFN synthesis by PBMC obtained from laboratory controls and HIV-1 infected subjects.

The culture parameters investigated to ascertain optimal SK/SD induced PBMC gIFN synthesis were based on the optimal conditions for SK/SD driven proliferation (see section 3.6.1.). As such, PBMC obtained from 3 SK/SD skin-test positive laboratory control subjects were adjusted to $2x10^6$ cells/ml and cultured with or without SK/SD for 7 days.

The 7 days culture period adopted here was identical to that employed by Hutchinson (1991) to study SK/SD driven PBMC gIFN production. Similarly, other workers have observed maximal gIFN synthesis between 5 and 8 days culture with viral antigen (Cunningham and Merigan, 1984; D'Andrea et al., 1986; Green et al., 1981; Lotz et al.,

1986). However Kelly et al (1987) observed peak gIFN levels at 48 to 72 hours following antigen stimulation; preliminary experiments for this report consistently found very low or non-detectable levels of gIFN 3 days after SK/SD stimulation (data not shown).

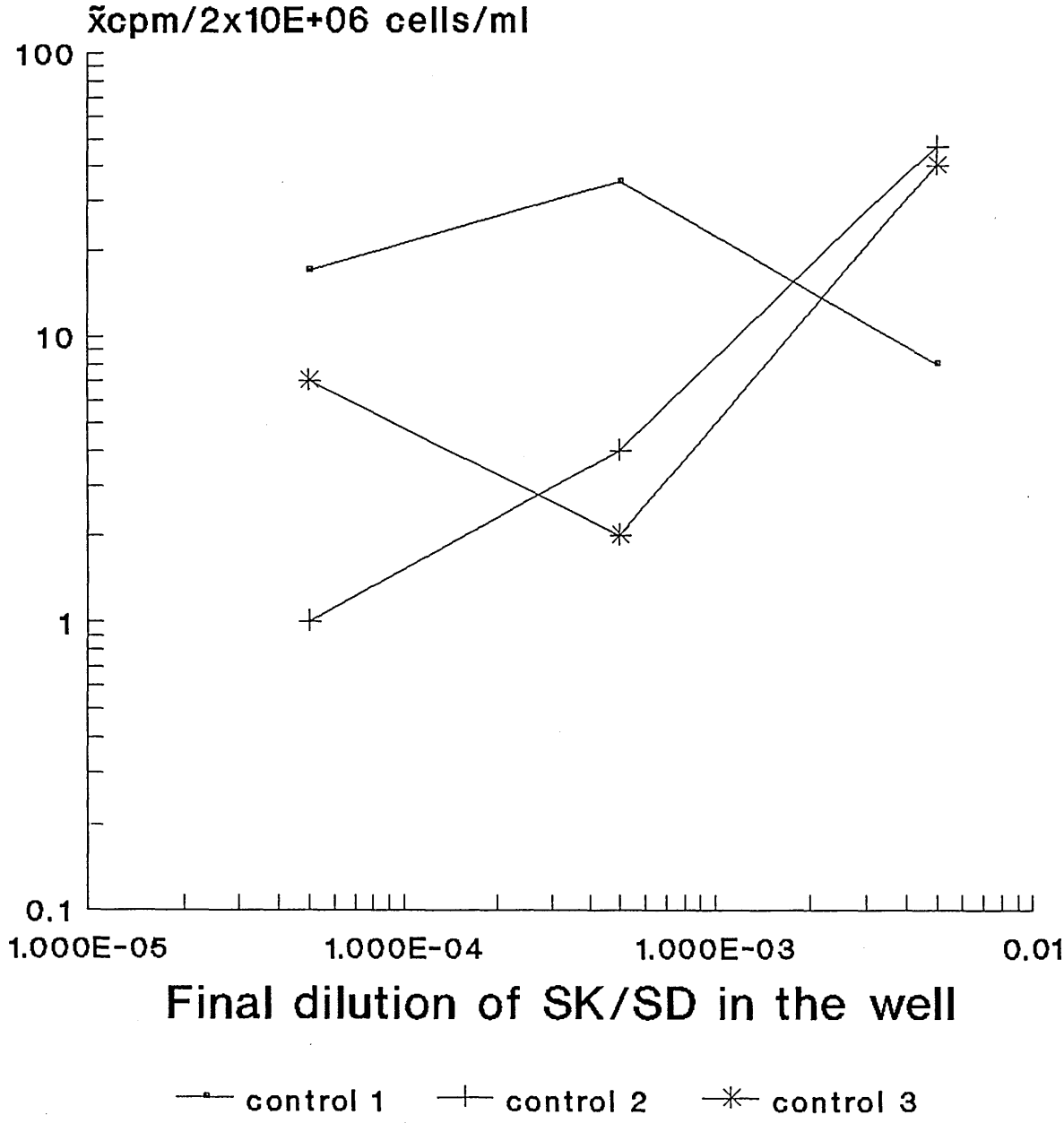
PBMC obtained from the 3 skin-test positive controls were cultured with the SK/SD skin-test preparation at final dilutions of 1/200, 1/2000 and 1/20000 for 7 days. The effect of these SK/SD concentrations on PBMC net gIFN production, calculated by subtracting the mean gIFN level obtained without SK/SD from the mean gIFN level obtained with SK/SD, is shown graphically by Figure 63. Two of the three skin-test positive subjects showed optimal in vitro net gIFN synthesis at a final SK/SD dilution of 1/200. The third control's response was optimal at 1/2000. The final dilution of SK/SD adopted throughout was 1/200, although the outlined discrepancy reiterates the need to assess individual response over a stimulant concentration range. It is noteworthy that the optimal SK/SD dilution for PBMC gIFN synthesis (final dilution: 1/200) differs to that for optimal proliferative response (final dilution: 1/2000) and suggests that SK/SD net PBMC proliferation does not correlate with SK/SD induced net PBMC gIFN production.

In summary, PBMC were cultured at 2×10^6 cells/ml with SK/SD (final dilution: 1/200) or without for 7 days; the culture supernatants were then harvested and assayed by Celltech Diagnostics SUCROSEP™ IRMA (see sections 2.6.1.b and 2.6.2.). Under these culture conditions, SK/SD was observed to cause significant production of gIFN by PBMC obtained from laboratory control subjects ($p < 0.01$; Figure 63). However, SK/SD induced PBMC gIFN levels were similar to spontaneous levels in 6 of the 8 AIDS subjects tested, irrespective of ZDV therapy (Figure 63).

4.8.2. The SK/SD induced net gIFN response pattern: cross-sectional study

The spontaneous, total and net SK/SD induced gIFN

Figure 62: The effect of SK/SD concentration on PBMC net gIFN production after 7 days culture



SK/SD induced PBMC gIFN production
Laboratory control subjects

Control	gIFN x̄U per 2x10E+06 cells		
	without SK/SD	with SK/SD	net response
1	16	51	35
2	3	29	26
3	1	19	18
4	6	46	40
5	3	3	NA
6	13	61	48
7	0.5	22	22

PBMC from 7 control subjects were cultured for 7 days without or with SK/SD at a final dilution of 1/200 in the well. NA: not applicable

Figure 63: The SK/SD induced gIFN response of PBMC obtained from laboratory controls and AIDS patients. HIV-1 infected individuals were subdivided by zidovudine therapy.

SK/SD induced PBMC gIFN production
AIDS, no zidovudine therapy

Patient	gIFN x̄U per 2x10E+06 cells		
	without SK/SD	with SK/SD	net response
1	3	10	7
2	9	9	NA
3	ND	2	2
4	ND	ND	NA

PBMC from 4 AIDS subjects were cultured for 7 days without or with SK/SD at a final dilution of 1/200 in the well. Patients 1, 3 and 4 were classified as CDC IVC1 and 2 as CDC IVD.
ND: not detectable
NA: not applicable

SK/SD induced PBMC gIFN production
AIDS, receiving zidovudine therapy

Patient	gIFN x̄U per 2x10E+06 cells		
	without SK/SD	with SK/SD	net response
1	ND	51	51
2	3	2	NA
3	1	2	1
4	ND	2	2

PBMC from 4 AIDS subjects were cultured for 7 days without or with SK/SD at a final dilution of 1/200 in the well. All patients were classified as CDC IVC1.
ND: not detectable
NA: not applicable

response of PBMC obtained from laboratory controls and ZDV untreated or treated AIDS patients is tabulated (Figure 63).

Although formal analysis of both patient groups was prevented by limited subject numbers (N=4), it is apparent that the AIDS patients' net PBMC gIFN response level to SK/SD was profoundly depressed relative to heterosexual control levels. This observation is in accordance with corresponding proliferative results (see section 3.6.2.), as is the observation that ZDV has no apparent effect on the AIDS subjects' net SK/SD induced gIFN response.

The patient gIFN results, irrespective of antiretroviral therapy, mostly lie at the very lower limit of Celltech Diagnostic IRMA detection. Hutchinson (1991) made similar observations and it may have been more appropriate to use the Medgenix system, which is designed to measure lower concentrations of gIFN (see section 4.1.2.). However, these observations suggest that SK/SD induced net gIFN response may be too insensitive a system to detect changes in functional capacity of PBMC obtained from AIDS patients. In addition and as previously mentioned (see section 3.6.2.), it may be that selective memory T-lymphocyte loss early in HIV-1 infection (Germain, 1988; Miedema et al., 1990) compounds problems with analysis by this antigenic system.

CHAPTER 5: RESULTS

LYMPHOCYTE FUNCTION STUDIES:
CHANGES IN THE PHOSPHATIDYL INOSITOL HYDROLYSIS PATHWAY

5. Lymphocyte Function Studies: Changes in the Phosphatidyl Inositol Hydrolysis Pathway of HIV-1 Infectd Subjects Receiving Zidovudine Therapy

Chronic HIV-1 infection of permissive H9 T-lymphoblastoid cells interferes with inositol polyphosphate (InsP) mediated signal transduction and with intracellular calcium ($[Ca^{2+}]_i$) regulation (Nye and Pinching, 1990). It has been subsequently observed that similar changes take place in HIV-1 infected subject donated lymphocytes (Nye et al., 1991).

Using methodoloy (see section 2.7.) defined and standardised by Nye and Riley (personal communiction), we have demonstrated in preliminary studies, that the lymphocyte InsP hydrolysis pathway defect seen in HIV-1 infected subjects is reversed with ZDV treatment and is coincident with improved cellular function (Nye et al., 1991).

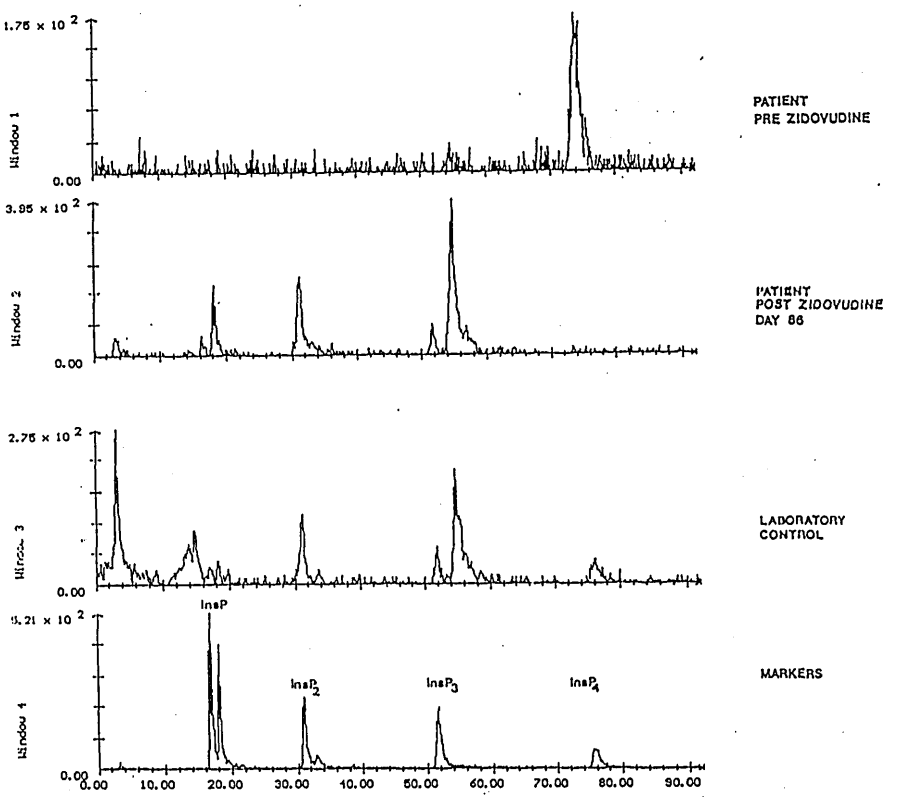
5.1. Analysis of PHA induced InsP response: an ARC subject pre and post zidovudine administration

PHA stimulation of control lymphocytes obtained from healthy laboratory donors is associated with increased levels of $[Ca^{2+}]_i$; and both inositol trisphosphate, $Ins(1,4,5)P_3$, and inositol tetrakisphosphate, $Ins(1,3,4,5)P_4$, these isomers being undetectable in resting cells. The $Ins(1,4,5)P_3$ increase is transient (Nye et al., 1991). A summarised description of the inositol polyphosphate pathway is given in section 1.3.4.b (Figure 01).

Figure 64 illustrates the InsP prolfile of PHA stimulated lymphocytes obtained from a laboratory control and from an HIV-1 infected patient pre ZDV and then at day 86 (3 months) post ZDV administration. This patient is subject (5) of the ARC serial functional and phenotypic studies and his InsP response is typical of several symptomatic patients studied.

At day zero of this patient's ZDV treatment, lymphocyte $Ins(1,4,5)P_3$ levels were barely detectable and

Figure 64: Analysis of Ins P hydrolysis of control and ARC (CDC IVC2) subject post zidovudine.



Isolated PBMC were incubated to isotopic equilibrium with 4.0 uCi/ml myo-[3H]inositol. Ins P stimulation with PHA (3 ug/ml) was amplified by previous incubation with Lithium chloride (10 mM). The reaction was terminated with 10% Trichloroacetic acid. Supernatant was run by HPLC using anion exchange. Ins P was displaced according to phophorylation: IP, IP2, 3 and finally 4. An in line beta counter recorded tritium emissions and then plotted by computer.

$Ins(1,3,4,5)P_4$ levels were strikingly elevated following 2 mins PHA stimulation. Interestingly, such defective metabolism of the inositol polyphosphates although evident in HIV-1 infected asymptomatic subjects, was much more profound in cells obtained from symptomatic individuals (K. Nye, personal communication).

However at day 86 of ZDV treatment, InsP metabolism appeared closer to that of the laboratory control subject: $Ins(1,3,4,5)P_4$ levels were normalised, while phosphatidyl-inositol 4,5-bisphosphate ($PtdIns(4,5)P_2$) and $Ins(1,4,5)P_3$ peaks became obvious and approached normal control levels.

5.2. Restoration of ARC subject PBMC lysate with [^{3}H]$Ins(1,3,4,5)P_4$

It appears that lymphocytes obtained from HIV-1 infected donors are unable to metabolise $Ins(1,3,4,5)P_4$ to $Ins(1,3,4)P_3$ by 5'-phosphatase (K. Nye, personal communication). This phenomenon was observed by incubating cell lysates, under the appropriate conditions, with [^{3}H]$Ins(1,3,4,5)P_4$ and separating the isomers formed by HPLC (see section 2.7.3.).

By this stratagem, the $Ins(1,3,4,5)P_4$, $Ins(1,4,5)P_3$ and $Ins(1,3,4)P_3$ levels were recorded at days 0, 26, 42 and 86 of the ARC subject's ZDV treatment (Table 29 and Figure 65).

The inositol polyphosphate profile shows little difference between day 0 and 26 (1 month) of therapy. However, after day 42 (2 months) there was a fall in $Ins(1,3,4,5)P_4$ levels and a slight rise in $Ins(1,4,5)P_3$; there was also a notable rise in the inactive metabolite $Ins(1,3,4)P_3$ which is part of the recycling pathway. By 86 days (3 months), further normalisation had occurred and inositol mono and bisphosphates appeared (data not shown). The profile seen after 86 days ZDV therapy approximated to that seen in HIV-1 infected asymptomatic subjects.

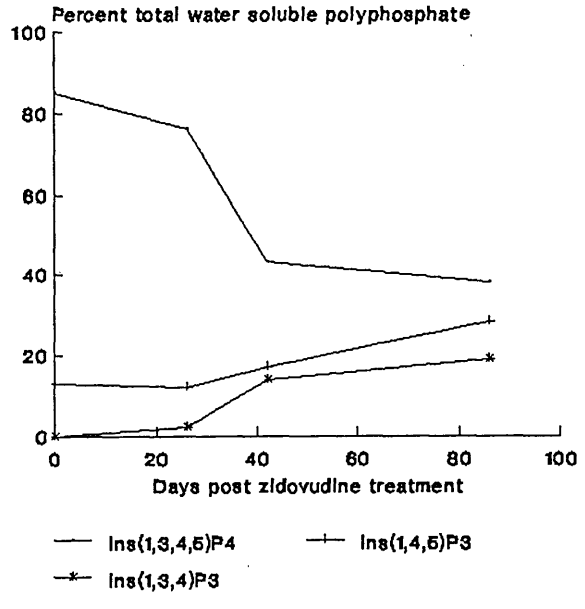
Parallel studies on absolute circulating

Table 29: Restoration of ARC (CDC IVC2) subject PBMC lysate with [3H]Ins(1,3,4,5)P4

	% total water soluble polyphosphate at day post zidovudine treatment			
	0	26	42	86
Ins(1,3,4,5)P4	85	76	43	38
Ins(1,4,5)P3	13	12	17	28
Ins(1,3,4)P3	0	2	14	19

ARC (CDC IVC2) subject donated PBMC were separated over lymphoprep. Lysis was by freeze/thaw in liquid nitrogen.
Restoration of inositol polyphosphate pathway with zidovudine treatment was visualised by addition of [3H]Ins(1,3,4,5)P4. This procedure was in buffer supplemented with ATP (2mM), free Mg^{2+} (4mM) and free Ca^{2+} (0.1uM).

Figure 65: Restoration of ARC (CDC IVC2) subject PBMC lysate with [3H]Ins(1,3,4,5)P4



T-lymphocyte subset numbers (see Figures 96 to 98); PHA and Con A induced proliferative response and gIFN production (see Figures 20, 29, 51 and 60) show no significant change in lymphocyte numbers but, some functional improvement at 1 month and marked improvement at 3 months of therapy.

5.3. Summary of results

These results demonstrate that the defect seen in inositol polyphosphate metabolism is probably at 5'-phosphatase and is reversible if HIV-1 replication is stemmed, in this case by ZDV. The time course of this reversal suggests that the factor responsible is removed from HIV-1 replication itself; this is consistent with a protein mediated defect (see section 1.3.4.b).

CHAPTER 6: RESULTS

LYMPHOCYTE FUNCTION STUDIES:
SPONTANEOUS AND PWM INDUCED PRODUCTION OF IMMUNOGLOULIN

6. Spontaneous and PWM Induced Production of Immunoglobulin Classes IgG, IgM and IgD

The potential antibody repertoire of individual donors was examined by stimulating isolated PBMC with pokeweed mitogen (PWM). PBMC were cultured for nine days in the presence or absence of the mitogen and the supernatant harvested from triplicate cultures. The concentration of IgM, IgG and IgD present was ascertained by ELISA as previously described (see section 2.8.). The ELISA methodology was employed by Rogers (1987) and Rogers et al (1989) and is a modification of the assay described by Farrant et al 1985.

6.1. Standardisation and sensitivity of the ELISA assay

Non-specific binding (NSB):

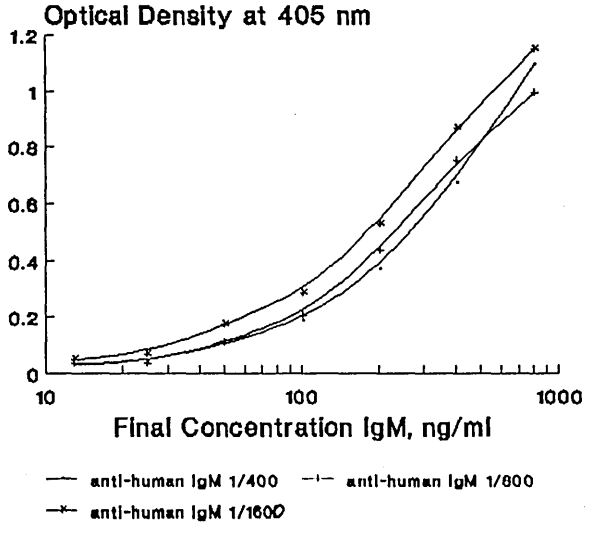
A. Mustafa (personal communication) has shown non-specific binding (NSB) to differ between microtitre plate batches. Consequently, a sample plate from four batches was tested for IgM and IgG NSB (see section 2.8.3.). Each batch was found to be acceptable, as the optical density (OD) at 405 nm was less than 0.15. These plates were used throughout. IgD NSB subsequently proved satisfactory.

Optimal coating antibody and antibody-enzyme conjugate concentrations:

To maximise the measurable range of total immunoglobulin the optimal concentration of coating antibody and antibody-enzyme conjugate was ascertained.

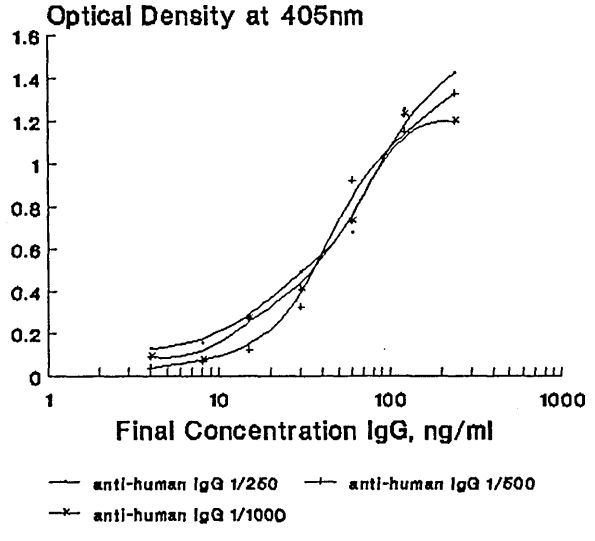
Figures 66 and 67 demonstrate little difference between the three concentrations of coating antibody used for both IgM and IgG, while varying the dilution of enzyme-conjugate causes perceptible change (Figures 68 and 69). In each case the figures are representative of two experiments. The dilutions consequently adopted throughout were: 1/800 for rabbit anti-human IgM and 1/ 500 for goat anti-human IgG coating antibody; 1/1000 and 1/800 for goat anti-human IgM and anti-human IgG alkaline phosphatases respectively. Employing this system IgM was measurable from

Figure 66
Titration of IgM Standard:
optimum concentration of anti-human IgM



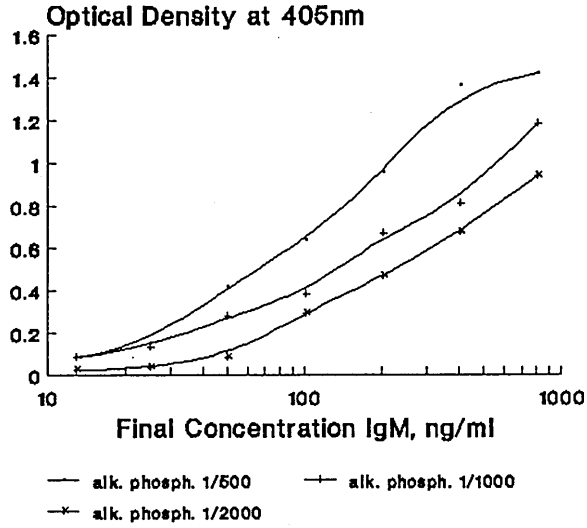
OD 405nm (corrected for NSB) for rabbit anti-human IgM diluted 1/400, 1/800, 1/1600 with BS.

Figure 67
Titration of IgG Standard:
optimum concentration of anti-human IgG



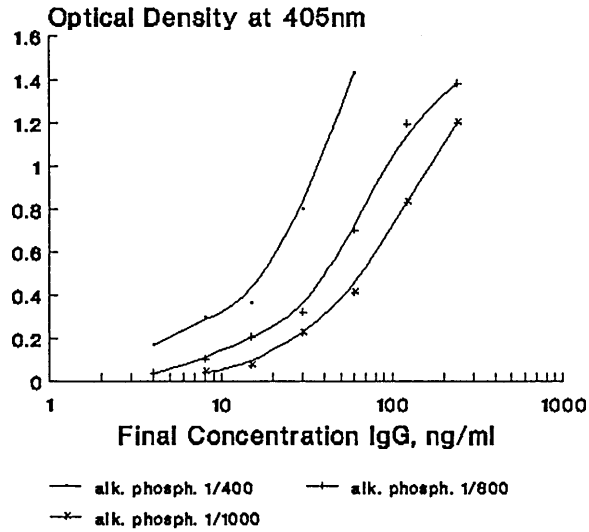
OD 405nm (corrected for NSB) for goat anti-human IgG diluted 1/250, 1/500, 1/1000 with BS.

Figure 68
Titration of IgM Standard: optimum anti-IgM-enzyme conjugate concentration



OD 405nm (corrected for NSB) for goat anti-human IgM-alkaline phosphatase diluted 1/500, 1000, 2000 with BS.

Figure 69
Titration of IgG Standard: optimum anti-IgG-enzyme conjugate concentration



OD 405nm (corrected for NSB) for goat anti-human IgG-alkaline phosphatase diluted 1/400, 800, 1000 with BS.

30 ng/ml to 1000 ng/ml and IgG from 8 ng/ml to 200 ng/ml.

The recommended dilutions of both rabbit anti-human IgD and goat anti-human IgD alkaline phosphatase detected 0.5 mU/ml to 12 mU/ml of the standard, as shown by a representative of three experiments in Figure 70.

6.2. Optimal condition of PBMC

It has been observed, with reference to an ELISA-spot assay, that a two hour period before separation procedure does not significantly effect the yield of antibody secreting cells from freshly drawn blood (A. Mustafa, personal communication). Similar observations have been reported by Blaese et al (1980). The out-patient system employed at St. Mary's implies that patient donated blood may not always have been separated within this period. However, the relevance of this observation is unclear since assay was after nine days culture and not two as for the ELISA-spot.

6.3. Optimal concentration of PWM

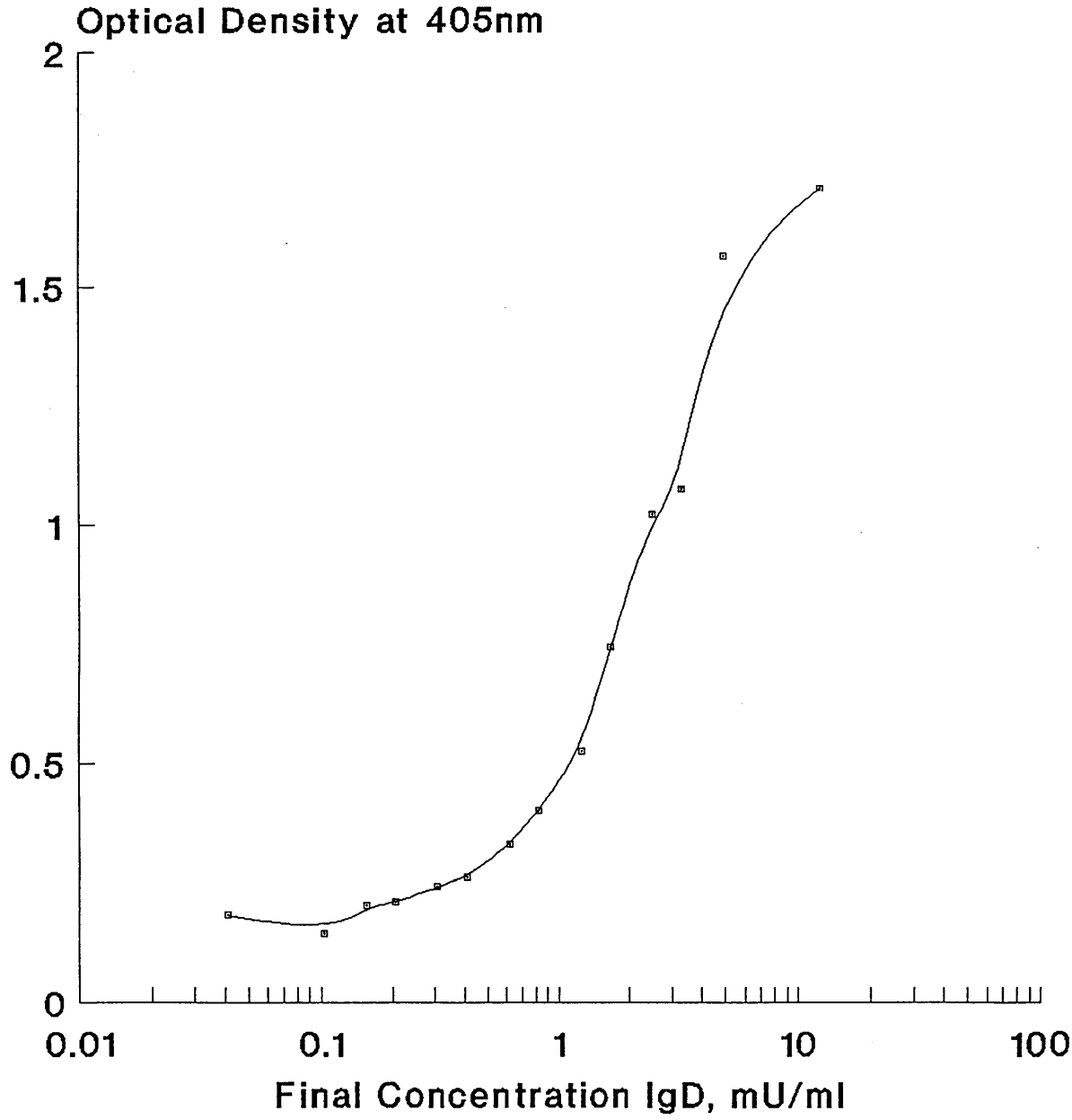
PBMC from three laboratory control subjects were cultured in triplicate with PWM at final dilutions in the microtitre plate ranging from 1/20 to 1/1000. PBMC were also cultured without PWM. The resultant supernatants were assayed for IgM and IgG by ELISA.

PWM induced IgM and IgG production most effectively at a final dilution of 1/200. Figures 71 and 72 record this effect on the net concentration of IgM and IgG. Net production is defined as total PWM driven minus spontaneous concentration of immunoglobulin. PWM was used at a final dilution of 1/200 throughout and caused significant PBMC IgM and IgG production in the control group ($p < 0.01$).

6.4. Spontaneous and PWM induced immunoglobulin production by HIV-1 infected subjects

Figures 73 to 77 collate spontaneous, total PWM and net PWM induced IgM, IgG and IgD concentrations for the five groups studied: laboratory control subjects, and HIV-1

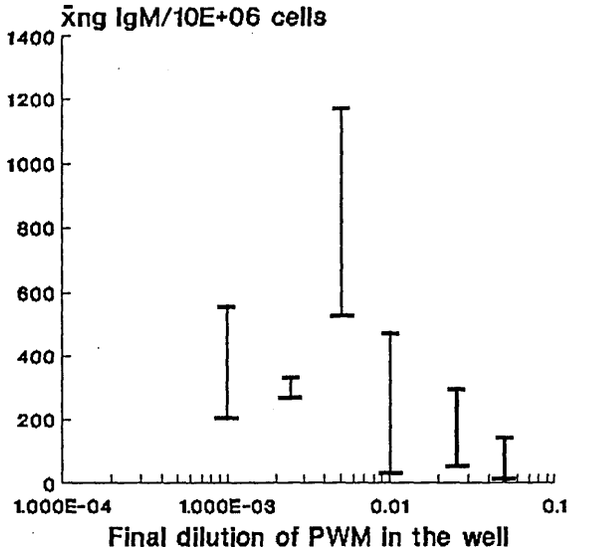
Figure 70
Titration of IgD Standard



OD 405nm (corrected for NSB) for IgD standard titration. Coating antibody and enzyme conjugate diluted 1/1000 with BS.

Figure 71

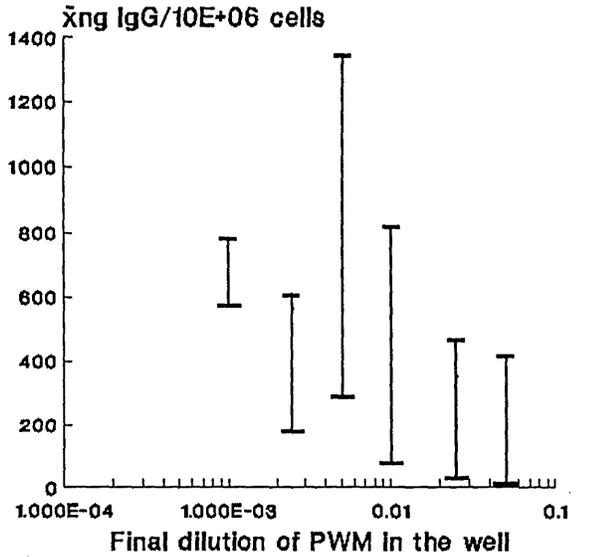
The effect of PWM concentration on the net production of IgM in culture



PBMC (1.0x10E+06/ml) were cultured for 9 days with or without PWM.
I: mean ± SEM

Figure 72

The effect of PWM concentration on the net production of IgG in culture



PBMC (1.0x10E+06) were cultured for 9 days with or without PWM.
I: mean ± SEM

infected subjects classified as ARC (CDC IVC2) or AIDS (CDC IVC1, IVD). The latter two groups were subdivided by ZDV therapy. Translation of the tables to graphs (Figures 78, 79, and 80) allows comparison of the five groups against the three Igs studied. Ig concentrations are expressed as $\bar{x}$ng per $1.0x10^6$ cells for IgM and IgG and as $\bar{x}$mU per $1.0x10^6$ cells for IgD.

6.4.1. Spontaneous production of IgM and IgG

Spontaneous IgM production was not significantly different between heterosexual controls and HIV-1 infected subjects. However, ZDV untreated AIDS patient levels appeared slightly depressed and treated ARC subject levels slightly elevated relative to the laboratory controls.

Similarly, IgG spontaneous production was no different between control and AIDS groups, irrespective of ZDV therapy. However, the ZDV untreated and treated ARC group levels were significantly elevated ($p < 0.01$ and $p < 0.05$ respectively) relative to laboratory control production.

In accordance with the results reported here, Rogers (1987) and Rogers et al (1989) previously observed in this laboratory no difference in spontaneous IgM and IgG concentrations between heterosexual controls and ZDV untreated HIV-1 seropositive subjects classified as CDC IVC1. In addition, these previous studies found significant elevation of spontaneous immunoglobulin production in less severe disease (CDC III and IVD) and this is consistent with our observations on ARC subjects.

6.4.2. Response to PWM: net IgM and IgG production

Rogers (1987) and Rogers et al (1989) reported significant depression of net IgM and IgG production, in response to PWM, with the onset of HIV-1 symptomatic disease. This is reflected by the data collectd here.

ARC and AIDS subjects' PWM induced IgM production was significantly decreased relative to controls ($p < 0.05$ and $p < 0.05$ respectively) and regardless of ZDV therapy.

Control Subjects, IgM
Response to PWM After 9 Days

x̄ng IgM/1.0x10E+06 cells

Subject	Spontaneous	Total PWM	Net PWM
1	285	335	50
2	468	1030	562
3	85	812	727
4	151	802	651
5	72	386	314
6	404	846	442
7	187	248	61
8	475	1403	928
9	702	1567	865
10	22	1026	1004

Control Subjects, IgG
Response to PWM After 9 Days

x̄ng IgG/1.0x10E+06 cells

Subject	Spontaneous	Total PWM	Net PWM
1	245	559	314
2	330	1867	1537
3	878	2199	1321
4	537	1818	1281
5	486	566	80
6	238	675	437
7	386	755	369
8	480	1321	841
9	409	3097	2688
10	125	8225	8100

Control Subjects, IgD
Response to PWM After 9 Days

x̄mU IgD/1.0x10E+06 cells

Subject	Spontaneous	Total PWM	Net PWM
1	3.07	1.84	-1.23
2	3.80	1.99	-1.81
3	1.53	3.42	1.89
4	1.99	2.01	0.02
5	1.53	2.04	0.51
6	2.98	3.22	0.24
7	3.86	2.78	-1.08
8	te	te	na
9	2.01	3.23	1.22
10	1.03	0.96	-0.07

Figure 73: Control Subjects

PBMC from 10 laboratory control subjects were cultured in triplicate with or without PWM. The supernatants were assayed by ELISA for IgM, IgG and IgD.
The IgD results for subject 9 were obtained on repeat.
Net Ig production: total PWM - spontaneous
te : technical error
na : not applicable

ARC, no zidovudine: IgM
Response to PWM after 9 days

x̄ng IgM/1.0x10E+06 cells

Subject	Spontaneous	Total PWM	Net PWM
1	78	136	58
2	297	133	-164
3	208	97	-111
4	500	575	75
5	122	61	-61
6	506	180	-326

ARC, no zidovudine: IgG
Response to PWM after 9 days

x̄ng IgG/1.0x10E+06 cells

Subject	Spontaneous	Total PWM	Net PWM
1	1002	695	-307
2	514	1449	935
3	1940	750	-1190
4	738	726	-12
5	1757	666	-1091
6	808	1670	862

ARC, no zidoudine: IgD
Response to PWM after 9 days

x̄mU IgD/1.0x10E+06 cells

Subject	Spontaneous	Total PWM	Net PWM
1	6.39	6.60	0.21
2	4.18	4.47	0.29
3	4.53	3.72	- 0.81
4	2.60	2.34	- 0.26
5	32.80	8.98	-23.82
6	1.38	1.46	0.08

Figure 74: ARC, no zidovudine

All 6 subjects were classified CDC IVC2 and were pre-zidovudine. Subjects 1 to 6 correspond with subjects 1 to 6 grouped ARC, on zidovudine. PBMC were cultured in triplicate with or without PWM and the supernatants assayed by ELISA for IgM, IgG and IgD.
Net Ig production: total PWM - spontaneous

ARC, on zidovudine: IgM
Response to PWM after 9 days

x̄ng IgM/1.0x10E+06 cells

Subject	Spontaneous	Total PWM	Net PWM
1	278	72	-206
2	170	467	297
3	273	720	447
4	804	475	-329
5	144	315	171
6	360	266	-94
7	120	176	56

ARC, on zidovudine: IgG
Response to PWM after 9 days

x̄ng IgG/1.0x10E+06 cells

Subject	Spontaneous	Total PWM	Net PWM
1	1088	607	-421
2	511	2304	1793
3	587	1143	556
4	1468	880	-558
5	520	1210	690
6	748	718	-30
7	461	543	82

ARC, on zidovudine: IgD
Response to PWM after 9 days

x̄mU IgD/1.0x10E+06 cells

Subject	Spontaneous	Total PWM	Net PWM
1	2.00	2.99	0.99
2	6.91	6.03	- 0.88
3	te	12.61	na
4	31.00	13.87	-17.13
5	3.12	2.91	- 0.21
6	1.09	1.90	0.81
7	1.63	1.47	- 0.16

Figure 75: ARC, on zidovudine

All 7 subjects were classified CDC IVC2 and were receiving zidovudine therapy. Subjects 1 to 6 correspond with subjects 1 to 6 grouped ARC, no zidovudine.
Subjects 4, 5, & 6 had received zidovudine therapy for 3 to 4 wks; subjects 1 & 2 for 7 to 10 wks; subject 3 for 14 wks and subject 7 for 29 wks.
te : technical error
na : not applicable
Net Ig production: total PWM - spontaneous

AIDS, no zidovudine: IgM
Response to PWM after 9 days

x̄ng IgM/1.0x10E+06 cells

Subject	Spontaneous	Total PWM	Net PWM
1	270	372	102
2	52	nd	na
3	38	270	232
4	1007	996	-11
5	69	164	95
6	nd	nd	na
7	49	134	85
8	57	171	114
9	42	nd	na

AIDS, no zidovudine: IgG
Response to PWM after 9 days

x̄ng IgG/1.0x10E+06 cells

Subject	Spontaneous	Total PWM	Net PWM
1	984	1296	312
2	107	297	190
3	268	757	489
4	1369	2105	736
5	195	307	112
6	358	1163	805
7	236	495	259
8	646	502	-144
9	523	522	-1

AIDS, no zidovudine: IgD
Response to PWM after 9 days

x̄mU IgD/1.0x10E+06 cells

Subject	Spontaneous	Total PWM	Net PWM
1	12.12	6.44	-5.68
2	1.57	1.31	-0.26
3	3.80	3.92	0.12
4	3.39	3.64	0.25
5	1.47	1.60	0.13
6	2.45	1.51	-0.94
7	8.96	4.64	-4.32
8	1.08	0.80	-0.28
9	0.68	1.35	0.67

Figure 76: AIDS, no zidovudine

Subject 1 was classified CDC IVD and subjects 2 to 9 CDC IVC1.
Subjects 1 to 3 correspond with 1 to 3 grouped AIDS, on zidovudine and were from the longitudinal study. Likewise individuals 4 to 6 were part of the longitudinal study and were pre-zidovudine. Subjects 7 to 9 were part of the cross sectional peripheral neuropathy study.
Net Ig production : total PWM - spontaneous
nd : not detected
na : not applicable

AIDS, on zidovudine: IgM
Response to PWM after 9 days

x̄ng IgM/1.0x10E+06 cells

Subject	Spontaneous	Total PWM	Net PWM
1	2458	1059	-1399
2	618	1140	512
3	nd	nd	na
4	448	285	-163
5	314	370	56
6	nd	nd	na
7	3	32	-2
8	71	123	52
9	101	148	47
10	70	nd	na

AIDS, on zidovudine: IgG
Response to PWM after 9 days

x̄ng IgG/1.0x10E+06 cells

Subject	Spontaneous	Total PWM	Net PWM
1	2282	2298	16
2	448	2275	1827
3	253	359	106
4	641	1025	384
5	634	1820	1186
6	612	1255	643
7	338	554	216
8	60	58	-2
9	928	941	13
10	302	926	624

AIDS, on zidovudine: IgD
Response to PWM after 9 days

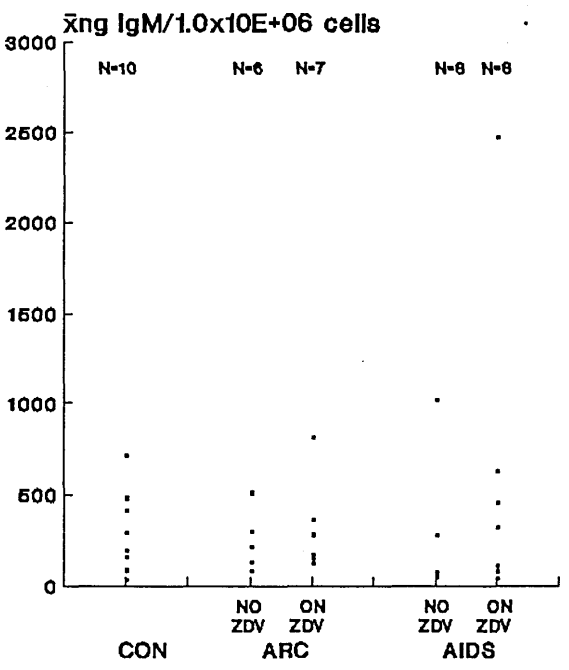
x̄mU IgD/1.0x10E+06 cells

Subject	Spontaneous	Total PWM	Net PWM
1	9.80	6.37	- 3.43
2	9.53	4.03	- 5.50
3	0.37	0.40	0.03
4	24.50	5.65	-18.88
5	0.95	1.18	0.03
6	7.80	5.01	- 2.79
7	4.05	2.39	- 1.66
8	0.39	0.36	- 0.03
9	1.70	0.95	- 0.75
10	2.28	2.21	- 0.07

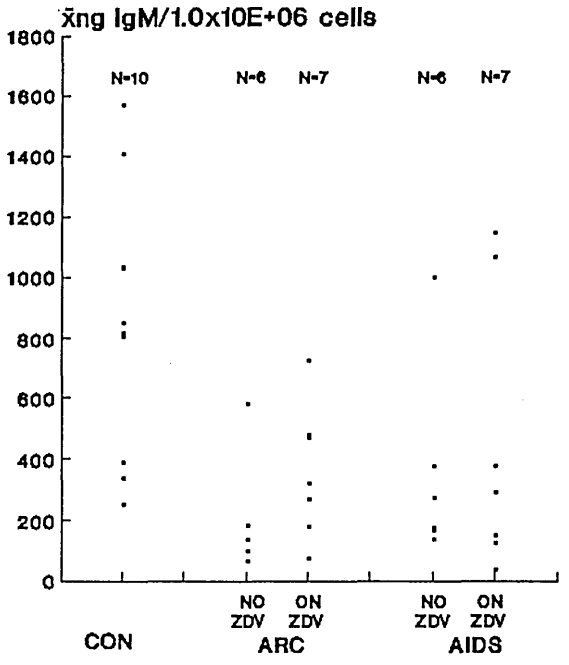
Figure 77: AIDS, on zidovudine

All but individuals 1 & 4 were classified CDC IVC1 and these were CDC IVD. Subject 1 to 3 correspond with 1 to 3 grouped AIDS, no zidovudine. Subjects 1 to 4 were from the longitudinal study. The remaining individuals were part of the cross sectional peripheral neuropathy study. Subjects 1 to 5 had received zidovudine therapy for 1 to 5 wks; subject 7 for 28 to 30 wks; subjects 8 & 10 for 76 to 80wks; subject 6 for 94 to 98wks a subject 7 for 128 to 132 wks.
Net Ig Production : total PWM - spontaneous
nd : not detectable
na : not applicable

Graph a :
Spontaneous Production of IgM
After 9 Days Culture



Graph b :
Total PWM-Driven Production of
IgM After 9 Days Culture



Graph c :
Net Production of IgM After 9 Days
Culture

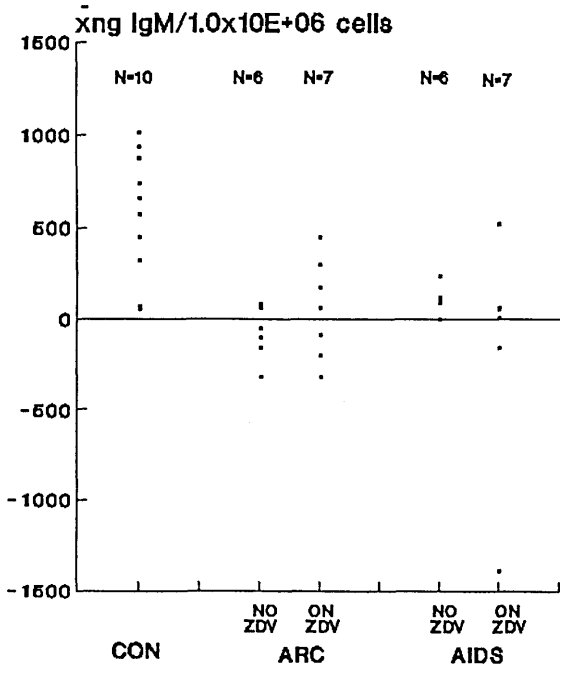
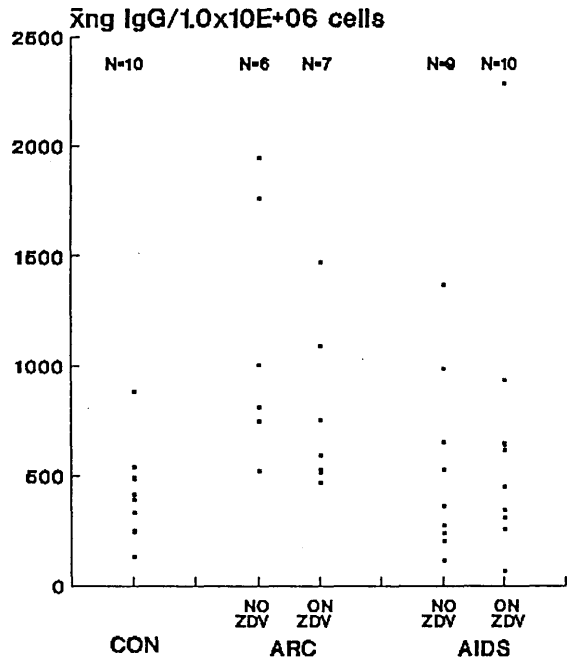
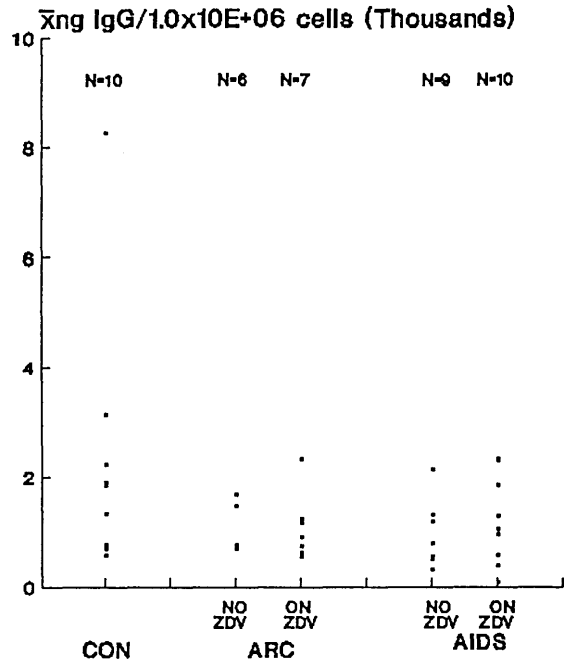


Figure 78: Compilation of spontaneous, total and net PWM-driven IgM production against CDC classification and zidovudine therapy.

Graph a :
Spontaneous Production of IgG
After 9 Days Culture



Graph b :
Total PWM-Driven Production of
IgG After 9 Days Culture



Graph c :
Net Production of IgG After 9 Days
Culture

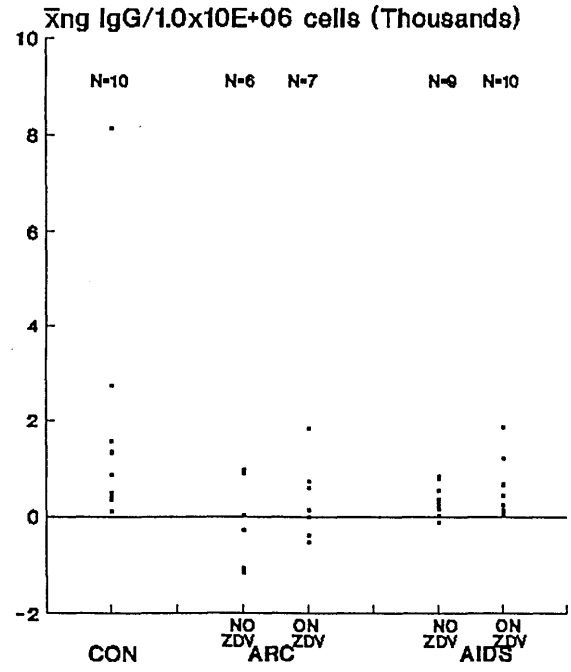
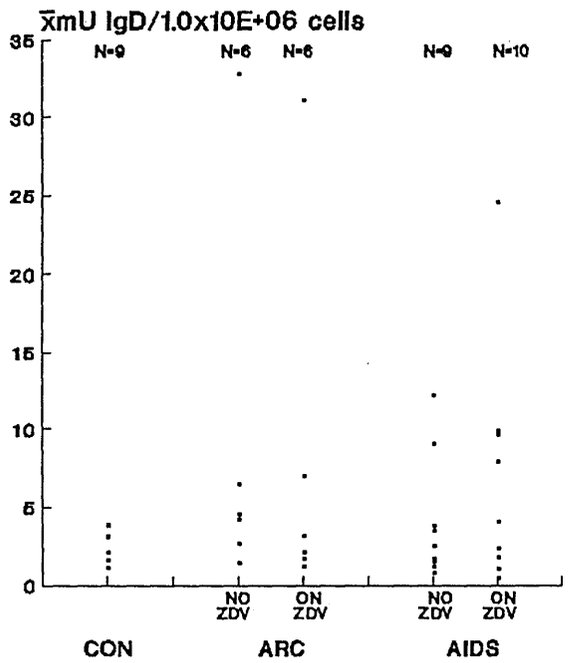
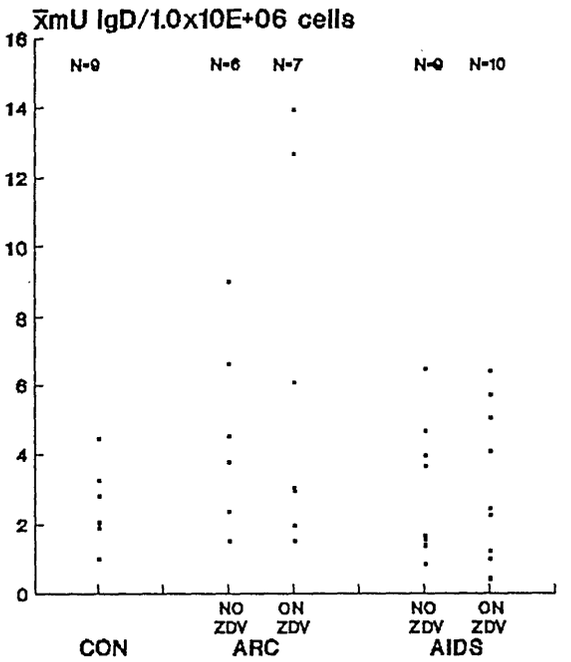


Figure 79: Compilation of spontaneous, total and net PWM-driven IgG production against CDC classification and zidovudine therapy.

Graph a :
Spontaneous Production of IgD
After 9 Days Culture



Graph b :
Total PWM-Driven Production of
IgD After 9 Days Culture



Graph c :
Net Production of IgD After 9 Days
Culture

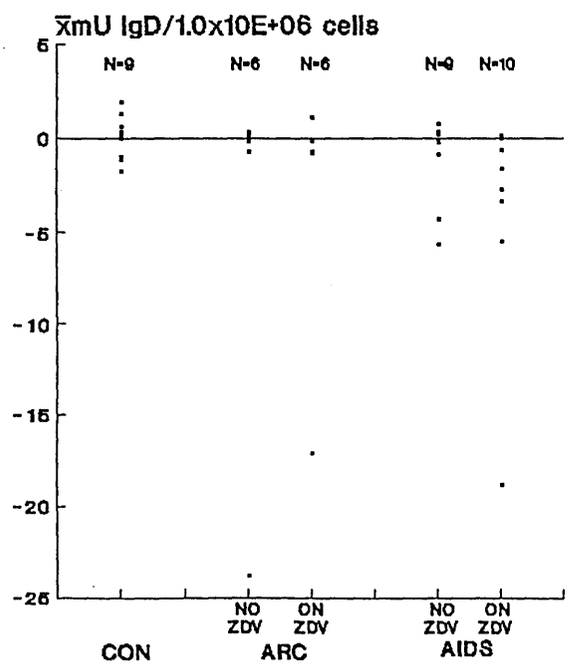


Figure 80: Compilation of spontaneous, total and net PWM-driven IgD production against CDC classification and zidovudine therapy.

IgG production was reduced in the ZDV untreated ARC and AIDS groups ($p < 0.05$). In the case of ARC subjects, this observation may be partially explained by the high spontaneous levels of IgG production. This defect may be resolved in part by ZDV therapy, as a slight trend to improvement was observed in ARC and AIDS subjects (Figure 79).

6.4.3. Summary of spontaneous and PWM induced IgM and IgG production

The data collected here revealed decreased net production of IgM and IgG PWM responses in ARC and AIDS subjects and has previously been observed in patients with severe HIV-1 disease (Rogers, 1987; Rogers et al., 1989). This defect was not significantly corrected by ZDV therapy.

6.4.4. Spontaneous and PWM induced IgD production

Similar to the observations of Rogers (1987) and Rogers et al (1989), spontaneous levels of IgD were not observed to differ noticeably between laboratory controls and HIV-1 infected subjects with AIDS. Although, a slight rise was seen in the ZDV untreated group.

The majority of HIV-1 infected subjects, especially those classified as AIDS, produced less IgD in response to PWM stimulation than spontaneously and has been observed previously by Rogers (1987) and Rogers et al (1989). This was also seen in four laboratory control subjects. ZDV treatment was not associated with any obvious trend.

N.B. Statistical analysis was by Mann-Whitney U-test.

CHAPTER 7: RESULTS

LYMPHOCYTE PHENOTYPIC STUDIES

7. Phenotypic Analysis: Enumeration of T-lymphocyte Subsets

Both immunocytochemical and flow cytometry methodologies were employed to enumerate total T-lymphocyte, T-helper/inducer and T-cytotoxic/suppressor populations. These subsets are defined by surface expression of protein receptor molecules CD3, CD4 and CD8 respectively. As stated, immunocytochemical labelling proved time-consuming and temperamental and was superseded when a suitable flow cytometry system became available in the department.

Absolute T-lymphocyte subset counts, especially for CD4+ cells, are reported here as a necessary adjunct to interpretation of the functional data.

7.1. T-lymphocyte enumeration by immunocytochemical labelling: standardisation

The immunocytochemical protocol employed has been precisely detailed within the Class 3 laboratory of this department. The choice of Mab, accuracy of intra-assay measurement, and effect of storage were of major consideration (Hutchinson, 1991). Therefore, it was inefficient of time and finances to repeat these standardisation experiments.

7.2. T-lymphocyte enumeration by flow cytometry (FCM): standardisation

Diagnostic Immunology established a suitable flow cytometry (FCM) system within the department during the summer and autumn of 1989. Extensive protocol standardisation, including regular national quality control, was discharged by C. Clark and T. Ekong. It became possible by direct staining and EDTA whole blood lysis to analyse each subject for T-lymphocyte subset relatively easily and reliably. A typical laboratory control read-out is shown (Figures 106 and 107, Pocket; see section 2.9.2.c).

7.3. Comparison of immmunocytochemical and FCM methods

T-lymphocyte subsets were enumerated for seven subjects, three normal laboratory controls and four HIV-1 infected individuals, by immunocytochemical and FCM methodology. The results are tabulated (Tables 30, 31 and 32). CD3+, CD4+ and CD8+ T-lymphocyte absolute counts were analysed as recommended by Altman and Bland (1983) and as already outlined (see section 4.6.1.b). CD4+ T-lymphocyte counts are emphasised.

The direct plot of data demonstrated a reasonable agreement of methodologies at the line of unity (Figures 81, 82 and 83). However, the plot of difference between methods against the method average revealed an obvious and unacceptable scatter for each subset population (Figures 84, 85 and 86). In the case of absolute CD4+ T-lymphocyte count, scatter may have been partly enhanced by the difference in Mab. Leu 3a3b used for immunocytochemistry detects two TcR epitopes while Leu 3a for FCM only one.

The T-lymphocyte subset counts quoted in the text were mainly obtained by FCM given the results of protocol comparison and the difficulties of immunocytochemical staining. In addition, national quality control tests have shown a wider scatter and less satisfactory results by lymphocyte separation than by direct staining and whole blood lysis (The December 1989 meeting of the Working Immunology Group, Immunology and Haematology MSLO's and members of the MRC HIV Clinical Trials Centre; T. Ekong, personal communication). Immunocytochemical results were used only when completely necessary and these are indicated.

7.4. Enumeration of T-lymphocyte subsets: cross-sectional study

Absolute T-lymphocyte CD3+, CD4+ and CD8+ subset counts were tabulated for HIV-1 low risk controls and HIV-1 infected donors (Tables 33 to 37). HIV-1 seropositive subjects were classified as ARC (CDC IVC2) or as AIDS (CDC IVC1, IVD) and were subdivided by ZDV therapy. ARC

Table 30

Subject	Absolute CD3+ T-lymphocyte x10E+09 cells/l	
	Immunocyt	FCS
1	1.45	1.56
2	1.40	2.13
3	1.19	1.22
4	0.74	0.84
5	0.36	0.44
6	0.23	0.40
7	0.50	0.38

COMPARISON OF ABSOLUTE CD3+ T-LYMPHOCYTE COUNT BY IMMUNOCYTOCHEMISTRY AND FLOW CYTOMETRY METHODOLOGY

Table 31

Subject	Absolute CD8+ T-lymphocyte x10E+09 cells/l	
	Immunocyt	FCS
1	0.53	0.59
2	1.33	0.99
3	-	0.54
4	0.48	0.66
5	0.28	0.39
6	0.20	0.23
7	-	0.31

COMPARISON OF ABSOLUTE CD8+ T-LYMPHOCYTE COUNT BY IMMUNOCYTOCHEMISTRY AND FLOW CYTOMETRY METHODOLOGY

Table 32

Subject	Absolute CD4+ T-lymphocyte count x10E+09 cells/l	
	Immunocyt	FCS
1	0.99	1.07
2	1.04	0.83
3	0.68	0.68
4	0.31	0.20
5	0.20	0.05
6	0.10	0.06
7	0.06	0.09

COMPARISON OF ABSOLUTE CD4+ T-LYMPHOCYTE COUNT BY IMMUNOCYTOCHEMISTRY AND FLOW CYTOMETRY METHODOLOGY

Figure 81: Comparison of absolute CD3+ T-lymphocyte count by immunocytochemistry and FCM

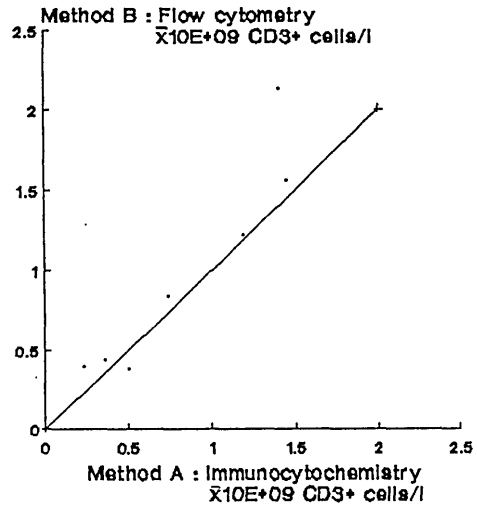


Figure 82: Comparison of absolute CD8+ T-lymphocyte count by immunocytochemistry and FCM

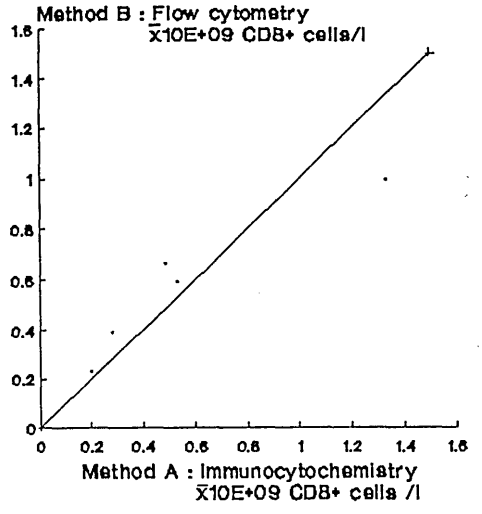


Figure 83: Comparison of absolute CD4+ T-lymphocyte count by immunocytochemistry and FCM

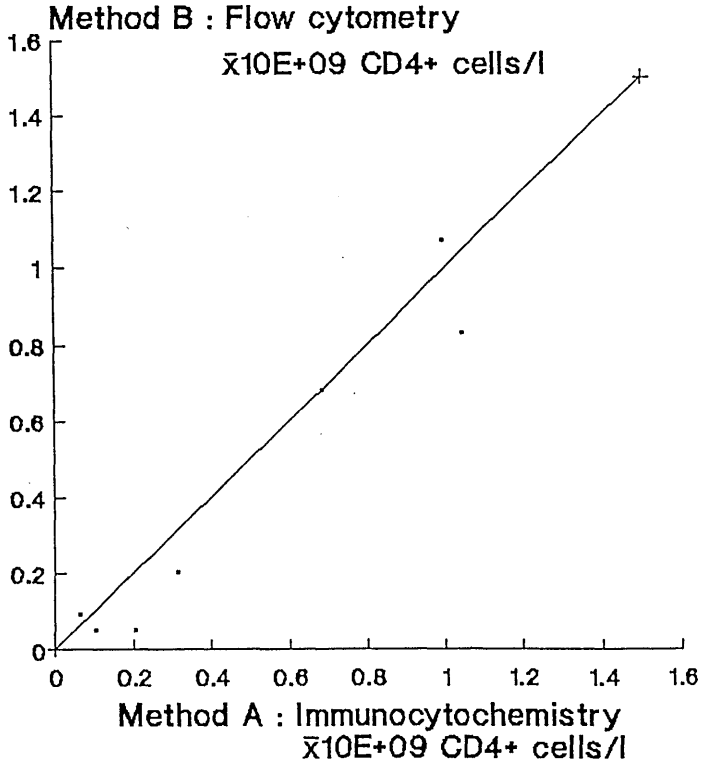


Figure 84: Comparison of absolute CD3+ T-lymphocyte count by immunocytochemistry and FCM

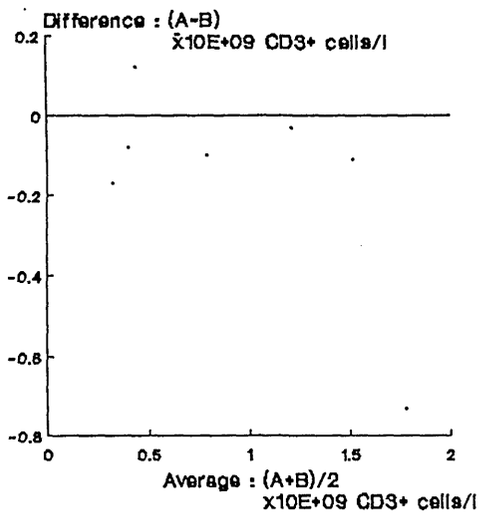


Figure 85: Comparison of absolute CD8+ T-lymphocyte count by immunocytochemistry and FCM

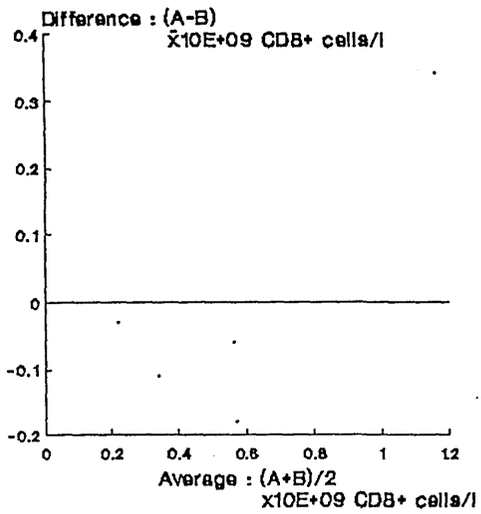
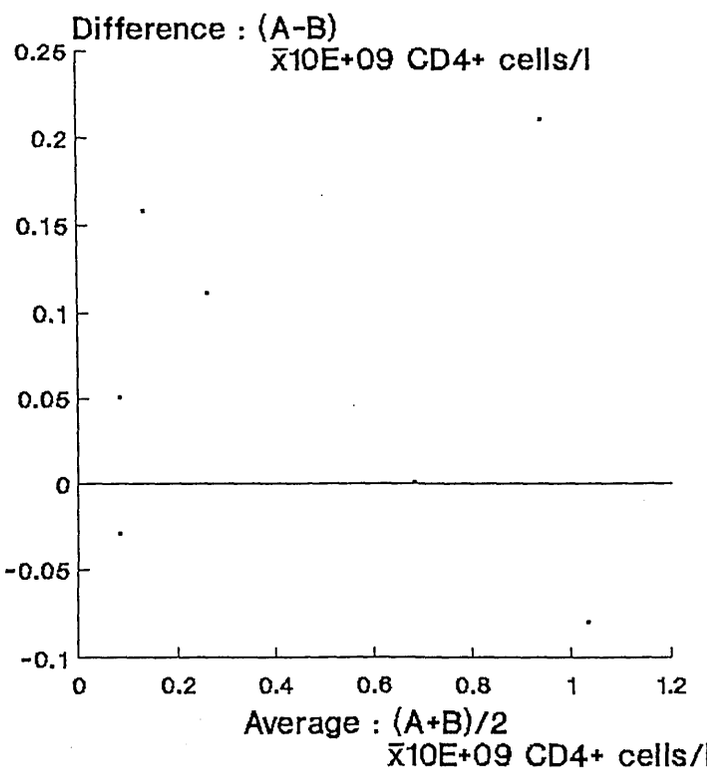


Figure 86: Comparison of absolute CD4+ T-lymphocyte count by immunocytochemistry and FCM



patients had received ZDV for a median of 1.5 months (range: 1 to 4 months) and AIDS subjects for a median of 4 months (range: 1 to 30 months) at the time of analysis. The collated data has been graphed, with emphasis placed on the CD4+ cell counts (Figures 87, 88 and 89).

All phenotypically analysed subjects participated in the functional studies.

7.4.1. T-lymphocyte subsets of non-zidovudine treated HIV-1 infected individuals: natural history

The ARC and AIDS patients' absolute CD4+ T-lymphocyte counts were substantially depressed in comparison with the laboratory controls ($p < 0.01$; Table 40) as was the AIDS subjects CD3+ numbers ($p < 0.01$; Table 38). However, ARC CD3+ and ARC and AIDS CD8+ subset counts (Table 39) were not significantly different to the control group.

Therefore, quantitative depression of peripheral CD3+ T-lymphocyte numbers occurred with severe disease, while CD4+ T-lymphocyte counts decreased earlier in the spectrum of HIV-1 disease. This has been documented previously (Seligmann et al., 1987; Eales and Parkin, 1988).

7.4.2. The effect of zidovudine treatment on T-lymphocyte subset counts

ZDV therapy exerted no significant effect on the circulating levels of CD3+ T-lymphocytes in AIDS and ARC patients. However, AIDS and ARC subjects' CD8+ numbers were elevated to and above control levels respectively. Similarly CD4+ ARC absolute numbers exhibited slight increase with ZDV in a median of 1.5 months administration, although this was not statistically significant. ZDV caused significant elevation of CD4+ numbers ($p < 0.05$; Table 40) to ARC patient levels, within a median period of 4 months treatment and similar observations have been made previously (Fischl et al., 1987; Pinching et al., 1989; Stambuk et al., 1989a,b).

Table 33: Absolute T-cell subset count Laboratory control subjects

Subject	Absolute count x10E+09 cells/l CD3+	CD4+	CD8+
1	1.23	0.68	0.58
2	2.13	0.83	0.99
3	1.66	1.08	0.65
4	0.88	0.48	0.37
5	0.85	0.43	0.46
6	1.67	1.14	0.70
7	1.25	0.69	0.61
8	1.58	0.94	0.62
9	1.16	0.71	0.66
10	0.84	0.59	0.45
x ± SEM	1.33 ± 0.14	0.76 ± 0.08	0.61 ± 0.05
(range) SEm	(1.19 - 1.47)	(0.68 - 0.84)	(0.56 - 0.66)

T-lymphocyte subset enumeration for ten laboratory control subjects was by flow cytometry.

Table 34: Absolute T-cell subset count ARC, no zidovudine therapy

Subject	Absolute count x10E+09 cells/l CD3+	CD4+	CD8+
1	0.57	0.02	0.59
2	0.40	0.05	0.23
3*	0.95	0.54	0.71
4	1.11	0.24	0.98
5	1.51	0.22	1.37
6	1.69	0.49	1.14
7	0.91	0.41	0.51
x ± SEM	1.02 ± 0.18	0.28 ± 0.08	0.79 ± 0.15
(range) sem	(0.84 - 1.20)	(0.20 - 0.36)	(0.64 - 0.94)

T-lymphocyte subset enumeration for seven ARC subjects all classified CDC IVC2 was by flow cytometry, except subject 3* where immunocytochemical labelling was employed. All subjects pre-zidovudine treatment.

Table 35: Absolute T-cell subset count ARC, receiving zidovudine

Subject	Absolute count x10E+09 cells/l CD3+	CD4+	CD8+
1	1.20	0.42	0.77
2	0.84	0.20	0.66
3	1.22	0.06	1.18
4	0.95	0.13	0.85
4#	1.26	0.15	1.19
5	1.50	0.65	0.82
6	1.48	0.48	1.06
7	1.47	0.46	1.04
7#	1.46	0.56	0.87
x ± SEM	1.26 ± 0.09	0.35 ± 0.08	0.92 ± 0.07
(range) sem	(1.17 - 1.35)	(0.27 - 0.43)	(0.85 - 0.99)

T-lymphocyte subset enumeration for seven ARC subjects all classified CDC IVC2 was by flow cytometry. All subjects were from the longitudinal study. As such the median result for each is quoted.

Table 36: Absolute T-cell subset count AIDS, no zidovudine therapy

Subject	Absolute count x10E+09 cells/l CD3+	CD4+	CD8+
1	0.08	0.01	0.09
2	0.42	0.02	0.05
3	0.32	0.02	0.31
4	1.88	0.08	1.42
5	0.10	0.02	0.31
6	0.68	0.09	0.54
7	0.32	0.07	0.27
8	0.47	0.03	0.44
9	0.48	0.02	0.50
10	0.64	0.07	0.59
11	0.42	0.02	0.43
x ± SEM	0.53 ± 0.15	0.04 ± 0.01	0.45 ± 0.11
(range) sem	(0.38 - 0.68)	(0.03 - 0.05)	(0.34 - 0.56)

T-lymphocyte subset enumeration for eleven AIDS patients was by flow cytometry. All subjects were classified CDC IVC1 except individuals 6 and 8 who were CDC IVD. Subects 1 to 5 were from the peripheral neuropathy study, and 6 to 11 were pre-zidovudine.

Table 37: Absolute T-cell subset count AIDS, receiving zidovudine

Subject	Absolute count x10E+09 cells/l CD3+	CD4+	CD8+
1*	0.56	0.21	0.46
2	2.04	0.22	1.80
3	0	0	0
4	1.04	0.31	0.83
5	0.77	0.06	0.68
6	1.47	0.14	1.50
7	0.35	0.02	0.34
8	2.35	0.58	1.88
9	0.98	0.41	0.51
x ± SEM	1.06 ± 0.26	0.22 ± 0.06	0.89 ± 0.23
(range)$_{sem}$	(0.80 - 1.32)	(0.16 - 0.28)	(0.66 - 1.12)

T-lymphocyte subset enumeration for nine AIDS subjects was by flow cytometry except individual 1* where immunocytochemical labelling was employed. All subjects were classified CDC IVC1 and all but subject 9 were from the peripheral neuropathy study. Subject 9 was from clinic.

Table 38
Absolute CD3+ T-lymphocyte count

	ARC no ZDV	ARC on ZDV	AIDS no ZDV	AIDS on ZDV
CONTROL	ns	ns	0.01	ns
ARC, no ZDV	-	ns	0.05	ns
ARC, on ZDV	-	-	0.01	ns
AIDS, no ZDV	-	-	-	ns

Comparison of absolute CD3+ T-lymphocyte count, the independent variables being CDC classification groups and zidovudine (ZDV) therapy.
Significant result are quoted as figures and represent probability less than 0.01 or 0.05 ($p < 0.01$ or 0.05).
Non significant results are represented as ns.

Table 39
Absolute CD8+ T-lymphocyte count

	ARC no ZDV	ARC on ZDV	AIDS no ZDV	AIDS on ZDV
CONTROL	ns	0.01	ns	ns
ARC, no ZDV	-	ns	ns	ns
ARC, on ZDV	-	-	0.01	ns
AIDS, no ZDV	-	-	-	ns

Comparison of absolute CD8+ T-lymphocyte count, the independent variables being CDC classification groups and zidovudine (ZDV) therapy.
Significant result are quoted as figures and represent probability less than 0.01 or 0.05 ($p < 0.01$ or 0.05).
Non significant results are represented as ns.

Table 40
Absolute CD4+ T-lymphocyte count

	ARC no ZDV	ARC on ZDV	AIDS no ZDV	AIDS on ZDV
CONTROL	0.01	0.01	0.01	0.01
ARC, no ZDV	-	ns	0.01	ns
ARC, on ZDV	-	-	0.01	ns
AIDS, no ZDV	-	-	-	0.05

Comparison of absolute CD4+ T-lymphocyte count, the independent variables being CDC classification groups and zidovudine (ZDV) therapy.
Significant result are quoted as figures and represent probability less than 0.01 or 0.05 ($p < 0.01$ or 0.05).
Non significant results are represented as ns.

N.B. Statistical analysis was by Student t-test.
Although we must be aware that the greater the number of separate t-tests the greater the risk of mistakenly rejecting the null hypothesis, the data shown here is unlikely to present this problem (J. Wadsworth, personal communication).

Figure 87: Cross-sectional summary of absolute CD3+ T-lymphocyte count

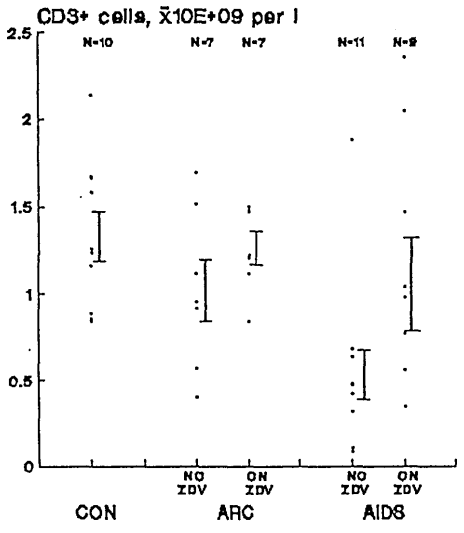


Figure 88: Cross-sectional summary of absolute CD8+ T-lymphocyte count

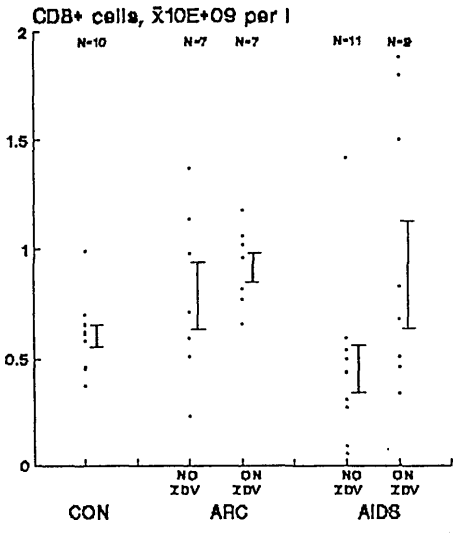
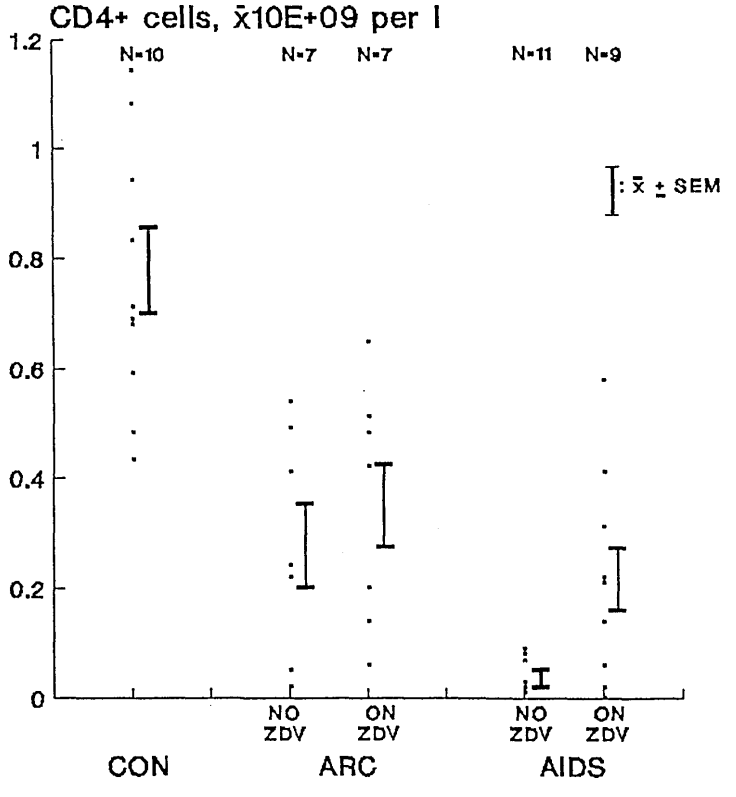


Figure 89: Cross-sectional summary of absolute CD4+ T-lymphocyte count



7.5. Enumeration of T-lymphocyte subsets: longitudinal study

The longitudinal absolute CD3+, CD4+ and CD8+ T-lymphocyte counts are tabulated with their respective coefficient of variations, or CVs (see section 2.10.2.) for each laboratory control (Figure 90) and HIV-1 infected subject. The ZDV treated ARC (Figures 94 and 95) and AIDS (Figures 99 and 100) patients have been numerically labelled to correspond with the serial functional studies.

7.5.1. Laboratory control subjects

The T-lymphocyte subset count of 4 laboratory control donors were monitored regularly over a median of 4.5 months (range: 3 to 7 months) and the data is shown graphically by Figures 91, 92 and 93. The CVs were found to range from 17 % to 29.5 % for CD4+ counts; from 8 % to 37.2 % for CD8+ counts; and from 10.3 % to 29.1 % in the case of CD3+ T-lymphocyte numbers.

7.5.2. Zidovudine treated ARC subjects

Seven ARC (CDC IVC2) patients, subjects 2 to 8 of the longitudinal functional studies, were followed over 1 to 7 months (median: 2 months) of ZDV treatment. The results of regular T-lymphocyte subset enumeration are delineated by Figures 96, 97 and 98.

The CD4+ T-lymphocyte counts of 4 of the 6 subjects (subjects 4 to 7) analysed at day zero were depressed in comparison with the control range, whereas the majority of CD8+ and CD3+ absolute counts were similar to the control values. These data concur with with cross-sectional results and such observations have been made in previous studies (see section 7.4.1.).

ZDV therapy was associated with an obvious increase of circulating CD4+ cells in 4 cases (subjects 4 to 7) within 1 to 2 months of administration. This was confirmed by their CVs of 70.7 %; 48.1 %; 65.1 % and 52.5 %. It is noteworthy that these subjects had the most depressed day

CONTROL 1

	Absolute subset count x10E+09 / l at Month 0	2	3	4	5	5.5	6	7	%CV
CD3+	0.94*	1.39	1.10	1.05	1.22	1.24	1.61	1.31	17.1
CD4+	0.63*	0.74	0.42	0.57	0.68	0.67	0.79	0.72	17.7
CD8+	0.49*	0.63	0.50	0.49	0.54	0.61	0.81	0.67	18.9

* : results by immunocytochemical labelling
(all other results by flow cytometry)

CONTROL 2

	Absolute subset count x10E+09 / l at Month 0	1	2	2.5	3	4	5	%CV
CD3+	2.14*	2.42*	2.02*	1.94*	1.73*	2.18	2.13	10.3
CD4+	0.96*	1.40*	1.18*	0.93*	0.76*	1.18	0.83	22.0
CD8+	1.02*	0.88*	1.02*	0.84*	te	0.98	0.99	8.0

te: technical error
* : results by immunocytochemical labelling
(all other results by flow cytometry)

CONTROL 3

	Absolute subset count x10E+09 / l at Month 0	1	2	3	3.5	4	%CV
CD3+	1.88	0.98	1.58	1.66	1.82	1.16	24.1
CD4+	0.93	0.68	1.07	1.08	1.15	1.04	17.0
CD8+	0.65	0.34	0.59	0.65	0.70	0.44	25.1

Results by flow cytometry.

CONTROL 4

	Absolute subset count x10E+09 / l at Month 0	1	2	3	%CV
CD3+	1.26	0.74	0.85	1.37	29.1
CD4+	0.68	0.38	0.43	0.68	29.5
CD8+	0.69	0.41	0.46	0.91	37.2

Results by flow cytometry.

FIGURE 90
ABSOLUTE T-LYMPHOCYTE SUBSET COUNT
LONGITUDINAL STUDY
LABORATORY CONTROL SUBJECTS

Figure 91: Longitudinal absolute CD3+ T-cell count of four laboratory control suubjects

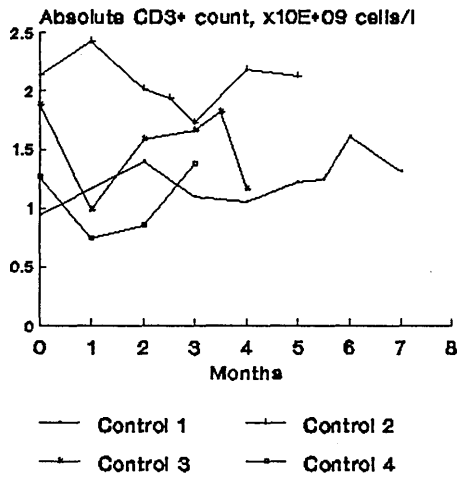


Figure 92: Longitudinal absolute CD8+ T-cell count of four laboratory control suubjects

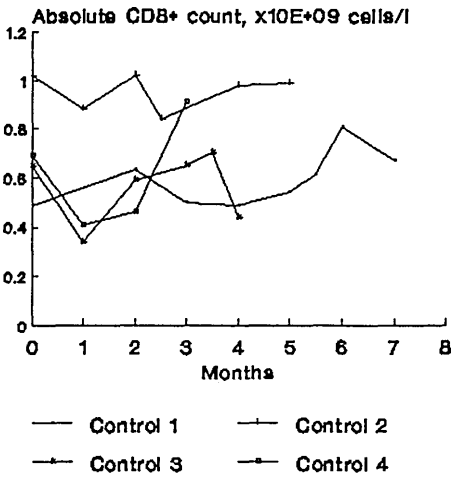
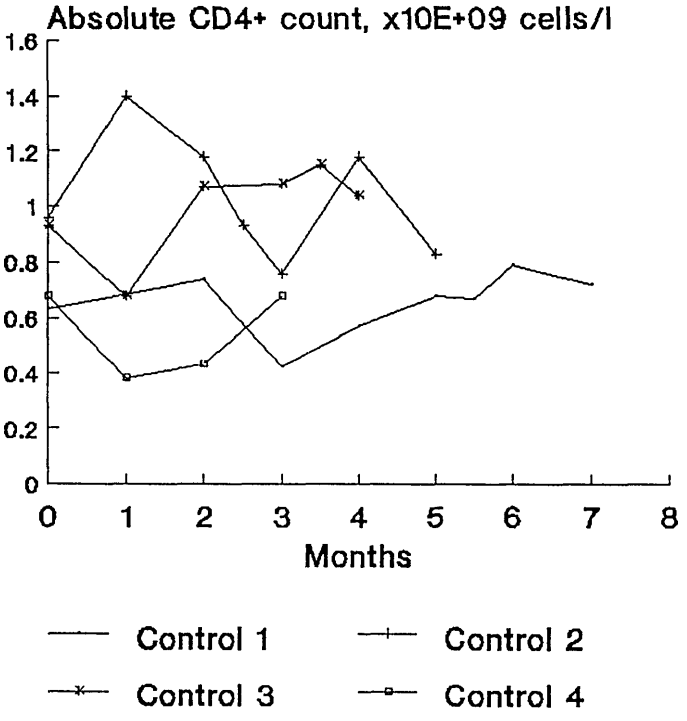


Figure 93: Longitudinal absolute CD4+ T-cell count of four laboratory control suubjects



zero CD4+ counts and that only 2 individuals (subjects 6 and 7) attained the laboratory control range with therapy. Likewise, subject 3 showed a slight increase of circulating CD4+ cells from months 1 to 2 of treatment; however numbers then decreased to month 7. These results are consistent with cross-sectional analysis (see section 7.4.2.) where a rise of peripheral CD4+ T-lymphocytes within a median of 1.5 months (range: 1 to 4 months) of ZDV therapy was seen. Other groups (Fischl et al., 1987; Pinching et al., 1989; Stambuk et al., 1989a,b) observed that ZDV therapy was associated with an increase of CD4+ cells within approximately 2 months of administration; although these trends were generally transient (see section 1.5.1.b). In contrast, the CD4+ T-lymphocyte levels of patients 2 and 8 decreased from day zero to month 1 of ZDV therapy. This trend was continued by the former patient to month 3, where his CD4+ counts increased towards pre treatment levels at month 7. Patient 2 and 8's counts were within the laboratory control range at all analysis time-points.

Similar to the observations made by cross-sectional analysis (see section 7.4.2.), ZDV therapy exerted no obvious effects on individual serial CD8+ and CD3+ T-lymphocyte absolute numbers. The numbers of circulating CD8+ and CD3+ cells increased slightly in 4 and 5 cases respectively, while a decrease was observed in the remaining subjects (Figures 96 and 97). However, calculation of subject CVs (Figures 94 and 95) suggests that the majority of these fluctuations may be attributed to individual or experimental variation. Furthermore, most patient absolute CD8+ and CD3+ cell counts were within the normal control range.

Clearly, the quantitative serial changes of peripheral CD8+ and CD3+ T-lymphocytes do not reflect the longitudinal changes of mitogenic PBMC functional response (see sections 3.3.2., 3.4.2., 4.5.2., and 4.6.2.) observed of these ZDV treated ARC subjects. However, a loose correlation of individual functional response with peripheral CD4+ numbers seems likely with short-term ZDV treatment.

SUBJECT 2: Absolute T-subset counts of an ARC subject receiving zidovudine

	Absolute subset count x10E+09 / l at						%CV
Month	0	1	2	3	4	7	
CD3+	0.95*	1.34	-	1.17	1.20	1.30	12.8
CD4+	0.54*	0.50	-	0.40	0.42	0.52	13.1
CD8+	0.71*	0.86	-	0.73	0.77	1.08	18.2

* : results by immunocytochemical labelling (all other results by flow cytometry)
- : absolute lymphocyte count not available

SUBJECT 3: Absolute T-subset counts of an ARC subject receiving zidovudine

	Absolute subset count x10E+09 / l at			%CV
Month	1	2	7	
CD3+	0.84	0.52*	0.99	30.6
CD4+	0.20	0.23*	0.21	7.2
CD8+	0.66	0.39*	0.82	34.9

* : results by immunocytochemical labelling (all other results by flow cytometry)

SUBJECT 4: Absolute T-subset counts of an ARC subject receiving zidovudine

	Absolute subset count x10E+09 / l at		%CV
Month	0	1	
CD3+	0.57	1.22	51.4
CD4+	0.02	0.06	70.7
CD8+	0.59	1.18	47.1

Results by flow cytometry.

SUBJECT 5: Absolute T-subset counts of an ARC subject receiving zidovudine

	Absolute subset count x10E+09 / l at				%CV
Month	0	1	2	3	
CD3+	0.40	0.95	1.26	-	50.1
CD4+	0.05	0.13	0.15	-	48.1
CD8+	0.23	0.85	1.19	-	64.3

Results by flow cytometry.
- : Absolute lymphocyte count not available

FIGURE 94
ABSOLUTE T-LYMPHOCYTE SUBSET COUNTS: LONGITUDINAL STUDY OF ARC SUBJECTS RECEIVING ZDV

SUBJECT 6: Absolute T-subset counts of an ARC subject receiving zidovudine

Month	Absolute subset count x10E+09 / l at 0	1	%CV
CD3+	1.11	1.50	21.1
CD4+	0.24	0.65	65.1
CD8+	0.98	0.82	12.6

Results by flow cytometry.

SUBJECT 7: Absolute T-subset counts of an ARC subject receiving zidovudine

Month	Absolute subset count x10E+09 / l at 0	1	%CV
CD3+	1.51	1.48	1.4
CD4+	0.22	0.48	52.5
CD8+	1.37	1.06	18.0

Results by flow cytometry.

SUBJECT 8: Absolute T-subset counts of an ARC subject receiving zidovudine

Month	Absolute subset count x10E+09 / l at 0	1	2	%CV
CD3+	1.69	1.47	1.46	8.4
CD4+	0.49	0.46	0.56	10.2
CD8+	1.14	1.04	0.87	13.4

Results by flow cytometry.

FIGURE 95
ABSOLUTE T-LYMPHOCYTE SUBSET COUNTS
LONGITUDINAL STUDY
ARC SUBJECTS RECEIVING ZIDOVUDINE

Figure 96: Longitudinal absolute CD3+ T-cell count of seven ARC subjects, CDC IVC2, receiving zidovudine

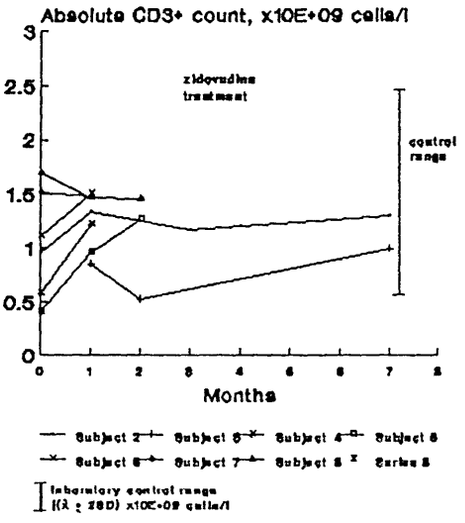


Figure 97: Longitudinal absolute CD8+ T-cell count of seven ARC subjects, CDC IVC2, receiving zidovudine

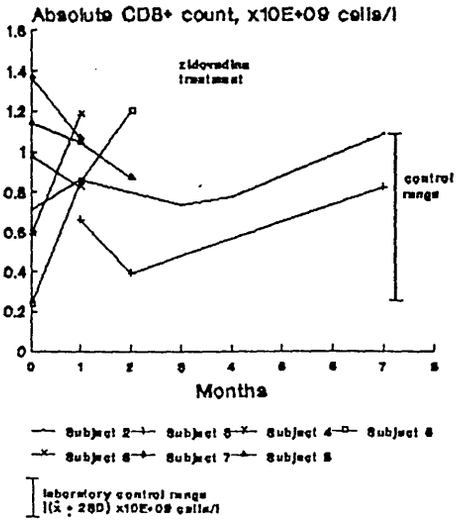
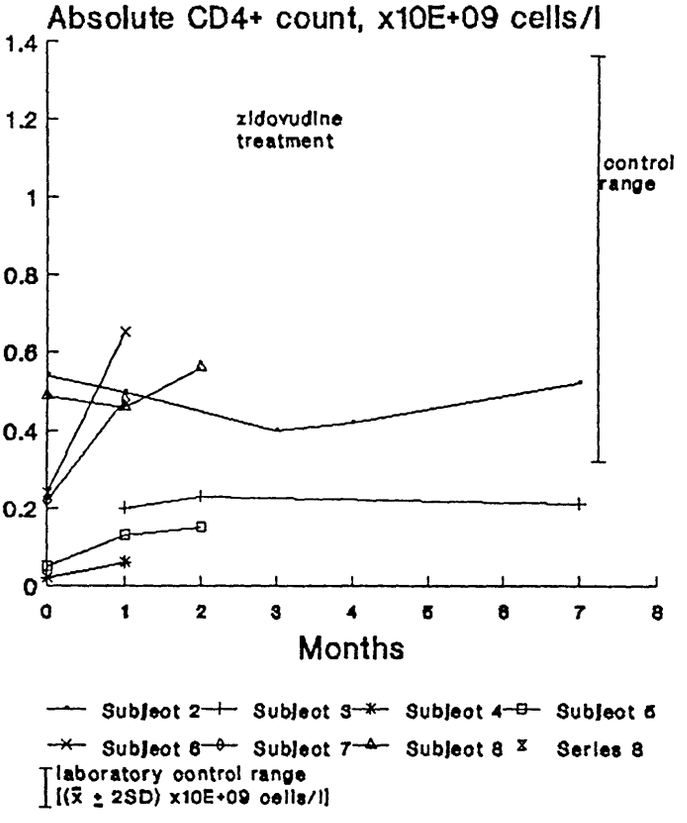


Figure 98: Longitudinal absolute CD4+ T-cell count of seven ARC subjects, CDC IVC2, receiving zidovudine



ZDV therapy was associated with an increase of CD4+ T-lymphocytes and an increase of PHA induced PBMC functional response in 3 subjects (patients 5, 6 and 7; see sections 3.3.2. and 4.5.2.) within 1 or 2 months of administration; although such an obvious pattern was not apparent in other subjects. Within 3 months of administration, subject 4 showed elevated CD4+ numbers and raised PHA PBMC proliferative response, although his gIFN response was erratic. However, subject 8's CD4+ counts did not change within 2 months of starting ZDV; during this time his Con A proliferative and Con A and PHA gIFN response improved markedly. A slight increase in CD4+ numbers was observed in subject 3 between months 1 and 2 of therapy - in contrast, a decrease of PHA induced PBMC proliferation and gIFN synthesis was noted; in addition a relatively steady CD4+ count to month 7 saw an improvement of functional response. Subject 2's CD4+ count fell from day zero to month 3 of ZDV administration, whereas a transient improvement of PHA functional response was noted - an increase of CD4+ numbers during months 3 to 7 was also paralleled by a continued improvement of functional response to above pre treatment levels.

7.5.3. Zidovudine treatment of AIDS subjects

Peripheral CD4+, CD8+ and CD3+ T-lymphocyte numbers were monitored serially for 5 patients with AIDS (CDC IVCI or IVD) receiving ZDV for 1 to 12 months (median: 2 months). The results of analysis are shown graphically by Figures 101, 102 and 103.

The CD4+ T-lymphocyte absolute counts of all 4 patients (subjects 3, 4, 5 and 6) analysed before therapy or at day zero of ZDV administration were markedly depressed relative to laboratory control levels. Three of these four subject's (patients 3, 5 and 6) CD3+ counts fell within the lower end of the laboratory control range; the same subjects had relatively normal circulating CD8+ numbers. The fourth patient's (subject 4) absolute CD3+ and CD4+ counts fell

SUBJECT 2: Absolute T-subset counts of an AIDS subject receiving zidovudine

	Absolute subset count x10E+09 / l at					%CV
Month	4	8	10	11	12	
CD3+	0.11*	0.07	0.07	0.07	0.06	25.6
CD4+	0.05*	0.004	0.004	0.006	0.004	150
CD8+	0.14*	0.13	0.12	0.12	0.10	12.2

* : results by immunocytochemical labelling (all other results by flow cytometry)

SUBJECT 3: Absolute T-subset counts of an AIDS subject receiving zidovudine

	Absolute subset count x10E+09 / l at		%CV
Month	0	1	
CD3+	0.68	0.94	22.7
CD4+	0.09	0.22	59.3
CD8+	0.54	0.78	25.7

Results by flow cytometry.

SUBJECT 4: Absolute T-subset counts of an AIDS subject receiving zidovudine

	Absolute subset count x10E+09 / l at			%CV
Month	-4	0	2	
CD3+	0.23	0.32	0.35	20.8
CD4+	0.06	0.07	0.10	27.2
CD8+	0.18	0.27	0.26	20.8

Results by flow cytometry.

FIGURE 99
ABSOLUTE T-LYMPHOCYTE SUBSET COUNTS
LONGITUDINAL STUDY
AIDS SUBJECTS RECEIVING ZIDOVUDINE

SUBJECT 5: Absolute T-subset counts of an AIDS subject receiving zidovudine

	Absolute subset count x10E+09 / l at			%CV
Month	0	2	4	
CD3+	0.45	1.01	0.63	41.0
CD4+	0.03	0.11	0.05	65.7
CD8+	0.44	0.89	0.59	35.8

Results by flow cytometry.

SUBJECT 6: Absolute T-subset counts of an AIDS subject receiving zidovudine

	Absolute subset count x10E+09 / l at			%CV
Month	0	1	2	
CD3+	0.64	0.76	1.51	48.6
CD4+	0.07	0.12	0.19	47.6
CD8+	0.59	0.67	1.21	41.0

Results by flow cytometry.

Figure 100
Absolute T-lymphocyte subset counts
Longitudinal study
AIDS subjects receiving zidovudine

Figure 101: Longitudinal absolute CD3+ T-cell count of five AIDS subjects receiving zidovudine.

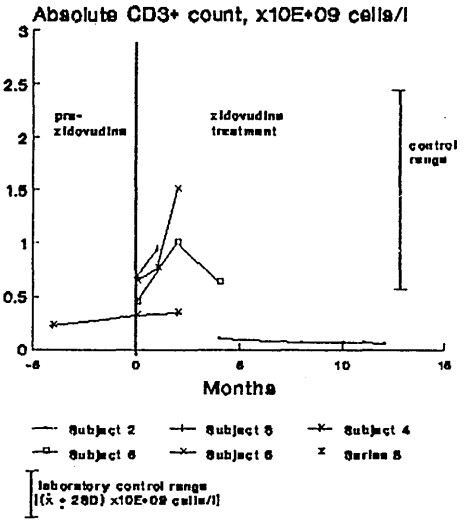


Figure 102: Longitudinal absolute CD8+ T-cell count of five AIDS subjects receiving zidovudine.

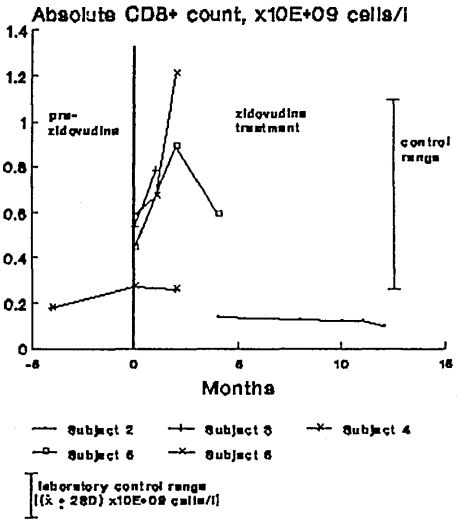
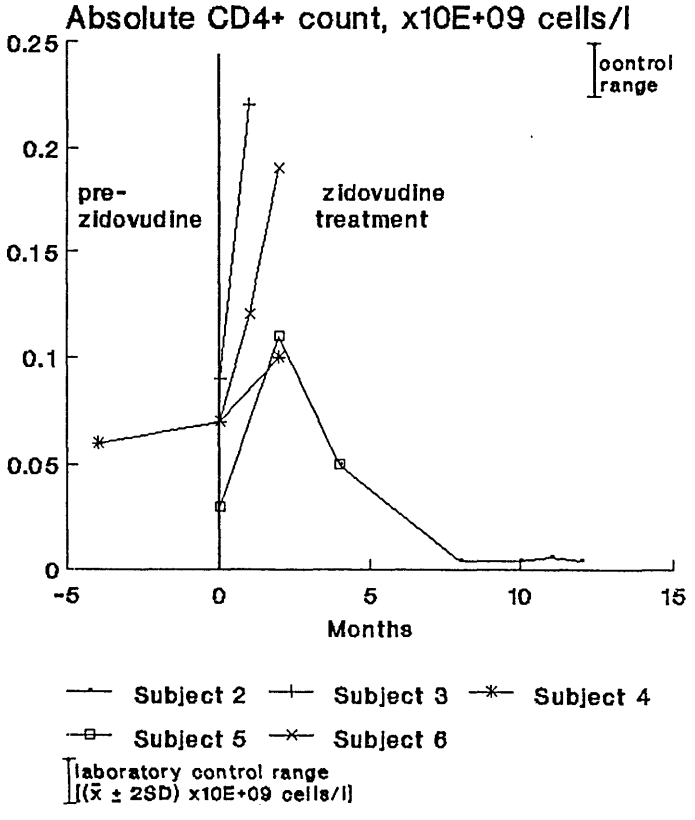


Figure 103: Longitudinal absolute CD4+ T-cell count of five AIDS subjects receiving zidovudine.



just below the control range. These results are consistent with those observations made by cross-sectional analysis (see section 7.4.1.).

The circulating CD4+ T-lymphocyte numbers of subjects 3, 4 and 6 were raised within 1 or 2 months of ZDV therapy. Although this was also true of subject 5, his CD4+ numbers decreased to almost pre treatment level by month 4 of administration. Subject 2's CD4+ counts deteriorated from months 4 to 12 of ZDV. Cross-sectional analysis (see section 7.4.2.) found that ZDV significantly elevated CD4+ counts within a median of 4 months administration.

The CD3+ and CD8+ counts of 3 of the 5 patients (subjects 3, 5 and 6) were observed to increase over 1 to 4 months of ZDV therapy. Similar to the situation observed of ARC subjects (see section 7.5.2.), these AIDS patients' CVs were similar to those of the laboratory controls, therefore, individual or experimental variation cannot be dismissed as influencing factors in their trends of improvement. Subject 4 also showed slight increase of CD3+ numbers within 2 months of ZDV; while his CD8+ counts remained relatively stable from day zero of treatment. Subject 2's CD3+ and CD8+ numbers paralleled the trend of progressive deterioration observed for his CD4+ counts from months 4 to 12 of ZDV. Likewise, subject 2's CD3+ and CD8+ counts were markedly depressed relative to the control range throughout testing.

As previously suggested of ARC subjects (see section 7.5.2.) at least some correlation seems likely between these AIDS patients' mitogen induced PBMC functional response and their circulating CD4+ cell numbers with short-term ZDV treatment. CD3+ and CD8+ numbers do not seem to correlate with PBMC function. Subjects 3, 4 and 6 increase of CD4+ numbers was paralleled by an improvement of PHA and Con A PBMC proliferative (see section 3.3.3. and 3.4.3.) and gIFN (see section 4.5.3. and 4.6.3.) responses.
However, while subject 4's CD4+ numbers increased from day zero of administration to 2 months of ZDV therapy and then decreased between 2 and 4 months, his PHA induced PBMC

functional responses improved throughout the whole of this treatment period. From 4 to 12 months of treatment, subject 2's CD4+ counts progressively declined; his PHA proliferative and gIFN response at this time was erratic but remained above their baseline.

7.5.4. Summary of serial T-lymphocyte subset enumeration in zidovudine treated ARC and AIDS patients

Serial studies of 7 ARC and 5 AIDS subjects revealed that ZDV therapy did not exert a marked effect on their circulating CD8+ and CD3+ T-lymphocyte numbers - although a trend to increase was noted within the first few months of therapy, particularly in AIDS patients (see sections 7.5.2. and 7.5.3.). These observations are similar to cross-sectional findings (see section 7.4.1.).

ZDV therapy was associated with an obvious increase of peripheral CD4+ T-lymphocytes in the majority of studied patients (4 ARC and 3 AIDS cases) within 1 or 2 months of administration. Again these results are consistent with cross-sectional observations (see section 7.4.2.).

The quantitative serial changes in peripheral CD8+ and CD3+ T-lymphocytes did not reflect the longitudinal changes of mitogenic PBMC functional responses. However in 3 of 7 ARC subjects and 3 of 5 AIDS subjects, a loose correlation of individual functional response with peripheral CD4+ T-lymphocyte numbers was apparent within the first 2 months of ZDV treatment. This correlation was not obvious in the remaining 6 subjects. In emphasis, ARC subject 8 showed marked improvement of functional response while his CD4+ counts remained relatively stable within the first 2 months of therapy. Testing over a longer treatment period for AIDS subject 4 revealed a transient increase of CD4+ numbers whereas functional response improved during the 4 months study period. ARC subject 3 was tested throughout 7 months of ZDV therapy: relatively stable CD4+ numbers were observed while functional responses improved.

These results show a ZDV associated rise in CD4+

lymphocyte counts within 2 months of administration and imply that they remain relatively stable or decline thereafter. In contrast, immunological benefit as perceived by mitogenic functional testing persists for longer and better correlates with temporal clinical trends (see section 3.5. and 4.7.). Therefore, PBMC mitogenic functional response may prove a better marker of ZDV or other antiretroviral associated immunological effects than the CD4+ phenotyping system.

7.6. Further investigation of a cell population with antigen expression CD3-, CD4-, DR+, CD8+ and CD57+ in an AIDS subject receiving zidovudine

A subject classified as CDC IVC1 and labelled (2) in previous longitudinal functional and phenotypic studies was found to have consistently depressed CD3+ and CD4+ circulating T-lymphocyte numbers relative to comparable ZDV treated AIDS patients, whereas his CD8+ counts were similar (see Figures 99 to 103). These observations were made by immunocytochemistry and flow cytometry methodologies over months 4 to 12 of ZDV therapy; unfortunately no phenotypic data were available previously. PBMC obtained from this subject showed erratic improvement in proliferative response (see Figures 21 and 23; section 3.3.3.) and deterioration in gIFN response (see Figures 52 and 54; section 4.5.3.) to PHA during 1 year of ZDV treatment. These functional and phenotypic parameters were inconsistent with his general clinical well-being on ZDV. However, he discontinued treatment earlier this year because of ZDV associated bone-marrow toxicity, manifested as low peripheral neutrophil counts and is presently receiving 2',3'-dideoxyinosine (B. Peters, personal communication).

This patient's flow cytometry scatter cytograms, collected from previous T-lymphocyte subset analysis, demonstrated unusual features suggesting an expanded peripheral CD57+ CD8+ natural killer (NK) cell population, as found in some other HIV-1 infected subjects (C. Clark, personal communication). A peripheral blood sample from this

patient was analysed for such a phenotypically defined NK population.

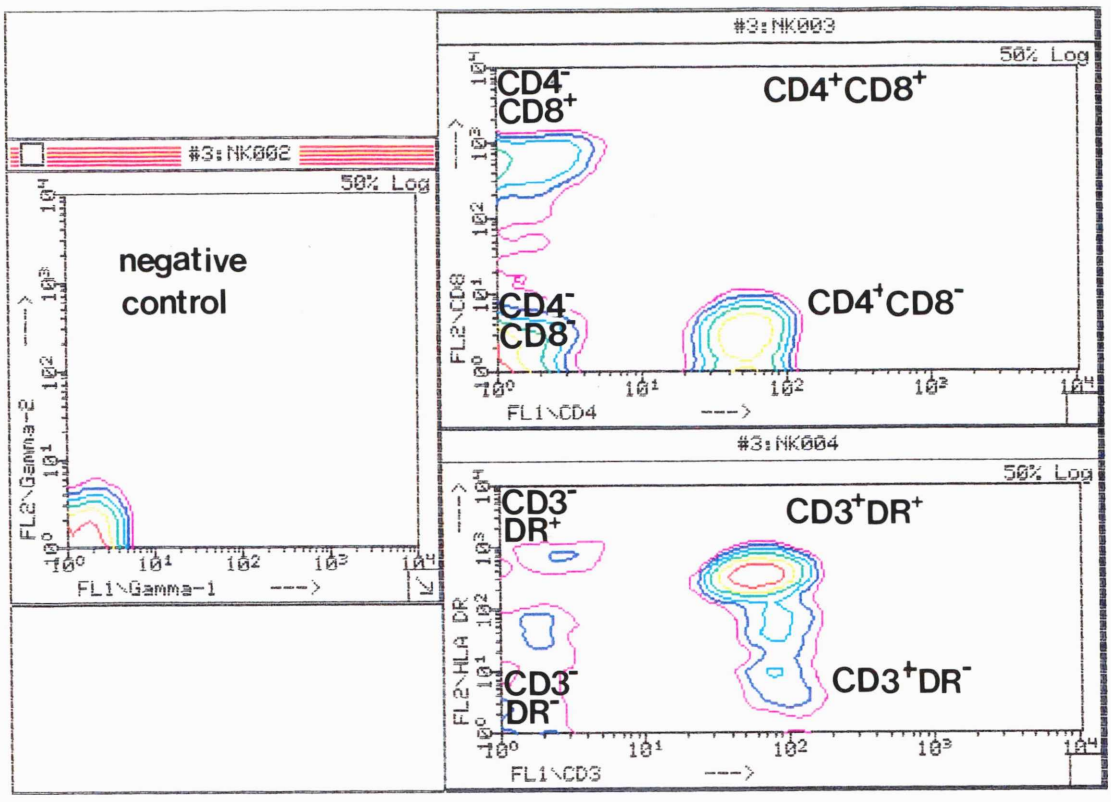
The basic protocol employed has been described previously (see section 2.9.2.). The Simulset reagent (95-0010, Becton Dickinson Immunocytochemistry Systems, Mountain View, USA) used to detect the CD57 NK antigen was the only FACScan characterised reagent allowing 2 colour analysis including NK cells available at the time. It consisted of fluorescein isothiocyanate (FITC)-conjugated Mabs to CD57 or Leu 7 (a carbohydrate antigen found on 20 % to 50 % of circulating NKs) and phycoerythrin (PE)-conjugated Mabs to the CD8 antigen. Testing for the antigens CD3, CD4, CD8 and HLA-DR was also performed as the CD57 marker is coexpressed by a subset of T-lymphocytes (Landay et al., 1990). Therefore other Mabs were required to assess what proportion of CD57+ cells represented NK cells and what proportion T-lymphocytes.

The CD3+, CD4+ and CD8+ absolute T-lymphocyte subset counts were consistent with previous enumeration (see Figure 99, section 7.5.3.). In addition, this AIDS patient was found to have an NK population amounting to 20 % of the gated population or 0.04×10^6 cells/ml. These cells had antigen expression CD3-, CD4-, DR+, CD8+ and CD57+. However, the CD8 and HLA-DR staining of the CD57+ CD8+ NK population was observed to be weak. The HLA-DR staining is typical of HIV-1 infected subjects (C. Clark, personal communication). Such findings were typical of 7 other HIV-1 infected subjects shown to express the DR+ CD57+ CD8+ NK phenotype (C. Clark, personal communication). Figures 104 and 105 delineate a colour print-out of results for one of these patients (an ARC subject classified as CDC IVC2); AIDS subject (2) was so lymphopenic as to make interpretation of the print-out unclear.

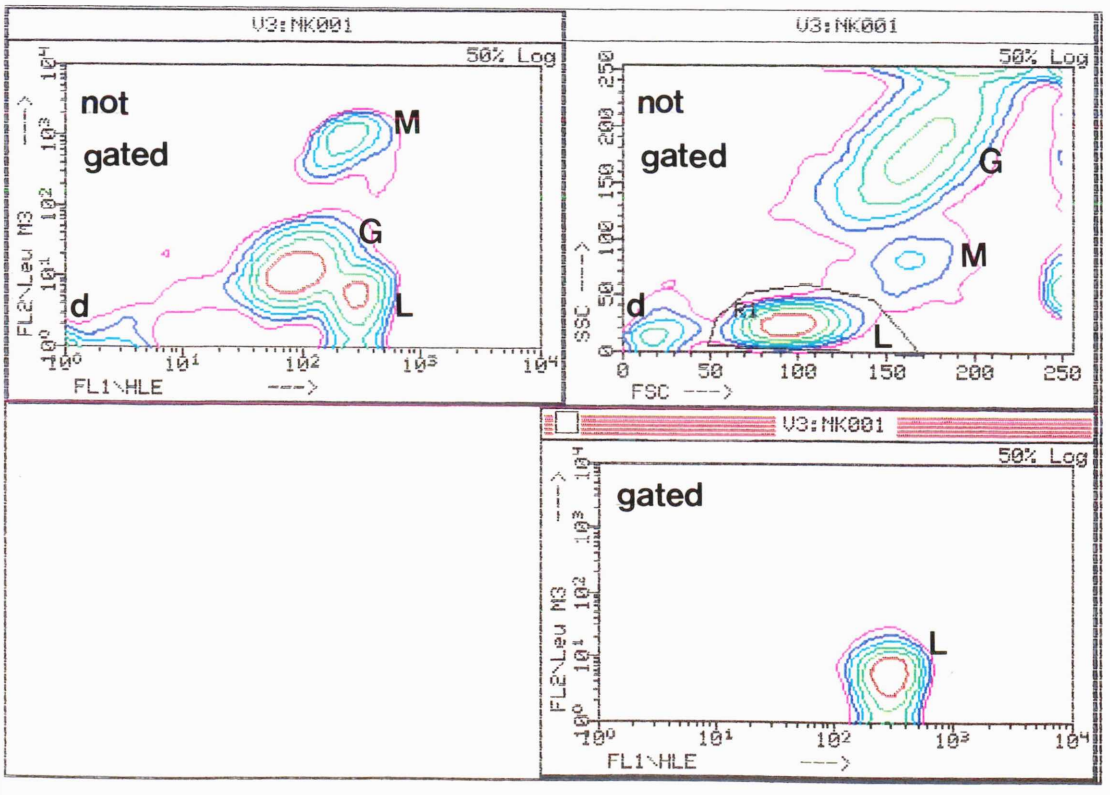
Several previous studies have documented an HIV-1 associated increase of the CD57+ CD8+ NK population in comparison with heterosexual controls (Lewis et al., 1985, Prince et al., 1985, Ziegler-Heitbrock et al., 1985). In this

FIGURE 104

1. Analysis of gated lymphocyte population: CD3, CD4, CD8, HLA-DR



2. Gating the lymphocyte population



d: debris

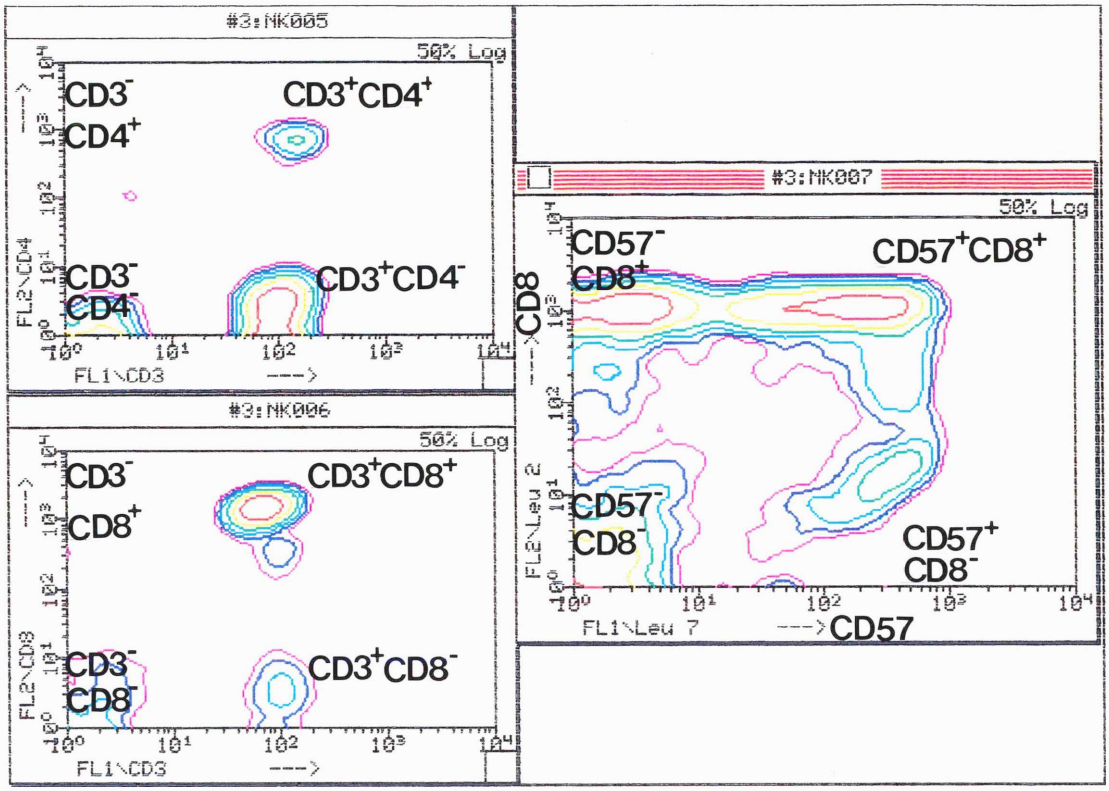
L: lymphocyte

G: granulocyte

M: monocyte

FIGURE 105

Analysis of gated lymphocyte population: CD3, CD4, CD8, CD57



limited study, it was not possible to determine whether our patient had a relatively expanded CD57+ CD8+ NK population or one which had newly arisen. However, circumstantial evidence does indicate the rarity of this observation: the scatter and fluorescence cytograms of AIDS subject (2) consistently showed an unusual and weakly stained CD8+ population, distinct from the classically defined T-lymphocyte subset cluster (CD3+ CD8+). This secondary population was not observed in heterosexual controls nor other HIV-1 patients analysed for this report. More indirectly, he had exhibited profound depression of CD3+ lymphocytes, even relative to comparable AIDS subjects (see Figures 99 to 103).

The presence of such a CD3- CD4- DR+ CD57+ CD8+ NK population, together with severe depression of total T-lymphocytes may have affected this subject's PBMC functional responses to PHA. This mitogen acts through the CD3 moiety of the T-cell receptor (Valentine et al., 1985). Therefore, erratic proliferative and gIFN responses may have been caused by the small numbers of PHA functional responsive cells (CD3+ T-lymphocytes) in the PBMC functional cultures; the comparatively large numbers of the NK cells relative to heterosexual controls and other HIV-1 infected subjects may have passively blocked PHA molecules access to the CD3+ lymphocytes; in addition, the CD57+ CD8+ NK population may have mediated lytic events or released lymphokines in vitro to the detriment of CD3+ T-lymphocyte cell viability or PHA induced functional capabilities. Assessment of usual NK mediated lysis or lymphokine release were not performed for this subject.

It is of interest, though of uncertain significance, that this patient had the very unusual AIDS opportunistic infection, disseminated strongyloidiasis, during the course of the above investigation (A.J. Pinching, personal communication).

CHAPTER 8

DISCUSSION

8.1. The principal findings and conclusions of this project

The central aim of this project was to evaluate labarotory parameters of immunological functional and phenotypic change as correlates of clinical efficacy following ZDV treatment of HIV-1 symptomatic disease. In fulfilment of this objective, various *in vitro* parameters of lymphocyte quantitative and qualitative immunocompetence were assessed by cross-sectional and longitudinal analysis.

Cross-sectional study revealed that absolute CD4+ T-lymphocyte numbers were substantially depressed in subjects with ARC or AIDS, as were AIDS patients' CD3+ counts. ZDV therapy exerted no significant effect on the level of circulating CD3+ cells, while CD4+ and CD8+ T-lymphocyte numbers increased, at least initially, following ZDV administration. These studies were confirmed by serial analysis.

Analysis of a cross-sectional natural history cohort showed that the proliferative and gIFN responses of peripheral lymphocytes to the T-cell mitogens PHA and Con A decreased with progressive HIV-1 disease. Substantial decline of proliferation was observed before the onset of clinical symptoms, while gIFN synthesis became significantly impaired with symptomatic disease. Both cross-sectional and longitudinal analysis of patients with ARC and AIDS demonstrated that ZDV substantially augmented these mitogenic responses in parallel with clinical improvement. In contrast, three subjects receiving AL 721, which showed no evidence of clinical benefit, exhibited either no change or deteriorating serial PHA proliferative and gIFN response patterns. Furthermore, one of these patients showed significant improvement when treated with ZDV after discontinuing AL 721.

Serial observations demonstrated that HIV-1 associated phosphatidylinositol metabolism defects are reversed with ZDV therapy and are coincident with the improvements seen in PHA induced proliferation and gIFN synthesis.

In essence, the mitogenic proliferative and gIFN response, particulary to PHA, may better parallel temporal trends of clinical efficacy than CD4+ T-lymphocyte enumeration following ZDV treatment, as the magnitude of functional improvement may better account for ZDV's profound clinical benefits. It indeed could be argued that these functional responses are less surrogates than they are direct measures of the relevant variables underlying changes in immunological status. Therefore, future evaluations of antiretroviral therapy, particularly of nucleoside analogues, may benefit from the addition of lymphocyte functional assays in addition to the now standard enumeration of CD4+ T-lymphocytes. Analysis of inositol polyphosphate metabolism may also prove useful in this context.

The T-lymphocyte proliferative and gIFN response to the soluble recall antigen, SK/SD, was profoundly depressed in AIDS subjects; however, in contrast to the mitogen system, ZDV therapy had no obvious effect.

ZDV also apparently failed to reverse the abnormal synthesis of IgG, IgM and IgD, produced spontaneously and in response to PWM by lymphocytes obtained from ARC and AIDS patients.

The in vitro response to SK/SD by T-lymphocytes and Ig production by B-lymphocytes do not appear useful markers of ZDV efficacy in symptomatic HIV-1 disease. These observations may reflect the insensitivity of the assay systems employed; alternatively, ZDV may exert no effect on such defects and thus provide a putative explanation of ZDV's inability to stem HIV-1 disease progression completely.

8.2. The utility of the studied immunological markers in the evaluation of ZDV therapy

In order to evaluate in vitro markers of immunological change following ZDV treatment of HIV-1 symptomatic disease, it was essential to ensure adequate control of the analysis systems utilised.

St. Mary's offers ZDV therapy to all patients with

AIDS and opportunistic infection (CDC IVC1) or Kaposi's sarcoma (CDC IVD) and to ARC patients with two or more features of CDC groups IVA or IVC2. Therefore, it was not possible serially to monitor ZDV untreated patients as control of longitudinally studied subjects receiving this therapy. An open pilot study of AL 721 provided an opportunity to conduct a comparative study and was appropriate since, in contrast to ZDV, clinical, virological and neuropsychological data has shown no evidence of benefit from AL 721 treatment (Peters et al., 1990; see section 1.4.2.a).

An ideal laboratory marker should accurately predict the ultimate clinical response of individuals receiving treatment soon after the initiation of therapy (Valentine and Jacobson, 1990); longitudinal studies, validated by cross-sectional analysis, attempted to evaluate the immunological markers employed with respect to this criteria. Furthermore, these studies provided the means to assess functional markers, in comparison with the now routine enumeration of CD4+ T-lymphocytes, as temporal correlates of ZDV efficacy.

Cross-sectional studies on the natural history of HIV-1 infection encompassed patients representing the spectrum of HIV-1 infection. Asymptomatic individuals were categorised by their CDC grouping and comprised subjects with or without generalised lymphadenopathy (CDC II or III); lymphadenopathy in the absence of constitutional symptoms such as weight loss, fever or diarrhoea is not considered as clinical immune deficiency, nor does it appear to signify a greater risk of progression to frank AIDS than for group II subjects. Symptomatic patients were classified as ARC (CDC IVC2) or AIDS (CDC IVC1 and IVD). In view of limited subject numbers, the AIDS group comprised subjects with opportunistic infection and a small number of patients with Kaposi's sarcoma, although the latter individuals have been observed to fare clinically and immunologically better than the former (Pinching, 1990). These studies provided essential

information on the detectability of immunological functional markers across the spectrum of HIV-1 infection; ideally, a laboratory marker for clinical efficacy should be detectable in the majority of individuals at all stages of the disease (Valentine and Jacobson, 1990).

A study on the effect of ZDV in symptomatic disease was also assessed by cross-sectional analysis of randomly selected individuals. Comparison with the natural history cohort provided data on the magnitude of marker change following ZDV administration.

8.2.1. Lymphocyte phenotypic studies

Circulating CD4+ T-lymphocytes are now routinely enumerated for patients with AIDS or at risk for AIDS. Although considerable controversy surrounds their value as a surrogate marker of HIV-1 disease progression and of clinical efficacy following ZDV therapy, most investigators would agree that it is useful to have periodic measurements of CD4+ T-cell level (see section 1.5.1.a,b). Since both CD4+ T-lymphocyte depletion and cellular dysfunction contribute to the pathogenesis of AIDS (see section 1.3.3.), this study reported absolute CD4+, as well as CD8+, cell counts in parallel with functional data.

The cross-sectional natural history cohort comprised patients classified as ARC or AIDS only; data on asymptomatic subjects was not reported in lieu of technical difficulties with the immunocytochemical procedure (see section 7.). However, CD4+ (Detels et al., 1988; Lang et al., 1989; see section 1.5.1.a,b) and CD8+ (Gaines et al., 1990; Lang et al., 1989; Pedersen et al., 1990; see section 1.5.2.) T-lymphocyte enumeration has been previously described for asymptomatic subjects.

As expected, in view of many past studies (see section 1.5.1.a), ZDV untreated ARC and AIDS patients' absolute CD4+ T-lymphocyte counts were substantially depressed relative to heterosexual control levels (see section 7.4.1.). The mean peripheral CD4+ counts reported by

this study (0.28 and 0.04 x 10^9 cells/ l for ARC and AIDS patients respectively) were lower than figures documented during an 18 months period prior to the development of AIDS (0.25 x 10^9 cells/l; Detels et al., 1988; Lang et al., 1989; Taylor et al., 1989; see section 1.5.1.a). This discrepancy likely indicates differences in experimental procedure and emphasises the need for stringent quality control in multicentre trials.

In contrast, CD8+ T-cell levels were apparently raised in ARC and depressed in AIDS patients in comparison with laboratory controls, although statistical significance was not achieved (see section 7.4.1.). CD8+ lymphocytosis has been previously observed shortly after seroconversion (Gaines et al., 1990; Pedersen et al., 1990), after which levels remain relatively stable until fall with the onset of severe clinical deterioration (Lang et al., 1989; Lau et al., submitted for publication; see section 1.5.2.); the results on CD8+ T-lymphocyte numbers presented here are consistent with these findings.

ZDV therapy (see Figure 88, section 7.4.2.) of ARC subjects for a median 1.5 months (range: 1 to 4 months) was associated with a slight, although statistically non-significant, increase in peripheral CD4+ cell numbers (mean difference: 0.07 x 10^9 cells/l). ZDV significantly augmented AIDS patient CD4+ counts to ARC patient levels (median difference: 0.18 x 10^9 cells/l) within a median period of 4 months (range: 1 to 30 months) administration. These observations are consistent with results obtained by the American placebo-controlled study over similar periods of ZDV administration; Fischl et al (1987) reported that the number of CD4+ cells was significantly increased in AIDS subjects (apparent from 1 month to 4 months therapy) and in ARC subjects (apparent from 1 month to 5 months therapy) receiving 250 mg ZDV four hourly, as compared with those patients receiving placebo (see section 1.4.1.b). Similar observations have been reported by open-label studies conducted in the UK (Pinching et al., 1989; Stambuk et al.,

1989a,b).

The quantitative increase of CD4+ T-lymphocytes observed by this study, as indicated above, seemed relatively small to explain the profound clinical benefits of ZDV treatment alone, especially since the clinical effect of ZDV was most evident in ARC patients. Fischl et al (1990b) has also found that patients with mildly symptomatic ARC, who had between 0.2 and 0.5 x 10^9 CD4+ cells/l on entry, was associated with a median rise of only 0.025 x 10^9 cells/l above pre treatment levels apparent from 1 month through 4 months ZDV treatment (see section 1.5.1.b). Valentine and Jacobson (1990) have postulated that improvement of lymphocyte function may provide a better explanation of ZDV's clinical benefit.

Previous studies have indicated that patients treated with ZDV show a transient rise in CD4+ lymphocyte count, which reaches a peak one month or two after the initiation of therapy and falls thereafter; however, clinical benefit from ZDV is apparent only after 6 to 8 weeks of therapy and persists for many months (Fischl et al., 1987; Pinching et al., 1989; Stambuk et al., 1989a,b). In view of this evidence, T-lymphocyte subset analysis was performed regulary for a number of ARC and AIDS patients participating in the longitudinal studies of functional response.

Serial CD4+ and CD8+ T-lymphocyte counts were determined for 7 ARC and 5 AIDS patients and 4 laboratory control subjects (see sections 7.5.2., 7.5.3. and 7.5.1. respectively). Restricted data prevented formal analysis of laboratory control and patient groups results by time series analytical techniques. However, informal comparison of the patient with control coefficient of variation, as a measure of experimental and individual variation (see section 2.10.2), provided an appropriate and useful gauge of patient response trends with therapy. The magnitude of patient response during ZDV therapy was assessed by setting the laboratory workers mean net response plus or minus 2 standard deviations as the normal range (see section 3.4.). These

procedures were also utilised in serial studies of CD8+ and CD3+ T-lymphocyte count and of functional response (see sections 3.3., 3.4., 4.5. and 4.6.).

Since there is a wide variation in range of T-lymphocyte subset counts between patients at a given disease state and in the range of response to ZDV, as shown by the cross-sectional study (see Tables 34 to 37), an alternative assessment would compare the slope of sequential observations for each subject prior to the institution of therapy. Similar calculations have been used in studies of CD4+ T-lymphocyte decline during the natural history of HIV-1 disease (Detels et al., 1988; Munoz et al., 1988). Changes in the slopes could then be validated by correlating with clinical outcome; this method has been used by Fischl et al (1990b) in analysis of the effect ZDV treatment on CD4+ absolute counts in patients with early symptomatic disease. However this analytical technique was not appropriate to the present study since typically analysis of one sample only was possible pre treatment.

Serial study revealed that ZDV therapy was associated with an obvious increase of peripheral CD4+ T-lymphocytes in 4 of 7 ARC and 3 of 5 AIDS patients within 1 or 2 months of ZDV administration. However, 2 ARC subjects who were studied for 7 months therapy (see Figure 97, section 7.5.2.) and an AIDS subject monitored for 4 months (see Figure 102, section 7.5.3.) showed only transient increase of CD4+ count within those periods. Although, these patients only were studied over extended drug administration times, the data obtained is consistent with that of larger cohorts (Fischl et al., 1987; Pinching et al., 1989; Stambuk et al., 1989a,b). In evidence, Fischl et al (1987) reported that after 3 months ZDV therapy, a gradual decline in the number of CD4+ cells was noted in patients with AIDS who were receiving ZDV; after 5 months, a return to base-line values occurred in many of these subjects and appeared to coincide with the development of neutropenia. In subjects with ARC who were receiving ZDV, increases in the number of CD4+ cells

persisted throughout most of the 6 month study, although a decline in the number of CD4+ cells was noted after 5 months among subjects with a pre treatment CD4+ T-lymphocyte count below 0.1 x 10^9 cells/l.

Therefore, CD4+ T-lymphocyte counts do not typically parallel the clinical benefits of ZDV therapy in symptomatic HIV-1 disease which is evident from 4 or 6 weeks to at least 6 months of administration (Fischl et al., 1987; Pinching et al., 1989; Stambuk et al., 1989a,b).

Cross-sectional analysis by the present study found that CD8+ T-lymphocyte numbers were elevated, although not significantly, by ZDV administration in both ARC (mean difference: 0.13 x 10^9 cells/l) and AIDS (mean difference: 0.44 x 10^9 cells/l) subjects. ARC and AIDS patients had received ZDV for a median of 1.5 months and 4 months respectively. A similar increase in absolute CD8+ count was noted in the majority of ARC and AIDS subjects studied longitudinally and was apparent within one or two months of ZDV administration. Unfortunately, only limited data are available on the maintenance of this increase and is inconclusive (see Figures 96 and 101, sections 7.5.2. and 7.5.3.). Interestingly, Via et al (1990a) has postulated, based on data from murine models of HIV-1 disease and on in vitro studies, that CD8+ cells play a crucial role in preventing the opportunistic viral infections observed in AIDS patients and that maintenance of CD8+ T-lymphocyte function may also be important for limiting the spread of HIV-1 infection. Therefore, it is conceivable that an increase of functionally active CD8+ T-lymphocytes may provide an immunological basis of ZDV clinical benefit. No evidence is available on this hypothesis as yet.

8.2.2. The mitogen induced proliferative response

Cross-sectional analysis of the natural history cohort demonstrated that the in vitro proliferative response of unfractionated lymphocytes to mitogen (PHA and Con A) declined with HIV-1 disease progression. This decrease

occurred early in the spectrum of HIV-1 infection, before the onset of symptomatic disease (see section 3.2.1.). These observations were consistent with previous studies of the PHA proliferative response (Lane et al., 1985; Rogers, 1987; Rogers et al., 1989). Hofmann et al (1985) attributed the defective response to mitogens PHA and PWM, and to antigens PPD and tetanus toxoid to CD4+ T-lymphocyte depletion and an intrinsic defect of those CD4+ cells remaining. Other work on the T-lymphocyte response in asymptomatic and symptomatic HIV-1 disease has subsequently confirmed these results in various systems of functional assessment (Gabrecht et al., 1987; Shearer et al., 1984, 1985, 1986; Smolen et al., 1985; see section section 1.3.4.a).

Data accumulated from the cross-sectional natural history cohort indicate the potential utility of mitogen induced proliferation as a marker of clinical efficacy following antiretroviral treatment. First, in vitro lymphocyte proliferation was measurable for all HIV-1 infected subjects participating in these studies. Second, the PHA response distinguished asymptomatic subjects from AIDS patients, as observed previously by Gluckman et al (1985), Rogers (1987) and Rogers et al (1989), and ARC patients from AIDS patients (see Table 10; Figure 11; section 3.2.1.). Although data accumulated from the Con A assay was consistent with the PHA response, the latter is of particular interest since Coates et al (1989), in a study of 143 asymptomatic subjects, found a significant association between PHA, but not Con A, proliferation and HIV-1 disease progression (see section 1.5.3.a). A possible explanation for this discrepancy, and one that is supported by data collected during further analysis (see section 8.2.3.), is that PHA is more sensitive to lymphocyte functional change than Con A. It is likely that differences in quantitative receptor expression or in the competence of signal transduction pathways underlie this observation.

Cross-sectional study revealed that the net PHA proliferative response of ARC and AIDS patients was

substantially augmented by ZDV treatment over median treatment times of 3 months (range: 1 to 18 months) and 4 months (range: 1 to 30 months) respectively. The Con A assay further indicated that ZDV elevated the proliferative response of AIDS subjects and a similar increase was observed in ARC subjects. The Con A response was ascertained over ZDV treatment periods of 3 months for both ARC and AIDS patients (range: 1 to 16 months for ARC subjects and 1 to 30 months for AIDS subjects; see section 3.2.2.).

As stated previously (see section 1.5.1.b) and evident from this study (see section 8.2.1.), the relatively small increase of CD4+ cell number following ZDV administration alone fails to adequately explain its profound clinical benefit; Valentine and Jacobson (1990) postulated that improvement of cellular function may better account for ZDV's clinical effect. In the present study, ZDV was shown to elevate the mitogen response levels of AIDS patients to or towards the untreated asymptomatic response level by ZDV therapy (see Table 10 and 11; section 2.3.3.). Furthermore, the PHA assay indicated that ZDV augments the ARC response to a slightly higher level than of asymptomatics. A similar although less obvious trend was observed of the Con A response assay. The mitogen response indicated a greater increase of functional immunocompetence for ARC subjects (PHA: mean net response difference: 12603 $\bar{x}$cpm per 1 x 10^6 cells/ml; see Table 08) than for AIDS patients (PHA: mean net response difference: 6909 $\bar{x}$cpm per 1 x 10^6 cells/ml; see Table 08) over similar treatment periods; this likely reflects the severity of HIV-1 immunosuppression, since patients started on ZDV soon after the diagnosis of symptomatic disease fare better than those started later (Fischl et al., 1987; Pinching et al., 1989; Stambuk et al., 1989a,b). However ZDV does not increase either ARC or AIDS proliferative response to heterosexual control levels; this may reflect the inability of the host immune system to fully reconstitue itself and in turn provide a reason for HIV-1 disease progression during ZDV treatment. However, a better

control here would have been HIV-1 seronegative homosexual men, as it has been shown that these individuals have similarly decreased immunological function to HIV-1 seropositive asymptomatics (see section 1.5.3.a).

From these studies it appeared that mitogen proliferation changed towards normal soon after administration of ZDV and that improvement was maintained thereafter, in line with the persistence of ZDV clinical benefit (Fischl et al., 1987; Pinching et al., 1989; Stambuk et al., 1989a,b). Temporal correlation of the mitogen proliferative response with the clinical efficacy of ZDV was assessed by longitudinal study of ARC and AIDS subjects and acted to validate this conclusion drawn from the cross-sectional study.

The longitudinal studies comprised 8 ARC subjects and 6 AIDS subjects in receipt of ZDV and as control, 5 heterosexual laboratory workers.

Of the 8 ARC subjects studied longitudinally, the net PHA proliferative response of 5 was augmented by ZDV administration over a range of 1 to 16 months (median: 3 months) to or towards the control range (see section 3.3.2). Similarly in 5 of 6 AIDS patients, the use of ZDV was associated with a striking trend of improvement over 1 to 12 months (median: 2 months) of ZDV adminstration (see section 3.3.3.). Serial data on the Con A response, although limited (see section 3.4.2. and 3.4.3), and results from the cross-sectional study (see section 3.2.2. and 3.2.3.) were consistent with these observations, for similar periods of ZDV treatment. The ZDV associated immunological improvement, perceived by the proliferative response to PHA, paralleled the general clinical improvement or maintenance of clinical status seen in these patients, even when PHA responses were studied for extended periods (B. Peters, personal communication).

Therefore, the mitogen proliferative response indicated individual clinical benefit soon after antiretroviral adminstration. In those patients where

improvement was observed, normalisation of the PHA proliferative response was seen within the first analysis time-point post treatment (range: 1 to 4 months). Although study of the Con A response was limited, the results reflected those of the PHA system.

The PHA proliferative response also discriminated subjects receiving antiretroviral who clinically deteriorated. ZDV treatment of AIDS subject (1), was associated with profound and progressive deterioration of net PHA proliferative response, and was evident from 1 or 2 months ZDV administration. This patient presented with pulmonary Kaposi's sarcoma which repeated and became severe with progressive pyogenic infection; during this time his clinical status deteriorated rapidly. The decline of his in vitro PHA proliferative response reflected his underlying clinical status and falling as it did before severe clinical deterioration may have acted as a predictor.

Further support that the PHA proliferative response accurately predicts the clinical response of treated HIV-1 infected subjects comes from the studies with AL 721 (see section 3.3.4.). The serial data on three symptomatic patients (see Figures 25 and 26) reveals little change or a progressive decrease in the net PHA proliferative response, evident from 1 month, during 4 to 11 months of AL 721 treatment. Similarly, an open study of AL 721 treatment of HIV-1 infected subjects classified as CDC group III noted no consistent or substantial trend in the in vitro proliferative response to PHA (Grieco et al., 1988; see section 1.4.2.a). These observations may reflect the failure of AL 721 to stem the progression of HIV-1 induced immunosuppression and therefore represent the natural progression of the disease. Nevertheless, the PHA proliferative response is in accordance with the observed clinical ineffectiveness of AL 721 (Peters et al., 1990).

Moreover one of these subjects who received ZDV after he discontinued AL 721 (see section 3.3.5.) showed clear improvement of the PHA proliferative response, in

contrast with the deterioration during AL 721 therapy. However, the magnitude of his proliferative response remained below the control range. This reflected his clinical improvement with ZDV treatment, since he did not do as well as comparable patients started on ZDV sooner after diagnosis of AIDS.

8.2.3. The mitogen induced gIFN response

In common with previous studies (Lane et al., 1985; Murray et al., 1984; Murray et al., 1988), the present investigation has shown reduced gIFN synthesis in response to T-cell mitogen in AIDS. Indeed, the mitogen induced lymphocyte synthesis of gIFN declined with HIV-1 disease progression (see section 4.4.1.) in a manner reminiscent of the proliferative response decrease (see section 3.2.1.). Whereas substantial decline of proliferation was observed before the onset of clinical symptoms, gIFN synthesis became significantly impaired with symptomatic disease. It is apparent that the proliferative response is more sensitive to the clinical stage of HIV-1 infection than corresponding gIFN assessment; this observation should be considered in the choice of functional markers in future assessment of antiretrovirals, especially where expense is limiting.

Nevertheless, an abnormal gIFN response to PHA can distinguish asymptomatic, ARC and AIDS patients, while Con A assay differentiates asymptomatic and AIDS subjects. The PHA stimulated production of gIFN was measurable, by an IRMA technique, in the culture supernatants of all asymptomatic, ARC subjects and in 86 percent of AIDS patients; 100 percent of supernatants obtained by Con A stimulation of patient lymphocytes, contained detectable levels of gIFN. Similar to previous comment (see section 8.2.2.), these observations indicate a difference in senstitivity of these mitogens to clinical status and suggest the PHA gIFN response as a useful marker of immunocompetence following antiretroviral therpay.

A median 3.5 to 4 months (range: 1 to 30 months) ZDV treatment of AIDS patients caused significant elevation

of both PHA and Con A induced gIFN synthesis. An increase of ARC patients mitogenic response was also apparent within 1.25 to 2.5 months (range: 1 to 18 months) (see section 4.4.2.). In common with results obtained by proliferation assay (see section 8.2.1.), the improvement of PHA induced gIFN production was greater for ARC (mean net response difference: 457 $\bar{x}$U gIFN/1x10^6 cells; see Table 23) than AIDS patients (mean net response difference: 311 $\bar{x}$U gIFN/1x10^6 cells; see Table 23). Again, this likely reflects the difference in clinical response shown between such patients following ZDV administration. This trend did not hold for the Con A system where improvement levels of ARC and AIDS subjects were similar (see Table 23), and is another indication of the relative insensitivity of the Con A response (see section 8.2.2.).

Serial determination of mitogen induced gIFN synthesis was performed for the cohort of ZDV or AL 721 treated ARC and AIDS patients previously described by the phenotypic and proliferative longitudinal studies (see sections 8.2.1. and 8.2.2.). The trends of individual gIFN response delineated (see sections 4.5.2. to 4.5.5. and 4.6.2. to 4.6.3.) closely reflected the proliferative results and confirmed the conclusions drawn from those functional observations (see section 8.2.1.).

In essence, the gIFN response indicated individual clinical outcome soon after the initiation of therapy. Where ARC and AIDS patients clinically benefited from ZDV treatment, the PHA and Con A response improved to or towards the control range within the first analysis time point (range: 1 to 3 months). Moreover, the gIFN response remained an accurate temporal reflection of clinical benefit over the some 1 to 7 months of assessed ZDV treatment. The PHA gIFN response was also observed to discriminate a patient (AIDS subject 1) whose clinical response deteriorated over 5 to 11 months treatment; this was also shown by the proliferative response (see section 8.2.1.). In further support, the PHA gIFN response reflected the clinical ineffectiveness of

AL 721 (Peters et al., 1990) since a trend of deterioration was associated with AL 721 treatment of 3 symptomatic subjects. The ability of this functional response to distinguish clinical benefit was emphasised by observation of a patient sequentially treated with AL 721 and ZDV, where deterioration followed by profound improvement of in vitro response was found (B. Peters, personal communication).

8.2.4. The phosphatidyl inositol hydrolysis pathway

Previous studies have shown that either HIV-1 infection of CD4+ lymphoblastoid cells or exposure of normal lymphocytes to the HIV-1 envelope glycoprotein, gp120, induce chronic activation of the inositol polyphosphate pathway of signal transduction with a consequent rise in intracellular calcium. Stimulation of such cells with PHA or anti-CD3 antibody leads to a markedly attenuated response in intracellular calcium and is associated with altered kinetics of metabolism and breakdown of inositol polyphosphates (Kornfeld et al., 1988; Nye and Pinching, 1990; see section 1.3.4.b).

The studies described here (see section 5.1. and 5.2.) and in collaboration with K. Nye (Nye et al., 1991) demonstrated that HIV-1 infected individuals show equivalent changes to those observed in vitro. This confirms the relevance of the in vitro studies and, since many studies have shown that few viable CD4+ lymphocytes show evidence of HIV-1 infection in vivo (see section 1.3.3.), implies that these cellular changes can occur without cellular infection. In view of work demonstrating that gp120 can induce similar changes (Kornfeld et al., 1988), it may be that gp120 binding to uninfected cells underlies the abberant inositol polyphosphate metabolism seen in patients. A possible explanation for these changes, and one which is supported by further experiments in our laboratory is that HIV-1 infection or gp120 exposure causes inhibition of the $Ins(1,4,5)P_3$/ $Ins(1,3,4,5)P_4$-5'-phosphatase (K. Nye, personal communication). This would lead to the observed accumulation

of the active second messengers $Ins(1,4,5)P_3$ and $Ins(1,3,4,5)P_4$, and reduced levels of the inactive intermediate metabolite $Ins(1,3,4)P_3$, production of which is dependent upon the 5'-phosphatase.

The effect of ZDV therapy in inositol polyphosphate metabolism (see section 5.2.and 5.3.) demonstrates that the defect, putatively the inhibition of the 5'-phosphatase, is reversible if HIV-1 replication is stemmed. The time course of this reversal suggests that the factor responsible for the inhibition is at one or more removes from HIV-1 replication itself; this is consistent with reduced gp120 binding to uninfected lymphocytes consequent on reduced HIV-1 replication and hence reduced gp120 production in infected cells.

The time course of the improvement of inositol polyphosphate metabolism following ZDV therapy parallels the clinical benefit and the changes in lymphocyte function responses.

8.2.5. The utility of T-lymphocyte functional response to mitogen as markers of antiretroviral efficacy

In essence, the functional data just discussed (see section 8.2.2. to 8.2.4.) indicate that ZDV can reverse, to some extent, previous HIV-1 immunosuppression.

Both the proliferative and gIFN response to T-lymphocyte mitogen, particularly to PHA, appear promising in vitro markers of clinical efficacy following antiretroviral treatment of symptomatic HIV-1 disease, and future evaluation of anti-HIV-1 agents may benefit from the inclusion of these assays in addition to the now standard enumeration of CD4+ T-lymphocytes. Analysis of inositol polyphosphate metabolism may also prove useful in this context. These functional tests are relatively simple cellular assays to perform and could easily be accommodated as routine. However, as with all cellular assays intralaboratory and interlaboratory variations may be enormous, such that participation in a quality assurance

programme would be essential, especially if assimilated into multicentre trials.

The simultaneous study of T-lymphocyte phenotype with function, by serial determination, further extends our knowledge of their quantitative and qualitative relationship within an HIV-1 infected individual.

In 3 of 7 ARC and 3 of 5 AIDS subjects, a correlation of individual functional response with peripheral CD4+ T-lymphocyte number was apparent within the first 2 months of ZDV treatment (see section 7.5.4.). Murray et al (1988) has also presented evidence, obtained from a natural history cohort, of an association between absolute peripheral CD4+ T-lymphocyte count and mitogen (Con A) or antigen (Toxoplasma gondii lysate and CMV antigen) induced gIFN synthesis by mononuclear cells obtained from ARC and AIDS patients.

Nevertheless, correlation of CD4+ count and lymphocyte function was not obvious in the remaining 6 subjects. Peripheral CD8+ T-lymphocyte numbers also failed to reflect the functional response to mitogen, by the criteria of change imposed in this study (see section 2.10.2). These results are corroborated by the work of Gluckman et al (1985). This group investigated the relationship between the proliferative response to PHA and IL-2 receptor, CD3, CD4 and CD8 antigen expression across the spectrum of HIV-1 infection. No correlation between variation in T-lymphocyte subset distribution and PHA responsiveness was found. Lack of correlation is not entirely unexpected since response to PHA is an indication of the potential of T-lymphocytes to be activated via the CD2 (O'Flynn et al., 1985) and/or the CD3 (Valentine et al., 1985) T-lymphocyte antigens. A similar case may be made for the Con A response, although there is no evidence to this end.

However, the limitations of utilising phenotypic or functional markers of clinical efficacy following antiretroviral therapy alone was demonstrated by a single subject, where the results of each analysis were inconsistent

with his general clinical well-being on ZDV (see section 7.6.). Thus, markers of clinical status may be best used in conjunction with careful physical examination of an individual in evaluation of antiretroviral treatment.

8.2.6. The proliferative and gIFN response to antigen

Previous studies have shown that similar to mitogen induced proliferation and gIFN synthesis, these lymphocyte responses to antigen decline with HIV-1 disease progression (Lane et al., 1985; Murray et al., 1984; Murray et al., 1988; Rogers, 1987). Despite limited subject numbers, this study also showed severe defect of the antigen stimulated functional responses of lymphocytes obtained from AIDS patients (see section 3.6.2. and 4.8.2.). Neither experimental approach demonstrated an effect of ZDV therapy, in contrast to the results with mitogen (see section 3.5. and 4.7.). Several explanations may underlie these observations.

In patients with far advanced disease on entry into a clinical trial, the clinical outcome may be sufficiently poor and response so low that drug induced changes in these parameters may not correlate with one another as well they might in patients with less advanced disease. Therefore, the results obtained by this study on AIDS patients should not be extrapolated to less severe HIV-1 disease; it has already been shown that ZDV associated improvement of mitogen response is greater in patients with less advanced HIV-1 disease than those with AIDS (see section 8.2.1. and 8.2.2.).

The implicit limitations of the assay system must also be considered within this context. Since the SK/SD induced PBMC proliferative or gIFN response, like that of any recall antigen, is determined by a small number of T-lymphocyte reactive clones, these systems may be too insensitive to detect changes in PBMC functional capacity. In evidence, SK/SD failed to induce PBMC proliferation significantly above spontaneous levels in the AIDS patients; similarly, three of four patients failed to synthesise

detectable levels of gIFN following SK/SD stimulation. This may be compounded by the selective loss of memory T-lymphocytes early in HIV-1 disease (De Paoli et al., 1988; Germain et al., 1988; Miedema et al., 1990) and which may be unaffected by ZDV therapy.

With the respect to data on gIFN production; patient results, irrespective of antiretroviral therapy, lay at the at the very lower limit of the IRMA detection range. Hutchinson (1991), also using SK/SD as a stimulus and the Celltech Diagnostics kit, made similar observations. In retrospect, the Medgenix kit may have been more appropriate to this system as it is designed to assay lower concentrations of gIFN than the Celltech Diagnostics IRMA (see section 4.1.2.). Murray et al (1988) also reported undetectable gIFN synthesis, as quantified by bioassay, on lymphocyte antigen stimulation (see section 1.5.3.d). An undetectable response is unsuitable as a marker of therapy (Valentine and Jacobson, 1990; see section 1.5.). Therefore, although a given marker may be a useful predictor in a study of natural history, as undetectable antigen induced gIFN synthesis has proved (Murray et al., 1984, 1988, 1989; see section (1.5.3.d), it may not prove as useful in clinical trial of new antiretrovirals.

8.2.7. Spontaneous and PWM induced production of Ig

The studies described here demonstrate that lymphocytes obtained from HIV-1 infected subjects with symptomatic disease are hyporesponsive to PWM, as perceived by decreased IgM and IgG production (see section 6.4.2.). Lane et al (1983), Rogers (1987) and Rogers et al (1989) have made similar observations.

In addition, previous studies have shown increased spontaneous production of Ig in vitro by lymphocytes obtained from patients with PGL and Kaposi's sarcoma; interestingly this dysfunction is not as marked in other disease states. Rogers (1987) and Rogers et al (1989) observed no difference between the spontaneous IgG and IgM concentrations of

heterosexual controls and HIV-1 seropositive subjects classified as CDC IVC1; whereas significant elevation of spontaneous IgG and IgM production was observed in less severe disease (CDC III and IVD). The data presented by this study is consistent with these results, since spontaneous production of Ig, especially IgG, was elevated in ARC but not AIDS patients (see section 6.4.1.).

The combination of decreased spontaneous production of IgG and IgM and defective PWM responses in patients with opportunistic infection may result from severe T-lymphocyte immunodeficiency with an associated reduction in B-cell activation.

The multiple defects in B-lymphocyte function reported in HIV-1 infection have been further and strikingly illustrated by the study of IgD. Mizuma et al (1987) found significant elevation of serum IgD levels in HIV-1 infected subjects classified as CDC II and III; although the serum level of IgD fell in AIDS patients predominantly diagnosed by opportunistic infection. Rogers et al (1989) subsequently demonstrated that spontaneous in vitro production of IgD was increased in HIV-1 infection, most strikingly in asymptomatic subjects and with the exception of patients with Kaposi's sarcoma. Similar to the results of Rogers (1987) and Rogers et al (1989), the spontaneous production of IgD was not observed to differ noticeably between heterosexual laboratory controls and symptomatic patients (see section 6.4.4.).

The heterosexual control group showed no increase in IgD production following PWM stimulation (see section 6.4.4.). This is not unexpected, since activation of resting B-lymphocytes results in low levels of IgD gene transcription (Tucker, 1985). The majority of HIV-1 infected subjects produced less IgD in response to PWM than spontaneously, as shown previously by Rogers (1987) and Rogers et al (1989). Suppression of Ig production by PWM is not without precedent; Takeuchi et al (1982) described a decrease in IgG production by lymphocytes from patients with active systemic lupus erthematosus.

ZDV therapy was shown to have no significant effect on spontaneous IgM, IgG or IgD production in ARC and AIDS patients following median treatment periods of 2 months (range: 1 to 8 months) and 6 months (range: 1 to 32 months) respectively (see section 6.4.1. and 6.4.3.). ZDV also failed to reverse defective IgM production by lymphocytes obtained from ARC or AIDS patients, although a slight trend of improvement was seen in the IgG response to PWM (see section 6.4.2.); ZDV was not associated with any obvious trend in IgD production (see section 6.4.3.). Evidence on the effect of antiretroviral therapy on these putative markers is scarce, and although this study was concerned wth total Ig production, in evidence of the present results, Weber and Jeffries (1989) report that successful clinical ZDV treatment did not result in the reappearance of anti-p24 antibodies. The continued attenuation of Ig response as seen in vitro and in vivo with ZDV treatment may at least in part account for disease progression observed despite treatment.

It is clear from the results obtained by this study that the in vitro quantification of IgM, IgG and IgD produced spontaneously or in response to PWM is not a useful marker of clinical efficacy following ZDV treatment of symptomatic HIV-1 disease. Several explanations may underlie these observations, and act as useful guides to future evaluation of antiretroviral therapy.

First and most obviously, it appears from previous in vivo and in vitro studies that abnormalites of Ig production are most apparent in patients with asymptomatic infection (IgD), with or without PGL (IgM and IgG), or those with Kaposi's sarcoma (IgM and IgG). Thus, the use of these markers in the present study may have failed to reflect ZDV's clinical benefit since they were inappropriate to the symptomatic disease state, as diagnosed by opportunistic infection. A study of Ig production concomitantly with ZDV treatment of asymptomatic disease would be of great interest and would also clarify the present situation. In essence, these observations serve to underline the importance of

utilising a laboratory marker appropriate to the clinical status of HIV-1 infection; this should be considered in future evaluations of antiretroviral agents.

The functional defects in Ig production described appear to be due to qualitative disturbances in T-lymphocyte immune responsiveness and/or to intrinsic B-cell dysfunction (Lane et al., 1983; Schnittman et al., 1986; see section 1.3.4.c). Therefore, it is probable that abnormal B-lymphocyte synthesis of Ig reflects the heterogeneous nature of the cellular defects present in HIV-1 infection, and that the attenuated spontaneous and PWM induced production of Ig in symptomatic patients is caused by a culmination of cellular dysfunction in end-stage disease. The failure of ZDV to have an effect on Ig production may reflect the multi-factorial pathogenesis of these defects.

The work of Martinez-Maza et al (1987) and Rogers et al (1989) (see section 1.3.4.c) suggests that in symptomatic HIV-1 disease, as defined by opportunistic infection, circulating B-cells show evidence of activation and of immaturity. In addition, there is likely to be a reduction in T-lymphocyte co-operation. Thus, ZDV may have no effect on the recruitment of immature B-lymphocytes into the circulation, or on the interaction of B-cells with T-lymphocytes thereafter, and this has been observed as the apparent ineffectiveness of ZDV.

8.3. Future studies

A number of future studies are clearly indicated by the results obtained in the present project.

The foremost of these requires that lymphocyte functional responses to mitogen, especially PHA, be included in larger and longer follow-up studies of clinical efficacy following ZDV therapy of asymptomatic as well as symptomatic HIV-1 disease. Such studies should define and validate the changes in functional response, alone or in combination with other immunological or virological markers, that would best predict the long term clinical outcome following

administration of experimental antiretroviral therapy.

Inositol polyphosphate metabolism should also be studied as outlined above and to equivalent goals, and in addition as a potential marker of HIV-1 disease progression. Clearly, the defect in 5'-phosphatase merits extensive investigation and results here may provide an exciting insight to the pathogenesis of HIV-1. Other components of signal transduction have not yet been investigated and study should perhaps include analysis of diacylglycerol generation and protein kinase activity. These studies should not be restriced to the T-lymphocyte since substantial data demonstrate B-lymphocyte and monocyte/macrophage dysfunction.

Study of the function and number of CD8+ T-lymphocytes, with repect to HIV-1 specific CTL activity, may be useful as a marker of antiretroviral therapy, and may also partly explain the benefits of ZDV therapy.

The antigen induced functional response and abnormal production of Ig, both spontaneously or in response to PWM, may be useful markers of immunorestorative therapies.

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APPENDIX I

RESULTS - THE PHA PROLIFERATIVE RESPONSE

Table 41: PHA proliferative response Laboratory control subjects

Subject	[3H]TdR incorporation $\bar{x}$cpm per 1x10E+06 cells/ml ± SD	
	without PHA	with PHA
1	308	58037 ± 2694
2	319	59108 ± 3671
3	411	66265 ± 5438
4	196	37477
5	1384	84825 ± 2510
6	294 ± 18	120050 ± 9882
7	151	64564 ± 3497
8	2507 ± 442	69240 ± 2523
9	3002 ± 57	102247 ± 1806
10	100 ± 45	63072 ± 446
11	277 ± 62	62683 ± 6720
12	533 ± 30	81810 ± 9309
13	147 ± 69	58219 ± 2139
14	327 ± 72	31483 ± 3347
15	240 ± 43	61821 ± 2510
16	335 ± 54	39439 ± 1312
17*	460 ± 31	67860 ± 2684
17**	757 ± 110	61714 ± 3844
18*	890	67702 ± 2286
19*	161 ± 39	30331 ± 3914
19**	314 ± 50	30040 ± 1433
20*	1000 ± 82	68803 ± 3858
21*	458 ± 13	58008 ± 1205
21**	254 ± 6	51972 ± 2596
22*	205 ± 21	75957 ± 333
23*	470 ± 23	87632 ± 1306

PBMC from 23 control subjects were cultured with PHA at 1 ug/ml or without for 72 h.
*: median result for control subjects tested more than once.

Table 42: PHA proliferative response Asymptomatic, no zidovudine therapy

Subject	[3H]TdR incorporation $\bar{x}$cpm per 1x10E+06 cells/ml ± SD	
	without PHA	with PHA
1	232 ± 16	5380 ± 429
2	197 ± 56	18802 ± 1550
3	169 ± 25	41202 ± 2511
4	139 ± 28	18852 ± 2289
5	691 ± 115	25038 ± 4081
6	253 ± 29	6428 ± 663
7	202 ± 22	31316 ± 6738

PBMC from 7 asymptomatic subjects (CDC II & III) were cultured with PHA at 1 ug/ml or without for 72 h. Five of the 7 subjects were from the peripheral neuropathy trial while subjects 4 & 5 were pre-surgery. Subjects 1-5 are CDC II and 6-7 CDC III. No subject was receiving zidovudine therapy.

Table 43: PHA proliferative response Asymptomatic, receiving zidovudine

Subject	[3H]TdR incorporation $\bar{x}$cpm per 1x10E+06 cells/ml ± SD	
	without PHA	with PHA
1	374 ± 21	16021 ± 2176
2	191 ± 27	55202 ± 899
3	236 ± 7	22247 ± 1915
4	229 ± 9	1814 ± 173

PBMC from 4 asymptomatic subjects (CDC III) were cultured with PHA at 1 ug/ml or without for 72 h. All subjects were from the peripheral neuropathy study and all were receiving zidovudine treatment.

Table 44: PHA proliferative response Asymptomatic, concorde trial

Subject	[3H]TdR incorporation $\bar{x}$cpm per 1x10E+06 cells/ml ± SD	
	without PHA	with PHA
1	131 ± 14	17722 ± 86
2	260 ± 48	21174 ± 1732
3	203 ± 35	44226 ± 2822

PBMC from 3 asymptomatic subjects (CDC III) were cultured with PHA at 1 ug/ml or without for 72 h. All subjects were from the concorde trial. It was therefore not known if these subjects were receiving zidovudine treatment.

CROSS-SECTIONAL STUDY: ASYMPTOMATIC, PHA RESPONSE

Table 45: PHA proliferative response ARC, no zidovudine therapy

Subject	[3H]TdR incorporation $\bar{x}$cpm per 1x10E+06 cells/ml ± SD	
	without PHA	with PHA
1	1440 ± 129	8520 ± 718
2	548 ± 71	10578 ± 499
3	139 ± 37	22611 ± 3924
3*	132 ± 35	17229 ± 633
4	184 ± 45	2118 ± 170
5	201 ± 119	26692 ± 2740
6	234 ± 11	12799 ± 2488
7	425 ± 42	47810 ± 3761
8	174 ± 110	814 ± 22

PBMC from 8 ARC subjects (CDC IVC2) were cultured with PHA at 1 ug/ml or without for 72 h. All subjects were pre-zidovudine, except subject 8 who was pre-AL 721. Subject 3 (3*) was tested twice pre-zidovudine.

Table 46: PHA proliferative response ARC, receiving zidovudine

Subject	[3H]TdR incorporation $\bar{x}$cpm per 1x10E+06 cells/ml ± SD	
	without PHA	with PHA
1	254 ± 66	21197 ± 1689
2	278 ± 4	13198 ± 2458
3	597 ± 97	28129 ± 3262
4	46 ± 19	25867 ± 6635
5	542 ± 119	71624 ± 2540
6	237 ± 7	21180 ± 1184
7	249 ± 21	25503 ± 1130
7*	1826 ± 605	31040 ± 15
8	369 ± 73	21020 ± 5442
8*	327 ± 60	14116 ± 3103
9	293 ± 75	31678

PBMC from 9 ARC (CDC IVC2) subjects were cultured with PHA at 1 ug/ml or without for 72 h. Subject 1 was from the peripheral neuropathy study, while the remaining individuals were from the longitudinal group. The median result was quoted for these.

Table 47: PHA proliferative response AIDS, no zidovudine therapy

Subject	[3H]TdR incorporation x̄cpm per 1x10E+06 cells/ml ± SD	
	without PHA	with PHA
1	214 ± 89	525 ± 135
2	1653 ± 380	2381 ± 111
3	473 ± 24	648 ± 17
4	235 ± 66	2380 ± 197
5	235 ± 18	1118 ± 110
6	426 ± 83	16648 ± 1669
7	649 ± 68	2776 ± 184
8	2630 ± 202	36134 ± 2712
9	357 ± 8	4870
10	358 ± 35	4002 ± 52
11	145	13325 ± 483
12	199 ± 73	11146 ± 94
13	1273 ± 200	7940 ± 247
14	102 ± 29	301 ± 83
15	256	3340 ± 256
16	813 ± 101	2390 ± 603
16#	134 ± 16	1773 ± 611
17	306 ± 4	833 ± 141
17*	354 ± 157	380 ± 111
18	222 ± 57	2094 ± 240
19	481 ± 91	10227 ± 2446
19*	231 ± 12	1889 ± 86
20	271 ± 81	14640 ± 1549
21	308	2156 ± 140
22	221 ± 29	427 ± 11
23	218 ± 75	331 ± 51

PBMC from 23 AIDS subjects were cultured with PHA at 1 ug/ml or without for 72 h. All subjects were classified CDC IVC1, except 18 and 19 who were CDC IVD. Individuals 1-13 were from the peripheral neuropathy study, 14-16 were pre-AL 721 and 17 to 23 pre-zidovudine. Subjects 17* and 19* were twice tested pre-zidovudine, and subject 16# was tested once pre-AL 721 and once pre-zidovudine.

Table 48: PHA proliferative response AIDS, receiving zidovudine

Subject	[3H]TdR incorporation $\bar{x}$cpm per 1x10E+06 cells/ml ± SD	
	without PHA	with PHA
1	410 ± 69	20958 ± 4862
2	375 ± 144	16125 ± 1414
3	286 ± 39	18122 ± 2681
4	176 ± 3	16613 ± 5833
5	133 ± 26	13786 ± 1236
6	97 ± 14	110 ± 3
7	865 ± 146	18219 ± 3780
8	202 ± 49	7339 ± 794
9	212 ± 39	6483 ± 253
10	251 ± 25	17814 ± 1345
11	374 ± 54	12643 ± 1689
12	259 ± 69	12810 ± 1216
13	1489	2149 ± 185

PBMC from 13 AIDS subjects were cultured with PHA at 1 ug/ml or without for 72 h. All subjects were classified CDC IVC1. Individuals 1-11 were from the peripheral neuropathy study, while 12 and 13 were from clinic.

APPENDIX II

RESULTS - THE CON A PROLIFERATIVE RESPONSE

Table 49: Con A proliferative response Laboratory control subjects

Subject	[3H]TdR incorporation $\bar{x}$cpm per 1x10E+06 cells/ml ± SD	
	without Con A	with Con A
1	450	36736 ± 7185
2	294 ± 18	30882 ± 6134
3	151	23962 ± 853
4	277 ± 62	22989 ± 2627
5	533 ± 30	25478
6	147 ± 69	23076 ± 1415
7	327 ± 72	23896 ± 861
8	240 ± 43	8502 ± 1275
9	335 ± 54	17458 ± 1771
10*	404 ± 68	22343 ± 4705
10**	188 ± 24	20336 ± 600
11*	257 ± 180	33417 ± 7439
12*	439 ± 64	11214 ± 479
13*	305 ± 136	25862 ± 1457
14	128 ± 24	27175 ± 1334
15	338 ± 62	42723 ± 977
16*	142 ± 17	26430 ± 2952

PBMC from 16 control subjects were cultured with Con A at 10 ug/ml or without for 72 h.
* : median result taken for control subjects tested more than once.

Table 50: Con A proliferative response Asymptomatic, no zidovudine therapy

Subject	[3H]TdR Incorporation x̄cpm per 1x10E+06 cells/ml ± SD	
	without PHA	with PHA
1	232 ± 16	1000 ± 200
2	197 ± 56	10255 ± 1120
3	139 ± 28	16215 ± 786
4	691 ± 115	17919 ± 2327
5	253 ± 29	6500 ± 263
6	202 ± 22	8980 ± 1703

PBMC from 6 asymptomatic subjects (CDC II & III) were cultured with Con A at 10 ug/ml or without for 72 h. Four of the 6 subjects were from the peripheral neuropathy study while subjects 3 & 4 were pre-surgery. No subject was receiving zidovudine treatment.

Table 51: Con A proliferative response Asymptomatic, receiving zidovudine

Subject	[3H]TdR Incorporation x̄cpm per 1x10E+06 cells/ml±	
	without Con A	with Con A
1	392 ± 16	8611 ± 872
2	236 ± 7	18916 ± 2046
3	229 ± 9	2149 ± 229

PBMC from 3 asymptomatic subjects (CDC III) were cultured with Con A at 10 ug/ml or without for 72 h. All subjects were from the peripheral neuropathy trial and all were receiving zidovudine treatment.

Table 52: Con A proliferative response Asymptomatic, concorde trial

Subject	[3H]TdR Incorporation x̄cpm per 1x10E+06 cells/ml ± SD	
	without Con A	with Con A
1	131 ± 14	35030 ± 1094
2	260 ± 48	6998 ± 448
3	203 ± 35	46488 ± 3719

PBMC from 3 asymptomatic subjects (CDC III) were cultured with Con A at 10 ug/ml or without for 72 h. All subjects were from the concorde trial. It was therefore not known if these subjects were receiving zidovudine treatment.

CROSS-SECTIONAL STUDY: ASYMPTOMATIC, Con A RESPONSE

Table 53: Con A proliferative response ARC, no zidovudine therapy

Subject	[3H]TdR incorporation $\bar{x}$cpm per 1x10E+06 cells/ml ± SD	
	without Con A	with Con A
1	548 ± 71	6673 ± 1854
2	184 ± 45	1937 ± 136
3	234 ± 11	5425 ± 561
4	425 ± 42	12069 ± 2700

PBMC from 4 ARC subjects (CDC IVC2) were cultured with Con A at 10 ug/ml or without for 72 h. All subjects were pre-zidovudine.

Table 54: Con A proliferative response ARC, receiving zidovudine

Subject	[3H]TdR incorporation $\bar{x}$cpm per 1x10E+06 cells/ml ± SD	
	without Con A	with Con A
1	278 ± 4	6493 ± 516
1*	203 ± 31	15559 ± 1897
2	597 ± 97	28099 ± 834
2*	195 ± 47	7970 ± 1352
3	166 ± 4	2594 ± 55
4	542 ± 119	55728 ± 9178
5	249 ± 21	8327 ± 190
5*	1826 ± 605	30629 ± 1514
6	232 ± 133	6815 ± 187
7	293 ± 75	4140 ± 1160

PBMC from 7 ARC subjects (CDC IVC2) were cultured with Con A at 10 ug/ml or without for 72 h. All subjects were from the longitudinal study. The median result for each individual receiving zidovudine is quoted.

Table 55: Con A proliferative response AIDS, no zidovudine therapy

Subject	[3H]TdR incorporation $\bar{x}$cpm per 1x10E+06 cells/ml ± SD	
	without Con A	with Con A
1	355 ± 78	2617 ± 365
2	191 ± 65	291 ± 60
3	198 ± 49	441 ± 86
4	308 ± 64	1171 ± 225
5	2630 ± 202	9954 ± 314
6	358 ± 35	840 ± 104
7	199 ± 73	4556 ± 411
8	222 ± 57	1853 ± 232
9	481 ± 91	5464 ± 436
10	308	570 ± 53
11	221 ± 29	10595 ± 991
12	134 ± 16	1265 ± 231

PBMC from 12 AIDS subjects were cultured with Con A at 10 ug/ml for 72 h. All subjects were classified CDC IVC1, except individual 8 who was CDC IVD. Subjects 1-7 were from the peripheral neuropathy study, and 8-12 were pre-zidovudine.

Table 56: Con A proliferative response AIDS, receiving zidovudine

Subject	[3H]TdR incorporation $\bar{x}$cpm per 1x10E+06 cells/ml ± SD	
	without Con A	with Con A
1	410 ± 69	5980 ± 880
2	202 ± 86	36967 ± 3069
3	286 ± 39	14062 ± 1132
4	865 ± 146	15094 ± 2187
5	202 ± 49	8980 ± 415
6	212 ± 39	1034 ± 285
7	251 ± 25	11331 ± 554
8	374 ± 54	19453 ± 1045
9	259 ± 69	6916 ± 924

PBMC from 9 AIDS subjects were cultured with Con A at 10 ug/ml or without for 72 h. All subjects were classified CDC IVC1. Individuals 1-8 were from the peripheral neuropathy study, while 9 was from clinic.

APPENDIX III

RESULTS - THE PHA gIFN RESPONSE

Table 57: PHA driven gIFN response Laboratory control subjects

Subject	gIFN $\bar{x}$U per 1x10E+06 cells	
	without PHA	with PHA
1*	nd	2858
2*	nd	2032
3*	6	1266
4*	nd	1457
5*	5	1445
6	0.2	1813
7	nd	431
8	nd	1537
9	0.2	396
10	13	1641
11	0.5	412
12*	nd	1398
13	6	729

PBMC from 13 laboratory control subjects were cultured with PHA at 1ug/ml or without for 72 h.
* : median result taken for control subjects tested more than once
nd: not detected

Table 58: PHA driven gIFN response Asymptomatic, no zidovudine therapy

Subject	gIFN x̄U per 1x10E+06 cells	
	without PHA	with PHA
1	7	228
2	3	884
3	2	1260
4	4	890
5	9	476
6	7	460
7	nd	2885

PBMC from 7 asymptomatic subjects (CDC II & III) were cultured with PHA at 1 ug/ml or without for 72 h. Five of the 7 subjects were from the peripheral neuropathy trial while subjects 4 & 5 were pre-surgery. Subjects 1-5 are CDC II and 6-7 CDC III. No subject was receiving zidovudine therapy.
nd: not detected

Table 59: PHA driven gIFN response Asymptomatic, receiving zidovudine

Subject	gIFN x̄U gIFN per 1x10E+06 cells	
	without PHA	with PHA
1	te	te
2	nd	407
3	2	1113
4	nd	185

PBMC from 4 asymptomatic subjects (CDC III) were cultured with PHA at 1 ug/ml or without for 72 h. All subjects were from the peripheral neuropathy study and all were receiving zidovudine treatment.
te: technical error
nd: not detected

Table 60: PHA driven gIFN response Asympotomatic, concorde trial

Subject	gIFN x̄U per 1x10E+06 cells	
	without PHA	with PHA
1	0.3	1061
2	nd	352
3	nd	2551

PBMC from 3 asymptomatic subjects (CDC III) were cultured with PHA at 1 ug/ml or without for 72 h. All subjects were from the concorde trial. It was therefore not known if these subjects were receiving zidovudine treatment.
nd: not detected

CROSS-SECTIONAL STUDY: ASYMPTOMATIC, PHA RESPONSE

Table 61: PHA driven gIFN response ARC, no zidovudine therapy

Subject	gIFN $\bar{x}$U per 1x10E+06 cells	
	without PHA	with PHA
1	nd	110
1*	nd	575
2	0.6	31
3	0.2	1150
4	5	372
5	nd	269
6	4	te
7	nd	83

PBMC from 7 ARC subjects (CDC IVC2) were cultured with PHA at 1 ug/ml or without for 72 h. All subjects were pre-zidovudine, except subject 7 who was pre-AL 721. Subject 1 (1*) was tested twice pre-zidovudine.
nd: not detected
te: technical error

Table 62: PHA driven gIFN response ARC, receiving zidovudine

Subject	gIFN $\bar{x}$U per 1x10E+06 cells	
	without PHA	with PHA
1	0.1	148
2	8	1265
3	1	161
4	1	1418
5	2	587
6	15	500
7	3*	1825*
7#	nd*	1749*
8	nd	810

PBMC from 8 ARC (CDC IVC2) subjects were cultured with PHA at 1 ug/ml or without for 72 h. Subject 1 was from the peripheral neuropathy study. while the remaining individuals were from the longitudinal group. The median result was quoted for these when receiving zidovudine.

* : results by the Medgenix IRMA

nd: not detected

Table 63: PHA driven gIFN response AIDS, no zidovudine therapy

Subject	gIFN $\bar{x}$U per 1x10E+06 cells	
	without PHA	with PHA
1	nd	nd
2	nd	10
3	4	173
4	0.2	4
5	nd	43
6	1	0.5
7	0.2	617
8	nd	nd
9	1	81
10	nd	177
11	0.2	312
12	3	5
13	nd	117
14	nd	3
14*	nd	5
15	23	122
16	nd	59
16*	nd	2
17	nd	5
18	2	31
18*	nd	5
19	8	104
20	nd	48
21	0.1	2
22	5	8

PBMC from 22 AIDS subjects were cultured with PHA at 1 ug/ml or without for 72 h. All subjects were classified CDC IVC1, except 17 and 19 who were CDC IVD. Individuals 1-12 were from the peripheral neuropathy study, 13 and 14 were pre-AL 721 and 15-22 pre-zidovudine. Subjects 16 and 18 were twice tested pre-zidovudine, subject 14 was tested once pre-AL 721 and then once pre-zidovudine.
nd: not detected

Table 64: PHA driven gIFN response AIDS, receiving zidovudine

Subject	gIFN $\bar{x}$U per 1x10E+06 cells	
	without PHA	with PHA
1	45	353
2	nd	13
3	3	233
4	7	999
5	nd	642
6	8	nd
7	0.1	249
8	0.6	71
9	13	316
10	3	32
11	2	421
12*	5	1135

PBMC from 13 AIDS subjects were cultured with PHA at 1 ug/ml or without for 72 h. All subjects were classified CDC IVC1. Individuals 1-11 were from the peripheral neuropathy study while subject 12 was from clinic.
12* results by Medgenix gIFN IRMA
nd not detected

APPENDIX IV

RESULTS - THE CON A gIFN RESPONSE

Table 65: Con A driven gIFN response Laboratory control subjects

Subject	gIFN $\bar{x}$U per 1x10E+06 cells	
	without Con A	with Con A
1	6	410
2	0.5	163
3	5	108
4	nd	1899
5	nd	208
6	0.2	841
7*	0.4	1444
8*	3	539
9*	nd	666
10*	0.3	1039
11	2	479
12*	nd	239

PBMC from 12 laboratory control subjects were cultured with Con A at 10 ug/ml or without for 72 h.
* : median result taken for control subjects tested more than once
nd: not detected

Table 66: Con A driven gIFN response Asymptomatic, no zidovudine therapy

Subject	gIFN $\bar{x}$U per 1x10E+06 cells	
	without Con A	with Con A
1	7	248
2	3	212
3	4	953
4	9	125
5	7	593
6	nd	892

PBMC from 6 asymptomatic subjects (CDC II & III) were cultured with Con A at 10 ug/ml or without for 72 h. Four of the 6 subjects were from the peripheral neuropathy study while subjects 3 & 4 were pre-surgery. No subject was receiving zidovudine treatment.
nd: not detectable

Table 67: Con A driven gIFN response Asymptomatic, receiving zidovudine

Subject	gIFN $\bar{x}$U per 1x10E+06 cells	
	without Con A	with Con A
1	2	656
2	nd	376

PBMC from 2 asymptomatic subjects (CDC III) were cultured with Con A at 10 ug/ml or without for 72 h. Both subjects were from the peripheral neuropathy study and all were receivind zidovudine treatment.
nd: not detected

Table 68: Con A driven gIFN response Asymptomatic, concorde trial

Subject	gIFN $\bar{x}$U per 1x10E+06 cells	
	without Con A	with Con A
1	0.3	1445
2	nd	305
3	nd	1064

PBMC from 3 asymptomatic subjects (CDC III) were cultured with Con A at 10 ug/ml or without for 72 h. All subjects were from the concorde trial. It was therefore not known if these subjects were receiving zidovudine treatment.
nd: not detected

CROSS-SECTIONAL STUDY: ASYMPTOMATIC, Con A RESPONSE

Table 69: Con A driven gIFN response ARC, no zidovudine therapy

Subject	gIFN $\bar{x}$U per 1x10E+06 cells	
	without Con A	with Con A
1	0.5	89
2	0.2	341
3	5	490
4	nd	177
5	4	440
6	nd	77

PBMC from 6 ARC subjects (CDC IVC2) were cultured with Con A at 10 ug/ml or without for 72 h. All subjects were pre-zidovudine, except subject 6 who was pre-AL 721.
nd: not detected

Table 70: Con A driven gIFN response ARC, receiving zidovudine

Subject	gIFN x̄U per 1x10E+06 cells	
	without Con A	with Con A
1	nd	1532
1#	nd	702
2	1	41
2#	2*	414*
3	nd*	944*
3#	1	870
4	2	247
5	15	191
6	3*	1135*
6#	nd*	1825*
7	nd	359

PBMC from 7 ARC (CDC IVC2) were cultured with Con A at 10 ug/ml or without for 72 h. All subjects were from the longitudinal group. The median result was quoted for these.
* : results by the Medgenix IRMA
nd: not detected

Table 71: Con A driven gIFN response AIDS, no zidovudine therapy

Subject	gIFN $\bar{x}$U per 1x10E+06 cells	
	without Con A	with Con A
1	0.2	15
2	0.5	32
3	0.2	31
4	nd	49
5	0.1	157
6	0.2	275
7	nd	125
8	23	78
9	nd	7
10	2	5
10*	nd	10
11	nd	97
12	0.1	365
13	nd	10

PBMC from 13 AIDS subjects were cultured with Con A at 10 ug/ml for 72 h. All subjects were classified CDC IVC1, except 9 who was CDC IVD. Subjects 1-7 were from the peripheral neuropathy study, 7 was pre-AL 721 and 8-13 pre-zidovudine. Subject 10 was twice tested pre-zidovudine treatment.
nd: not detected

Table 72: Con A driven gIFN response AIDS, receiving zidovudine

Subject	gIFN $\bar{x}$U per 1x10E+06 cells	
	without Con A	with Con A
1	45	271
2	nd	114
3	3	589
4	0.1	1699
5	0.6	143
6	13	350
7	3	43
8	2	738
9*	5	413

PBMC from 9 ARC subjects were cultured with Con A at 10 ug/ml or without for 72 h. All subjects were classified CDC IVC1. Individuals 1-8 were from the peripheral neuropathy study, while subject 9 was from clinic.

9* results by Medgenix gIFN IRMA

nd not detected

APPENDIX V

SAFETY PROCEDURES

1. Safety procedures

The Advisory Committee on Dangerous Pathogens (ACDP) issued interm guidlines on the handling of HIV-1 infected specimens in January, 1985. In the revised ACDP guidlines of June 1986, HIV-1 was classified as a group III pathogen, since infection with this retrovirus typically causes a lethal disease for which there is as yet no cure, but does not appear to present a high risk to individuals outside the known risk groups (ACDP, 1986).

The committee felt that whilst epidemiological evidence suggested that infection by an airborne route was not substantiated, the risk of infection from a high respiratory challenge could not be dismissed. In accordance, all samples at risk of being HIV-1 infected were handled in a class 3 containment laboratory. Gloves and gowns were worn for all procedures carried out in this laboratory. All waste material was autoclaved in double bags in metal boxes which were swabbed with 70 percent ethanol (superseded by 2 percent activated glutaraldehyde; see below) before leaving the laboratory. The waste material was then incinerated.

2. Cell Culture

Peripheral blood mononuclear cells obtained from patients at risk for HIV-1 were cultured in a class II microbiological cabinet (Medical Air Technology Ltd, Manchester). All centrifugation procedures were carried out in centrifuge buckets with screw top lids; these were opened and closed in the tissue culture cabinet to protect from potential aerosol generation.

All cell preparations were cultured in an incubator within the class 3 laboratory.

3. Cell harvesting

Cultures were stored at -20^{o}C, after 4 hour incubation with [^{3}H]TdR (see section 2.5.4.); frozen culture plates were then thawed and harvested by batch weekly. The cultures were harvested with a Skatron cell

harvester assembled within a Class I microbiological cabinet. The electric pump and water reservoir attached to the harvester remained outside the cabinet.

It was originally shown that HIV-1 is inactivated after as little as 1 minute exposure to 70 percent ethanol (Resnick et al., 1986; Spire et al., 1984). However, Hanson et al (1989) showed that 70 percent alcohol failed to inactivate dried cell free and cell associated virus within 20 and 15 minutes respectively; whereas both 2 percent and 1 percent alkaline glutaraldehyde inactivated cell free HIV-1 within 1 minute and a 2 percent solution also inactivated cell free virus in serum within 2 minutes.

For this reason the harvested filter papers were allowed to dry within the cabinet; individual disks were then placed in the scintillation vials and saturated with 70 percent ethanol and allowed to redry overnight before scintillant was added. Scintillation disks saturated in 2 percent activated glutaraldehyde (ASEP; Galen Ltd, N. Ireland) took over 36 hours to dry in this system and were therefore not used. This procedure did not affect the level of β-emissions. The scintillation vials were subsequently placed in plastic trays which were swabbed with 2 percent glutaraldehyde before being taken from the class 3 laboratory to the β-counter.

Following β-emission determination, the scintillation vials were autoclaved and incinerated as described above.

4. Quantitative assay of hu gIFN and Ig isotypes

Human gIFN (see section 2.6.) and Ig isotypes (see section 2.8.) were determined quantitatively in cell culture supernatants by IRMA and ELISA respectively. All procedures were carried out with class I or II microbiological cabinets in the class 3 containment laboratory, except the final stages of gamma-counting for gIFN assay and OD determination for Ig isotype ELISA. Possible HIV-1 residue was inactivated by 10 percent formol saline (Martin et al., 1985) following

gIFN assay and by 3M NaOH (J. Bridges, personal communication) after ELISA, which was also utilised to neutralise the substrate conjugate reaction.

5. T-lymphocyte subset enumeration by flow cytometry (FCM)

Samples were prepared for T-lymphocyte subset analysis by FCM (see section 2.9.2.) within a class I or II microbiological cabinet within the class 3 laboratory. Before prepared samples were removed from the containment laboratory for analysis, residual HIV-1 activity was inactivated a 1 percent paraformaldehyde solution (Martin et al., 1985).

The cell suspension was taken up through a biohazard nozzle, which prevents dripping, on the Becton-Dickinson flow cytometer.

Following excitation of the fluorochromes by the laser and analysis of the signal generated, the cell suspension was conducted to a waste flask containing 2 percent activated glutaraldehyde.

APPENDIX VI

REAGENTS

1. White cell counting fluid (WCCF)

Materials

1.0ml 2% Methylene blue (in distilled water)
1.0ml Glacial acetic acid
0.4ml Ethyl alcohol
17.6ml distilled water

Method

1. Prepare stock solution, filter with Whatman filter paper 50 (W & R Balston Ltd, UK), and store at room temperature.
2. Dilute 25ul of cell suspension with 225ul WCCF, mix and apply to haemocytometer.

Note

This solution lyses contaminating erythrocytes and stains leucocyte nuclei blue.

2. Trypan blue

Materials

0.1% Trypan blue (in Phosphate buffered saline)

Method

1. Prepare stock soluton, filter with Whatman filter paper 50 (W and R Balston Ltd, UK), and store at room temperature.
2. Dilute 25ul of cell suspension with 75ul 0.1% trypan blue, mix and apply to haemocytometer.

3. ELISA blocking solution

Materials

2g Gelatine (BDH Chemicals Ltd, Poole, England)

2g Bovine Serum Albumin, BSA (Sigma A 7030 98.99% Albumin)

1g Sodium azide, NaN_3 (BDH Chemicals Ltd, Poole, England)

Method

1. Dissolve the Gelatine in 20ml distilled water, using a magnetic stirrer and hot plate.
2. Dissolve BSA by sprinkling over 250ml beaker of distilled water.
3. Pool Gelatine and BSA solutions, add the Sodium azide and dilute to 1l with distilled water.
4. Store at 4 ^{o}C.

4. 0.5M Tris-buffered saline (TBS), pH 7.6

Materials

60.55g Tris(hydroxymethyl)-methylamine [2-amino-2-(hydroxymethyl) propane-1,3-diol,(tris)] (BDH Chemicals Ltd, Poole, England)

87.66g Sodium chloride (BDH Chemicals Ltd, Poole, England)

Method

1. Dissolve ingredients in 1000ml distilled water and adjust to pH 7.6 with HCl.
2. Dilute 1:10 with distilled water before use.

5. 0.5M Tris buffered saline (TBS), pH 8.2

Method

1. Prepare Tris-buffered saline as above but adjust to pH 8.2 with HCl.
2. Dilute 1:5 with distilled water before use.

6. Napthol AS-MX phosphate / Fast red mixture

Materials

10mg Napthol AS-MX phosphate (free acid) (N-4875, Sigma, St. Louis, USA)
200ul Dimethyl formamide
9.8ml TBS, pH 8.2
30ul 1M Levamisole (L-9756, Sigma, St. Louis, USA)
10mg Fast red TR salt (5-Chloro-2-toluenediazonium chloride hemi [zinc chloride]) (F-1500, Sigma, St. Louis, USA)

Method

1. In a glass container, dissolve Napthol AS-MX phosphate in Dimethyl formamide and dilute with TBS, pH 8.2.
2. Add Levimasole solution and Fast red TR salt.
3. Prepare just prior to use and filter through Whatman No. 1 filter paper (Whatmann Ltd, Maidstone, UK).

7. Haematoxylin counterstain

Materials

5g Haematoxylin (monohydrate) (Gurr-BDH Chemicals Ltd, Poole, England)
50ml Ethanol
100g Ammonium or Potassium alum (Aluminium ammonium/potassium sulphate)
1000ml distilled water
2.5g Mercuric oxide red [mercury (II) oxide red] (BDH Chemicals Ltd, Poole, England)
40ml Glacial acetic acid

Method

1. Dissolve Haemotoxylin in alcohol.
2. Dissolve alum in hot water.
3. Add Haematoxylin/alcohol mix to alum and bring to boil.
4. Add Mercuric oxide.
5. Allow to cool and filter.
6. Add Glacial acetic acid.

8. FacScan lysing solution

Method

1. Dilute FACScan lysing solution (Becton Dickinson, San Jose, USA) 1:10 with distilled water.
2. Store in the dark at room temperature.

9. 1.0% Paraformaldehyde solution

Materials

200ml Phosphate buffered saline

2g Paraformaldehyde (BDH Ltd, Poole, England)

Method

1. Disslove Parformaldehyde in Phosphate buffered saline at 56°C.
2. Allow to cool and adjust to pH 7.2.
3. Filter through 0.2um filter (Sartorius, West Germany for Flow Laboratories).
4. Store at 4°C.

APPENDIX VII

Publications related to this study

KNOX KA; PETERS B; PINCHING AJ. (1989) Anti-viral agents for AIDS: lymphocyte function as a marker of response. V International Conference on AIDS, Montreal. Abstract C557

KNOX KA; WINER J; GOMPELS M; BANG B; PINCHING AJ. (1991) Immunodeficiency and peripheral neuropathy. British Society of Immunology Spring Meeting. Poster 77

NYE KE; KNOX KA; PINCHING AJ. (1991) Lymphocytes from HIV infected individuals show abberant inositol polyphosphate metabolism which reverses after zidovudine therapy. AIDS 5:(in press)

PETERS BS; BENNETT JM; JEFFRIES D; KNOX K; KOCIS A; PINCHING AJ. (1990) Ineffectiveness of AL 721 in HIV disease. Lancet i:545-546

KIRSTINE A. KNOX

Ph.D. THESIS 1991

CORRECTIONS

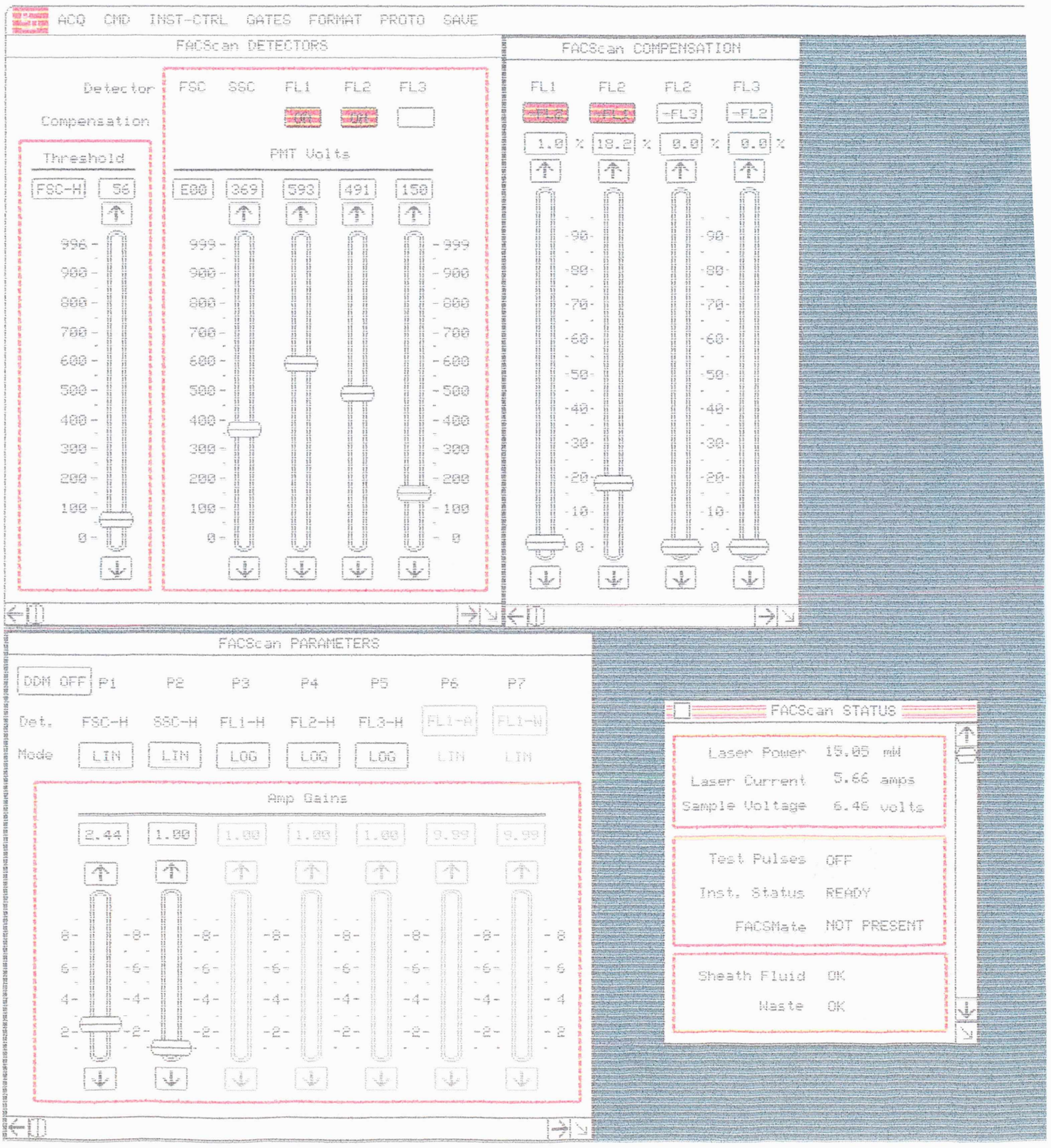
Kirstine A. Knox
Dept. Immunology
The Medical School
University of Birmingham
Vincent Drive
Birmingham B15 2TJ

CHAPTER	PAGE	CORRECTION
1	36	focused
	45	Kornfeld
	46	(PtdIns(4,5)P2)
	46	phosphoninositidase
	60	1987
	77	NF-kappaB
	82	focused
	88	Valentine and Jacobson 1990
	89	Valentine and Jacobson 1990
	91	Valentine and Jacobson 1990
	92	CD4
	93	Valentine and Jacobson 1990
	94	Valentine and Jacobson 1990
	105	Valentine and Jacobson 1990
	111	Valentine and Jacobson 1990
	113	Valentine and Jacobson 1990
	115	Valentine and Jacobson 1990
2	122	Glaxo
	129	of
	129	standard
	129	control
	142	Appendix VI.6
	142	Appendix VI.7
	144	Appendix VI.8
	144	Appendix VI.9
4	229	subject 1
	238	AIDS
6	252	1/500
7	282	8
8	303	Valentine and Jacobson 1990
	304	Valentine and Jacobson 1990
	305	x10 cells/l
	306	Valentine and Jacobson 1990
	310	Valentine and Jacobson 1990
	318	4.7
	319	Valentine and Jacobson 1990
References	353	Valentine and Jacobson 1990
Appendix V	387	glutaraldehyde
	388	glutaraldehyde
	388	V.4
	389	V.5

BECTON DICKINSON

LYSYS II Version 1.0 11/90

DATE: 30-JUL-91 TIME: 9:59:49



FACScan ANALYSIS PANEL

FIGURE 106

BECTON
DICKINSON

SimulSET Software Version 2.2 4/89

Operator : oper
Patient ID : KK-1
Preparation: Lysed Whole Blood
Panel Name : SUBSET

Cytometer: FACSCAN

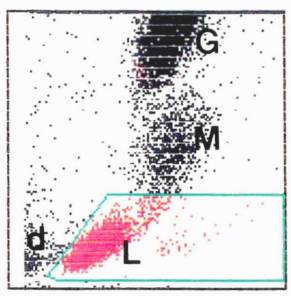
Date: 18-Jul-90
Time: 2:09
Date Collected: 7-Jun-90

LeucoGATE

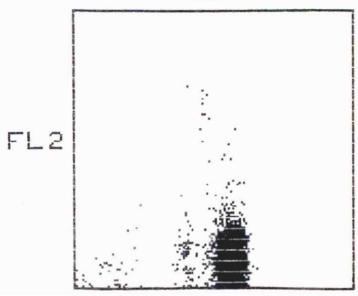
#3:SS07069001

Gate:	FSC	SSC
	42	6
	255	6
	255	86
	91	86
	35	10

(1)



(2)



>> Operator defined gates <<

FSC Mean 81
SSC Mean 38

Events 3758

% L	% M	% G	% D	% Gated Lymphs
97	0	2	1	98

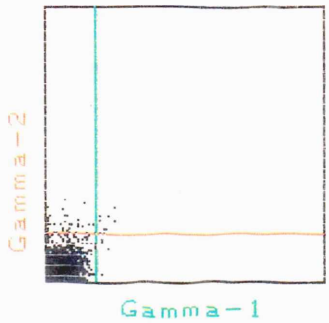
Three Part Differential	% of Leucocytes
Lymphocytes	25
Monocytes	7
Granulocytes	67

Control

#3:SS07069002

Markers:
FL1 44
FL2 44

(3)



Quad	Cell Type	Corr % L
1	NSS PE	1
2	NSS ++	0
3	Unstained	98
4	NSS FITC	0

FSC Mean 84
SSC Mean 38

Events 2605

FIGURE 107

BECTON DICKINSON SimulSET Software Version 2.2 4/89

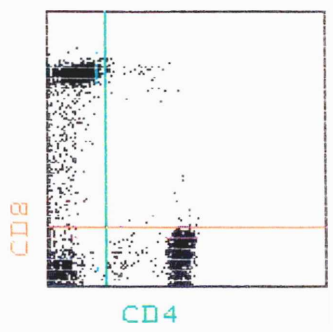
Operator : oper Cytometer: FACSCAN Date: 18-Jul-90
Patient ID : KK-1 Time: 2:09
Preparation: Lysed Whole Blood Date Collected: 7-Jun-90
Panel Name : SUBSET

(1)

CD4/CD8

#3:SS07069003

Markers:
FL1 52
FL2 54



Quad	Cell Type	Corr % L
1	CD4-CD8+	36
2	CD4+CD8+	2
3	CD4-CD8-	24
4	CD4+CD8-	38

FSC Mean 86 Events 2777
SSC Mean 39

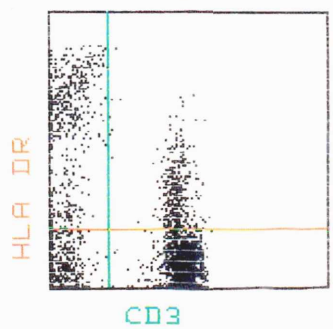
>> Operator defined markers <<

(2)

CD3/HLA DR

#3:SS07069004

Markers:
FL1 52
FL2 54



Quad	Cell Type	
1	CD3-DR+	17
2	CD3+DR+	15
3	CD3-DR-	10
4	CD3+DR-	59

FSC Mean 87 Events 2852
SSC Mean 39

>> Operator defined markers <<

For research use only. Not for use in diagnostic or therapeutic procedures.