NOTICE

The quality of this microform is heavily dependent upon the quality of the original thesis submitted for microfilming. Every effort has been made to ensure the highest quality of reproduction possible.

If pages are missing, contact the university which granted the degree.

Some pages may have indistinct print especially if the original pages were typed with a poor typewriter ribbon or if the university sent us an inferior photocopy.

Reproduction in full or in part of this microform is governed by the Canadian Copyright Act, R.S.C. 1970, c. C-30, and subsequent amendments.

AVIS

La qualité de cette microforme dépend grandement de la qualité de la thèse soumise au microfilmage. Nous avons tout fait pour assurer une qualité supérieure de reproduction.

S'il manque des pages, veuillez communiquer avec l'université qui a conféré le grade.

La qualité d'impression de certaines pages peut laisser à désirer, surtout si les pages originales ont été dactylographiées à l'aide d'un ruban usé ou si l'université nous a fait parvenir une photocopie de qualité inférieure.

La reproduction, même partielle, de cette microforme est soumise à la Loi canadienne sur le droit d'auteur, SRC 1970, c. C-30, et ses amendements subséquents.

CD4 T CELLS AS TARGETS FOR THE CYTOTOXIC T LYMPHOCYTE ASSAY: A STUDY OF ENHANCERS OF HIV-1 INFECTION IN THE TARGET CELL PREPARATION

By

Elizabeth H. Fullmer

THESIS

Submitted to the Faculty of Graduate Studies in partial fulfillment of the requirements for the degree of

Master of Science

Department of Microbiology and Immunology

Faculty of Medicine

University of Ottawa

ISBN 0-315-75101-0

UNIVERSITÉ D'OTTAWA
UNIVERSITY OF OTTAWA

ABSTRACT

It has been proposed that the cytotoxic T lymphocyte (CTL) response to the Human Immunodeficiency Virus (HIV)-1 plays an important role in delaying progression of the disease in infected individuals. These CTLs bear the CD8 cell-surface marker and kill virus-infected cells in an MHC-restricted way. The characterization of the viral epitopes recognized by these CTLs is valuable in the design of a vaccine. A frequent problem in the analysis of the CTL response is the lack of an adequate target cell. My objective was to establish the conditions necessary to measure CTL activity. The approach was to clone peripheral blood mononuclear cells (PBMC) from HIV-1^+ asymptomatic patients to obtain $CD8^+$ clones and test these cells against autologous $CD4^+$ T lymphoblasts. The infection of the CD4 cells was problematic and experiments were performed to improve the infection of $CD4^+$ T cell lymphoblasts. Titration and quantization of virus stocks were carried out in normal T cells and in the HUT-78 cell line. Detection methods of infection included: 1) the p24 Antigen assay (Abbott), 2) immunofluorescence (IFA) and 3) the Cytopathic Effect (CPE). The results demonstrate that the supernatant used for infection should contain at least 100,000 pg/ml of p24 antigen. I also tested the following enhancers of viral infectivity: DEAE-Dextran (DD), Polybrene and Tumor Necrosis Factor-Alpha (TNFα) in normal T cells and HUT-78 cells and found that DD greatly increases infection when used as pretreatment and during infection at 10 μg/ml. Polybrene (2.5-5 μg/ml) also increases infection when used as pretreatment and during infection but not as much as DD. It proved to be less toxic than DD and would be useful when a slow, less acute infection is desired. The effect of TNF α at 5 ng/ml was not noticeable in normal T cells the first few days after the infection but the infection increased six or seven days later. TNFα did not have any significant effect on the HUT-78 cell line. The improved infection protocol with the use of enhancers will be useful in the production and maintenance of high titer virus stocks in the laboratory and in the infection of sensitive target cells for CTL assays.

I was also able to determine that the infection of the $CD4^+$ T cells should be done 3 days after Phytohemagglutinin (PHA) stimulation. Cells that were infected 6 days after PHA stimulation had very low levels of infection as determined by p24 Ag assay and IFA.

This new improved infection protocol was tested in CTL assays with PBMC from HIV-1^+ asymptomatic patients. The results were not promising and I concluded that the $CD4^+$ T lymphocyte is not a sensitive target to detect HIV-1-specific CTL from fresh blood of asymptomatic patients. The reason is that CD4 T lymphocyte numbers are quite low in patients and the cells have to be cultured for 5 or 6 days in order to have sufficient numbers for the CTL assay. By then, however, in spite of the fact that viral entry is not affected, the cells become relatively resistant to viral infection and perhaps CTL killing. The mechanisms for such resistance remain to be determined.

ACKNOWLEDGEMENTS

My sincere gratitude to Dr. Carlos A. Izaguirre for his constant guidance and for his excellent advice in analyzing the results and editing the thesis.

I am grateful to Dr. Lionel G. Filion for carefully reading this thesis and for his constructive comments throughout the research project.

My thanks also to Dr. Syed A. Sattar, who made my arrival in Canada and the initial orientation at the University such a pleasant experience.

Alice Sherring and Dragica Bogdanović, at the Federal Center for AIDS, will be specially remembered for their friendship, invaluable help and support.

Many thanks to Françoise Beaudoin and Sylvia Frenette for doing the p24 Ag assays and to Michèle Bergeron and Peggy Seely for their assistance in FACScan analysis.

I was supported by a grant from the National Health Research and Development Program (NHRDP), Health and Welfare, Canada.

To my mother, my husband Robert and my sons
for their unfailing support.

TABLE OF CONTENTS

PAGE

PAGE

PAGE

PAGE

LIST OF TABLES

LIST OF FIGURES

PAGE

LIST OF ABBREVIATIONS

ADCC	Antibody-Dependent Cell mediated Cytotoxicity
AIDS	Acquired Immunodeficiency Syndrome
AMT	Aminomethyl 4,5',8-Trimethylpsoralen
CD(3,4,8)	Cluster of Differentiation
CPE	Cytopathic Effect
^{51}Cr	Chromium 51
CTL	Cytotoxic T Lymphocyte
DEAE-Dextran	Diethylaminoethyl-Dextran
E:T Ratio	Effector to Target Ratio.
FCS	Fetal Calf Serum
gp	Glycoprotein
HIV-1	Human Immunodeficiency Virus-Type 1
IFA	Immunofluorescence Assay
IFNγ	Interferon-gamma
IL-2	Interleukin 2
IMDM	Iscove's Modified Dulbecco's Medium
LTR	Long Terminal Repeat
2-ME	2-Mercaptoethanol
MHC	Major Histocompatibility Complex
MW	Molecular Weight
NF-kB	Nuclear Factor-kappa B cell

p24 Ag	protein 24 Antigen
PBL	Peripheral Blood Lymphocyte
PBMC	Peripheral Blood Mononuclear Cells
PBS	Phosphate Buffered Saline
PPD	Para-Phenylene Diamine
RT	Room Temperature
TNFα	Tumor Necrosis Factor-Alpha
PHA	Phytohemagglutinin (Phaseolus spp.)

LIST OF REAGENTS

Anti -Leu-3a (CD4) Anti -Leu 2a (CD8)	COULTER
AMT	LEE BIOMOLECULAR
Castanospermine	GENZYME
Chromium-51	NEN
DEAE-Dextran	SIGMA
Dynabeads M-450	DYNAL
FCS	HYCLONE
Ficoll-Hypaque	PHARMACIA
Galactose Oxidase	SIGMA
Gentamicin	FLOW LABORATORIES
Goat Serum	GIBCO
Goat anti-Human IgG -FITC	TAGO or JACKSON IMMUNORESEARCH
Heparin	SIGMA or GIBCO
HUT-78 Cell line	AMERICAN TYPE CULTURE COLLECTION
IFNγ	AMERSHAM
IL-2	AMERSHAM
Indomethacin	SIGMA
IMDM	GIBCO
Mouse Serum	CEDARLANE
MsIgG1-RDI/MsIgG1-FITC	COULTER
Neuraminidase	SIGMA

Paraformaldehyde	BDH BIOCHEMICALS.
PBS	GIBCO
PHA-HA16	WELCOME DIAGNOSTICS
Polybrene	SIGMA
Polyclonal Human IgG anti-HIV Standard	NIH-AIDS Research and Reference Program
PPD	BAKER
RPMI-1640	GIBCO
Streptomycin	GIBCO
Tumor Necrosis Factor-α	AMERSHAM

I. INTRODUCTION

I.1. Human Immunodeficiency Virus-Type 1.

The Acquired Immunodeficiency Syndrome (AIDS) was first described in 1981 (Gottlieb, et al., 1981). The Human Immunodeficiency Virus, type 1 (HIV-1) was identified as the etiologic agent of AIDS in 1983 (Barré-Sinoussi et al., 1983). Seroepidemiological studies suggested Central Africa to be the cradle of HIV-1.

In 1986, a second human immunodeficiency virus (HIV-2) was isolated from West Africa. Both HIV-1 and HIV-2 are retroviruses belonging to the lentivirus group. The inclusion in this group is based on genetic and morphologic criteria. Assuming a constant rate of evolution comparable to that of other retroviruses, it is estimated that HIV-1 entered the human population approximately 50 years ago. HIV-2, was found to be more closely related to the simian immunodeficiency virus isolated from macaques in captivity (SIVmac) than to HIV-1. Nonetheless, the major biological and genetic features are parallel although HIV-2 may be less virulent (Wong-Staal, 1990).

I.1.1. The HIV-1 genome and viral proteins.

One feature that distinguishes the lentiviruses from other retroviruses is the remarkable complexity of their viral genome. The HIV-1 genome encodes not only the virion proteins *gag*, *pol*, and *env* which are the three structural genes essential for virus replication common to all retroviruses, but also two different proteins required for virion morphogenesis and maturation (*vif* and *vpu*), a protein of unknown function (*vpr*) and three nonstructural, regulatory proteins (*tat*, *rev*, and *nef*) (Cullen & Greene, 1989).

The *gag* gene, named in honor of the first recognition of the proteins encoded by it as *group-specific antigens*, encodes a polyprotein precursor that is subsequently cleaved by the viral protease during maturation into the following core proteins: p24 which is the major core antigen, p18 which is the myristoylated *gag* protein and p15

which may be the RNA-binding protein. The phosphorylated p24 polypeptide forms the chief component of the inner shelf of the nucleocapsid

The *pol* gene encodes a precursor protein which is cleaved to yield the enzymes: protease, reverse transcriptase, integrase and ribonuclease.

The *env* gene encodes a glycosylated polypeptide precursor (gp160) that is processed to form the exterior glycoprotein (gp120) and the transmembrane glycoprotein (gp41). This processing of gp160 appears to be essential for infection and syncytia formation (Wong Staal, 1990). The *env* gene is the one that shows greater variability. This degree of variability among independent virus isolates is one of the principal features of the HIV-1 genome and one of the key obstacles in the development of a vaccine.

The *tat* gene stimulates expression from the HIV-1 Long Terminal Repeat (LTR). It has a trans-activating function through a "transactivation response element" located in the HIV-1 mRNAs and it is essential for the replication of HIV-1.

The *rev* gene regulates expression of the virion structural proteins by inducing the cytoplasmic expression of unspliced (*gag,pol*) and singly spliced (*env, vif*) viral mRNA while simultaneously reducing the expression of the doubly spliced RNAs that encode the viral regulatory proteins; thus the Rev protein down regulates its own production; it acts through an RNA motif termed Rev response element (Cullen and Greene, 1989).

The *nef* gene, or "negative factor" was thought to reduce transcription driven by the HIV-1 LTR. But recently some authors report that it does not act as a negative factor (Kim et al., 1989; Hammes et al., 1989). This protein is found mainly outside the nucleus in the cytoplasm and is absent from infectious HIV-1 virions (Chenciner et al., 1989).

The *vif* gene or "virus infectivity factor" is the product of a short open-reading frame that encodes a protein necessary for virion infectivity. It appears to have important functional roles during late stages of virion morphogenesis.

I.1.2. Life Cycle

The receptor for HIV-1 is the CD4 molecule present mainly in T cells and macrophages. The binding of viral envelope gp120 to the CD4 receptor is the first step of viral entry into the cell. Studies with enveloped viruses have defined two distinct pathways by which virus can enter the cell: direct fusion of the viral envelope with the plasma membrane and internalization of the virus-receptor complex by receptor-mediated endocytosis (Kielian and Helenius, 1986). In the low pH of the endosome that is formed, the viral envelope fuses with the limiting membrane of this vacuole and the nucleocapsid enters the cytoplasm of the cell.

The CD4 molecule is internalized after exposure of $CD4^+$ T cells to either phorbol esters or appropriate antigen-bearing target cells (Hoxie et al. 1986). These observations had led to the suggestion that the HIV-1-CD4 complex on the cell surface was internalized via acidic endocytic vesicles (endosomes). However, studies with agents that raise the pH of these intracellular organelles failed to inhibit HIV-1 entry via the endocytic pathway. This suggests that entry of HIV-1 results from direct fusion of the viral envelope with the cell membrane (McClure et al., 1988; Maddon et al., 1988).

After internalization, the HIV-1 virion is rapidly uncoated. Viral replication begins with the generation of a first strand DNA copy of viral RNA mediated by the HIV-1 encoded reverse transcriptase (RT). Second-strand DNA synthesis is also controlled by the RT but proceeds after the ribonuclease H (a product of *pol* gene) partially degrades the original RNA template. The action of RT, when completed, yields a double stranded DNA replica of the original RNA genome flanked by directly repeated sequences termed long terminal repeats (LTRs) (Greene, 1991). The viral DNA intermediate migrates to the nucleus, where it is covalently integrated into a host chromosome by the viral integrase, the third enzymatic product of the *pol* gene. This gives rise to the HIV-1 provirus. The viral mRNAs reach the cytoplasm and encode various regulatory proteins including *Tat* and *Nef*. This is the early phase of expression

of the HIV-1 genes which is characterized by cytoplasmic expression of the viral mRNAs. For the assembly of infectious HIV-1 virions, however, the retroviral structural and enzymatic proteins must also be produced. These proteins are uniquely encoded by incompletely processed viral transcripts, including the unspliced *gag-pol* mRNA and the singly spliced *env* mRNA. The transition between the synthesis of early regulatory genes and late structural gene products is critically dependent on the HIV-1 *Rev* protein which activates the cytoplasmic expression of the HIV-1 RNA that encoded the products of the *gag*, *pol* and *env* genes. The products of the *gag* and *pol* genes form the core of the mature HIV-1 virion, and the products of the *env* genes are the principal exterior-coat proteins. After the cores are assembled the final budding process takes place by cleavage events mediated by the HIV-1 protease and the p17 of *gag* protein. The protein products of the *vif* and *vpu* appear to have important functional roles during these late stages of virion morphogenesis. Specially, the *Vpu* protein promotes the efficient release of the budding virions from the surface of the cell.. The product of the *vif* gene also appears to be necessary for full infectivity of the released HIV-1 virions (Greene, 1991).

I.1.3. Latency

In some cases, viral infection of resting lymphocytes results in a latent viral state which can be converted to a productive form of infection following cellular activation. Cells in the growth zero (G_0) phase of the cycle appear to lack cellular factors required for the complete synthesis of the full-length DNA provirus and may be unable to support effective integration of the DNA provirus into the host genome. These unintegrated virions can be stably maintained in resting cells extrachromosomally for a significant period of time *in vitro* (Stevenson et al., 1990). After the activation state, which is transient, the T lymphocyte cycles back into the resting phase as a memory cell. But this time the virion is established in a second latent form of an integrated but quiescent HIV-1 provirus (Cullen & Greene, 1989).

I.1.4. HIV-1 tropism

The virus is tropic for cells which bear the CD4 antigen on their surface. Among these cells is included one of the principal regulatory cells in the immune system, the T-helper cell. Several other cells, such as dendritic cells, glial cells, macrophages, EBV-transformed B cells and myeloid progenitor cells, which express low levels of the CD4 molecule, can also be infected by HIV-1.

HIV-1 can produce a persistent nonproductive infection of Epstein-Barr Virus (EBV)-transformed human B lymphocytes where virions accumulate within cytoplasmic vesicles. These vesicles are similar in morphology but less abundant than those described in HIV-1 infected monocyte lines (Dahl et al., 1990). The persistent infection in EBV-transformed B lymphocytes can also be productive (Tozzi et al., 1989). This raises the possibility that B cells may constitute reservoirs for HIV-1 *in vivo*, similar to that reported for macrophages (Ho et al., 1986). HIV-1 can also infect CD4-negative fibroblastoid cells (Tateno et al., 1989), and human bone marrow stromal fibroblasts (Scadden et al., 1990). There is one report (Tsubota et al., 1989b) indicating that CD8+ lymphocyte lines can harbor the AIDS virus *in vitro*. Recently it has been reported that HIV-1 can also infect Natural Killer (NK) cells *in vitro* (Chehimi et al., 1991). These cells do not express the CD4 molecule and infection was not blocked by anti-FcγRIII, which raises the possibility that HIV-1 can enter cells via a receptor other than the CD4 glycoprotein.

In vitro infection of $CD4^+$ T cells with HIV-1 can result in downregulation of CD4 expression, but within peripheral blood mononuclear cells (PBMC), HIV-1 is expressed *in vivo* predominantly in the T cell subpopulation which continues to express the CD4 molecule on the surface. The frequency of infected cells in AIDS patients is at least 1/100 cells including latently infected cells; this is a high viral burden as compared to asymptomatic HIV-1-positive individuals which have a frequency of 1/10,000 infected cells (Schnittman et al., 1989).

I.1.5. Cytopathic Effects of HIV-1.

The cytopathic effects of HIV-1 in the CD4+ T lymphocytes contribute in great part to the disregulation of the entire immune system including the various cytokines produced by the CD4 T lymphocyte and the other cells involved in immune responses. One of the cytopathic mechanisms involves the cell fusion and the formation of syncytia. This only requires the presence of the *env* gp120 of HIV-1 and CD4-expressing cells. The multinucleated syncytia of these uninfected CD4-cells eventually die. Other mechanisms of cytopathicity include membrane injury with changes in permeability, accumulation of unintegrated HIV-1 DNA and destruction of T cells infected with HIV-1 by both antibody and cytotoxic T-cell-mediated mechanisms (Greene, 1991).

Some strains of HIV-1 are associated with virulence in the host. The characteristics associated with this are:1) enhanced cellular host range, 2) rapid kinetics of replication, 3) high titers of virus production, 4) efficient cell killing, 5) sensitivity to antibody-mediated enhancement and 6) lack of sensitivity to suppression by the *nef* gene; different strains with distinct features can emerge in the same individual and be found particularly in certain tissues; the cytopathogenicity appears to be linked to the envelope gene, most likely the glycosylation pattern of the HIV gp120 (Levy, 1990). It has been argued that the HIV-1-specific CTL activity can be deleterious to the patient. In HIV-1-infected humans, specific CTL have been isolated from tissues like the lung where inflammatory reactions are clearly occurring (Guillon et al, 1988).

I.2. The Immune Response to HIV-1.

The immune system has two main branches to respond to an antigenic challenge: 1)Humoral, which is the production of antibodies by B cells and 2) Cellular which is the killing of virally or parasite-infected cells by cytotoxic cells. Despite the detection of both humoral and cellular immune responses in HIV-1-infected people, the exact role of the immune system in the pathogenesis of AIDS has not been totally ellucidated.

Evidence suggests that the immune response plays a critical role in delaying the clinical manifestations of the disease. Proof of this is that a great number of infected people remain asymptomatic for years after the infection despite persistent viremia. This is in contrast with the perinatal transmission where the immunologically immature infant usually develops pathological disease by the second year of life (Auger et al.,1988).

I.2.1. Humoral Response

HIV-1 elicits a broad-spectrum B cell immune response in infected individuals. Between 1 and 3 months after infection, antibodies specific for all the major proteins are generally present. Typically, reactivity with the p24 and gp41 products of the *gag* and *env* genes respectively is associated with seropositivity. Protein products of the HIV *pol* gene (protease, reverse transcriptase and endonuclease) are also detected on immunoblots by most patient sera. Anti-core (p24) antibodies frequently fall off with increasing illness and poor prognosis in AIDS (Sattentau, 1989; O'Shea et al., 1990). Low antibody reactivity to accessory gene products *nef, rev,* and *tat* also appears to be associated with rapid progression to AIDS after infection with HIV-1 (Reiss et al., 1990).

Neutralizing antibodies, even though they can be detected in the serum of most HIV-1 positive patients, are not enough to control the progression of the disease; the majority of these neutralizing antibodies are directed against the major envelope glycoprotein, gp120. Transmission of HIV-1 from cell to cell makes virus destruction difficult by means of antibodies only; thus, neutralizing titers of antibodies are generally low in HIV-1 infected patients (Sattentau, 1989).

In some cases HIV-1 infection is enhanced *in vitro* via an antibody-dependent enhancement mechanism. Both Fc and complement receptor might be involved in the internalization of HIV -1 in some cases (Fanger et al., 1989; Levy, 1990).

Classic Antibody-Dependent Cell mediated Cytotoxicity (ADCC) is also operative in seropositive patients. FcR-bearing cells "armed" *in vivo* with anti-gp120

antibodies can mediate this gp120-specific cytotoxicity. This cytolytic activity is not MHC-restricted. When gp120 is liberated from cells during virus replication, it associates with CD4-bearing cells in the absence of infectious virus. This renders these cells susceptible to a form of lympholysis which destroys the so-called "innocent bystander" cells. The antibodies in patients' antisera which correlate best with ADCC are those against p24 rather than against the envelope glycoproteins.

I.2.2. Cellular Response.

The cellular response is mediated by cytotoxic cells. This cytotoxic response is one of the early host defenses against intracellular infections including viruses and parasites. It can be non specific, mediated by macrophages, natural killer cells (NK) and Lymphocyte Activated Killers (LAK), and it can be specific, mediated by cytotoxic T lymphocytes (CTL). The specific response has two major requirements to kill a cell: 1) both killer cell (effector) and target cell have to share the same molecules of the major histocompatibility complex (MHC restriction) and 2) the target has to present processed antigen (viral proteins) on the surface as a complex with the MHC molecule. The MHC molecule can be of Class I, present in most cells, and Class II present mainly in macrophages, B cells and antigen presenting cells. The majority of cytotoxic T cells are usually of the CD8 phenotype restricted by Class I (A,B,C) MHC but they can be CD4 T cells restricted by Class II (DP, DR, DQ) MHC. The CD8 CTL specialize in killing cells expressing endogenous antigens present in the cytoplasm which are processed and presented on the surface with Class I MHC molecule. The CD4 CTL will kill cells that present exogenous antigens processed and presented with Class II MHC. Since CTL typically recognize viral antigen which has been processed and presented at the cell surface as a short peptide fragment, the use of synthetic viral peptides has been used to sensitize targets to virus-specific lysis *in vitro*. The first studies regarding epitopes recognized by HIV-1-reactive CTL were performed in mice immunized with a

recombinant vaccinia virus expressing the gp160 envelope protrein (Takahashi et al.,1988). In studies of HIV-1-infected people (Plata et al., 1987b), a different envelope epitope was reported from the C terminal portion of gp 120 HIV-1 (Bru), restricted by HLA Class I antigen A2 which is highly conserved among sequenced isolates. In addition to CTL epitopes within the envelope protein, *gag* epitopes have also been identified restricted by HLA B27 (Nixon et al., 1988). Four additional *gag* epitopes from the p14 and p24 proteins have been identified using a different methodology. In these experiments, proteins representing computer-predicted *gag* T-cell epitopes were used (Claverie et al., 1988). Another internal viral protein which serves as target for HIV-1-specific CTL is viral RT (Walker et al., 1988 and 1989). To define these epitopes of RT, target cells are infected with recombinant HIV-1-vaccinia viruses expressing truncated RT gene inserts or synthetic peptides (Walker et al., 1989). Other proteins of the virus like *nef* and *vif* can serve as targets for CTL (Chenciner et al., 1989; Plata, 1989). The internal viral proteins of HIV-1 (*gag*, RT) exhibit far less heterogeneity among sequenced isolates than does envelope glycoprotein and the charactization of those epitopes is very important from the vaccine development standpoint.

HIV-1-specific human CTL recognize the HIV-1 envelope glycoprotein 120 in association with HLA-A2 transplantation antigen. Analyses of other patients indicate that HLA-A1,-A3, A9, A11, B8 and B27 can also associate with HIV-1 antigens for CTL recognition to occur (Langlade-Demoyen, 1988; Nixon et al., 1988; Walker et al., 1989). A hierarchy of HLA antigens concerning their capacity to associate with HIV-1 proteins remains to be established.

Even though the cytolytic machinery of $CD8^+DR^+$cells is still functioning in patients with AIDS, it seems that their HIV-1-specific cytolytic activity is lost in the last stages of the disease. The reason for this decrease in CTL activity with disease progression may be due to several factors. Reports demonstrating that the CD8+ T lymphocytes may be infected with HIV (Tsubota et al., 1989b) suggests one possible

mechanism for this decrease . Another possible factor involved in the progressive decline of CTL activity is not sufficient helper cell function as CD4 depletion becomes more severe. HIV-1-specific CTL contributes to the gradual loss of CD4+ cells by killing these virally infected cells. Also CD4 T lymphoblasts and monocytes adsorb HIV-1 gp120 on their cell surface and become adequate targets for CTL attack or for antibody-dependent cell mediated cytotoxicity (ADCC). The decrease in cytotoxicity may result at least in part from a progressive decrease in the pool of HIV-1-specific cytotoxic T lymphocytes belonging to the $CD8^+DR^+CD25^-$ subset. These cells do not express the IL-2 receptor (CD25) and show a defect of proliferation *in vitro* in response to stimuli such as PHA or monoclonal antibodies directed against the CD3/T-cell receptor complex, CD2 and CD28 molecules. What happens *in vitro* may reflect a defect in the ability of these cells to amplify *in vivo*. This might explain the diminished CTL function in AIDS patients as disease progresses (Pantaleo et al., 1990b) and the decrease in the frequency of HIV-1-specific CTL precursors as infection progresses (Hoffenbach et al., 1989; Gruters et al., 1990). Interleukin-2 can restore the diminished levels of HIV-1-specific cytotoxic cells in the advanced stages of the disease. This indicates that IL-2-producing T cells are being affected during the course of the disease and that the defect is not in the $CD8^+$ CTL function (Via et al., 1990).

Many of the initial CTL studies tried to characterize the specific CTL response directed at the HIV-1 envelope glycoprotein. Using recombinant vaccinia viruses expressing the *env* gene to infected EBV-transformed B cells, a response was demonstrated using fresh peripheral blood (PBMC) from seropositive subjects (Walker et al., 1987a). Other laboratories have reported the use of this EBV/ recombinant vaccinia system (Chakrabarti et al., 1986; Hu et al., 1986; Kieney et al., 1986). Others have used alveolar macrophages (Plata et al., 1987a; Hoffenbach et al., 1989; Autran et al., 1988), or murine cell lines transfected with human Class I antigens (Plata et al., 1987a; Langlade-Demoyen, 1988). HIV-1-specific CTL response has also been derived from

cerebrospinal fluid (CSF) of AIDS patients with neurologic disorders. This was done by stimulating CSF T cells *in vitro* with autologous irradiated PBMC coated with recombinant gp120 (Sethi et al.,1988). CD4 T lymphocytes have also been used as targets, but there is only one published report (Hoffenbach et al., 1989).

I.3. Cytotoxic T-Lymphocyte Assay.

The detection of CTL-specific for HIV-1 requires the following steps:

1. Preparation of the Target Cell.
2. Preparation of effector cells or killer lymphocytes.
3. A good ^{51}Cr release assay to detect cytotoxicity

I.3.1. Preparation of the Target Cell.

One of the factors that complicates the study of direct cell-mediated cytotoxicity (CTL) is the requirement for an infected target that stably expresses HIV-1. Another requirement is that the target's MHC molecules should match those of the effector cell. Selecting autologous HIV-1-infected $CD4^+$ lymphocytes as target meets this requirement. These cells are very susceptible to the cytopathic effects of the virus.

As already indicated, target cells for these assays have included CD4 T-lymphoblasts , alveolar macrophages infected in vivo, Epstein-Barr virus (EBV)-immortalized lymphoblasts and EBV-immortalized lymphoblasts infected with vaccinia virus (VV) recombinants that incorporate some of the genetic sequences of the HIV-1 genome as mentioned in I.2.1.

Therefore, a number of aspects needed to be evaluated in the preparation and infection of T cells. I needed to know the titer of virus stocks, to select the best assay for the detection of the infection, to evaluate the role of enhancers of infectivity such as Polybrene, Tumor Necrosis Factor α and DEAE-Dextran, and to determine the best time of infection after PHA stimulation of the sample.

i. Stimulation of the cells with Phytohemagglutinin (PHA) prior to infection.

T cells have to be activated and in the log phase of growth to be properly infected by the virus. The eventual transcription of the virus-specific sequences is controlled by both cellular factors and HIV-1-encoded regulatory proteins. Activation of the cell permits the integration of the HIV-1 provirus and stimulates transcription. For this reason, Phytohemagglutinin (PHA), which stimulates DNA replication and cellular proliferation of T lymphocytes, has been used to stimulate the cells and amplify the number of infected cells (Castro et al., 1988). Moreover, this mitogen stimulates the release of virus by the cells so that culture supernatants have levels detectable by standard reverse transcriptase assays (Barré-Sinousi et al., 1983; McDougal et al., 1985). The increased proliferation of the cells after activation with PHA is detected by the ^{3}H Thymidine incorporation assay.

ii. Enhancers of infectivity.

I selected two polycations: DEAE-Dextran and Polybrene which facilitate viral entry and a cytokine, Tumor Necrosis Factor-Alpha (TNFα), produced mainly by activated macrophages.

A. DEAE-Dextran (Diethylaminoethyl-Dextran) is a polycation or basically charged DEAE- glucose polymer. A direct interaction between the polymer and various nucleic acids protects them against nuclease degradation. RNA is only partially protected against ribonuclease degradation but the protection is enough to enhance the infectivity of viral RNA, as is the case with poliovirus; the bonds established between nucleic acids and DEAE-Dextran take place at the phosphate backbone and can be loosened by a strong polyanion such as Dextran Sulfate. (Maes et al., 1967). A similar enhancing effect is seen for the infectious DNA of Simian Virus 40 (SV40). When cells are exposed to DEAE-Dextran before infection with SV40, the level of infectivity is less

than that obtained by exposure of the cell cultures simultaneously to DEAE-Dextran and virus DNA (McCutchan and Pagano, 1968). DEAE- Dextran enhances the infectivity of certain intact viruses in cell cultures. It also enhances virus plaque formation, the cellular uptake of infectious viral deoxyribonucleic acid and ribonucleic acid as well as virus growth and dissemination in animals. The simplest view of how DEAE-Dextran acts to produce enhancement of infectivity of nucleic acids is that it restores to the deproteinized virus the ability to stimulate pinocytosis. This result depends on the high molecular weight (MW) and the cationic charge of the polymer and its affinity not only for nucleic acids but also for tissue cells (McCutchan and Pagano, 1968).

DEAE-Dextran has been used as pretreatment of cells to be infected (Coombs et al., 1989; Ikeuchi et al., 1990; Levy et al., 1985; Duc-Nguyen, 1968; McCutchan and Pagano, 1968).

B. Polybrene (1,5-Dimethyl-1,5-diazaundecamethylene polymethobromide); Hexadimethrine bromide. The polycation polybrene is routinely used to increase the infection efficiency of both retroviruses and retroviral vectors in vitro. It has been utilized more recently instead of DEAE-Dextran because of its lower toxicity. The enhanced infection seen with this cation is presumably through a charge-mediated mechanism that affects virus absorption, penetration or both. (Cornetta and W. French Anderson, 1989).

Polybrene has been used as pretreatment before infection (Schmitt et al., 1990; Cheng-Mayer et al., 1990; Sethi et al., 1988), during infection (Gregersen et al., 1990; Sundqvist et al., 1989) and to recover HIV-1 from PBMC of patients by cocultivation with PHA-stimulated normal blasts with the addition of polybrene in the culture medium. (Castro et al., 1988; Cann et al., 1990).

C. Tumor Necrosis Factor-α.

Both Tumor Necrosis Factor-α and Interleukin-1 stimulate the HIV-1 enhancer by activation of the nuclear factor (NF)-kB. (Duh et al., 1989; Osborn et al., 1989; Lacoste et al., 1990).

The activation of the HIV-1 provirus is influenced by the action of both constitutively expressed host transcription factors, and inducible cellular transcription factors, such as NF-kB. NF-kB has been shown to bind to the duplicated HIV-1 enhancer element and to activate transcription from the HIV-1 Long Terminal Repeat (LTR). This factor was originally identified as a constitutive DNA binding protein present in kappa chain-immunoglobulin producing B cells, which bound to the k gene enhancer. It is also produced in a broad array of cells, including T lymphocytes and macrophages, where it is expressed in an inducible manner.

Tumor Necrosis Factor-α enhances replication of the virus not only in T cells but also in primary macrophages (Mellors et al., 1991). It can be toxic to a variety of malignant cell lines at high concentrations, but at low concentrations it has induced expression of HIV-1 in a chronically infected T-cell line. (Folks et al., 1989).

The action of TNF-α, a product of activated macrophages, requires interaction with specific cell membrane receptors that are expressed on activated T cells, fibroblast cell lines and various tumor cells (Scheurich et al., 1987). Resting T lymphocytes do not express TNF receptors, but they are induced upon activation of T cells with anti-CD3 antibody. TNF-α enhances expression of HLA-DR antigens, as well as of IL-2 receptors. It synergizes with IL-2 to stimulate T cell proliferation and production of interferon-gamma (IFNγ). It has also been reported that HIV-1 induces peripheral blood mononuclear cells (PBMC) and $CD4^+$ T lymphocytes to secrete TNF-α, TNF-ß and IFNγ (Vyakarnam et al., 1990).

iii. Methods to titrate and quantify infectivity.

in spite of the obvious importance of the quantitative aspects of HIV-1 infection *in vivo* and *in vitro*, developing standards for HIV-1 titration and for quantization of the virus expression has proven to be difficult. Physical methods as well as methods that measure biological activity have been implemented.

1. Physical methods:

Immunofluorescence: Detection of virus both in the membrane and intracellularly with the use of polyclonal human immunoglobulin G (IgG) or heat inactivated HIV-1 seropositive human serum. It determines the number of cells productively infected with virus.

ELISA: Enzyme-linked immuno-sorbent assay: antigen capture. Measures HIV-1 p24 core antigen in serum, plasma, and cell culture media.

Polymerase Chain Reaction (PCR): Detection of HIV-1-specific DNA by amplification. It is more sensitive than other methods, detecting also proviral forms of HIV-1.

RNA-RNA liquid hybridization (Volsky et al., 1990) RNA is the first indicator of virus expression. Quantitative and reproducible measurement of HIV-1 RNA levels provides an index of viral expression. Previous determination of viral RNA by Northern blot technique or in situ hybridization were impractical because of the large amounts of cellular material required, instability of RNA and complexity of the hybridization procedure.

The physical methods measure the amount of virus but not the degree of infectivity.

2. Measures of Biological Activity:

Syncytia formation (Cytopathic Effect - CPE). This assay does not measure the number of infectious virus particles/units in the inoculum. Instead it measures the cyto-

morphologic alterations of the indicator cells depending on the titer and cytopathic effect of the HIV-1-containing supernatant used for the infection of the target cell.

Plaque assays: Provide a direct measurement of virus infectious units. Not suitable for titration or quantization of biological activity of slowly replicating and non-cytopathic HIV strains.

Viability.

Reverse transcriptase assay. It is used to determine viral particle production and growth kinetics of a virus, not infectivity. The RT-to-infectivity ratio will vary depending on the HIV isolate and cell line used.

These techniques measure virus infectivity but are not strain-specific and do not correlate with the number of virus particles.

Individuals infected with HIV-1 carry multiple molecular and functional variants of the virus. The potential for variability in the cytopathic function of HIV-1 may be of importance in the pathogenesis of AIDS. Many isolates from healthy seropositive individuals exhibit a low-cytopathic, non-virulent phenotype (Asjö et al., 1986; Cheng-Mayer et al., 1988; Tersmette et al., 1988). Delayed processing or expression of HIV-1 genome during the early phase of the virus replicative cycle is an important determinant in noncytopathic infection (Ma et al., 1990). There are also variations in the growth capacity of HIV-1 in peripheral blood mononuclear cell (PBMC) preparations from different individuals (Yamada et al., 1990).

Accurate measurements are therefore complicated by the great variability in biological activity among the HIV-1 isolates. This functional heterogeneity among HIV-1 isolates includes differences in antigenic properties of HIV-1 *env* glycoprotein, host range, kinetics of infection and cytopathicity. Optimal time for the titration of virus varies according to the virus strain and the cell line. In some cases the optimal time would be 3 days and in others it may be 6 days or up to 2 weeks after infection. In

receptive cell lines, like HUT-78, which express high levels of surface CD4 molecules, immunofluorescence (IFA) is very sensitive and can result in 100% fluorescence by day 7 post infection if cells are infected with a high titer virus supernatant. If a low-titer virus is used for infection, IFA is not sensitive enough to detect this low level of infection. In strains that are non-cytopathic and with slow kinetics of infection, the determination of infectious titers may require a minimum of 3 weeks incubation in CEM cells to achieve at least 60% IFA (Volsky et al., 1990).

HIV-1 expression ranges from a low-level synthesis of viral products during latency to high-level production of particles during cytopathic infection. When the virus is in a state of latency, no virus genome products can be detected by immunofluorescence or reverse transcriptase. The induction of viral RNA expression by PHA activation of the cell is paralleled by an increase in p24 antigen and the appearance of immunofluorescent-positive cells. But in some cases this correlates poorly with intracellular HIV-1 RNA levels (Volsky et al., 1990).

An approximation of virus titer can be obtained by measuring HIV-1 p24 antigen, but this measure cannot be extrapolated to the number of viral particles. Neither viral RNA nor p24 antigen provide information about the biological activity of virus stocks and biological titration is required. For this purpose the Cytopathic Effect (CPE), reverse transcriptase (RT) and infectivity assays are used. These are not perfect either, since HIV-1 isolates may differ in their infection kinetics . For more complete virus titer information it is therefore essential to include at least one physical and one biological parameter of virus content.

Of the methods mentioned above to quantify virus detection, I decided to use immunofluorescence which measures individual cell events, the p24 assay which measures the production of virus by a given cell population and the cytopathic effect, or syncytia formation, to evaluate the biological activity.

A. Indirect Immunofluorescence Assay (IFA)

IFA is a useful means of determining the number of cells productively infected with virus. The assay is inexpensive, quantitative, and can be done using fixed cells or live cells. Not only membrane associated but also intracellular antigens can be detected.

B. p24 Ag assay

The p24 antigen is virtually undetectable and antibody is in excess during the asymptomatic stage. The p24 antigen can be detected right after the infection before the appearance of antibodies. In the last stages of the disease there is a marked decrease in antibody, which is followed by an increase in antigen. The measurement of p24 is an approximation to measure the amount of virus in fluids. The ability to consistently detect infectious HIV-1 in the plasma of infected persons at the different stages of infection is very useful in monitoring pathogenesis and therapy. Since the HIV-1 p24 antigens can be present as immune complexes with the antibodies in most sera from HIV-1-infected individuals, the assays are faced with this limitation (Nishanian et al., 1990). Commercially available kits offer a specific antibody neutralization step, followed by the solid phase enzyme immunoassay to detect the antigen.

C. Cytopathic Effect (CPE).

The cytopathic effect which can be induced by HIV-1 *in-vitro* is characteristically the formation of multinucleated giant cells and balloon degenerative forms followed by cell death. The cytopathic effect of HIV-1 in CD4 cells is linked to the last stage of virus infection (Leonard et al., 1988). The CD4 molecule and the envelope glycoproteins of the virus appear to be directly involved in the formation of syncytia. The appearance of these giant cells in the culture is therefore a good indicator of infection for strains that are cytopathic.

Some strains of HIV-1 are not cytopathic. It is believed that a mutation affecting the amino-terminus of the HIV-1 gp41 transmembrane envelope glycoprotein results in a virus that is less cytopathic than the wild-type HIV-1 (Kowalski et al., 1991).

I.3.2. Preparation of the Effector Cell.

One surprising finding among various researchers is the high frequency in which CTLs specific for HIV-1 appear in PBMC of infected patients. For this reason, mononuclear cells (MNC) have been used as effector T-lymphocytes in a ^{51}Cr release assay. These MNC can be separated into their $CD8^+$ and $CD4^+$ fractions which can also be used separately as effector cells. The majority of CTL are typically antiviral, expressing the CD8 surface marker and restricted by HLA Class I antigens. The presence of HIV-1-specific CTL in the blood of patients is different from other human viruses where specific antigenic stimulation must first be provided *in vitro* to ensure expansion of the CTL population before it can be detected.

Aside from fresh PBMC, HIV-1-specific CTL have also been obtained from bronchoalveolar lavage from the lungs of infected patients (Plata et al., 1987a) and from cerebrospinal fluid (CSF) of AIDS patients with neurologic disorders (Sethi et al., 1988). They can also be derived from seronegative donors by *in vitro* stimulation with viral antigen (Hoffenbach et al., 1989). This indicates that humans have high frequencies of circulating precursor cells capable of differentiating into HIV-1-specific CTL.

These CTL can be cloned and kept in culture for long periods. The essential ingredient for long-term growth of T lymphocytes is Interleukin 2 (IL-2), originally called T cell growth factor (TCGF). The approach to derive and maintain T cell clones employs stimulating antigen and "filler cells" in addition to IL-2. The filler cells are irradiated allogeneic cells. They provide a source of other growth factors as well as the right cell density. To promote the expansion *in vitro*, various stimuli are used:

Phytohemagglutinin (PHA), infected autologous or heterologous irradiated lymphoblasts, concanavalin A and anti-CD3 monoclonal antibody.

I.3.3. ^{51}Cr Release Assay.

The test system that has been adopted universally to measure cytotoxicity is the ^{51}Cr release assay. It involves the radioactive labeling of target cells with ^{51}Cr. Cell lysis can be evaluated by the amount of ^{51}Cr released into the culture medium. This release is directly proportional to the number of "lethal hits". The Effector to Target ratio can vary from 3:1 to 50:1 or more, depending on the number of cells available.

I.4. Objectives

The study of HIV-1-specific cell-mediated immunity requires an assay where the effector cells (cytotoxic T cells) are cocultured for a short period with target cells that express HIV-1-derived antigens and are labelled with ^{51}Cr. The release of ^{51}Cr to the supernatant indicates the presence of CTL activity when the release is above background levels. An absolute requirement to measure HIV-1-specific CTL acticivity is that the target cell has to be MHC compatible with the effector cell. For studies of HIV-1-specific CTL this has been achieved by using autologous EBV-transformed B cells that have been infected with recombinant vaccinia virus containing portions of the HIV-1 genome. There are some disadvantages using the EBV/Vaccinia target cell system such as the need to generate EBV transformed autologous cell lines for each patient, the large number of vaccinia-derived T-cell antigen peptides that could compete with HIV-1 peptides, plus the fact that vaccinia is a lytic virus that causes cell damage thus increasing the background release of ^{51}Cr.

1.4.1. General Objectives

For the reasons discussed above I decided as the general objective of this thesis to study the suitability of autologous CD4+ T cells, infected with a laboratory strain of HIV-1, as target cells in the HIV-1-specific CTL assay.

1.4.2. Specific Objectives

To assess: 1) the production of autologous CD4+ T cells, 2) the ability to infect such cells with HIV-1, 3) the role of enhancers of viral entry, 4) the evaluation of these target cells in the HIV-1-specific ^{51}Cr release assay, and 5) the cloning of HIV-1-specific CTL.

II. MATERIALS AND METHODS

II.1. Isolation of Peripheral Blood Mononuclear Cells (PBMC).

PBMC were obtained by Ficoll-Hypaque density gradient centrifugation. Basically, a fresh, heparinized 25 ml sample of blood from healthy seronegative donors or asymptomatic HIV-I seropositive subjects, was diluted 1:1 with sterile Phosphate Buffered Saline (PBS) and mixed gently. It was underlayered with 12 ml of Ficoll-Hypaque. The sample was centrifuged (Hettich-Rotanta/RP, Model 3504, DIAMED Lab Supplies) at 1,800 rpm (605 x G) for 25 min, brake off. Using a pipette, the top layer of plasma was removed. The interphase containing the lymphocyte layer was collected in a fresh tube, washed with PBS by centrifuging for 10 min at 1,180 rpm (260 x G) at room temperature (RT). Pellet was resuspended in PBS for a second wash and centrifuged at 1,790 rpm (600 x G) for 10 min. at RT. Cells were then resuspended in medium containing IMDM, 10% FCS, IL-2 (100 U/ml), and 1 µg/ml (5×10^{-5} M) of 2 Mercaptoethanol (2-Me), Phytohemagglutinin (PHA)-HA16 (1 µg/ml) was added to stimulate T-cell proliferation. The optimal level of PHA used was determined by measuring the concentration that induced the best replication of PBMC. This measure was done a ^{3}H-Thymidine incorporation assay. In some cases only the non-adherent cells were used. To obtain these, the PBMC were incubated in a petri dish for 2 hours. The supernatant containing most of the non-adherent cells was collected and treated as mentioned for PBMC.

II.2. Immunomagnetic separation of CD4 and CD8 T cells.

Lymphoblastoid populations enriched in CD4 or CD8 cells were prepared using magnetic beads. The CD8 fraction was used as the effector population and the CD4 as the target in CTL assays.

Magnetic polystyrene beads (Dynabeads) coated with mouse monoclonal antibody specific for the CD8 or CD4 cell membrane antigens were used for the positive selection of these cell populations. Beads were washed before use to remove sodium azide which is present as a preservative. The cell sample can be whole blood, buffy coat, or mononuclear cells (MNC) isolated by density gradient centrifugation. To accomplish an effective depletion the bead to target ratio should be high and in accordance with manufacturer's stipulations (Whole blood= 20-50:1, buffy coat and MNC= 10:1). After Dynabeads were added to a cooled cell sample, it was incubated 30 min. at 2-4^0 C on a rotator. The CD8 or CD4 T-cells were rosetted with the beads depending on which were used. After incubation the tubes were placed in a Magnetic Particle Concentrator (MPC) for 2-3 minutes. Free cells were collected with a small pipette and the bead-attached cells were resuspended in medium. If CD8 beads were used, the CD8$^+$ T cells would be rosetted with the beads and the CD4$^+$ T cells would be in the medium. Usually 99% depletion was achieved. Two consecutive incubations with Dynabeads gave a more complete depletion. The Dynabeads detached from the cells after an overnight incubation at 37^{0}C. The isolated cells are washed two times in PBS/1%FCS and placed in culture.

II.3. Phenotyping for Flow Cytometry.

PBMC as well as clones were phenotyped by flow cytometry (Becton Dickinson FACScan Research Software version 2.1) for CD4 and CD8, surface molecules. To phenotype for CD4 and CD8, 10^6 cells were washed and incubated in ice in the dark for 30 min. with anti-Leu-3a (CD4) phycoerythrin (PE) conjugate and anti-Leu-2a (CD8) fluorescein isothiocyanate (FITC) conjugate. For control, mouse IgG1-FITC conjugate and IgG1-PE conjugate were used. After incubation, cells were washed 3 times in PBS and resuspended in 0.5 ml of PBS. Cold paraformaldehyde at 2% (1 ml per 10^6 cells) was added to fix cells. This also inactivated the virus.

II. 4. Preparation of viral stocks.

HUT-78, a human cutaneous T cell lymphoma line derived from peripheral blood of a patient with Sezary syndrome, has properties of a mature T cell line with inducer/helper phenotype that releases T Cell Growth Factor (IL-2). The cells were grown in RPMI-1640 supplemented with 10% Fetal Calf Serum (FCS), antibiotics (Penicillin-Streptomycin 100 U each/ml) and 1.16% of L-Glutamine 200 mM.

Viral infection of HUT-78 was done with HXB2 a prototypic laboratory strain of HIV-1. Cells were washed and resuspended at 3 x 10^6 cells/ml of virus supernatant containing at least 100,000 pg/ml of p24 antigen.. They were placed in a rotator (Scientific Industries, Inc., Model 151, Bohemia, N.Y.) for 2 hours at room temperature. After infection cells were washed 2 times in RPMI and resuspended in RPMI/10% FCS at 1 x 10^5/ml. After 3 days in culture they were split once and grown until day 8 to 14. When syncytia appeared in the form of "balloons" (Cytopathic Effect-CPE), cells were spun down at 2,000 rpm for 10 minutes. Virus-containing supernatant was collected. FCS was added at 20% v/v before freezing aliquots at -80^{0}C. Infection was monitored by measuring the levels of HIV-1 p24 antigen in the supernatant. To evaluate the appearance of syncytia, 200 µl aliquots of cells were set up in a 96-well tissue culture plate.

II.4.1. Method of Infection.

***In vitro* infection.** Filtered supernatant from the HIV-1 infected HUT-78 cell line containing >100,000 pg/ml of p24 antigen, was added to HUT-78 or three-day-old, PHA-stimulated Peripheral Blood Mononuclear Cells (PBMC). PBMC were separated as explained in II.1 and washed 2 times in Iscove's Modified Dulbecco's Medium (IMDM) before infection with HIV-1-containing virus supernatant. Infection was carried out the same way as for HUT-78 cells (see II.4), with the exception that for PBMC the cell concentration was 5 x 10^6 cells per ml of virus supernatant and the medium used was

IMDM instead of RPMI. Once infected, PBMC were resuspended at 2 x 10^5/ml in IMDM/10% FCS., IL-2 (100 U/ml) and 2-Me (5 x 10^{-5} M).

II.4.2 Enhancers of Infection.

To enhance the infectivity of the virus, the following reagents were used alone or in combination:

a) DEAE-Dextran (Diethylaminoethyl-Dextran).

This enhancer was tested at the following concentrations: 1, 2.5, 5, 10, 20 and 25 µg/ml. It was used in various combinations: as pretreatment, during and after the infection. Pretreatment meant an incubation of the cells before infection for 30 minutes at 37°C with DEAE-Dextran. After the cells were infected, they were washed twice and set in culture with or without DEAE-Dextran as required for each particular experiment.

b) Polybrene (1,5-Dimethyl-1,5-diazaundecamethylene polymethobromide); Hexadimethrine bromide.

The same type of experiments were done with Polybrene following the same procedure as for DEAE-Dextran. It was tested at 1, 2.5, and 5 µg/ml.

c) Tumor Necrosis Factor-α. (Stock at 10 µg/ml= 10,000 U/ml).

It was always added after infection and tested at 1, 2.5, 5, 10, 50 and 100 ng/ml. It was used with cells that had been infected in the presence or absence of DEAE-Dextran or Polybrene.

II.5. Methods of Detection of Viral Infection.

To assess HIV-1 expression, I measured the p24 antigen, the biological activity (CPE or syncytia formation) and the percentage of infected cells by immunofluorescence (IFA). The assays were done on days 3, 5 and 7 post-infection.

II.5.1. p24 Ag assay.

The HIV-1 p24 antigen was measured in a solid-phase sandwich-type enzyme-linked immunosorbent assay (Abbott Laboratories). This assay used a polyclonal human HIV antibody and a purified viral lysate as a standard. The concentrations of p24 antigen present in serum samples were interpolated from a standard curve. For the assay, a 2 ml cell sample was spun down at 2,000 rpm for 10 minutes. The supernatant was collected and stored at -20^{O}C until the day of the assay. This was done according to manufacturer's instructions.

II.5.2. Indirect Immunofluorescence Assay (IFA).

The virus in the sample was first inactivated (see below). After inactivation, cells were washed 2 times in PBS. The pellet was resuspended in 100 µl of PBS or more depending on the number of cells. Smears were prepared in multiwell slides and dried. Slides were fixed in cold acetone for 5-10 min. They can be stored for 1 week at room temperature (RT), at 4^{O}C for up to 2 weeks or frozen individually wrapped in plastic foil at -20^{O}C for longer periods. When ready to use, slides were warmed up to RT for at least 15 minutes. They were hydrated and blocked with PBS/5% Goat Serum (GS) for 10 min. in a humidified chamber (e.g., a covered petri dish with a wet gauze pad inside). PBS/GS was blotted with a paper blotter. The first antibody was added: 20 µl of a 1:100 dilution of 10 mg/ml Polyclonal Human IgG anti-HIV Standard. Slides were incubated for 30 min. in a humidified chamber, washed 3 times (5 min. each time) in coplin jars with PBS and blotted around the wells. The secondary antibody was added (20 µl per well of a 1:40 dilution of Goat anti-human IgG-FITC at 1mg/ml), and incubated a further 30 min. in the dark. Slides were washed 3 times more in PBS alone and once in water. They were dried for at least 2 hours. A drop of PPD-Glycerol (Para-Phenylene Diamine) was added, followed by the coverslip before fluorescent microscopy viewing (Zeiss, model Axiophot El-Einsatz, 45-1888, West Germany). At least 200 cells were counted

per field including the positive ones. These were divided by total number of cells counted and multiplied by 100 to obtain the percentage.

i. Inactivation of the HIV-1 Virus.

To inactivate the virus I followed the protocol described by Watson et al., (1990). Basically, HIV-1 infected cells were harvested and 5 µg/ml of AMT were added. Cell density can range from 4 x 10^6 to 12 x 10^6/ml without affecting inactivation. Cells were added to tissue culture multiwell plates or flasks such that the depth of fluid was no greater than 3-3.5 mm. To this effect, I used 4 ml in T25 flasks. Plates were cooled for 30 min. at 4^{o}C to offset the exposure to radiant heat from the UV source. Cells were irradiated with long-wave UV irradiation for 5 min. (Spectroline Transilluminator -365 nm- Ultraviolet, Model TR-365A, Spectronics Corporation, Westbury, N.Y.). Following irradiation cells were harvested and washed 2 times in PBS before preparing the slides for IFA.

II.5.3. Cytopathic Effect (Syncytia formation).

Assays were performed in 96-well plates, using ten wells containing 4 x 10^4 infected cells/well in 200 µl aliquots. They were incubated at 37^{o}C in 5% CO_2 (Steri-Cult 200 Incubator, Forma Scientific, Inc., Model 3033) and left undisturbed for 7 days. Syncytia formation (number of balloon forms) from 3-4 wells were counted and averaged for each sample on days 3, 5 and 7 post-infection.

II.5.4. Viral binding and entry assay.

After cells were infected as described in II.7.1., a sample was washed 5 times in PBS. The cell pellet was lysed with 1 ml of PBS/1% Triton X-100. After a 2-hour incubation with the Triton the cells were spun down at 10,000 rpm (Brinkmann Eppendorf Centrifuge 5415C) for 10 minutes at 4^{o}C. The supernatant was collected and

stored at -80^0 C for the p24 Antigen assay. Controls were uninfected cells and lysed pelleted virus.

II.6. Generation of CTL clones.

To derive human alloreactive T cell clones, mononuclear peripheral blood lymphocytes (PBL) were isolated by Ficoll-Hypaque gradient centrifugation. Blood, anticoagulated with preservative-free heparin, usually yielded 0.5 to 1 x 10^6 lymphocytes per ml. The actual clones were derived by limiting dilution. For this, PBMCs were seeded at 10, 4 or 2 cells per well in 96-well plates. Allogeneic PBMC from normal donors were irradiated at 6,000 rad in a Cobalt 60 source (Gamma Cell, Model 220, Atomic Energy of Canada Ltd.) and added as feeders at 4 x 10^5 cells per ml, in a final volume of 200 µl (per well) in medium containing IMDM (supplemented with 4mM glutamine, 50 µg/ml of Gentamicin) 20% FCS, 100 Units of recombinant IL-2 per ml., 1 µg/ml of Indomethacin, l µg/ml (5 x 10^{-5} M) of 2-Me, 200 Units per ml of Interferon-γ. PHA-HA16 (1 µg/ml) was added as stimulation for T-cell proliferation (Expansion/cloning medium). For subsequent re-stimulations the feeder cells were treated with Neuraminidase/Galactose oxidase instead (see below). The irradiated feeders or "filler cells" provide a source of growth factors as well as the needed cell density. Plates were incubated at 37^0 C in a humidified chamber in 5% CO_2. On day 4 after cloning they were fed with medium exchanges of 0.1 ml containing IMDM, 20% FCS, IL-2 (100 U/ml) and 2-Me (feeding medium-no feeders). After 11 or 15 days from the onset of cloning, growing clones were transferred to a total of 5-10 wells of a 96-well plate in fresh expansion/cloning medium. Clones were expanded into 24-well plates one or two weeks later. The expansion/cloning medium with feeders was used every two weeks. In between expansions, medium exchanges were done every 3 to 4 days with feeding medium only (see above). Clones were phenotyped by Flow Cytometer. Those

that were more than 60% $CD8^+$ were kept in culture to be tested in a ^{51}Cr release assay for cytotoxic activity.

Feeder cells treated with Neuraminidase/Galactose Oxidase function as oxidized stimulator cells and are used for non-specific propagation of antigen-dependent proliferative or cytotoxic human T cell clones. This system is as efficient as the use of mitogenic antibodies or lectins but avoids the toxic effects of polyclonal T cell activators like PHA. Feeders were irradiated and incubated for 90 min. at 37^oC with 0.02 U/ml of Neuraminidase and 0.05 U/ml of Galactose Oxidase in serum-free RPMI. Cells were washed 3 x in RPMI/0.01M Galactose. Treated feeders (3×10^6) were used for each 5×10^5 cultured cells (clones) (Fleischer, 1988).

II.7. Cytotoxic T-Lymphocyte Assay.

II.7.1. Preparation of the CD4 T lymphocyte as Target Cell

Autologous $CD4^+$ T lymphocytes were used as target cells. Mononuclear cells were obtained by Ficoll-Hypaque, stimulated with PHA-HA16 (1 µg/ml) and kept in culture for 3 days with IL-2 (100 U/ml) and 2-Me (5×10^{-5} M). In the case of patients' samples, 25 µg/ml of Castanospermine (CSP) were added. The mechanism of anti-HIV-1 activity of castanospermine involves prevention of the correct glycosylation of viral envelope glycoproteins, resulting in diminished virion infectivity as well as decreased HIV-1 *env*-induced syncytium formation (Walker et al., 1987b; Ruprecht et al., 1991). This permits the proliferation of the CD4 T lymphocytes.

Heparinized blood from HIV-1-infected asymptomatic individuals was separated as described in section II.1. Three days after the PHA stimulation, cells were separated into their CD4 and CD8 fractions with the use of magnetic Dynabeads (section II.2). The $CD4^+$ fraction was kept in culture for 24 hours without CSP. Cells were pretreated with

DEAE-Dextran (10 µg/ml) and incubated for 30 minutes at 37°C. After this, cells were washed and infected with an HIV-1 virus supernatant containing >100,000 pg/ml of p24 antigen following the infection protocol described in section II.4. Infection was done in the presence of DEAE-Dextran at 10 µg/ml (see Results). Cells were washed three times and kept in medium without CSP for 24-or 48 hours. They were tagged with ^{51}Cr and incubated with the effector cells ($CD8^+$ T cells) for 4-6 hours before the ^{51}Cr assay.

II.7.2. Preparation of the macrophage as target cell for the CTL Assay.

A 20 ml whole blood sample from an HIV-1-positive asymptomatic patient was spun down at 2,000 rpm (747 x G) for 10 minutes. The buffy coat was collected (approx. 4 ml). and separated into $CD8^+/CD8^-$ fractions with the use of CD8 magnetic beads at a ratio of 6 beads to 1 cell. The $CD8^+$ cells attached to the beads were put in culture with PHA and IL-2 (no CSP). The beads were separated the following day with the Magnetic Particle Concentrator (MPC). The $CD8^-$ cells were depleted once more of $CD8^+$ T cells with Dynabeads at a ratio of 6:1. This new depletion was discarded. These $CD8^-$ cells were isolated by Ficoll-Hypaque and put in culture (IMDM/20%FCS) in 12-well plates at 2×10^6 cells per ml in 1 ml volume. Granulocyte Macrophage-Colony Stimulating Factor (GM-CSF) at 100 U/ml was added and kept in the culture medium. Supernatant was collected on days 5, 7, 10 and 14 for p24 Antigen assay. Between days 7 and 10 macrophages were harvested using PBS/10%EDTA. They were washed and labelled with ^{51}Cr. The $CD8^+$ cells were used as effectors. A cell pellet was lysed before the assay to determine virus presence by p24 Ag assay.

II.7.3. Preparation of the Effector Cell.

The effector cells ($CD8^+$ T lymphocytes, PBMC, or $CD8^+$ clones from same donor) were subcultured 2 days earlier with fresh medium without IL-2 or IFNγ (In the case of clones, these were kept without feeders for at least 7 days before the assay). A 0.1 ml aliquot of effector cells was placed over the ^{51}Cr-labelled target in an effector:target (E:T) ratio of 5:1 to 50:1, or 100:1 if enough cells were available.

II.7.4. ^{51}Cr Release Assay.

Target cells were labelled with ^{51}Cr (100 uCi per 10^6 dry cell pellet) and incubated for 1 hour at 37^0 C. Cells were washed in IMDM 3 times to get rid of unincorporated ^{51}Cr. They were aliquoted at 10,000 cells/well in 0.1 ml aliquots in triplicate in a V-bottom 96-well plate. Effector cells were added. After 4-6 hour incubation at 37^0C in 5% CO_2, plates were spun down 2 minutes at 2,000 rpm. Supernatant (100 µl) was harvested from each well and placed in a tube with 100 µl of sodium hypochlorite (Javex) to inactivate the virus. The tubes were read in a gamma counter (LKB, Wallac, Model 1260 Multigamma II, Allied Fisher Scientific). Percent lysis was calculated as follows:

$$\% \text{ lysis} = \frac{(\text{experimental release - spontaneous release})}{(\text{maximum release - spontaneous release})} \times 100$$

Maximum release was obtained by incubating target cells with 1M HCl or detergent. Spontaneous release was determined by the incubation of targets with culture medium alone. Spontaneous ^{51}Cr release was always 10-20% of the maximum release value.

III. RESULTS

III.1. Preparation of HIV-1-containing supernatants.

The infection protocol described in Materials and Methods was used to prepare several batches of HIV-1-containing supernatants (Table 1). HIV-1 virus content was evaluated by measuring p24 antigen. The p24 antigen content varied considerably from lot to lot from 13,000 to 500,000 pg/ml.

TABLE 1
HIV-1 Virus Stocks

Batch No.	p24 Antigen (pg/ml)	Harvested Days Post-Infection
1	13,000	Original Stock
2	55,000	12
3	190,000	12
4	500,000	12
5	140,000	10
6	200,000	10
7	170,000	12
8	300,000	14
9	285,000	14

Virus stocks were produced by infecting HUT-78 cells as described in Materials and Methods. Three days after infection cells were subcultured 1:3 in RPMI/10% FCS. Supernatants were collected 10 to 14 days post-infection. They were spun down, filtered and stored at -80° C.

III.2. Titration of HIV-1 viral supernatants on HUT-78 cell line.

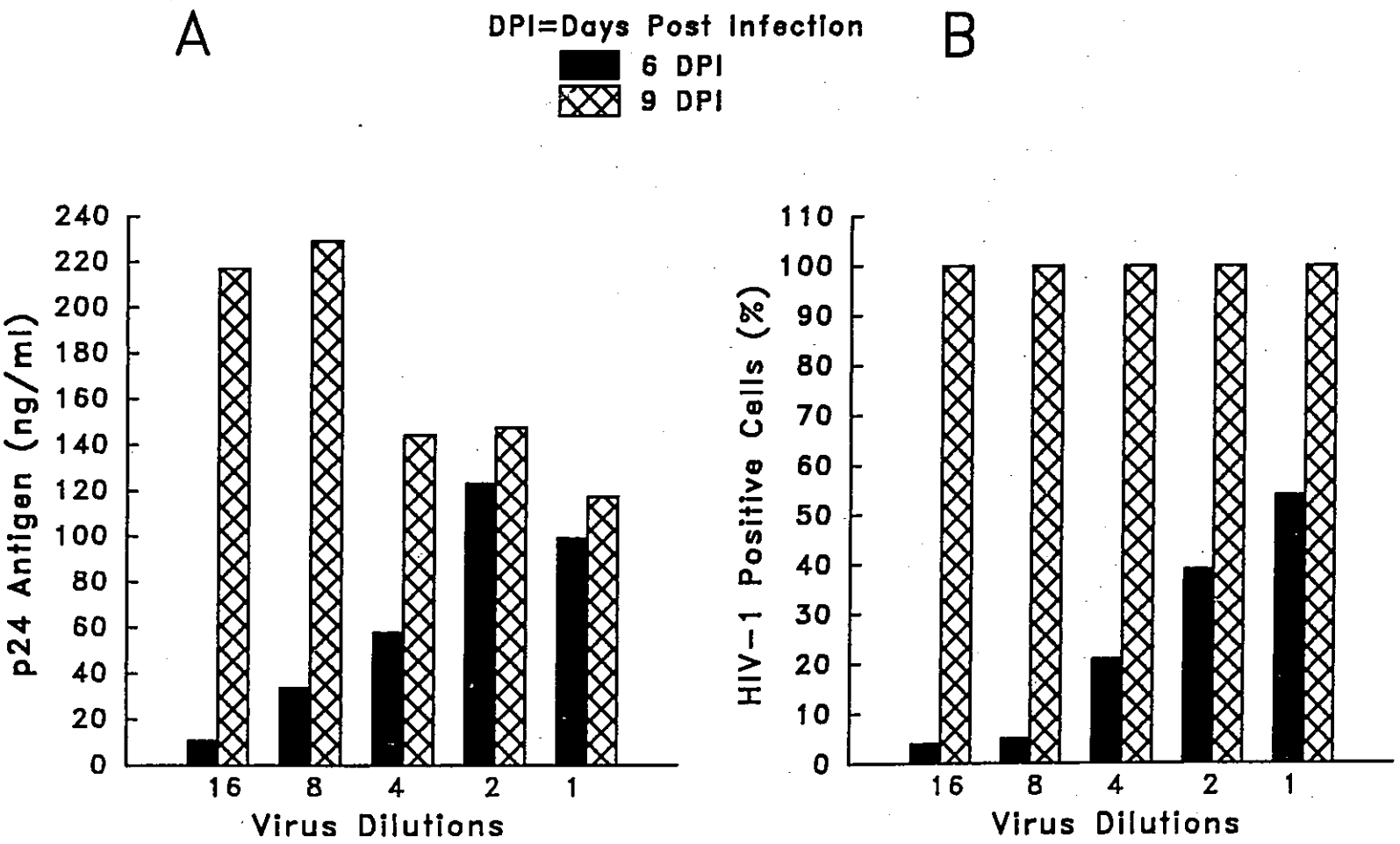
Viral lots (n=3) were selected for analysis of their infectivity. The p24 antigen levels of these lots were 13,000, 209,000 and 500,000 pg/ml.

A preliminary evaluation was done with the low and high p24-Ag content supernatants. The cell line HUT-78 was infected for 2 hours at room temperature in a rotator, with serial dilutions of the virus-containing supernatants . Infection was evaluated by IFA on days 3, 6 and 9 post-infection. HIV-1 was not detected on these days when using the supernatant containing 13,000 pg/ml whereas HIV-1 was detected on day 9 post-infection when using the 500,000 pg/ml supernatant.

The success of the infection appeared to depend on the amount of virus present in the supernatant; nevertheless, the infectivity of the supernatant may vary according to the viability of the virus and other factors that may affect its infectivity such as toxic effect of the spent supernatant on the target cells. Thus, my first task was to do a detailed characterization of the infectivity of the virus stock. Infectivity was measured using 3 assays: IFA, p24 antigen and CPE. A virus stock containing 200,000 pg of p24 antigen/ml was selected. Serial dilutions of the supernatant were used to infect HUT-78 cells. Fig. 1 shows a representative experiment. There is a dose-response effect for both detection assays. P24 antigen appears to be a more sensitive measurement than IFA because it becomes positive at a higher dilution on day 6 post-infection. By day 9 post-infection all dilutions gave 100% positivity by IFA. However, the p24 antigen showed an inverse correlation with the dilution. I interpreted the decline in p24 antigen concentration as due to the highly cytopathic effect of the virus on the HUT-78 cell line. I observed a large number of balloons in the cultures by day 6 and visual inspection by inverse microscope revealed that by day 9 there was a large number of dead cells; thus, the decline of p24 production was likely due to decreased number of viable cells. Therefore measurement of p24 antigen is not a reliable marker of infection if extensive cytopathic effect occurs in the infected cultures.

Fig. 1 Titration of HIV-1 viral supernatant on HUT-78 cell line.

Virus stock was obtained by infecting HUT-78 cells with a HIV-1 virus stored at -80^{O}C. (p24 antigen= 200,000 pg/ml). Titration was done using undiluted virus and serial dilutions: 1:2, 1:4, 1:8 and 1:16, expressed as reciprocal of the dilution in the graph. Cells were infected with the virus supernatant for 2 hours at room temperature in a rotator, washed once and placed in culture for 7 days. Medium exchanges (RPMI/10% FCS) and subculturing were done on day 3 post-infection or as required. The cultures were established in 12-well plates in duplicate . The infection was monitored on days 3, 6 and 9 post-infection by p24 antigen assay (A) and immunofluorescence on slides (B). For the p24 assay, a 2 ml sample was collected on the days indicated on the graph, spun down and the supernatant stored at -20^{O}C until the assay was done. The cell pellet of each sample was inactivated with AMT/UV irradiation (see Materials and Methods) and used to make smears on slides for IFA. Results of p24 Assay are shown in ng/ml.


A
DPI=Days Post Infection
6 DPI
9 DPI
B
p24 Antigen (ng/ml)
240
220
200
180
160
140
120
100
80
60
40
20
0
16
8
4
2
1
Virus Dilutions
HIV-1 Positive Cells (%)
110
100
90
80
70
60
50
40
30
20
10
0
16
8
4
2
1
Virus Dilutions

III.2.1. Effect of long-term storage at -80°C on the viability of viral stocks.

The effect of storage on the viability of the virus stock was determined. A second titration was done with the same virus stock (200,000 pg/ml of p24 antigen) after 6 weeks of storage at -80°C. (Fig. 2). Table 2 compares the titration experiments represented in Figures 1 and 2. The data is for days 6 and 7 post-infection respectively showing a decrease in infectivity of the virus stock after 6 weeks of storage at -80° C.

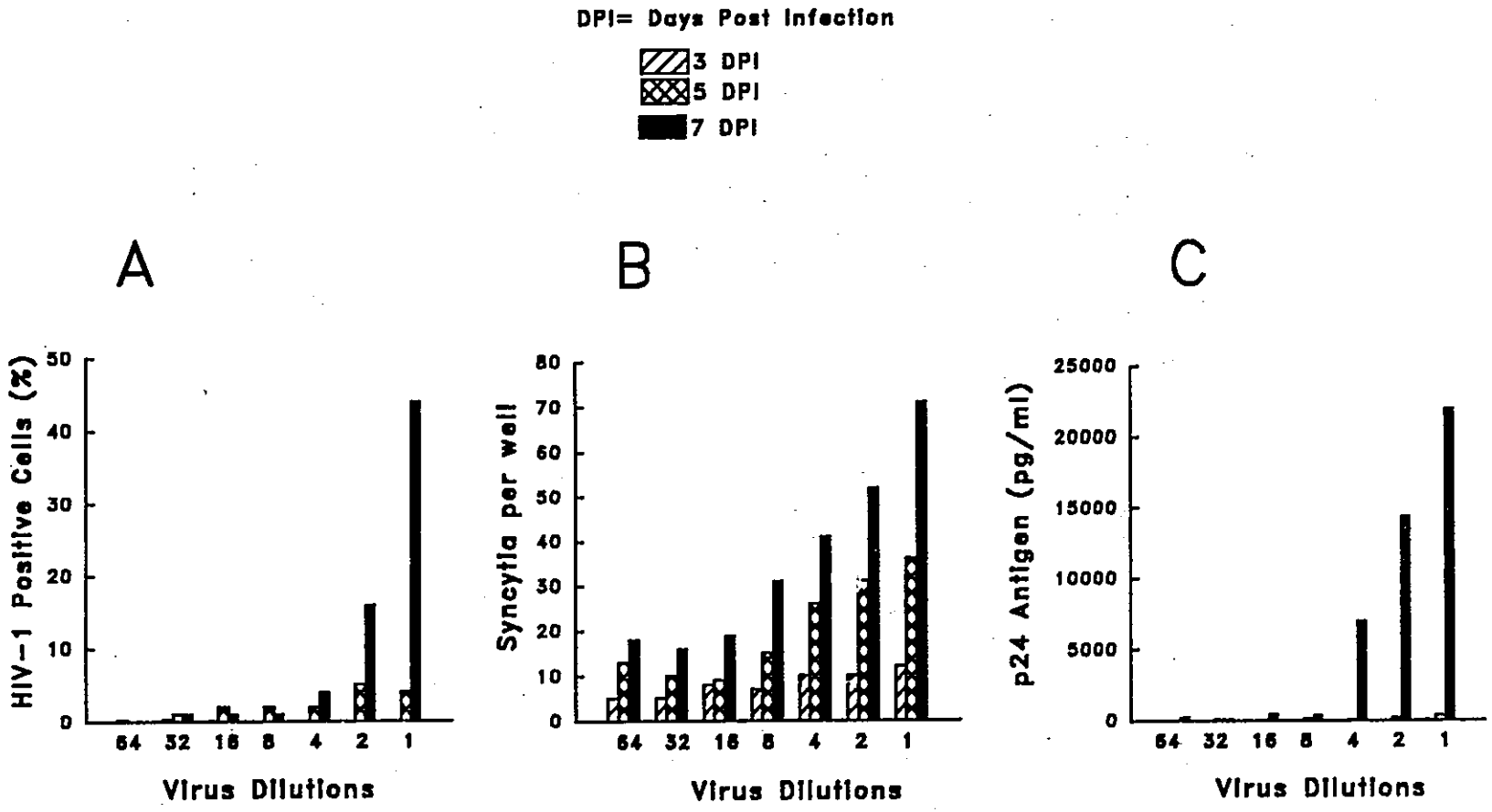
TABLE 2
Effect of long-term storage on viral stock infectivity.

Reciprocal of Dilution*	IFA(%) Exp.#1	IFA(%) Exp. #2	p24 Ag (ng/ml) Exp#1	p24 Ag (ng/ml) Exp#2
1	54	44	99	22
2	39	16	123	14
4	21	4	58	7
8	5	1	34	0.3
16	4	1	11	0.4

This table compares the titration experiments represented in Figures 1 and 2.
* Reciprocal of Dilution: 1 (Neat), 2 (1:2), 4 (1:4), 8 (1:8), 16 (1:16).

Fig. 2 Titration of HIV-1 viral supernatant on HUT-78 cell line. Effect of long-term storage on the viability of the virus stock.

Virus stock was obtained by infecting HUT-78 cells with a HIV-1 virus-supernatant stored at -80^{o}C (p24 Antigen= 200,000 pg/ml). Titration was done using virus stock neat and at serial dilutions: 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, expressed as reciprocal of the dilutions in the graph. The virus supernatant was the same used for the titration depicted on Fig. 1 with the exception of longer storage at -80^{o}C. Infection was done for 2 hours at room temperature in a rotator; cells were then washed once and placed in culture for 7 days, with medium exchanges (RPMI/10% FCS) and subculturing on day 3 post-infection or as required. The cultures were set up in 12-well plates in duplicate. The infection was monitored on days 3, 5 and 7 post-infection by Immunofluorescence on slides (A), Cytopathic Effect (CPE) (B) and p24 Ag assay (C). For the CPE, ten-200 µl aliquots were set up in a 96-well plate for each dilution. They were left undisturbed for 7 days and the number of syncytia in 4 wells were counted and averaged for each culture. For the p24 Ag assay, 2 ml of each cell culture were collected on the days of the assay, spun down and the supernatant collected and stored at -20^{o}C until the assay was done. The cell pellet of each sample was inactivated with AMT/UV irradiation (see Materials and Methods) and used to make smears on slides for IFA.


DPI= Days Post Infection
3 DPI
5 DPI
7 DPI
A
HIV-1 Positive Cells (%)
0
10
20
30
40
50
64 32 16 8 4 2 1
Virus Dilutions
B
Syncytia per well
0
10
20
30
40
50
60
70
80
64 32 16 8 4 2 1
Virus Dilutions
C
p24 Antigen (pg/ml)
0
5000
10000
15000
20000
25000
64 32 16 8 4 2 1
Virus Dilutions

III. 3. Titration of HIV-1 viral supernatant on normal T cell blasts.

To determine if comparable levels of infection could be obtained with normal T cell lymphoblasts, HIV-1-containing viral supernatant was tested on T cell lymphoblasts. Mononuclear cells (MNC) were obtained by Ficoll-Hypaque, stimulated with PHA (0.5 μg/ml) and the activated T cell lymphoblasts were collected 3 days after PHA stimulation and used as targets for infection. The infection was done with the same viral stock that had been used for the HUT-78 experiments (200,000 pg/ml of p24 antigen). After infection the cells were cultured in IL-2 (100 U/ml)-containing growth medium.

A representative titration experiment is shown in Fig. 3 (A-B). The lymphoblasts were infected with neat viral stock and six serial dilutions. Detection of infection was done by p24 Ag assay and Immunofluorescence (IFA). The titration was done with virus stock that had been stored-frozen at -80^{o}C for 6 weeks. The figure shows a dose-response effect with the highest infectivity at the highest concentration of virus used. In this experiment, IFA appeared to be more sensitive than the p24 Ag measurement.

In contrast to the large syncytia observed in HIV-1 infected HUT-78 cells, the normal T cell lymphoblasts did not show evidence of syncytia formation. (Fig. 4). This observation has been confirmed in 3 additional experiments. In a fashion similar to that found with HUT-78 cells 7 days after infection, the levels of p24 Ag decreased markedly even at the highest dilution of virus used to infect the cells (Fig. 5A). Because the levels were already high on day 5 after infection (no dose response effect) the data was interpreted to mean that a cytophatic effect had occurred resulting in low p24 production by fewer viable cells. Fig.5 compares titrations done on normal T cells (A) and HUT-78 cells (B).

Fig. 3. Titration of HIV-1 viral supernatant on normal T cell blasts.

The virus stock (200,000 pg/ml of p24 Ag) had been frozen at -80^{O}C for 6 weeks. Titration was done using virus stock neat and at serial dilutions: 1:2, 1:4, 1:8, 1:16, 1:32 and 1:64, expressed as reciprocal of the dilutions in the graph. Mononuclear cells were obtained by Ficoll-Hypaque separation, stimulated with PHA (0.5 µg/ml) and the activated T cell lymphoblasts infected 3 days after PHA stimulation. The infection was carried out as described in Materials and Methods. After the infection cells were washed once and resuspended in medium containing IL-2 (100 U/ml). The cultures were set up in 12-well plates in duplicate for each dilution. For the p24 assay, a 2 ml sample was collected on days 3 and 5 post-infection and spun down. Cell-free supernatants were collected and stored at -20^{O}C until the day of the p24 Ag assay. The cell pellet of each sample was inactivated with AMT/UV irradiation (see Materials and Methods) and used to make smears on slides for IFA. Results are shown for p24 Ag assay (A) and for IFA (B).

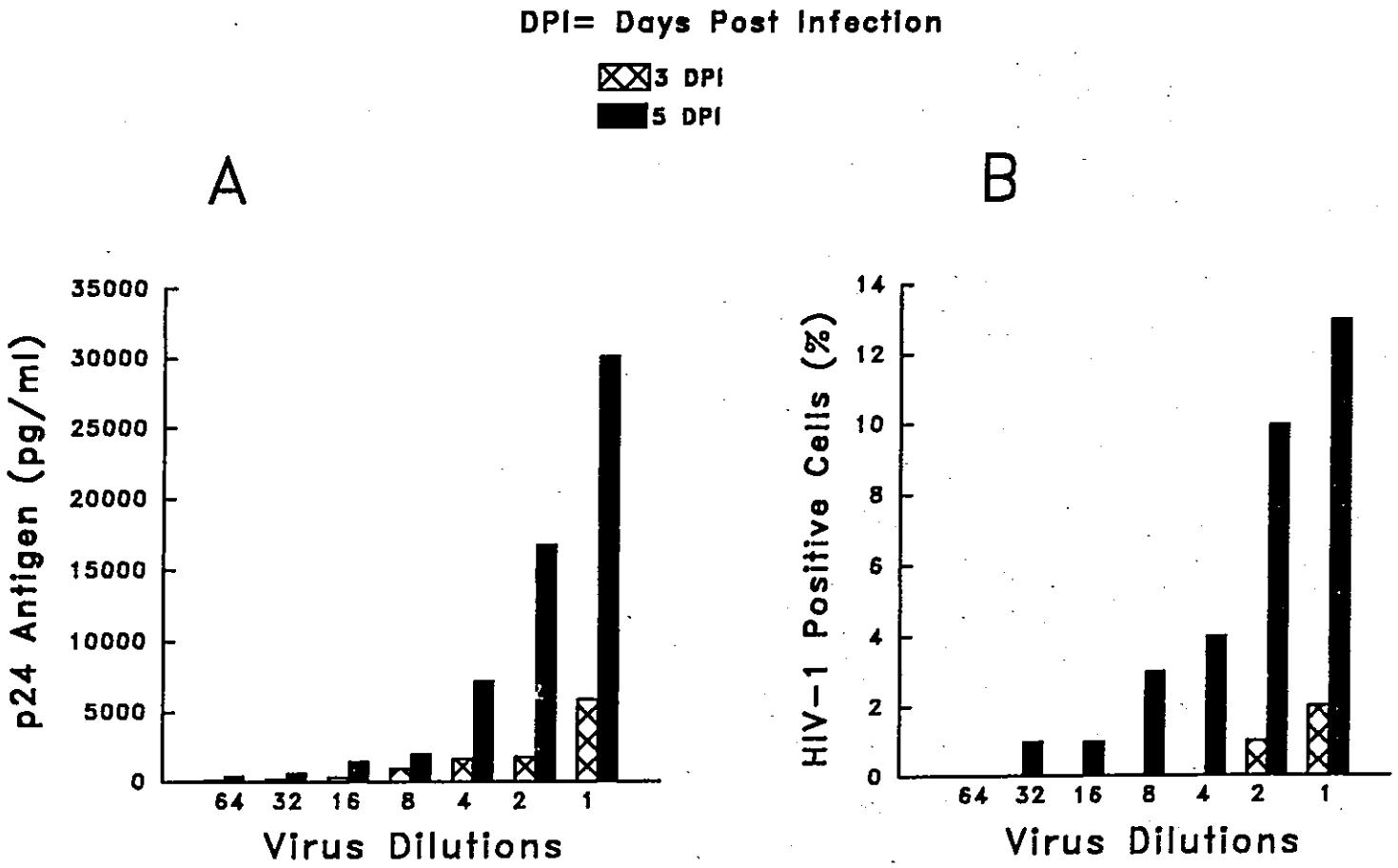
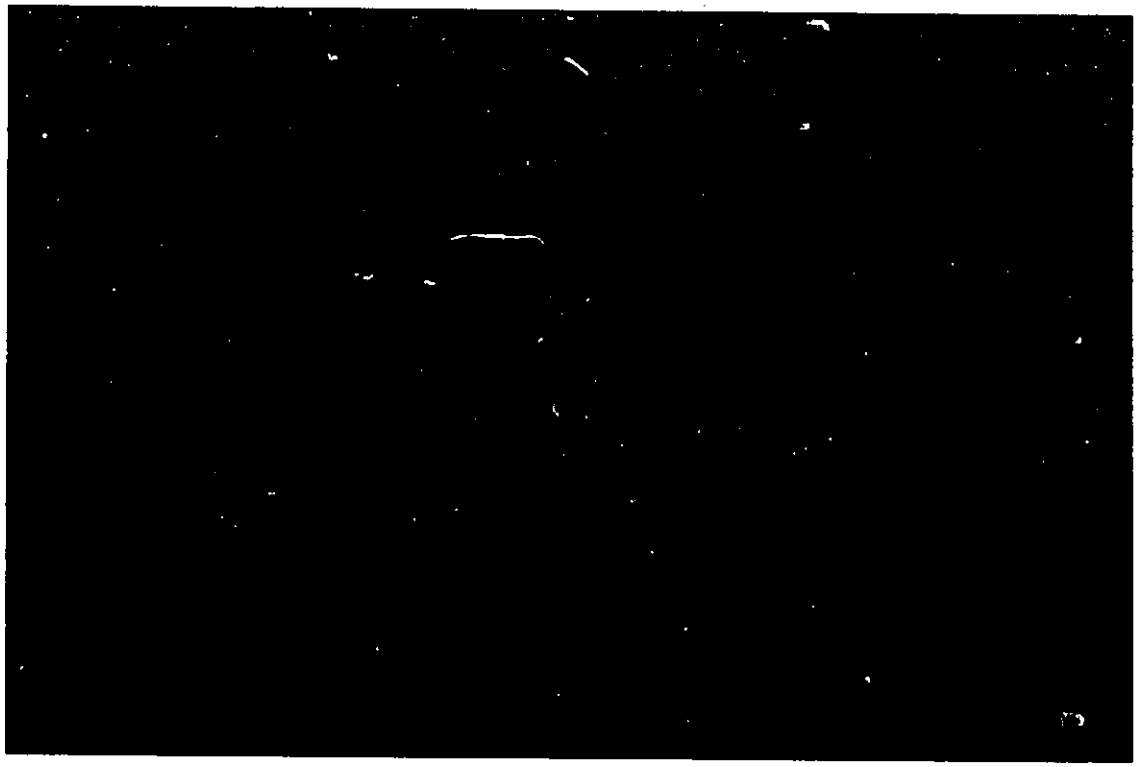

DPI= Days Post Infection
3 DPI
5 DPI
A
B
p24 Antigen (pg/ml)
35000
30000
25000
20000
15000
10000
5000
0
64 32 16 8 4 2 1
Virus Dilutions
HIV-1 Positive Cells (%)
14
12
10
8
6
4
2
0
64 32 16 8 4 2 1
Virus Dilutions

Fig. 4. Syncytia formation (Cytopathic Effect).

Pictures of Immunofluorescence Assay (IFA) taken of: A= HIV-1-infected normal T lymphocytes and B= HIV-1-infected HUT-78 cells. Indirect IFA was done on slides using human polyclonal IgG anti-HIV and goat anti-human IgG-FITC. The virus strain used for the infection had a cytopathic effect on the HUT-78 cells with the production of big syncytia, but it did not produce this effect on normal T-lymphocytes. Nevertheless, these cells were also infected in spite of the lack of syncytia formation.

A



B

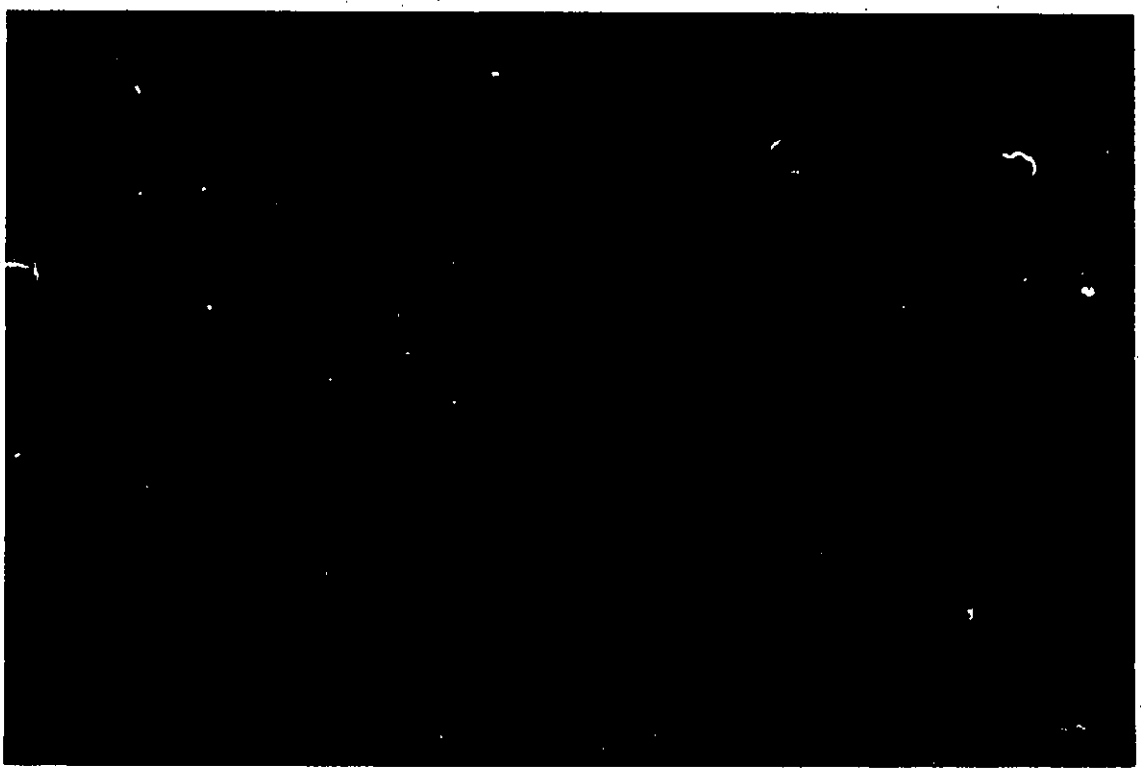
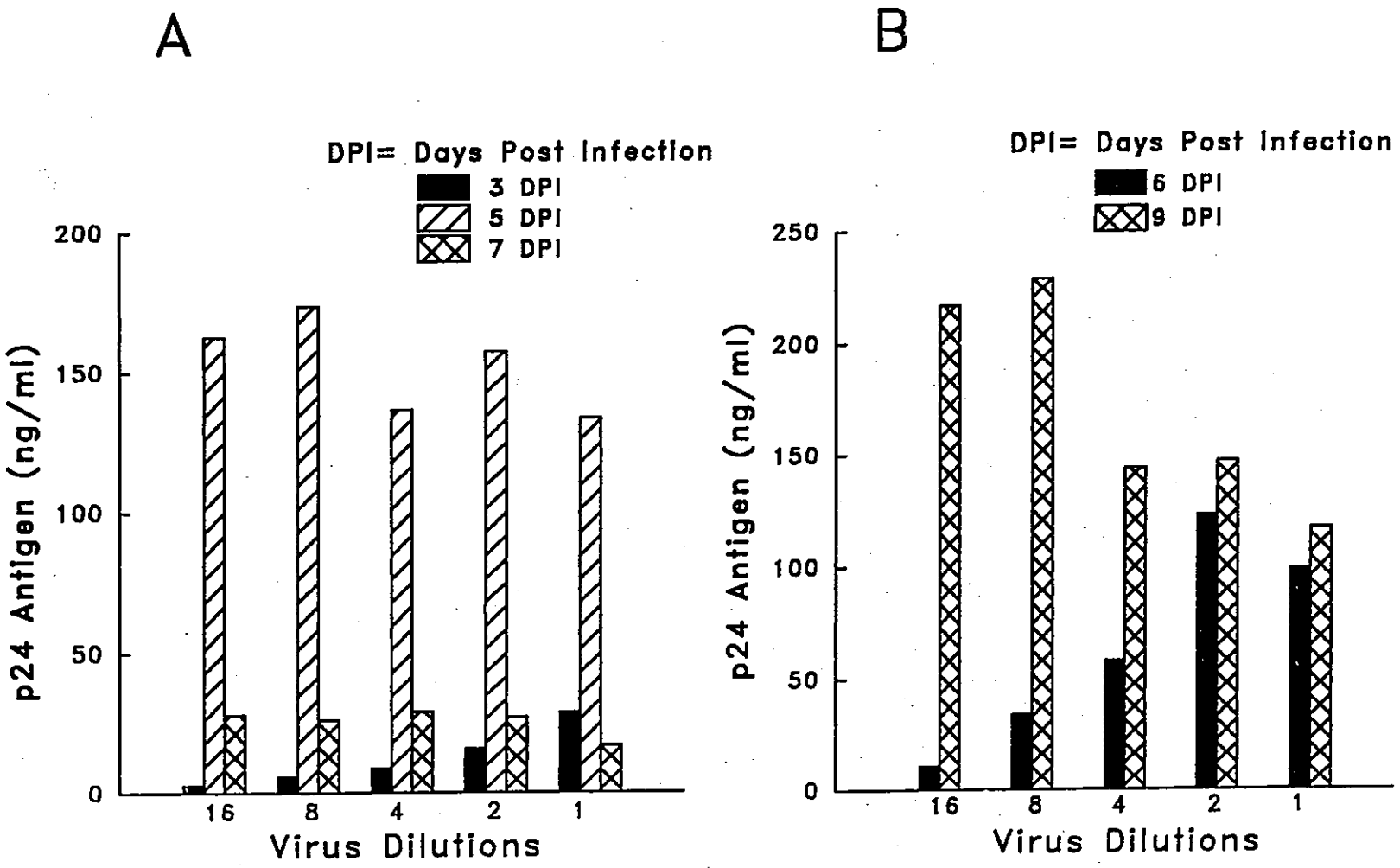


Fig. 5. Comparison of titration of HIV-1 viral supernatant on HUT-78 and normal T cell blasts.

The graphs compare titrations done in HUT-78 cells (B) and in normal T cell lymphoblasts(A). Virus stock used for the infection contained 200,000 pg/ml of p24 Ag. Titration was done using virus stock neat and at four dilutions: 1:2, 1:4, 1:8 and 1:16, expressed as reciprocal of the dilutions in the graphs. The results of the p24 Ag assay are given in ng/ml. Results indicate that in normal T cells infection peaks on day 5 post-infection and in HUT-78 cells on day 9 post-infection.


A
DPI= Days Post Infection
3 DPI
5 DPI
7 DPI
p24 Antigen (ng/ml)
200
150
100
50
0
16
8
4
2
1
Virus Dilutions
B
DPI= Days Post Infection
6 DPI
9 DPI
p24 Antigen (ng/ml)
250
200
150
100
50
0
16
8
4
2
1
Virus Dilutions

III.3.1. Kinetics of virus replication in PBMC preparations from different individuals

Table 3 shows the difference in the kinetics of HIV-1 replication in PBMC in three different normal donors. The infections were done with the same viral stock containing 19,250 pg/ml of p24 antigen. In two cases the infection peaked on day 5 and in another case on day 7 post-infection. Because of this difference in the virus replication, the p24 levels were measured on days 3, 5 and 7 post-infection. In the great majority of my experiments the peak occurred on day 5.

TABLE 3

Kinetics of virus replication in PBMC preparations from different individuals
p24 Antigen levels in culture fluid (ng/ml)

Virus titer used for infection (pg/ml)	DAYS POST INFECTION		
	3	5	7
19,250	8	150	136
19,250	2	177	163
19,250	1	15	40

Kinetics of virus replication. HIV-1 yield from PBMC cultures of healthy individuals infected with the same strain of HIV-1 virus.
p24 Ag levels of culture fluid are shown for days 3, 5 and 7 post-infection, as well as the titer of virus supernatant used for infection.

III.4. Effect of enhancers of HIV-1 infectivity on HUT-78 cells.

After establishing the optimal concentration of virus for infectivity assays and the kinetics of infection for both the HUT-78 cell line and normal human lymphoblasts I tested the effect of reagents that have been reported to enhance viral entry and virus production. The polycations DEAE-dextran and Polybrene are commonly used to promote viral entry; the reported protocols are variable. Tumor Necrosis Factor-Alpha (TNF-α) has been shown to activate transcription of the virus from the Long Terminal Repeat (LTR) by activating the nuclear factor NF-kB, thus resulting in markedly increased virus production. All three reagents were used to determine their effect on infectivity and viral production caused by the viral stock supernatants. The purpose was twofold: 1) to improve the virus production protocol and 2) to produce a fast acute infection of $CD4^+$ T lymphoblasts for their use in CTL assays.

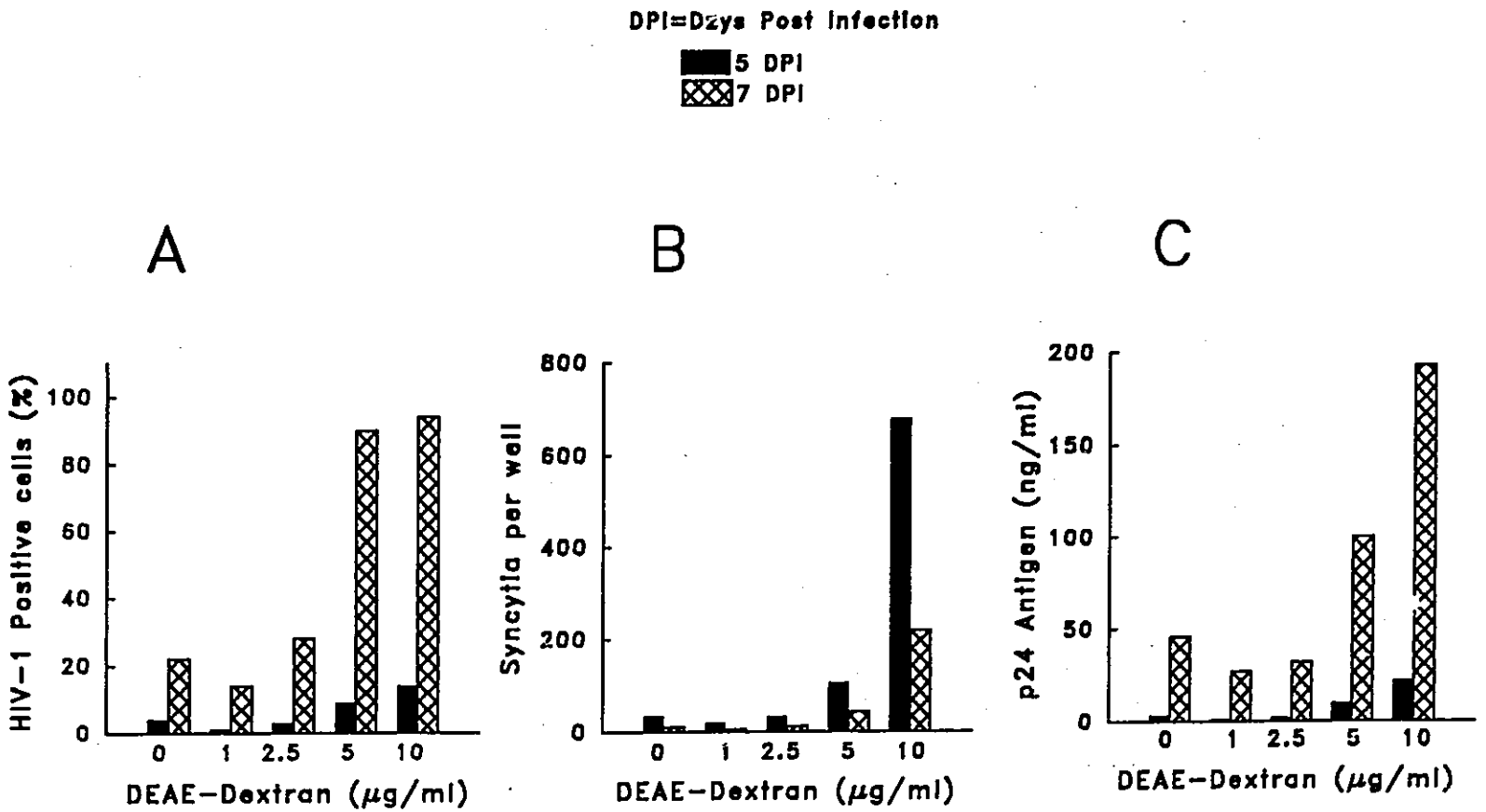
Experiments were done in triplicate. Results varied because of differences in the infectivity of the virus stocks used but the pattern was the same. A typical experiment is analyzed next. Cells were infected as described in Materials and Methods with a virus stock containing 100,000 pg/ml of p24 Ag (a 1/2 dilution of the stock containing 200,000 pg/ml of p24 Ag). After infection, cells were washed once and resuspended in RPMI/10% FCS.

III.4.1. DEAE-Dextran (DD).

DD was added to the cells during the period of incubation with the virus-containing supernatant and kept in the culture medium after infection. The concentrations tested were: 1, 2.5, 5 and 10 µg/ml. (Fig. 6 A-B-C). Virus infection measured in the presence of 10µg/ml of DD by CPE peaked on day 5 post-infection whereas virus levels, quantified by IFA and p24Ag expression, peaked on day 7 post-infection. No difference in viral production as measured by IFA was observed when using 5 or 10 µg/ml of DD.

Fig. 6. Effect of DEAE-Dextran on HIV-1 infection of the HUT-78 cell line.

HUT-78 cells were infected with virus stock containing 100,000 pg/ml of p24 Ag. DEAE-Dextran was titrated at 1, 2.5, 5 and 10 µg/ml. It was added during the infection and kept in the culture medium after infection. Controls were uninfected cells and cells infected without DEAE-Dextran. The infection was monitored by Immunofluorescence on slides (A), Cytopathic Effect (Syncytia formation) (B), and p24 Ag assay (C). The assays were done on days 3, 5 and 7 post infection. The results for days 5 and 7 are shown.


DPI=Dzys Post Infection
5 DPI
7 DPI
A
HIV-1 Positive cells (%)
0
20
40
60
80
100
0 1 2.5 5 10
DEAE-Dextran (μg/ml)
B
Syncytia per well
0
200
400
600
800
0 1 2.5 5 10
DEAE-Dextran (μg/ml)
C
p24 Antigen (ng/ml)
0
50
100
150
200
0 1 2.5 5 10
DEAE-Dextran (μg/ml)

III.4.2. Polybrene.

Polybrene was used during the exposure to HIV-1 and was not removed after the infection. The concentrations tested were: 1, 2.5 and 5 µg/ml (Fig.7 A-B-C). Except for a slight increase in syncytia formation, it did not have an enhancing effect as determined by IFA and p24 assays; on the contrary, it appeared to have a deleterious effect.

III.4.3. Tumor Necrosis Factor-α

TNFα was added to cultures of infected cells at the following concentrations: 0.5, 1, 5, 10 and 50 ng/ml. Controls were uninfected cells and cells infected without the addition of TNF-α. The results of a representative experiment (Figure 8) are for days 5 and 7 post-infection . On day 3 infection was still not detectable. The results of the IFA assay (Fig.8A) did not show any enhancing activity. The CPE assay (Fig. 8B) revealed a slight enhancing effect when used at 10 ng/ml and above. The p24 assay (Fig.8C) shows a slight increase in infectivity on day 7 post-infection when used at 5 ng/ml. In conclusion, the HUT-78 cell line appears to be relatively insensitive to TNF-α at least in the first 7 days after infection.

III.4.4. Comparison of enhancers of HIV-1 infectivity.

Fig.9 (A-B) compares the results of the best concentrations of the enhancers of infectivity from the above experiment. To summarize, the best results were obtained with DEAE-Dextran at 10 µg/ml. Polybrene did not have an effect but one could see an increase in the number of syncytia when used at 2.5 µg/ml. TNFα had a slight effect on day 7 post-infection when used at 5 ng/ml.

Fig. 7. Effect of Polybrene on HIV-1 infection of the HUT-78 cell line.

HUT-78 cells were infected with virus stock containing 100,000 pg/ml of p24 Ag. Polybrene was titrated at 1, 2.5, and 5 µg/ml. It was added during the infection and kept in the culture medium after infection. Controls were uninfected cells and cells infected without Polybrene. The infection was monitored by IFA on slides (Fig. 7A), CPE (Syncytia formation) (Fig. 7B), and p24 Ag assay (Fig. 7C). The assays were done on days 3, 5 and 7 post-infection. The results for days 5 and 7 are shown.

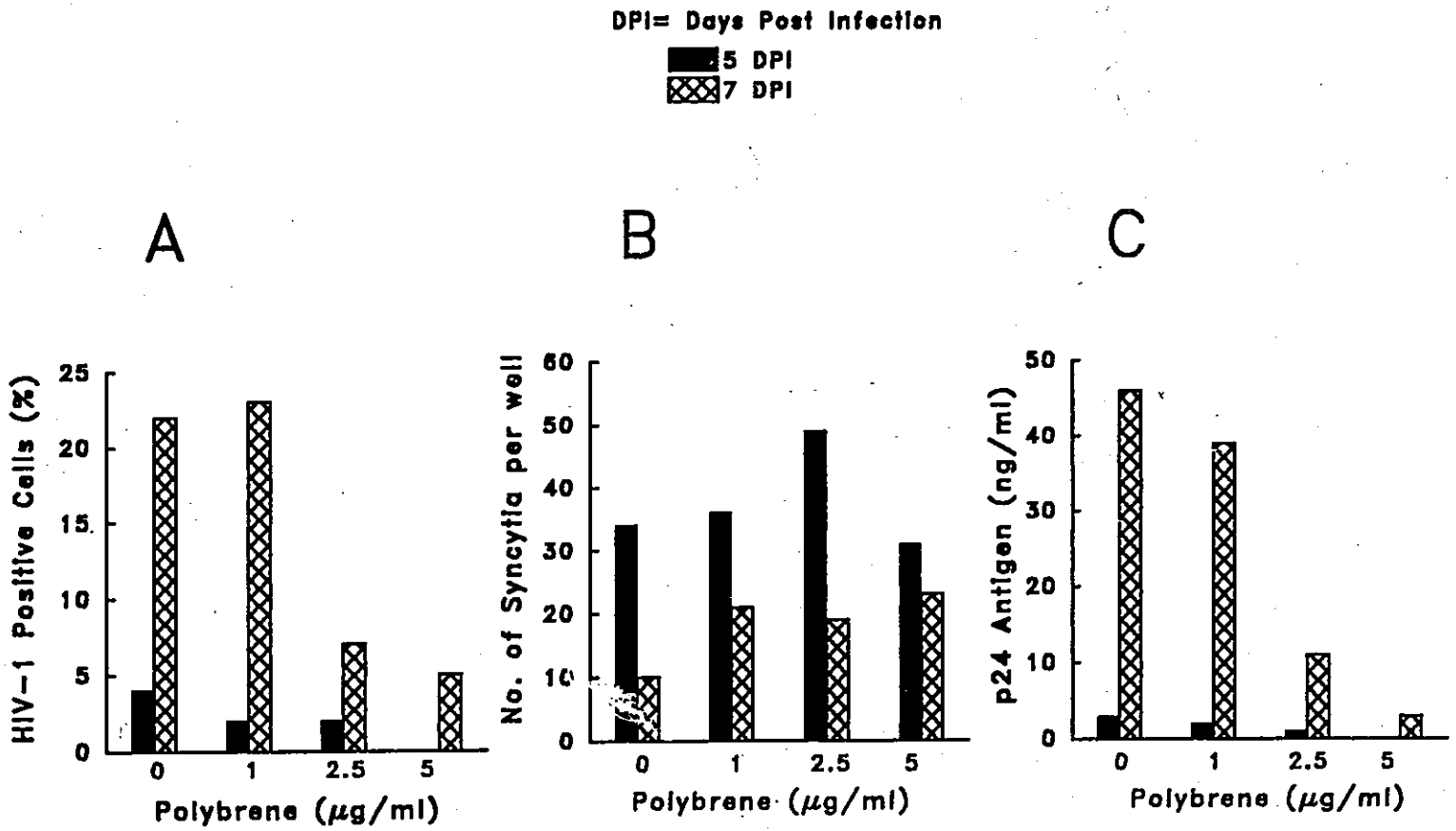

DPI= Days Post Infection
5 DPI
7 DPI
A
HIV-1 Positive Cells (%)
0
5
10
15
20
25
0
1
2.5
5
Polybrene (μg/ml)
B
No. of Syncytia per well
0
10
20
30
40
50
60
0
1
2.5
5
Polybrene (μg/ml)
C
p24 Antigen (ng/ml)
0
10
20
30
40
50
0
1
2.5
5
Polybrene (μg/ml)

Figure 8. Effect of Tumor Necrosis Factor Alpha (TNFα) on HIV-1 infection of the HUT-78 cell line.

The cells were infected with a virus stock containing 100,000 pg/ml of p24 Ag. TNFα was used at the following concentrations: 0.5, 1, 5, 10 and 50 ng/ml. It was added to the cell cultures after the infection of the cells (without DEAE-Dextran or Polybrene). The control were cells infected and cultured in the absence of TNFα. The infection was monitored by IFA (Fig. 8A), CPE (Fig. 8B) and p24 Ag assay (Fig. 8C). Results for days 5 and 7 post-infection are shown.

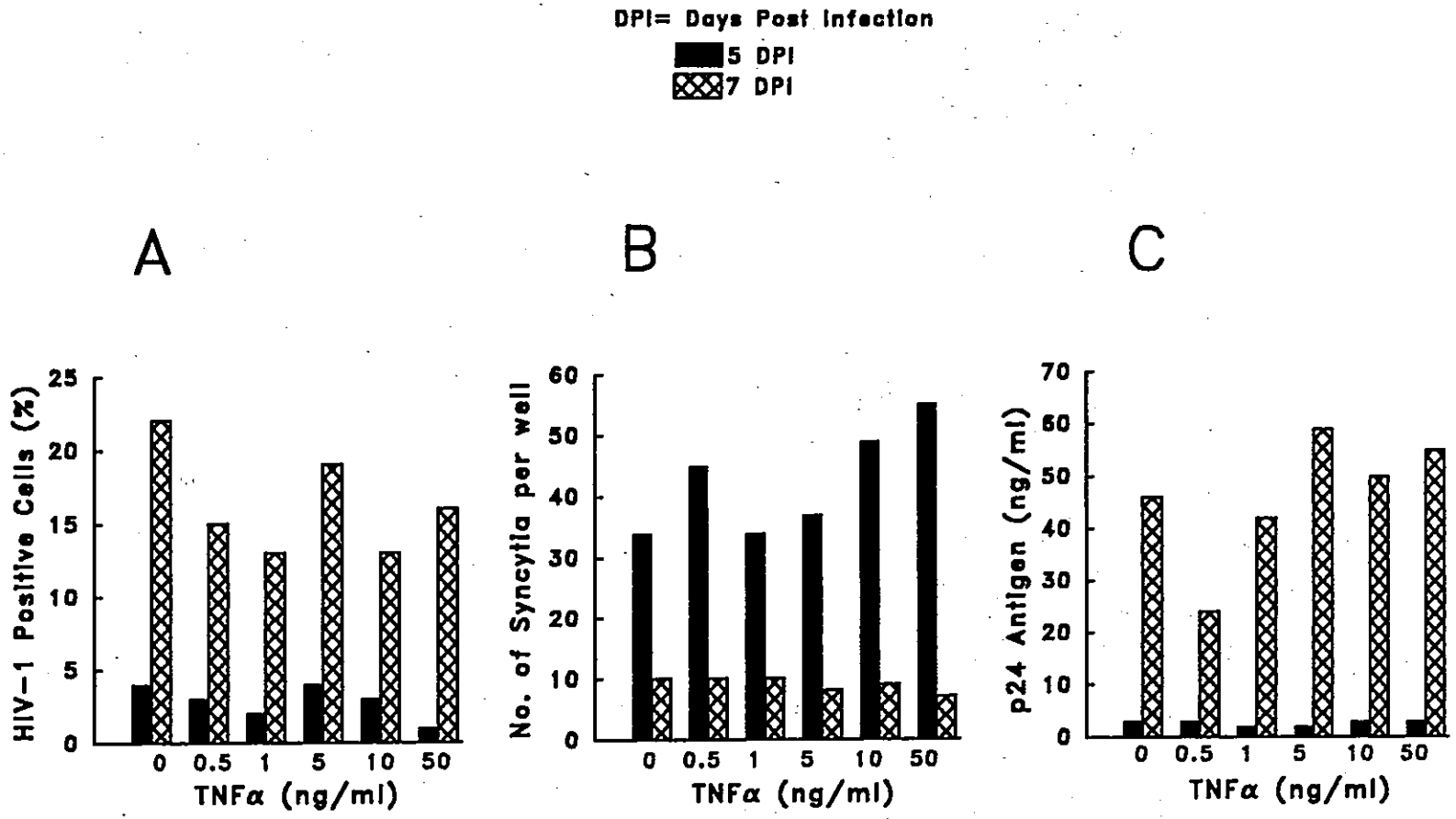
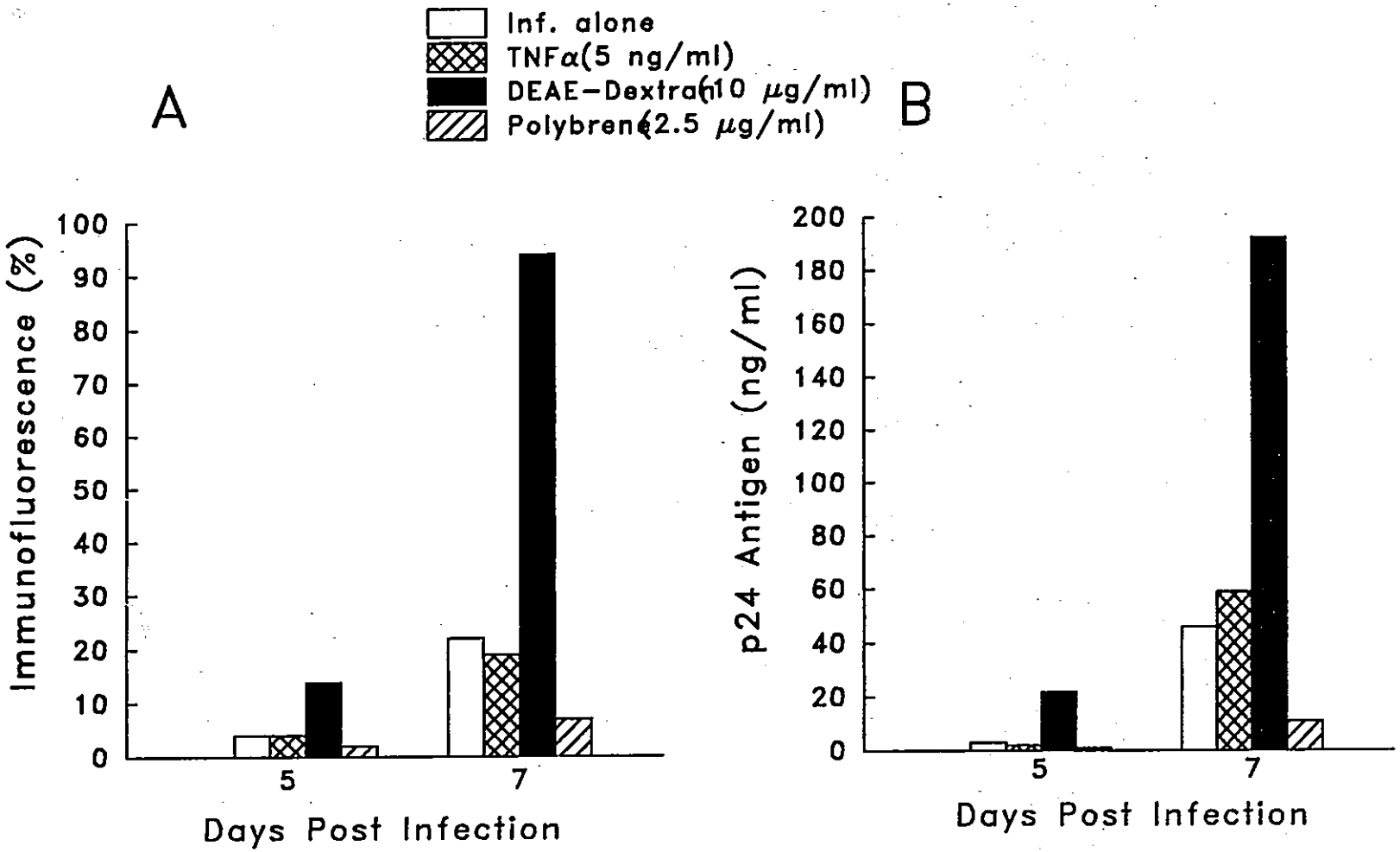

DPI= Days Post Infection
5 DPI
7 DPI
A
HIV-1 Positive Cells (%)
0
5
10
15
20
25
0 0.5 1 5 10 50
TNFα (ng/ml)
B
No. of Syncytia per well
0
10
20
30
40
50
60
0 0.5 1 5 10 50
TNFα (ng/ml)
C
p24 Antigen (ng/ml)
0
10
20
30
40
50
60
70
0 0.5 1 5 10 50
TNFα (ng/ml)

Figure 9. Comparison of the optimal dose of DEAE-Dextran, Polybrene and Tumor Necrosis Factorα on HIV-1 infection of the HUT-78 cell line

These graphs compare the best concentrations of these enhancers from the experiment in HUT-78 cells depicted in Figures 6, 7, and 8. The cells were infected with virus stock containing 100,000 pg/ml of p24 Ag. TNFα (5ng/ml) was always added to the culture medium after cells were infected and washed once. DEAE-Dextran (10 µg/ml) and Polybrene (2.5 µg/ml) were added during the infection and in the culture medium after infection. Results of IFA (Fig. 9A) and p24 Ag assay (Fig. 9B) are shown for days 5 and 7 post-infection.


Inf. alone
TNFα(5 ng/ml)
DEAE-Dextran(10 μg/ml)
Polybrene(2.5 μg/ml)
A
B
Immunofluorescence (%)
100
90
80
70
60
50
40
30
20
10
0
5
7
Days Post Infection
p24 Antigen (ng/ml)
200
180
160
140
120
100
80
60
40
20
0
5
7
Days Post Infection

III.5. Comparison of different modalities of use of DEAE-Dextran (DD) as enhancer of HIV-1 infectivity in HUT-78 cells.

As noted in published reports, DD has been used in various ways. In the preceding experiments DD was used in a single modality, during and after infection with HIV-1. In the following experiments, the effect of DD was compared when used in 5 ways: a) as pretreatment of the target cells before infection with HIV-1, b) as simultaneous exposure to DD and virus, c) as a combination of pretreatment and simultaneous exposure, d) a combination of exposure during and after infection, and e) a combination of pretreatment and exposure during and after infection. Pretreatment was done by incubation of the cells with DD for 30 min. at 37^{o}C before exposure of the cells to HIV-1. The optimal concentrations from my previous experiments (5 and 10 µg/ml) were tested, as well as 25 µg/ml. This concentration, as pretreatment, has been reported to be an effective dose for enhancement of viral infectivity (Ikeuchi et al., 1990; Tateno et al., 1989; Levy et al., 1985). The 25 µg/ml concentration was not used after infection because in preliminary experiments this concentration proved to be toxic to the cells.. Experiments were done in triplicate. Results varied in terms of levels of p24 Ag, percentage of IFA and number of syncytia due to differences in the infectivity of the virus stocks, but the pattern was the same. A typical experiment is shown in Fig. 10 (CPE), Fig. 11 (p24 Assay) and Fig. 12 (IFA). A virus dilution containing 19,250 pg/ml of p24 antigen was used.

III.5.1. Evaluation of viral production by CPE, p24 Ag assay and IFA.

As shown in Figures 10 to 12, optimum viral production was observed when 10 µg/ml of DD were employed before and during infection with HIV-1 (Fig. 10c, 11c and 12c). The use of DD at 25 µg/ml during infection increased infectivity in comparable levels as production of p24 Ag on day 5 post infection shows (Fig. 11) but this concentration was too toxic for the cells as evaluated by the morphology of the cells in

culture (CPE) and on the immunofluorescent slides. Pretreatment alone and the combinations where DD was kept in the culture after infection gave poorer viral levels as measured by the assays used. Only the combination of pretreatment and during infection with 10 µg/ml gave consistent results.

Fig. 10. Effect of DEAE-Dextran (DD), used in several modalities, on HIV-1 infection of HUT-78 cells. Results of Cytopathic Effect (CPE).

The virus stock (308,000 pg/ml of p24 Ag) used for infection was diluted to 1:16 (19,250 pg/ml) before infecting HUT-78 cells. DD was used at 5, 10 or 25 µg/ml as follows:

A. During infection.

B. Pretreatment.

C. Pretreatment and during infection.

D. During and after infection.

E. Pretreatment, during and after infection.

Controls were uninfected cells and cells infected without DD. Pretreatment consisted of an incubation period of 30 min. at 37^{O}C with DD at the various concentrations. After this, cells were spun down and infected with virus in the presence or absence of DD. After infection the cells were washed once and resuspended in culture medium with or without enhancer as required. To analyze the CPE, aliquots of 200 µl each were set up in a 96-well microtiter plate to observe the formation of syncytia per well (Cytopathic Effect). These wells were not disturbed for 7 days and syncytia of 3 wells for each sample were counted and averaged on days 3, 5 and 7 post-infection. Results for days 5 and 7 are shown.

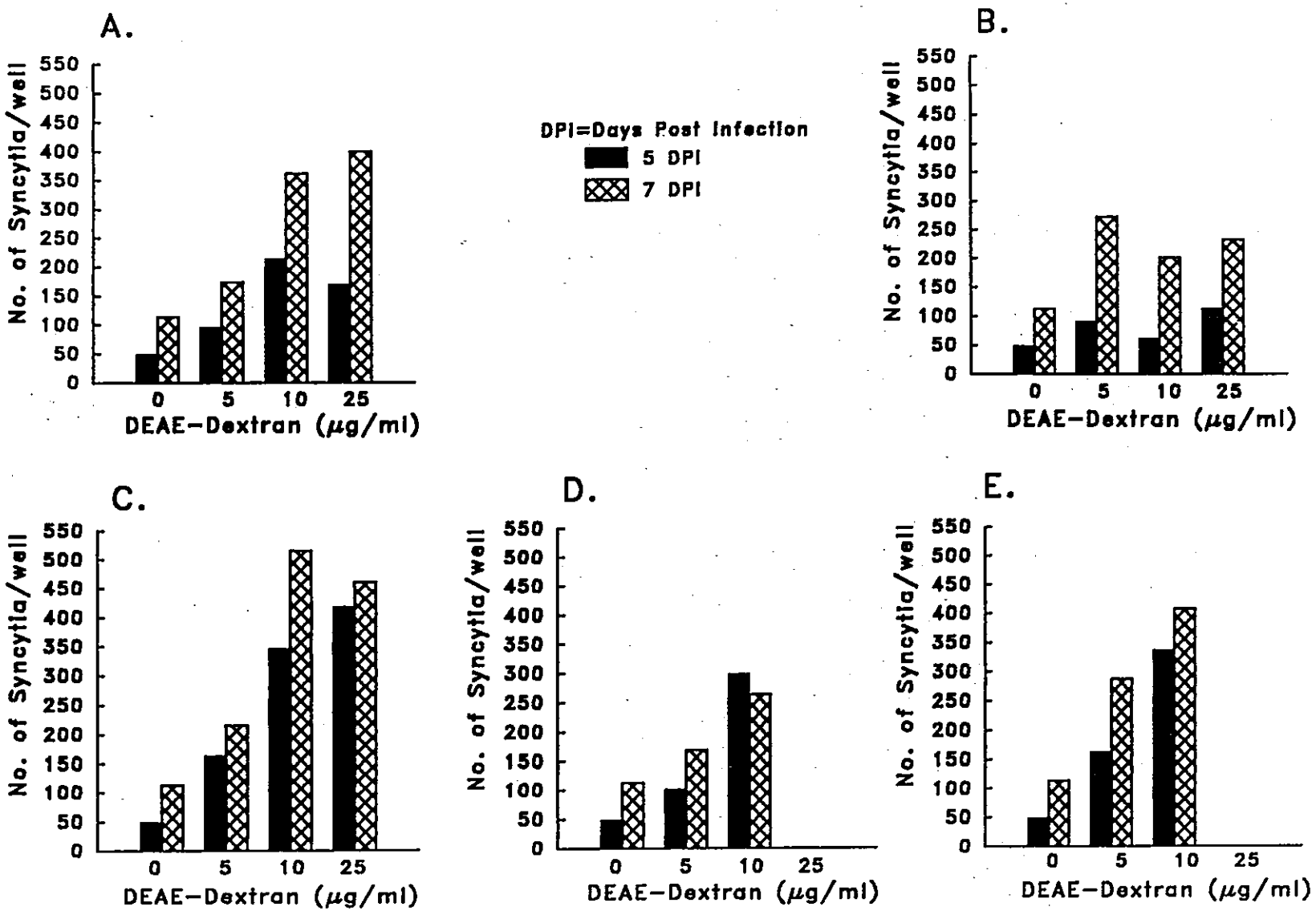

A.
B.
C.
D.
E.
No. of Syncytia/well
550
500
450
400
350
300
250
200
150
100
50
0
0
5
10
25
DEAE-Dextran (μg/ml)
DPI=Days Post Infection
5 DPI
7 DPI

Fig. 11. Effect of DEAE-Dextran, used in several modalities on HIV-1 infection of HUT-78 cells. Results of p24 Ag assay.

The virus stock (308,000 pg/ml of p24 Ag) used for infection was diluted to 1:16 (19,250 pg/ml) before infecting HUT-78 cells. DD was used at 5, 10 or 25 μg/ml as follows:

A. During infection.

B. Pretreatment.

C. Pretreatment and during infection.

D. During and after infection.

E. Pretreatment, during and after infection.

Controls were uninfected cells and cells infected without DD. Pretreatment consisted of an incubation period of 30 min. at 37^{0}C with DD at the various concentrations. After this, cells were spun down and infected with virus in the presence or absence of DD. After infection the cells were washed once and resuspended in culture medium with or without enhancer as required. A 2 ml sample was collected on days 3, 5 and 7 post infection, spun down and the supernatant stored at -20^{0}C until the day of the p24 antigen assay. Results for days 5 and 7 are shown.

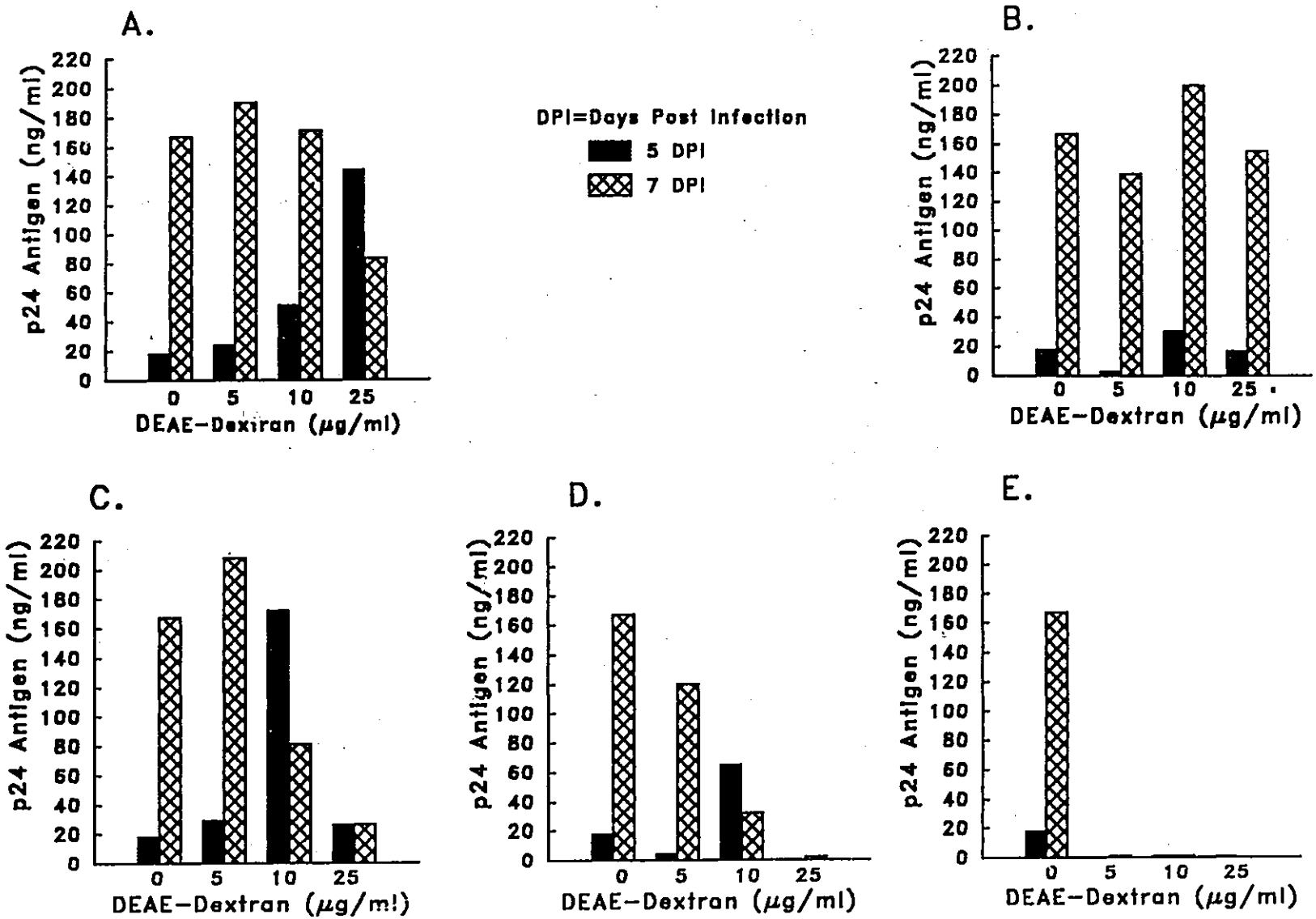

A.
B.
C.
D.
E.
p24 Antigen (ng/ml)
220
200
180
160
140
120
100
80
60
40
20
0
0
5
10
25
DEAE-Dextran (μg/ml)
DPI=Days Post Infection
5 DPI
7 DPI

Fig. 12. Effect of DEAE-Dextran, used in several modalities on HIV-1 infection of HUT-78 cells. Results of the Immunofluorescence Assay (IFA).

The virus stock (308,000 pg/ml of p24 Ag) used for infection was diluted to 1:16 (19,50 pg/ml) before infecting HUT-78 cells. DD was used at 5, 10 or 25 µg/ml as follows:

A. During infection.

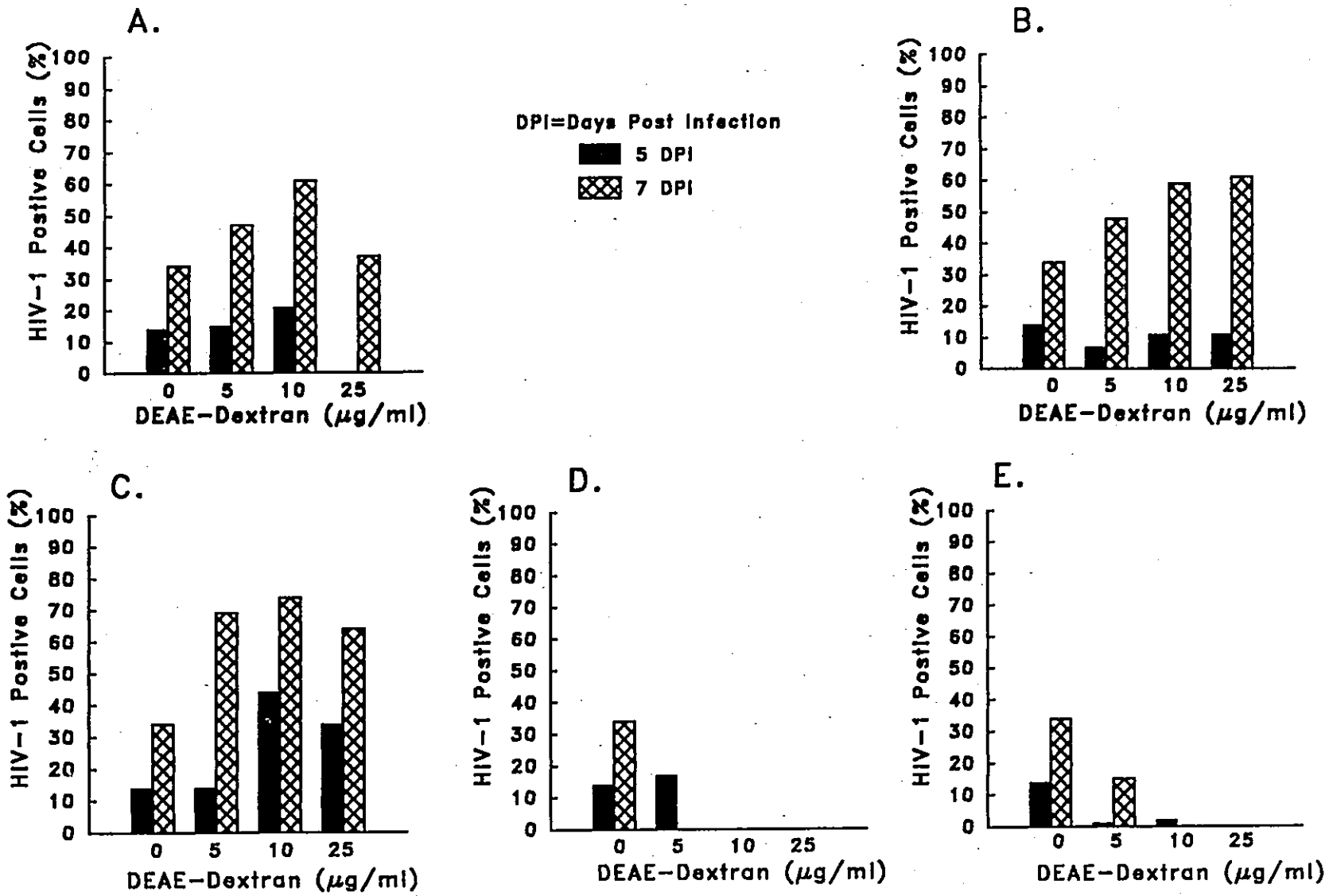
B. Pretreatment.

C. Pretreatment and during infection.

D. During and after infection.

E. Pretreatment, during and after infection.

Controls were uninfected cells and cells infected without DD. Pretreatment consisted of an incubation period of 30 min. at 37°C with DD at the various concentrations. After this, cells were spun down and infected with virus in the presence or absence of DD. After infection the cells were washed once and resuspended in culture medium with or without enhancer as required. A 2 ml sample was collected on days 3, 5 and 7 post-infection, spun down and the cell pellets were inactivated with AMT/UV irradiation (see Materials and Methods), washed and resuspended in PBS. Smears were prepared on slides for IFA staining. Results for days 5 and 7 are shown.


A.
B.
C.
D.
E.
HIV-1 Postive Cells (%)
100
90
80
70
60
50
40
30
20
10
0
0 5 10 25
DEAE-Dextran (μg/ml)
DPI=Days Post Infection
5 DPI
7 DPI

III.6. Comparison of different modalities of use of Polybrene as enhancer of HIV-1 infectivity in HUT-78 cells.

In the following experiments Polybrene was tested in 5 ways: a) as pretreatment of the target cells before infection with HIV-1, b) as simultaneous exposure with virus, c) as a combination of pretreatment and simultaneous exposure, d) a combination of exposure during and after infection, and e) a combination of pretreatment and exposure during and after infection. Pretreatment was done by incubation of the cells with Polybrene for 30 min. at 37^{o}C before exposure of the cells to HIV-1. I tested the best concentrations from my previous experiments, 2.5 and 5 µg/ml. Experiments were done in triplicate. Results varied in terms of levels of p24 Ag, percentage of IFA and number of syncytia due to differences in the infectivity of the virus stocks, but the pattern was the same. A typical experiment is analyzed next and shown in Fig. 13 (CPE), Fig. 14 (p24 Ag assay) and Fig. 15 (IFA). A virus dilution containing 19,250 pg/ml of p24 Ag was used .

III.6.1. Evaluation of viral production by CPE, p24 Ag assay and IFA.

As shown in Figure 13, none of the modalities used enhanced infection of HUT-78 cells with HIV-1 when CPE was used as an indicator. The p24 Ag assay indicates that Polybrene slightly enhanced viral infectivity on day 7 post-infection when used as pretreatment (5 µg/ml) (Fig. 14B) or as pretreatment and during infection at 2.5 µg/ml (Fig. 14C). The results of IFA (Fig. 15C) indicate that Polybrene slightly enhanced viral infectivity on day 5 post-infection when used as pretreatment and during the infection at 5 µg/ml.

Fig. 13. Effect of Polybrene, used in several modalities, on HIV-1 infection of HUT-78 cells. Results of Cytopathic Effect (CPE).

The virus stock (308,000 pg/ml of p24 Ag) used for infection was diluted to 1:16 (19,250 pg/ml) before infecting HUT-78 cells. Polybrene was used at 5, 10 or 25 µg/ml as follows:

A. During infection.

B. Pretreatment.

C. Pretreatment and during infection.

D. During and after infection.

E. Pretreatment, during and after infection.

Controls were uninfected cells and cells infected without Polybrene. Pretreatment consisted of an incubation period of 30 min. at 37^{o}C with Polybrene at the various concentrations. After this, cells were spun down and infected with virus in the presence or absence of Polybrene. After infection the cells were washed once and resuspended in culture medium with or without enhancer as required. To analyze the CPE, aliquots of 200 µl each were set up in a 96-well microtiter plate to observe the formation of syncytia per well (Cytopathic Effect). These wells were not disturbed for 7 days and syncytia of 3 wells for each sample were counted and averaged on days 3, 5 and 7 post-infection. Results for days 5 and 7 are shown.

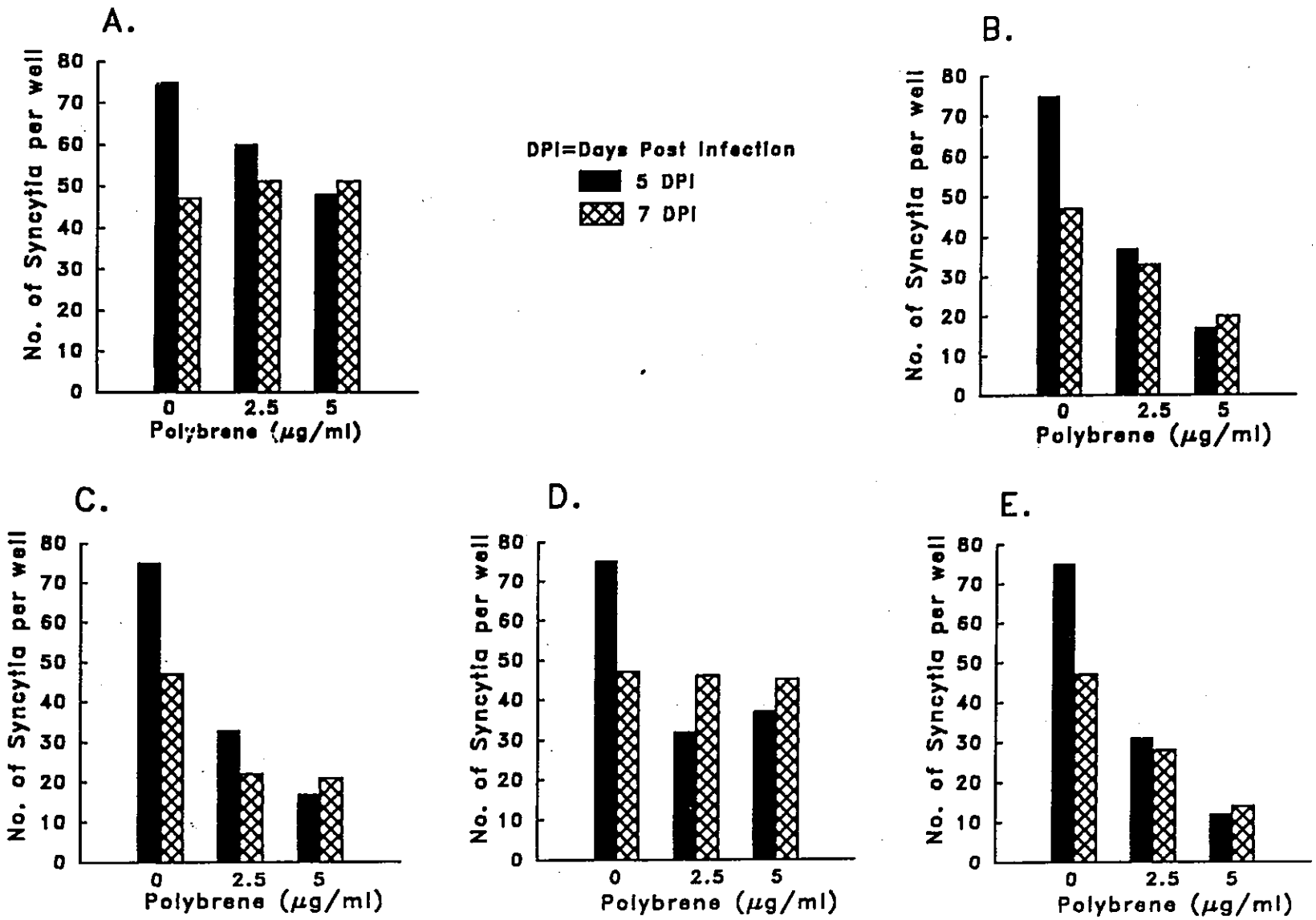

A.
B.
C.
D.
E.
No. of Syncytia per well
0
10
20
30
40
50
60
70
80
0
2.5
5
Polybrene (μg/ml)
DPI=Days Post Infection
5 DPI
7 DPI

Fig. 14. Effect of Polybrene, used in several modalities on HIV-1 infection of HUT-78 cells. Results of p24 Ag assay.

The virus stock (308,000 pg/ml of p24 Ag) used for infection was diluted to 1:16 (19,250 pg/ml) before infecting HUT-78 cells. Polybrene was used at 5, 10 or 25 µg/ml as follows:

A. During infection.

B. Pretreatment.

C. Pretreatment and during infection.

D. During and after infection.

E. Pretreatment, during and after infection.

Controls were uninfected cells and cells infected without Polybrene. Pretreatment consisted of an incubation period of 30 min. at 37^{O}C with Polybrene at the various concentrations. After this, cells were spun down and infected with virus in the presence or absence of Polybrene. After infection the cells were washed once and resuspended in culture medium with or without enhancer as required. A 2 ml sample was collected on days 3, 5 and 7 post-infection, spun down and the supernatant stored at -20^{O}C until the day of the p24 antigen assay. Results for days 5 and 7 are shown.

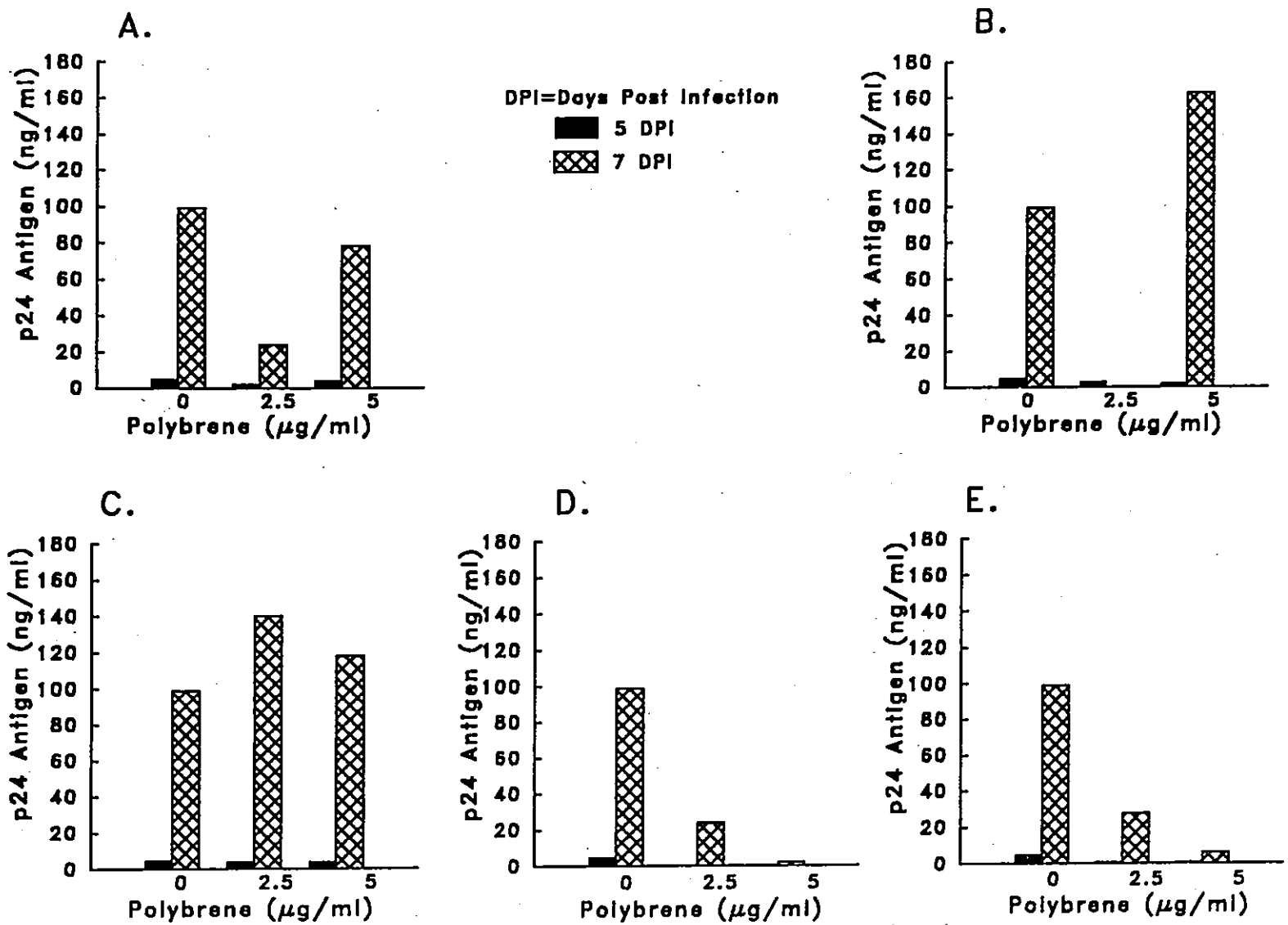

A.
B.
C.
D.
E.
DPI=Days Post Infection
5 DPI
7 DPI
p24 Antigen (ng/ml)
180
160
140
120
100
80
60
40
20
0
0
2.5
5
Polybrene (μg/ml)

Fig. 15. Effect of Polybrene, used in several modalities on HIV-1 infection of HUT-78 cells. Results of the Immunofluorescence Assay (IFA).

The virus stock (308,000 pg/ml of p24 Ag) used for infection was diluted to 1:16 (19,250 pg/ml) before infecting HUT-78 cells. Polybrene was used at 2.5, and 5 µg/ml as follows:

A. During infection.

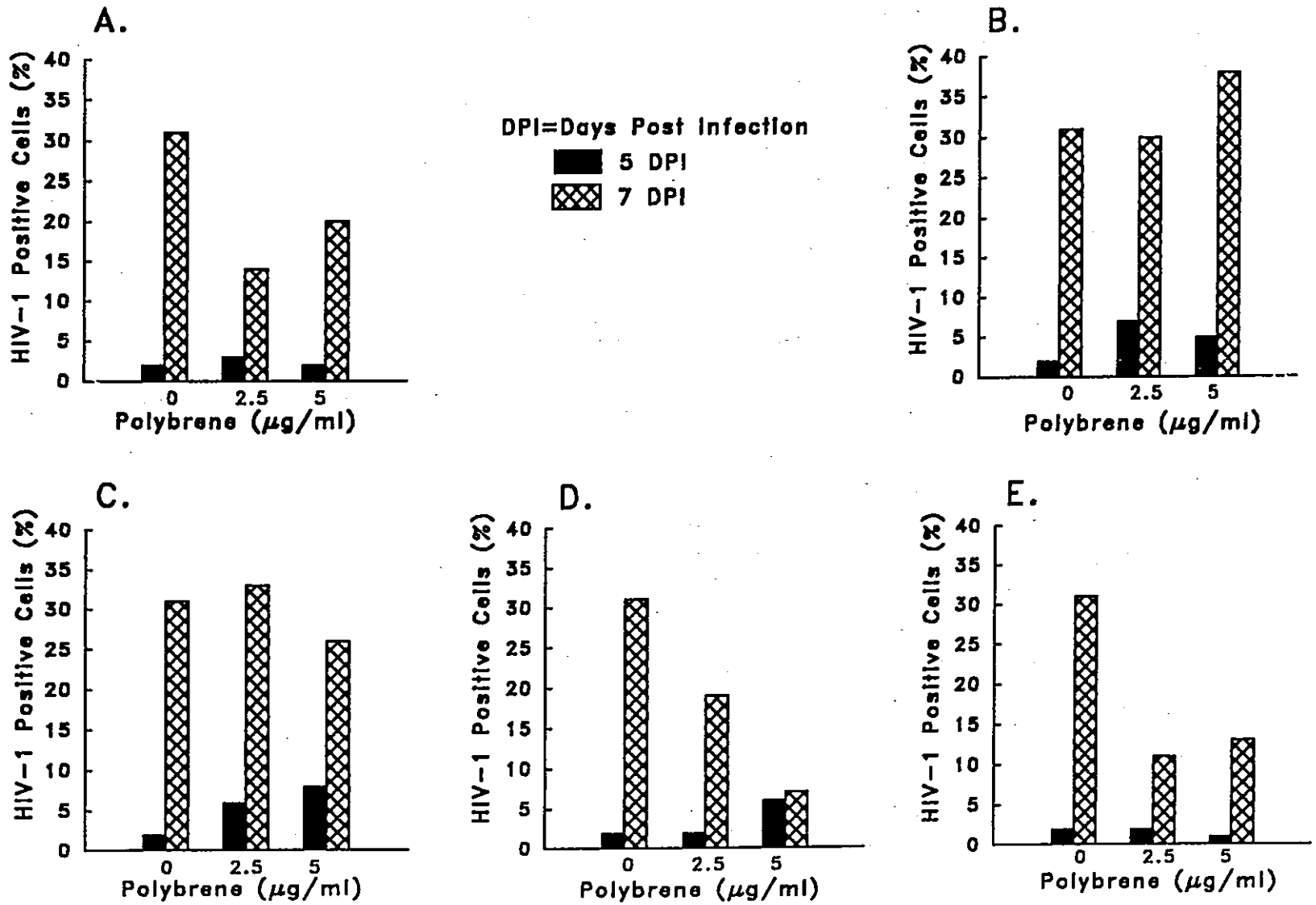
B. Pretreatment.

C. Pretreatment and during infection.

D. During and after infection.

E. Pretreatment, during and after infection.

Controls were uninfected cells and cells infected without polybrene. Pretreatment consisted of an incubation period of 30 min. at 37^{O}C with Polybrene at the various concentrations. After this, cells were spun down and infected with virus in the presence or absence of Polybrene. After infection the cells were washed once and resuspended in culture medium with or without enhancer as required. A 2 ml sample was collected on days 3, 5 and 7 post-infection, spun down and the cell pellets were inactivated with AMT/UV irradiation (see Materials and Methods), washed and resuspended in PBS. Smears were prepared on slides for IFA staining. Results for days 5 and 7 are shown.


A.
B.
C.
D.
E.
DPI=Days Post Infection
5 DPI
7 DPI
HIV-1 Positive Cells (%)
0
5
10
15
20
25
30
35
40
0
2.5
5
Polybrene (μg/ml)

III.7. Effect of enhancers of HIV-1 infectivity in normal T cell blasts.

I repeated the experiments described in section III.4 with some modifications using normal T cell lymphoblasts as target cells rather than the cell line HUT-78. The stocks of virus used in these experiments were titered in normal lymphoblasts without using enhancers. The virus dilution used for infection contained 50,000 pg/ml of p24 Ag. Since DEAE-Dextran and Polybrene are polycations that facilitate viral entry, they were used during and after the infection, TNFα was always added after the infection since it increases transcription of the integrated virus. Infection was evaluated by IFA and p24 Ag assay on days 2, 3 and 4 post-infection. On day 2, infection was undetectable. Results are presented for days 3 and 4 post-infection.

III.7.1. Tumor Necrosis Factor-α

Maximum viral production was obtained using TNFα at 5 ng/ml (Fig.16 A-B). The effect was dose-dependent up to 5 ng/ml and dropped with the use of higher concentrations. The enhancement was more noticeable on day 4 than on day 3 post-infection. The results of IFA (Fig.16A) correlate well with the p24 Ag assay (Fig. 16B).

III.7.2. DEAE-Dextran.

As shown in Figure (16 C-D), DEAE-Dextran enhanced viral production at concentrations from 2.5 to 10 µg/ml; 10 µg/ml was slightly better in both the p24 Ag and IFA assays.

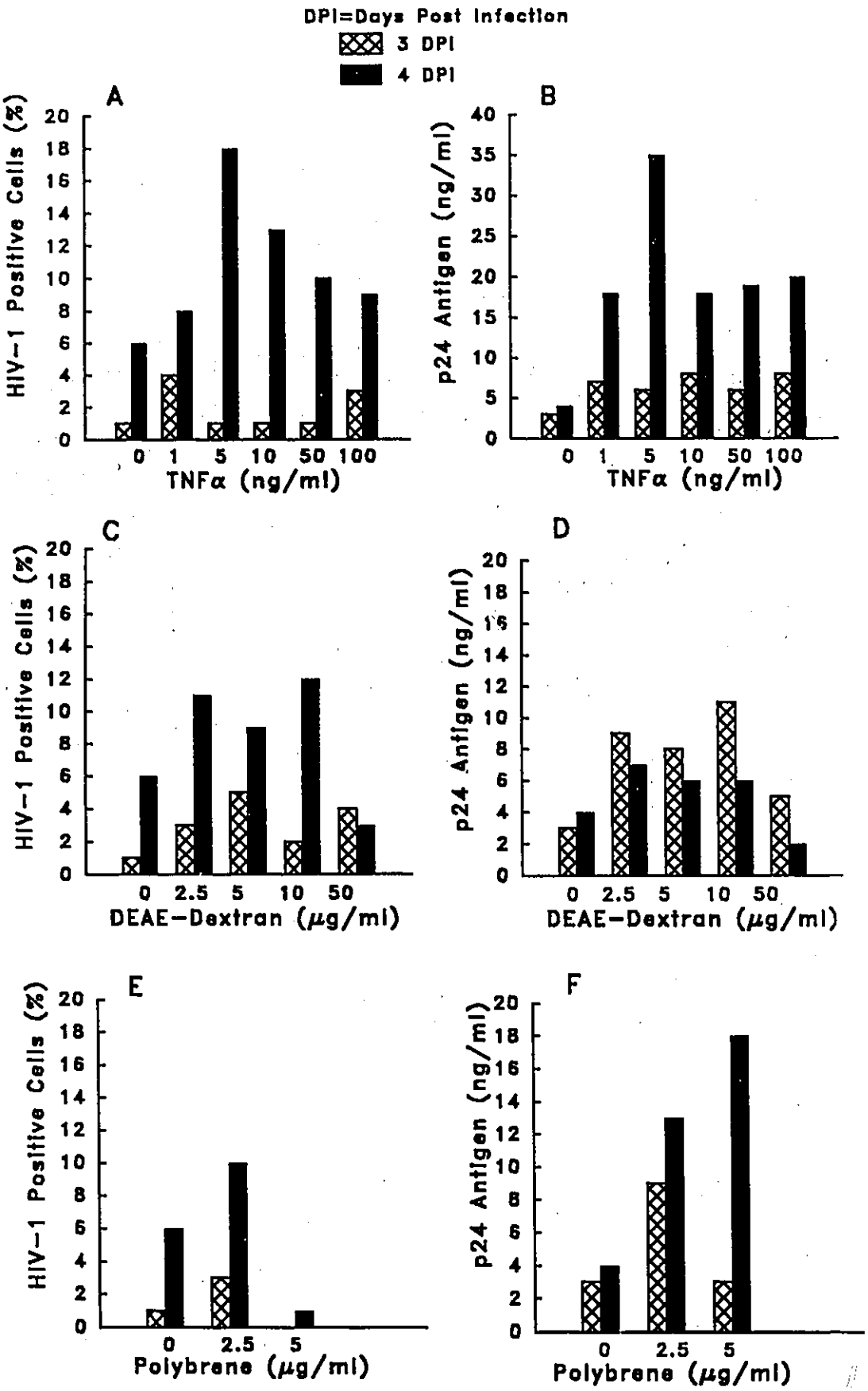
III.7.3. Polybrene

As shown in Figure 16 (E-F), Polybrene increased infectivity when used at 2.5 or 5 µg/ml as shown by the results of the p24 Antigen Assay (Fig 16F). It increased from 4 ng/ml for the control to 13 and 18 ng/ml respectively. The results of IFA (Fig. 16-E) only show this increase at the 2.5 µg/ml concentration.

Fig. 16. Effect of enhancers of HIV-1 infectivity on normal T cell blasts.

Mononuclear cells (MNC) from normal donors were obtained by Ficoll-Hypaque separation. Cells were activated with PHA (0.5 µg/ml) and set in culture with IL-2 (100 U/ml) and 2-Me (5×10^{-5} M). Three days later these T cell lymphoblasts were infected with HIV-1 virus supernatant containing 50,000 pg/ml of p24 Ag.

Tumor Necrosis Factor-α (TNFα) was always added after the infection of the cells and tested at 1, 5, 10, 50 and 100 ng/ml. (A-B). DEAE-Dextran was used during and after the infection of the cells. It was tested at 2.5, 5, 10 and 20 µg/ml. (C-D), and Polybrene was also used during and after the infection of the cells and tested at 2.5 and 5 µg/ml (E-F). The experiment was evaluated by IFA (A-C-E) and by p24 Ag assay (B-D-F). A 2ml sample was collected on days 3 and 4 post-infection, spun down and the supernatant stored at -20°C until the day of the p24 Ag assay. The cell pellets were inactivated with AMT/UV irradiation (see Materials and Methods), washed and resuspended in PBS. Smears were made on slides from these cells for IFA staining.


DPI=Days Post Infection
3 DPI
4 DPI
A
HIV-1 Positive Cells (%)
0 1 5 10 50 100
TNFα (ng/ml)
B
p24 Antigen (ng/ml)
0 1 5 10 50 100
TNFα (ng/ml)
C
HIV-1 Positive Cells (%)
0 2.5 5 10 50
DEAE-Dextran (μg/ml)
D
p24 Antigen (ng/ml)
0 2.5 5 10 50
DEAE-Dextran (μg/ml)
E
HIV-1 Positive Cells (%)
0 2.5 5
Polybrene (μg/ml)
F
p24 Antigen (ng/ml)
0 2.5 5
Polybrene (μg/ml)

III.8. Comparison of different modalities of use of DEAE-Dextran (DD) and Polybrene as enhancers of viral infectivity in normal T cell lymphoblasts.

In the preceding experiments DEAE-Dextran and Polybrene were used in a single modality: during and after infection with HIV-1. In the following experiments the effect of DD and Polybrene was tested in 5 ways: a) as pretreatment of the target cells before infection with HIV-1, b) as simultaneous exposure to DD or Polybrene and virus, c) as a combination of pretreatment and simultaneous exposure, d) a combination of exposure during and after infection, and e) as a combination of pretreatment and exposure during and after infection. Pretreatment was done by incubating the cells with DD or Polybrene for 30 min. at 37^{o}C before exposure of the cells to HIV-1. DD was tested at 5, 10 and 25 µg/ml. The 25 µg/ml concentration was not used after infection because in preliminary experiment this concentration proved to be toxic to the cells. Polybrene was tested at 2.5 and 5 µg/ml. Experiments were done in triplicate. Results varied in terms of levels of p24 Ag and percentage of immunufluorescent cells due to differences in the infectivity of the virus stocks used for the infection but the pattern was the same. A typical experiment is analyzed next and shown in Table 4 (IFA) and Table 5 (p24 Ag assay). A single virus dilution containing 19,250 pg/ml of p24 Ag. was used for the infection. Both enhancers were tested on the same unit of blood to avoid introducing the variable of a different donor.

III.8.1. Evaluation using the Immunofluorescence Assay (Table 4).

Maximum viral infectivity was obtained when the lymphoblasts were treated with DD and Polybrene during the period of infection. For DD optimal results were obtained 5 days after infection at concentrations of 10 and 25 µg/ml. For Polybrene the optimal dose was 2.5 µg/ml but there was a delay in reaching maximum IFA positivity (day 7 post-infection).

III.8.2. Evaluation using the p24 antigen assay (Table 5).

The highest values of p24 Ag were obtained when the cells were exposed to both virus and DD at the same time. To obtain an early maximum production the dose of 25 µg/ml appears optimal. For maximum infection on day 7, the dose of 5 µg/ml was the best. When used as pretreatment and during the infection at 10 µg/ml, infectivity was also greatly enhanced on day 5 post-infection. For practical purposes, a dose of 10 µg/ml appears a safe compromise because it is not as toxic as the 25 µg/ml concentration. Polybrene also has an enhancing effect on viral production at day 7 post-infection at 5 µg/ml when used as pretreatment and during the infection, or during and after infection.

TABLE 4. Comparison of different modalities of use of DEAE-Dextran and Polybrene as enhancers of HIV-1 infection on normal PHA-stimulated T cell lymphoblasts. Results of Immunofluorescence (IFA).

The virus stock (308,000 pg/ml of p24 Ag) was used at a 1:16 dilution (19,250 pg/ml). DEAE-Dextran was used at 5, 10 or 25 µg/ml. This last concentration was not used after infection as it had proved toxic to the cells. Polybrene was tested at 2.5, and 5 µg/ml. Both enhancers were used as follows:

A. During infection.

B. Pretreatment.

C. Pretreatment and during infection.

D. During and after infection.

E. Pretreatment, during and after infection.

Controls were uninfected cells and cells infected without enhancers. Pretreatment indicates an incubation period of 30 min. at 37^{0} C with the enhancers at the specified concentrations. Subsequently, cells were spun down, washed once and infected with virus in the presence or absence of enhancers as required for each culture. After infection cells were washed once and set in culture with or without enhancers as specified. A 2ml sample was collected, spun down and the supernatants stored for the p24 Antigen Assay. The cell pellets were inactivated with AMT/UV irradiation, washed in PBS and smears made on slides for IFA staining on days 3, 5 and 7 post-infection. Results for days 5 and 7 post-infection are shown.

TABLE 4
Immunofluorescence
(% of Positive Cells)

	5 DPI	7 DPI
Control (Infected alone)	7	13
A. During Infection		
- DD (5µg/ml)	6	11
- DD (10 µg/ml)	10	9
- DD (25 µg/ml)	15	9
- Polybrene (2.5 µg/ml)	2	20
- Polybrene (5 µg/ml)	7	13
B. Pretreatment		
- DD (5µg/ml)	2	5
- DD (10 µg/ml)	2	10
- DD (25 µg/ml)	1	8
- Polybrene (2.5 µg/ml)	4	12
- Polybrene (5 µg/ml)	4	10
C. Pretreatment and during		
- DD (5µg/ml)	6	10
- DD (10 µg/ml)	7	13
- DD (25 µg/ml)	5	10
- Polybrene (2.5 µg/ml)	6	17
- Polybrene (5 µg/ml)	5	7
D. During and after		
- DD (5µg/ml)	3	10
- DD (10 µg/ml)	5	10
- DD (25 µg/ml)	nd	nd
- Polybrene (2.5 µg/ml)	5	12
- Polybrene (5 µg/ml)	3	15
E. Pretreatment, during and after		
- DD (5µg/ml)	6	15
- DD (10 µg/ml)	10	11
- DD (25 µg/ml)	nd	nd
- Polybrene (2.5 µg/ml)	2	7
- Polybrene (5 µg/ml)	2	7

nd= not done DD=DEAE-Dextran

TABLE 5. Comparison of different modalities of use of DEAE-Dextran and Polybrene as enhancers of HIV-1 infection on normal PHA-stimulated T cell lymphoblasts. Results of the p24 Ag assay.

The virus stock (308,000 pg/ml of p24 Ag) was used at a 1:16 dilution (19,250 pg/ml). DEAE-Dextran was used at 5, 10 or 25 µg/ml. This last concentration was not used after infection as it was toxic to the cells. Polybrene was tested at 2.5, and 5 µg/ml. Both enhancers were used as follows:

A. During infection.

B. Pretreatment.

C. Pretreatment and during infection.

D. During and after infection.

E. Pretreatment, during and after infection.

Controls were uninfected cells and cells infected without enhancers. Pretreatment indicates an incubation period of 30 min. at 37^{o} C with the enhancers at the specified concentrations. Subsequently, cells were spun down, washed once and infected with virus in the presence or absence of enhancers as required for each culture. After infection, cells were washed once and set in culture with or without enhancers as specified. A 2ml sample was collected, spun down and the supernatants stored for the p24 Ag assay. Results for days 5 and 7 post-infection are shown.

TABLE 5
p24 Assay (ng/ml)

	5 DPI	7 DPI
Control (Infected alone)	15	40
A. During Infection		
- DD (5µg/ml)	22	94
- DD (10 µg/ml)	32	76
- DD (25 µg/ml)	94	59
- Polybrene (2.5 µg/ml)	6	33
- Polybrene (5 µg/ml)	2	49
B. Pretreatment		
- DD (5µg/ml)	12	35
- DD (10 µg/ml)	22	41
- DD (25 µg/ml)	11	27
- Polybrene (2.5 µg/ml)	10	23
- Polybrene (5 µg/ml)	12	40
C. Pretreatment and during		
- DD (5µg/ml)	36	87
- DD (10 µg/ml)	83	81
- DD (25 µg/ml)	14	19
- Polybrene (2.5 µg/ml)	21	42
- Polybrene (5 µg/ml)	21	52
D. During and after		
- DD (5µg/ml)	17	72
- DD (10 µg/ml)	22	41
- DD (25 µg/ml)	nd	nd
- Polybrene (2.5 µg/ml)	9	34
- Polybrene (5 µg/ml)	7	54
E. Pretreatment, during and after		
- DD (5µg/ml)	22	49
- DD (10 µg/ml)	52	45
- DD (25 µg/ml)	nd	nd
- Polybrene (2.5 µg/ml)	19	60
- Polybrene (5 µg/ml)	8	53

nd= not done DD=DEAE-Dextran

III.9. Effect of DEAE-Dextran and Polybrene on HIV-1 viral entry in normal T lymphoblasts and in T lymphoblasts from HIV$^+$ asymptomatic patients.

The previous experiments were concerned with the establishment of optimal conditions for infection of cell lines and normal T cell lymphoblasts. However, such experiments are not the best indicators of viral entry since a high viral dose can lyse the cells and high doses of polycations can also be toxic to the cells. Thus, the effect of the polycations on viral entry was determined soon after infection. The binding and uptake of virus immediately after exposure to the virus was measured. Uptake of virus was quantified by washing the cells followed by lysis of the cell pellet with Triton. The p24 antigen was measured in the cell lysate. As shown in Table 6, DEAE-dextran enhanced viral entry with p24 antigen values up to 100-fold higher than control values without enhancer. Polybrene is a modest enhancer by comparison, the p24 values increased only 8-fold over control values.

My main concern was to determine if the CD4$^+$ T cells from HIV-1$^+$ asymptomatic patients were also able to take up the virus. This was of utmost importance in CTL assays where these cells are used as targets. Table 7 indicates that the virus was incorporated and that DEAE-Dextran increased the viral entry. Results of the p24 Ag assay done right after infection and 24 hours later are shown in the table.

TABLE 6

Effect of DEAE-Dextran and Polybrene on HIV-1 viral uptake by normal T lymphoblasts immediately after infection and 24 hours later p24 Ag assay (pg/ml).

	Exp.#1		Exp. #2	
	After inf.	**24 h.after**	**After inf.**	**24h after**
A) Uninfected	0-	0	0	0
B) Infected alone	35	14	38	0
C) DEAE-Dextran				
1. During Infection (25 µg/ml)	3481	16	1420	15
2. Pre and during (10 µg/ml)	2140	930	1490	31
D) POLYBRENE				
3. Pre and during (5 µg/ml)	284	135	76	0

Mononuclear cells from normal donors were infected with a viral supernatant containing 95,000 pg/ml of p24 Ag. In some cases, as specified in the Table, the enhancers of infectivity were used as pretreatment and during the infection. For pretreatment, cells were incubated with DEAE-Dextran or Polybrene for 30 min. at 37° C. After the infection, the sample was washed 5 times in PBS. The cell pellet was lysed with 1 ml of PBS/1% Triton X-100. Cells were then spun down and the supernatant collected for the p24 Ag assay.

TABLE 7
Effect of DEAE-Dextran and Polybrene on HIV-1 viral uptake by T lymphoblasts from HIV-1 + asymptomatic patients

	p24 Ag (pg/ml)	
	After inf.	24 h.after
A) Uninfected		
Ctrl. (HIV-1 Negative)	0	0
Pt. 1.	Weak	0
Pt. 2	0	0
Pt. 3	0	0
Pt. 4	Weak	0
B) Infected alone		
Ctrl. (HIV-1 Negative)	41	0
Pt.1.	49	Weak
Pt. 2	50	0
Pt. 3	38	27
Pt. 4	71	Weak
C) DEAE-Dextran (Pre and during (10 µg/ml)		
Ctrl. (HIV-1 Negative)	725	58
Pt. 1	625	86
Pt. 2	715	19
Pt. 3	740	92
Pt. 4	2055	89
D) DEAE-Dextran (During Infection (25 µg/ml)		
Ctrl. (HIV-1 Negative)	960	109
Pt. 1	1105	196
Pt. 2	995	101
D) Polybrene (Pre and during (5 µg/ml)		
Ctrl. (HIV-1 Negative)	64	Weak
Pt. 1	143	Weak
Pt. 2	60	0
Pt. 3	141	25
Pt. 4	157	19

Mononuclear cells from patients were infected with a viral supernatant containing 95,000 pg/ml of p24 Ag. In some cases, as specified in the Table, the enhancers of infectivity were used as pretreatment and during the infection. For pretreatment, cells were incubated with DEAE-Dextran or Polybrene for 30 min. at 37° C. After the infection, the sample was washed 5 times in PBS. The cell pellet was lysed with 1 ml of PBS/1% Triton X-100. Cells were then spun down and the supernatant collected for the p24 Ag assay.

III.10. The susceptibility to infection by HIV-1 of normal T cell lymphoblasts varies with time in culture.

In several experiments, lymphoblasts from HIV-1 infected patients appeared to become resistant to infection after being in culture for more than 6 days. An experiment testing the susceptibility of lymphoblasts to HIV-1 kept in culture from 3 to 6 days was evaluated. Using the same lot of virus the cells were infected on day 3 and on day 6 after PHA stimulation. A representative experiment is shown in Fig. 17. The virus stock used for the infection contained 200,000 pg/ml of p24 Ag. As shown in the Figure 17, when the cells were infected 6 days after PHA stimulation, there was very little evidence of infection when compared with cells infected 3 days after PHA stimulation. The infection was undetectable by IFA on day 6. This experiment confirmed my observation and strongly suggested that infectivity experiments should be done 3 days after PHA stimulation.

In another experiment, the effect of enhancers of infectivity and viral replication was evaluated. As shown in Figure 18, on day 5 post-infection, although there was evidence of infection, the amount of p24 antigen produced by cells infected 6 days post-PHA stimulation was lower than the amount produced by cells that had been infected on day 3 (Fig. 18A). On the contrary, the results by immunofluorescence indicated that the infection of cells on day 6 post-stimulation was successful although still less effective than on day 3 post-stimulation (Fig.18B). There was an apparent discrepancy between the p24 antigen values and the immunofluorescence assay, suggesting that on cells infected on day 6 post-stimulation the release of virus to the supernatant was markedly decreased, but that enough virus was produced and retained inside the cell to give clearly positive results by immunofluorescence. In the experiments shown in Figure 18, DEAE-Dextran again appears to be the most effective enhancer of infectivity. The effect of TNFα was noticeable on day 7 post-infection in cells that were infected 6 days after PHA

stimulation (Fig.18C). This was seen only in increased production of p24 antigen when compared with infected but untreated control. The infection reached almost the same levels as those seen on day 5 post-infection with the use of DEAE-Dextran. On day 7 post-infection there was also an increase in the percentage of positive cells that were exposed to TNFα. (Fig.18D). The cells appeared to stain brighter than control cells suggesting that the amount of virus per cell may be higher. This seems to indicate that TNFα takes effect in low titer infections or infections that are kept in culture for longer periods.

Fig.17. The susceptibility to infection by HIV-1 of normal T cell lymphoblasts varies with time in culture.

Infections with a virus supernatant containing 200,000 pg/ml of p24 Ag, were done on the same blood sample 3 and 6 days after PHA stimulation. Samples were collected for the p24 Ag assay on days 0, 2, 3, 4 and 5 post-infection. The infection done 6 days after PHA stimulation shows very low levels of infection.

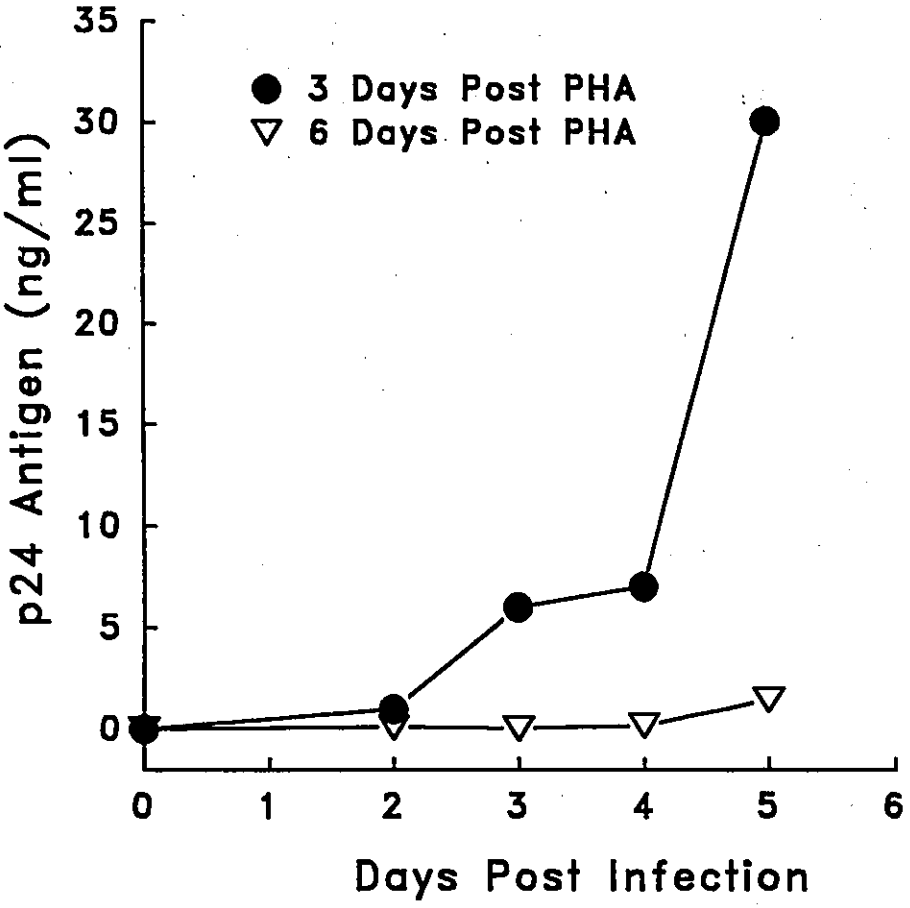
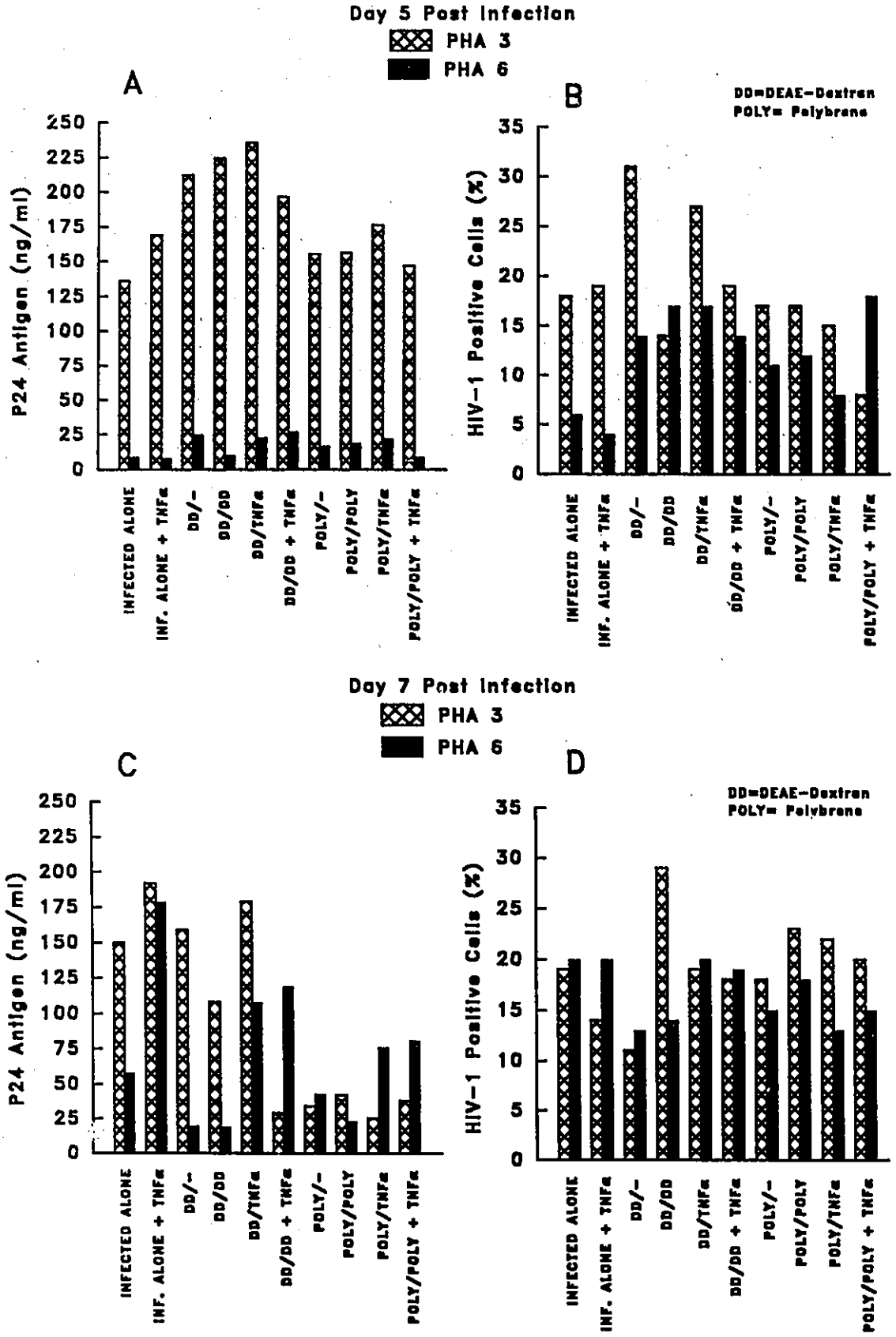

35
30
25
20
15
10
5
0
p24 Antigen (ng/ml)
3 Days Post PHA
6 Days Post PHA
0
1
2
3
4
5
6
Days Post Infection

Figure 18. The susceptibility to infection by HIV-1 of normal T cell lymphoblasts varies with time in culture. Effect of enhancers on infectivity and viral replication.

A virus stock containing 308,000 pg/ml of p24 Ag was employed. A dilution of 1:16 (19,250 pg/ml) was used for infection on day 3 or 6 post-PHA stimulation of cells. Enhancers were added as shown in the figure. Cells were infected for 2 hours at room temperature in the presence of either DEAE-Dextran (10 µg/ml) or Polybrene (2.5 µg/ml). They were washed once and set in culture medium containing IL-2 and 2-Mercaptoethanol with or without the enhancer. TNFα (5 ng/ml), when used, was added to the culture medium after infection. Three days later, cultures were split 1:3 with medium exchanges containing the same enhancers. Controls were uninfected cells and cells infected without enhancers. For the p24 Ag assay (A, C), a sample was collected, spun down and the cell culture supernatant tested on days 3, 5 and 7 post-infection. The cell pellets from the samples were inactivated with AMT/UV irradiation (see Materials and Methods), washed and resuspended in PBS. Smears were prepared for IFA staining (B, D). Results for days 5 and 7 post-infection are shown.


Day 5 Post Infection
PHA 3
PHA 6
A
B
DD=DEAE-Dextran
POLY= Polybrene
P24 Antigen (ng/ml)
HIV-1 Positive Cells (%)
INFECTED ALONE
INF. ALONE + TNFα
DD/-
DD/DD
DD/TNFα
DD/DD + TNFα
POLY/-
POLY/POLY
POLY/TNFα
POLY/POLY + TNFα
Day 7 Post Infection
PHA 3
PHA 6
C
D
DD=DEAE-Dextran
POLY= Polybrene
P24 Antigen (ng/ml)
HIV-1 Positive Cells (%)
INFECTED ALONE
INF. ALONE + TNFα
DD/-
DD/DD
DD/TNFα
DD/DD + TNFα
POLY/-
POLY/POLY
POLY/TNFα
POLY/POLY + TNFα

III. 11. Studies of Cytotoxic T lymphocytes (CTL)

In the following sections the results analyzing the specific CTL activity against HIV-1 from asymptomatic patients are described.

III.11.1 Cloning of Cytotoxic T Lymphocytes (CTL).

$CD8^+$ T cells from HIV-1^+ asymptomatic patients were cloned by limiting dilution of blood mononuclear cells. The generation of clones requires the presence of irradiated autologous or heterologous feeders or "filler cells" which provide the necessary accessory molecules and cell density. Cells were initially activated with PHA (0.5 μg/ml). Feeder cells were added the first day and at two-week intervals. Instead of using PHA for the required periodic re-stimulation of the clones, feeders were treated with Neuraminidase/Galactose oxidase (see Materials and Methods). This is a non-antigen-specific stimulation for CTL clones. The method is a substitute for the use of mitogens like PHA which can be toxic for cells in long-term culture (Fleischer, 1988). This medium with feeder cells is called expansion/cloning medium. IL-2 (100 U/ml) and IFNγ (200 U/ml) were also added to the medium. These cytokines synergize in producing proliferation and differentiation into CTL. Between expansions, medium exchanges were done with feeding medium (no feeders). This method of cloning was developed based on reports from the literature (Walker et al., 1989) as well as on my own experience over many cloning experiments. With this method the cloning efficiency was very high and we were able to maintain clones for weeks, allowing testing for CTL activity. Table 8 shows the efficiency of the cloning system for 7 experiments.

TABLE 8
Cloning of CTL

Experiment #	Total # of wells	No. of wells with clones	Cloning Efficiency (Percentage %)
1	80	25	31
2	80	15	19
3	180	79	44
4	180	34	19
5	240	93	39
6	240	111	46
7	310	132	43

PBMC from HIV-1^+ asymptomatic patients were seeded at limiting dilution in 96-well plates in a final volume of 0.2 ml containing IMDM/20% FCS, irradiated autologous feeder cells, IL-2 (100 U/ml), 2-Me, and IFNγ (200 U/ml). Between 11 and 14 days later, proliferating clones were subcultured and expanded into 5 or 10 wells each and later in 24-well plates.

PBMC from the same blood sample were stimulated with PHA and separated 7-14 days later into CD4 and CD8 fractions with the use of magnetic beads. The $CD4^+$ T lymphocytes were kept in culture with Castanospermine (25 µg/ml). Castanospermine is an anti-HIV drug that acts by extensive modification of the glycosylation of the envelope glycoprotein. The abnormal glycoprotein can no longer be processed to its gp120 and gp41 portions. Such molecule renders the virus unable to gain entry to target cells thus inhibiting the spread of virus to uninfected cells. The CD4 cells were used as targets in a CTL assay. The target cells were prepared without using enhancers of infection. The effector cells were clones that were more than 60% $CD8^+$.

Table 9 shows the percentage of positive clones in the CTL assays.

TABLE 9

CTL assay

Experiment #	Percentage of Positive Clones
1	40% (10/25)
2	27% (4/15)
3	6% (2/31)
4	0% (0/3)
5	13% (3/24)
6	0% (0/12)
7	5% (3/61)

The CD4 T lymphocytes used as targets were infected with HIV-1 virus supernatant for 2 hours. The infection was done between 18 and 43 days after the initial PHA stimulation of the cells. The clones were tested for their CTL activity in a 6 hr. ^{51}Cr assay. Spontaneous release was always 18-20% of maximum release. Effector:Target ratio was 3:1. Cytotoxicity of the clones varied from 3 to 88%.

The preparation of the CD4 T lymphocyte as target cell was problematic. These cells died rapidly in culture. Fresh cultures had to be started from frozen stocks. In some cases they were difficult to label with ^{51}Cr. Results were not easily reproducible due to these difficulties.

III.11.2. CTL assays with improved infection protocol of the CD4 T-lymphocytes used as Target Cells.

The results in the previous section demonstrate that in order to obtain CD4+ targets, enhanced infectivity of these cells had to be achieved. The results in Figure 18 showed that infection of cells 3 days after PHA stimulation, with or without enhancers, was successful and these cells could be employed in CTL assays. Based on these results, cells from HIV-1+ asymptomatic patients were infected 3 days after PHA stimulation with the optimal titer of a virus supernatant (1/3 dilution of 285,000 pg/ml of p24 Ag). One experiment was done without enhancers of infectivity, one with DEAE-Dextran (DD), 25 μg/ml during infection and two more with DD as pretreatment and during the infection at 10 μg/ml. To determine viral entry a sample of cells was lysed 24 hours after infection and the presence of the virus tested with a p24 Ag assay (Table 10). Incubation of the cells after infection with the virus was either 24 or 48 hours and the time of the ^{51}Cr release assay was at 4, 6 or 18 hours. The effector cells were $CD8^+$ T cells obtained by separation of $CD8^+$ T cells using CD8 Dynabeads at low bead to cell ratio (3-5:1). The beads were removed after 24 hours in culture. The $CD8^+$ T cells were maintained in culture with IL-2-containing growth medium. For the CTL assay the effector-to-target ratio varied from 40:1 to 5:1. In some experiments (6 or 18 h Cr. test) spontaneous release was more than 55% of total release. This invalidated these results which are not included in Table 11. This Table 11 shows the results of the experiments in which the percentage of the spontaneous release was between 4 and 25% of total release. No CTL activity was observed in spite of the improved preparation of the target cell.

TABLE 10

Viral Entry Assay in CD4$^+$ T lymphocytes from HIV-1$^+$ asymptomatic patients to be used in a CTL Assay.

Subject	p24 Ag. (pg/ml)	Enhancer of Infectivity
Control. (HIV$^-$)	243	DD (25)
Pt. 1	939	"
Pt. 2	147	"
Pt. 3	376	"
Control (HIV$^-$)	4225	DD.(10)
Pt. 4	2260	"
Pt. 5	4300	"
Pt. 6	9130	"
Pt. 7	4205	"

DD (25)=DEAE-Dextran, 25 µg/ml during infection.
DD (10)=DEAE-Dextran, 10 µg/ml as pretreatment and during infection.
Cells were washed 5 times 24 hours after infection and lysed with PBS/1% Triton X-100. They were spun down and the supernatant collected for the p24 Ag assay.

TABLE 11

HIV-1-specific CTL Assays from asymptomatic patients.

Subject	^{51}Cr.Release %				Virus	^{51}CrTest (hours)	DD
E:T*	40:1	20:1	10:1	5:1			
No DD**							
Ctrl. (HIV$^-$)	n.d.	1.4	1	1.4	Pellet	4	no
Pt. 1	n.d.	0	0	1	"	"	"
Pt. 2	n.d.	3	0	0	"	"	"
Pt. 3	n.d.	0	0	1	"	"	"
DD**(25 µg/ml)****							
Ctrl. (HIV$^-$)	n.d.	n.d.	0	0	S.N.	4	DD (25)
Pt. 4	n.d.	0	0	0	"	"	"
Pt. 5	n.d.	n.d	0	0	"	"	"
Pt. 6	n.d.	1	0	1	"	"	"
DD***(10 µg/ml)*****							
Ctrl.	0	0	1.3	0	S.N.	6	DD (10)
Pt. 7	6	1.3	0	0	"	"	"
Pt. 8	0	0	0	0	"	"	"
Pt. 9	8	1	1	2	"	"	"
Pt. 10	2	0	3	1.2	"	"	"

S.N.= virus supernatant n.d.= not done DD= DEAE-Dextran

Virus S.N.= 95,000 pg/ml of p24 Ag.

* E:T=Effector/Target Ratio

** 24 hrs of incubation with virus

*** 48 hrs of incubation with virus

**** DD during the infection (2hrs)

***** DD as pretreatment (30 min.) and during infection (2hrs).

III.12. Macrophage as Target Cell in a CTL Assay.

The results reported in the previous sections strongly indicate that $CD4^+$ T cells from HIV-1 infected patients are not suitable target cells. Experiments were performed to determine if the blood monocyte could be employed. In experiments done in this laboratory, blood monocytes from HIV-1 infected patients, when placed in culture in the presence of Granulocyte-Macrophage Colony Stimulating Factor (GM-CSF), show evidence of HIV-1 infection (multinucleated giant cells, HIV-1^+ by immunoperoxidase) 7 to 14 days after culture initiation. Thus, macrophages were cultured without reinfection in the presence of $CD4^+$ T cells but depleted of $CD8^+$ T cells. These latter cells were cultured separately to be used as effector cells. Peripheral blood mononuclear cells (PBMC) from HIV-1-positive asymptomatic patients were obtained by Ficoll-Hypaque. HIV-1-negative donors were used as controls. Monocytes were obtained from buffy coat by $CD8^+$ T-cell depletion (CD8 Dynabeads) and adherence to plastic. At no point were they superinfected *in vitro* with HIV-1. Supernatant was collected on days 5, 7 and 10 to determine p24 Ag expression. CTL relative frequencies were derived by incubating effector CD8 T-lymphocytes with ^{51}Cr-labelled macrophages at different effector:target ratios. ^{51}Cr release was measured after an 18-hour incubation because macrophages are relatively resistant to cytolysis (Hoffenbach et al., 1989). Table 12 shows that after 10 days in culture PHA-stimulated CD8 T-lymphocytes contained HIV-1-specific CTL. These results suggest that blood-derived macrophages are suitable target cells for the CTL assay. Another advantage is that we can use the virus that has infected the patient rather than a laboratory strain of virus.

In future experiments using this type of assay, several controls should be included. The most important one should be able to determine if the cytotoxicity is due to Natural Killer (NK) cells instead of CD8 T lymphocytes. To this effect the effector cells should be depleted of NK cells with the use of magnetic beads expressing the

specific marker for NK cells. Another control should include the inhibition of the specific cytotoxic response with the use of mAbs against the CD3/T-cell receptor complex. A third control should include heterologous non-infected macrophages as targets as well as autologous macrophages infected with a different virus in order to define the MHC-restriction and HIV-1-specificity of the response.

TABLE 12

HIV-1-specific CTL from PBMC from infected individuals.
Target Cell: Macrophage.

Subject	Status	Assay Time (hours)	Percent ^{51}Cr. Release		
			20:1	10:1	5:1
1.(Ctrl)	HIV-Neg.	18	0	0	0
2.	HIV-Pos.	18	52	11	5
3.	HIV-Pos.	18	12	11	6

PBMC were obtained by Ficoll-Hypaque. To obtain macrophages, the buffy coat was depleted of CD8 T-lymphocytes with the use of magnetic beads and adherence to 12-well plates at 2 x 10^6 cells per ml in 1 ml volumes. On day 10 they were harvested with PBS/10%EDTA, labelled with ^{51}Cr and tested in an 18-h assay. Effector cells were the CD8-positive T-lymphocytes from the same patient, stimulated with PHA (1 µg/ml) and kept in 20%FCS/IMDM and IL-2 (50 U/ml) for 10 days.

IV. DISCUSSION

IV.1. HIV-1-specific Cytotoxic T Lymphocytes (CTL).

The cell-mediated immune response gives protection against viruses or parasite infections by killing the infected cells. In the case of HIV-1, there is a strong HIV-1-specific CTL response. CTL have been detected in the lungs of infected patients against alveolar macrophages (Plata et al., 1987a), in the lymph nodes and in the cerebrospinal fluid of patients with neurological disorders (Sethi et al., 1988). CTL have been detected in fresh PBMC from HIV-1-positive patients, either asymptomatic or with AIDS. A surprising finding (Hoffenbach et al., 1989) is that peripheral blood lymphocytes (PBL) from seronegative donors also respond to HIV-1 stimulation *in vitro* and large numbers of HIV-1-specific CTL can be generated from PBL. The relevance of CTL for protection against HIV-1 is a controversial subject. Even though the virus appears to generate a strong CTL response, this response does not seem to be enough to control the spread of the virus. The CTL response also mediates inflammatory damage in the host's tissues.

CTL belong to the CD8 subset of T lymphocytes and are restricted by Class I MHC. A subset of CD8 T lymphocytes can control HIV-1 infection *in vitro* by suppressing virus replication (Tsubota et al., 1989a; Walker et al., 1986; Joly et al., 1989; Kannagi et al., 1988). The HIV-1 specific CTL response drops to undetectable levels in the final stages of the disease. It is unclear why this happens. The patients maintain normal cytolytic function, but seem to lose the HIV-1-specific cytotoxicity (Pantaleo et al., 1990b). This is probably due to an increase in a subset of $CD8^{+}DR^{+}$ cells that lack the receptor for Interleukin-2 (CD25). These T lymphocytes no longer respond to the HIV-1 antigen stimulation. The expansion *in vivo* of $CD8^{+}DR^{+}$ cells is common to other viral infections such as Epstein Barr Virus (EBV) and Cytomegalovirus. It also occurs in bone marrow transplantation and certain chronic inflammatory diseases such as

systemic lupus erythematosus and rheumatoid arthritis. Thus, the appearance of this subset may not be specific for HIV-1 infection, but appears to be common whenever a chronic stimulation of the immune system occurs (Pantaleo et al., 1990a).

A good CTL assay would help to define the epitopes in the target cell that are being recognized by the cytotoxic T cells. A major part of human CTL responses to HIV-1, *gag*, *pol*, *vif* and *nef* proteins seem to be directed against conserved epitopes. The viral antigens to be incorporated in the design of a future vaccine should be selected from these regions. The *env* protein exhibits a great degree of variability with the exception of a small sequence in the V3 loop. The variable part of the *env* protein has the disadvantage that it can cross-react with self antigens (Cheng-Mayer et al., 1988b). Therefore, more detailed definition of these epitopes is of great importance.

Since the $CD8^+$ T cells can suppress HIV-1 replication in infected lymphocytes, the cloning of lymphocytes has a dual purpose: First, the $CD8^+$ clones can be used in CTL analyses. Second, they can be kept frozen for later use in cell therapy for the same patient via adoptive transfer.

The $CD4^+$ population in a patient decreases with time during the HIV-1 infection. Therefore, the CTL assay using the autologous CD4 T cell as target can only be useful during the asymptomatic phase or in the early stages of the disease.

In CTL assays, the following cells have been used as targets: Autologous $CD4^+$ T lymphocytes (Hoffenbach et al., 1989), EBV-immortalized lymphoblasts (Malkowsky et al., 1988; Blumberg et al., 1987a&b; Dahl et al., 1987), alveolar macrophages (Plata et al., 1987a; Hoffenbach et al., 1989; Autran et al., 1988), HLA-matched macrophages adsorbed with UV inactivated HIV-1 or purified gp120 (Weinhold et al., 1988; Sethi et al., 1988). In this case the viral proteins are captured, processed and presented as peptides. HLA-matched EBV-immortalized B cells infected with Vaccinia virus recombinants that express genetic sequences of *gag*, *rev* or RT of the HIV-1 genome (Walker et al., 1987a&b and 1988; Nixon et al., 1988). The problem with this type of

target is the extreme toxicity of Vaccinia virus to the cells which leads to increased background in the ^{51} Cr release assay. Human blood monocytes are also excellent targets for the CTL Assay. Their convenience lies in the fact that they can be radiolabelled immediately after isolation or after culture for as long as 7 days. They also take up the ^{51}Cr completely with low spontaneous release (Young and Steinman, 1987).

A major problem in determining CTL specificities is the lack of appropriate target cells expressing well-defined HIV-1 antigens. Since the CTL killing is MHC restricted, both effector and target cells have to share some of the HLA Class I or Class II antigens. HLA A1, A2, A3 and A9 antigens have been shown to associate with HIV-1 antigen for CTL recognition (Langlade-Demoyen et al., 1988). A single HLA antigen can serve as the restricting element for more than one epitope (Walker et al., 1989). Selecting the autologous CD4 T-lymphocyte as target complies with this requisite.

My approach consisted of generating clones from HIV-1-positive asymptomatic patients. These clones were tested for their cytotoxic activity against autologous CD4$^+$ T lymphocytes that had been superinfected *in vitro* with HIV-1. The advantage of cloning is that a single CTL cell is expanded into a clone. If the clone is HIV-1-specific the CTL response is greatly enhanced.

IV.1.1. Cloning of Cytotoxic T-Lymphocytes.

The frequency of HIV-1 specific CTL among the circulating lymphocytes of HIV-1 infected patients is very high in the early stages of the disease. In contrast to other viruses, there is no need for specific antigenic stimulation *in vitro* to detect these HIV-1 specific CTL. This, however, is a requirement to derive CTL from seronegative donors. Several stimulation strategies are used to promote the expansion of CTL *in vitro*. These include: Phytohemagglutinin (PHA), concanavalin A, anti-CD3 monoclonal antibody, HIV-1-infected autologous or heterologous irradiated lymphoblasts and irradiated whole virus.

In my experiments to derive CTL clones I used PBMC from HIV-1 positive asymptomatic patients. The initial stimulation was done with PHA. Subsequent stimulations were done every two weeks with heterologous irradiated feeder cells treated with Neuraminidase/Galactose Oxidase (see Materials and Methods). This is a non-antigen-specific stimulation for CTL clones. By introducing aldehydes into galactose residues on stimulator cells, clones are stimulated without the use of lectins like PHA. This avoids the toxic effects of this mitogen. Also the use of lectins can lead to mutual killing if the clones possess cytotoxic activity (Fleischer, 1988). The clones I generated were not exposed to exogenous viral antigen at any time.

IFNγ was added to the cloning medium the first day of PHA stimulation. IFNγ has been shown to synergyze with IL-2 to induce proliferation and differentiation into CTL (Chen et al., 1986; Maraskovsky et al., 1989).

The results I obtained with this cloning protocol were promising. I had a high percentage of proliferating clones which were kept in culture for several weeks. Clones were phenotyped by Flow Cytometry and those that were more than 60% CD8-positive were selected to be used as effectors in the CTL assay. They were tested 4-6 weeks after cloning for their CTL activity. The percentage of wells that showed CTL activity varied from 0 to 40%. Even though a response could be generated, results were not consistent. I encountered problems with the autologous $CD4^+$ T lymphocytes that were used as targets. For this reason the clones that had been generated from the HIV-1^+ asymptomatic patients were cryopreserved in liquid nitrogen for future evaluation.

IV.1.2. CD4 T-lymphocyte as Target Cell in the CTL Assay.

The autologous CD4 T lymphocytes that I used as target cells proved to be a difficult cell to grow and properly reinfect with HIV-1 before the CTL assay. In some experiments the $CD4^+$ T lymphocyte could not be properly labelled with the ^{51}Cr.

An investigation of these problems led to a series of experiments to improve the HIV-1 infection of T cells. With this objective, experiments were carried out in a human T cell line, HUT-78, and in normal blood T lymphocytes. This project represents a major aspect of this thesis.

I defined the following areas that needed further evaluation: 1) Titration and characterization of the HIV-1 viral supernatants used for infection. 2) Evaluation of the effect of long-term storage on the viability of virus supernatants. 3) Evaluation of the use of DEAE-Dextran, Polybrene and TNFα as enhancers of viral infectivity. 4) Determination of the best time for infection after PHA stimulation of the target cell.

Since HIV-1 strains are heterogenous in their host range, infections are best defined if the virus is studied in a cell line and is compared to the corresponding primary cell. For this reason I selected a T-cell line, HUT-78, and normal T lymphocytes to titrate the virus and to test the enhancers of infectivity.

IV.2. Target Cell Preparation

IV.2.1. Titration and Quantization of viral supernatants.

In spite of the importance of quantitating HIV-1 infection *in vivo* and *in vitro*, developing standards for titration and quantization of virus expression has been difficult. One needs to evaluate the infection with more than one assay.

Various assays can be used to detect the infection. The p24 antigen assay provides an idea of the amount of virus particles produced, but it is not an indicator of the viability of the virus supernatant. In a patient, HIV-1 antigenemia is significant at the period of seroconversion, before the appearance of HIV-1 antibodies and at the terminal stages of infection. Consequently, the p24 Ag assay is a good indicator of the progression of the disease. IFA gives an approximation of the number of cells infected, but not of the infectious particles produced. A drawback with this assay is that infected cells are not detected until 3 days after the infection. The CPE is an indicator of the biological

activity. In strains of virus that produce this effect, it is a good early indicator of the titer and viability of the viral stock. The CPE consists of syncytium formation and single-cell lysis. It occurs by the fusion of the HIV-1 envelope glycoprotein gp120 with the CD4 molecule. These membrane fusion events can be disrupted by mutations in the transmembrane region of the gp41 (Kowalski et al., 1991).

HIV-1 strains may differ in pathogenicity. They may even evolve to increased virulence in the course of infection in the same individual. For this reason it is important to develop a sensitive, specific, quantitative infectivity assay for HIV-1 in an established cell line. In my case I found that the virus strain I used was highly cytopathic for the HUT-78 cell line, even though it did not produce syncytia formation in normal T cells. In HUT-78 cells, syncytia formation begins to appear 3 days after infection and peaks by day 9 or 10 post-infection. One can correlate the amount of p24 antigen used for the infection with the CPE produced.

The titration experiments were done in HUT-78 cells and normal T cell blasts. Virus supernatants (stocks) were produced in our laboratory by infecting HUT-78 cells. Various lots were kept frozen at -80^{0}C. I concluded from the titration of some of these stocks that a viral supernatant of >100,000 pg/ml of p24 antigen is required to obtain a strong infection that is easily detectable.

I monitored the infections done in all experiments with the three assays mentioned above: p24 Ag assay (Abbott), IFA and CPE.

IV.2.2. Effect of long-term storage on the viability of viral stocks.

The viability of the virus supernatants decreases with long-term storage at -80^{0}C. I proved this by repeating titrations with the same stock at 6-week intervals. It is therefore recommended that the virus stock be titrated for each experiment. The infection should be evaluated by observing the CPE to determine the viability of the lot. An advantage of the CPE assay, in cases where the virus is cytopathic and the cell line is

susceptible to the cytopathic effect, is that an easier titration can be made without further processing or the measurement of other parameters (IFA, P24 Ag). The HUT-78 cell line is more receptive to infection if it is kept in culture only for a maximum of 20 passages (approximately one month). Therefore new cultures should be started at one-month intervals.

IV.2.3. Enhancers of viral infectivity.

My purpose in increasing infectivity of the HIV-1 virus was twofold: 1) To obtain a quick, strong infection that would produce high titer viral supernatants and thereby permit us to maintain fresh viable stocks in the Lab. 2) To obtain a fast, acute infection of the $CD4^+$ T lymphocyte to be used as target cell in a CTL Assay.

I chose three enhancers of infectivity for this purpose: DEAE-Dextran and Polybrene, two polycations that facilitate virus entry into the cell, and a cytokine, Tumor Necrosis Factor-Alpha.

i. DEAE-Dextran.

DEAE-Dextran is a polycation that enhances the cellular uptake of infectious viral RNA, DNA or intact viruses. Levels of DEAE-Dextran as low as 30 µg/ml are sufficient to lend some protection against nuclease action (Maes et al., 1967). As early as 1968 its use was reported to enhance infectivity in plaque assays of Simian Virus 40 (McCutchan & Pagano, 1968) and in Murine Sarcoma Virus (MSV) (Duc-Nguyen, 1968). The infectivity depends on the molecular weight (MW) of the polycation. In these early experiments the highest titer of infectivity was obtained in experiments with the standard compound of MW 2,000.000. The DEAE-Dextran I used has a MW of 5.000,000. Duc-Nguyen, in his work with MSV in 1968, reported the use of DEAE-Dextran (25 µg/ml) as pretreatment for 90 minutes at 37^0 C. He reported that a two-hour incubation was too toxic for the cells. The pretreatment was followed by three washings

of the cells to remove all traces of the DEAE-Dextran before the infection. He found that DEAE-Dextran at 50 µg/ml is very toxic for the cells.

Recently DEAE-Dextran has been used to isolate HIV-1 from peripheral blood mononuclear cells (PBMC) from infected individuals. For this technique, DEAE-Dextran is used at 10 µg/ml (Coombs et al., 1989). Its use has also been reported as pretreatment (25 µg/ml - 30 min at 37^{o} C) before the infection of fibroblasts and epithelial cells with HIV-1 (Ikeuchi et al., 1990; Tateno et al., 1989; Levy et al., 1985).

I wanted to explore the use of DEAE-Dextran as pretreatment, during and after the infection at various concentrations. The testing was done in a human T cell line, HUT-78 and in normal T cell lymphoblasts. Based on the previous reports in the literature, I chose the 30-minute pretreatment and the highest concentration I tested was 25 µg/ml. My results were consistent with the previous findings in terms of toxicity. If DEAE-Dextran is kept in the culture medium after the infection (>10 µg/ml), cells die quickly. At 25 µg/ml it could only be used either as pretreatment, or during the infection without toxic effects. But in terms of infectivity, I found that pretreatment alone with all the concentrations tested did not have any effect. Optimal viral infectivity was obtained using 10 µg/ml as pretreatment and during the infection. A suboptimal viral infection was obtained using 25 µg/ml during infection followed by extensive washing of the cells. I prefer the first option since cells look healthier in morphology as seen by microscope both on the IFA slides and in culture when a less toxic concentration is used. The fast increase in infectivity obtained with the use of DEAE-Dextran, as mentioned above, was very useful for my particular goals.

ii. Polybrene

The use of this polycation has been reported by a number of investigators in the infection of cells with HIV-1. For example, it has been used at 2-2.5 µg/ml in the co-cultivation method to isolate virus from PBMC from seropostive individuals (Castro, et

al., 1988; Sundqvist et al., 1989), to infect the CEM line at 2 µg/ml as pretreatment for 24 hours (Schmitt et al., 1990) or for 2 hours (Sethi et al., 1988), and to infect a CD4 cell line, being kept in the culture medium for as long as 3 weeks (Gregersen et al., 1990). For other immortalized $CD4^+$ cell lines like Jurkat, HUT-78 and MOLT-4, it has been used during the infection at 10 µg/ml (Cann et al., 1990) or at 1 µg/ml as pretreatment (Malkowsky et al., 1988). For $CD4^+$ T cells or peripheral blood macrophages it has been used at 2 µg/ml as pretreatment (Cheng-Mayer et al., 1990). For EBV-immortalized B cells, it has been used at 2 µg/ml during infection (Tozzi et al., 1989).

In view of the various reported possibilities, I decided to titrate Polybrene in HUT-78 cells and normal T cell blasts. I tested it as pretreatment as well as during and after the infection. I found that Polybrene was less toxic for the cells, as evaluated by viability and general appearance of the cells in culture and in the IFA slides. This explains its use in the co-cultivation method for virus isolation which requires several days in culture. In my experiments, pretreatment alone for 30 min. at 37^0 C did not harm the cells, but did not increase the infectivity of the virus either. When it was used as pretreatment and during the infection at 2.5 or 5 µg/ml, infectivity increased by day 7 post- infection in normal T cells. This peak of infection, as determined by the p24 antigen expression, happens 2 days later than when DEAE-Dextran is used. I noticed that in the HUT-78 cells, the use of Polybrene produced bigger and brighter syncytia formation, but the degree of infection was lower than when DEAE-Dextran was used. The use of Polybrene is therefore recommended when cells are going to be kept in culture for several days and when a less acute infection is required.

iii. Tumor Necrosis Factor (TNF).

There are two types of TNF: TNFα (Cachectin) secreted by activated macrophages and TNFß (lymphotoxin) secreted by activated lymphocytes. They share the same receptors which appear on virtually all somatic cells. TNFα has higher affinity

for the receptor than TNFß. They have pleiotropic activities in vitro and in vivo, including cytotoxic effects against tumors and virus-infected cells. TNF is a common mediator of inflammation and the wasting syndrome (cachexia), which is commonly observed in chronic infections and neoplastic disease. TNFα has been shown to enhance HIV-1 replication in T cells and in primary macrophages (Vyakarnam et al., 1990; Lacoste et al., 1990; Mellors et al., 1991; Folks et al., 1989). The mode of action is by activation of the nuclear factor kB (NFkB), which then stimulates the HIV-1 enhancer at the long terminal repeat (LTR). This leads to increased virus transcription (Osborn et al., 1989; Duh et al., 1989). Since HIV-1 infected patients secrete significant higher levels of TNFα than controls, this autocrine production can increase transcription of the virus leading to enhancement of the infection.

It seems that in normal T lymphocytes, events linked to T-cell activation, in addition to the translocation from the cytoplasm of the NFkB, are necessary for the interaction of NFkB with the HIV-1 enhancer (Hazan et al., 1990). This is to be expected since resting T cells lack specific binding capacity for TNFα. The receptors are induced upon activation of the T cells. Maximum expression occurs at day 6 and subsequently declines (Scheurich et al., 1987). This finding correlates well with my finding that TNFα increased infectivity in cells that had been infected 6 days after PHA stimulation. The titration experiments indicated that 5 ng/ml was the optimal concentration for normal T cell blasts. I also noticed an increase in infectivity with TNFα on cells that had been infected in the presence of DEAE-Dextran, but the increase was not significant. The HUT-78 cell line was relatively insensitive to the effect of TNFα.. In general, I can say that the effect of TNFα is not noticeable during the first days after infection but would benefit virus infectivity in cultures that are kept for longer periods.

One of the most important findings was the fact that activated $CD4^+$ T lymphocytes do uptake the virus immediately. The p24 Ag assay done on lysed cells right after infection and 24 hours later showed high levels of this antigen. The highest

value of p24 Ag expression was obtained when DEAE-Dextran was used as pretreatment and during infection at 10 μg/ml. The use during infection at 25 μg/ml also gave good results in terms of p24 Ag levels but I prefer the first option because it is less toxic to the cells. Polybrene also increased the uptake of the virus, but not as much as DEAE-Dextran. This was expected from the results of my previous experiments.

In summary, there appeared to be both experiment-to-experiment and donor-to-donor variation. Despite this biological variation, there was a consistent pattern within each experiment, both in HUT-78 and normal T lymphocytes. Namely, DEAE-Dextran at 10 μg/ml as pretreatment and during the infection, or at 25 μg/ml during the infection, produced the highest levels of infection. Polybrene came next with infections peaking on the average 2 days later and cells looking healthier in morphology as seen by microscope both in the IFA slides and in culture. The optimal combination for Polybrene was at 2.5 or 5 μg/ml as pretreatment and during the infection. TNF-α took effect later. Its use at 5 ng/ml would benefit infections that have to be kept for weeks.

IV.2.4. CTL Assays with the improved infection protocol of the $CD4^+$ T-lymphocyte.

After completing the experiments with the enhancers of infectivity, the CTL assays were repeated with HIV-1^+ asymptomatic patients' samples. The $CD4^+$ T cells were infected alone or in the presence of DEAE-Dextran (10 or 25 μg/ml during infection). The infection was done with newly titrated virus supernatant 3 days after PHA stimulation of the cells. The infected cells were kept in culture for 24 or 48 hours before the CTL assay to allow for the expression of the antigen on the surface of the cells. I established that the virus was internalized after infection but could not determine whether or not it was expressed on the surface since by IFA, the earliest I had detected the HIV-1 antigens in my previous experiments was day 3 post-infection.I believe that further studies of the kinetics of virus replication and its expression on the cell surface are

required. My assumption, based on studies done in antigen processing, is that antigen is expressed on the surface in 18 to 36 hours even if it is not detected by IFA. For this reason the CTL experiments were done 24 or 48 hours after re-infection of the target cells. Cytotoxicity assays were performed with a 4 or 6-hour incubation period. Some researchers use an 18-hour incubation period for resistant target cells like the CD4 T-lymphoblast and the human macrophage (Hoffenbach et al., 1989; Chenciner et al., 1989; Plata et al., 1987a). Nevertheless, in several of my experiments using the CD4 T-cell as target, the 18-hour incubation period gave me very high spontaneous release (>50% of Total Release) which invalidated the results. The labelling of the CD4 T cells with ^{51}Cr was not consistent. I cannot explain these differences in the uptake of ^{51}Cr which were present even with the uninfected controls. It seems that the problem lies with the T cell regardless of whether it is HIV-1 infected or not. With the 48-hour incubation, after the infection of the cells and before the ^{51}Cr assay, I obtained a low percentage of cytotoxicity in some experiments. Nevertheless, from all the evidence so far in my experiments I believe that the CD4 T-cell is not a very sensitive target to detect spontaneous HIV-1-specific CTL activity in the peripheral blood of patients. HIV-1-specific CTL precursor cells decrease in number or lose their specificity as the infection progresses toward AIDS. This could explain in part the lack of positive results. Another possible explanation for the lack of CTL response is the fact that the patients' CD4 T lymphocytes are re-infected *in vitro*. The HIV-1 strain used for the infection has been passaged and selected for growth in an immortalized cell line. The patient's viral strain and the laboratory strain have differences in the viral T cell epitopes which may not permit recognition by the CTL from the patient. It is similar to what happens to the antigenic variability of B cell antigens in infected individuals. The immunodominant epitopes induce clonal amplification of certain CTL which prevent or suppress the differentiation of other CTL that could recognize other epitopes. This explains the inability of the CTL response to control the spread of the virus. Nevertheless, since I

was using HIV-1/HXB2, a prototypic laboratory strain whose reactivities may be different from the most common strain HIV-MN, I decided to use whole virus for the infection expecting enough cross-reactive epitopes to activate the CTL response. I believe that to obtain a CTL response, this would have to be generated *in vitro* by culturing the CD8 cells with autologous irradiated cells infected with the HIV-1 strain used in the Laboratory.

IV.3. Monocyte/Macrophage as alternative Target Cell for the CTL Assay.

A number of investigators (Collman et al., 1989; Young and Steinman, 1987; Massari et al., 1990, Mann et al.,1990; Koyanagi et al., 1988 and others) have demonstrated that the monocyte/macrophage can be infected *in vitro* with HIV-1. The infection of macrophages does not result in the cytopathic effect that is seen in $CD4^+$ T lymphocytes. To obtain these cells for a CTL assay, monocyte-enriched populations (depleted of $CD8^+$ T lymphocytes) from HIV-1-positive asymptomatic patients are cultured for 10 to 14 days in the presence of Granulocyte/Macrophage- Colony Stimulating Factor (GM-CSF). Without additional *in vitro* superinfection, these monocytes will transform into macrophages and get infected by the autologous infected $CD4^+$ T cells present in the culture. HIV-1 spreads by cell-to-cell transmission as well as by free-infectious virions released in the culture medium. These cells are excellent targets for the CTL. They uptake the ^{51}Cr totally and with very low spontaneous release (Young and Steinman, 1987). For the first 7 days in culture, the human macrophage expresses both MHC I and II molecules. This permits both MHC I and II-restricted CTL recognition.

In preliminary experiments in our Laboratory using the macrophage as target cell (see Results), I obtained positive results by culturing the monocytes from PBMC of asymptomatic patients for 10 days (See materials and methods). These monocytes

transformed to macrophages and were infected *in vitro* by the infected CD4 T-lymphocytes present in the culture medium without the need for HIV-1 re-infection. This is a different case from the CD4 T lymphocyte used as target which requires re-infection *in vitro* for the CTL assay thereby being exposed to a different strain of virus. With the macrophage as target, I was able to detect the CTL activity in an 18-hour ^{51}Cr release assay. I find these results very encouraging. The longer culture period required for the macrophage permitted me at the same time to grow the CD8 T-lymphocytes that were used as effector cells. This allows for higher effector to target ratios to be tested. The macrophage was easier to keep in culture with the addition of Granulocyte-Macrophage/Colony Stimulating Factor. I also found that the ^{51}Cr uptake was better and more consistent than with the CD4 T-lymphocyte.

To summarize, the experimental work presented in this thesis shows that in spite of the optimized infection, the CD4 T cells are not good targets to detect HIV-1-specific CTL activity in peripheral blood of patients using a CTL assay. This leads us to speculate about the possible reasons for this resistance of the CD4 T cell to killing under these conditions. To name a few:

1) CD4 T cells from HIV-1^+ patients are not equivalent to normal T cells. They are already activated *in vivo* as a consequence of the continuous antigenic challenge during the disease process. I noticed in experiments done to determine the best time of infection after PHA stimulation, that cells infected 6 days after the stimulation had a very low production of p24 Ag. I believe that the CD4 T cells from patients may behave in a similar way. This slower type of infection is not due to problems with viral binding and penetration as I was able to prove with the viral entry assays.

2) HIV-1 could be inducing abnormalities in antigen processing and/or presentation.

3) The CD8 effector population could be composed mostly of suppressor cells.

4) The CD8 cytotoxic cells from the patients may not recognize the laboratory strain of HIV-1 used for reinfection *in vitro* due to genetic variability.

5) A lack of spontaneous circulating HIV-1-specific CTLs in the peripheral blood of these patients.

6) The CD4 T cells infected with the virus and labelled with ^{51}Cr may become resistant to killing in this type of assay.

The search should continue for the proper target as well as for the implementation of the assay to study the natural history of the HIV-1-specific CTL response in cohorts of patients.

REFERENCES

Asjö, B., L. Morfeldt-Manson, J. Albert, G. Biberfeld, A. Karlsson, K. Lidman, and E.M. Fenyo. 1986. Replicative capacity of HIV from patients with varying severity of HIV infection. Lancet **II**: 660-662.

Auger, I., P. Thomas, V. DeGruttola 1988. Incubation periods for pediatric AIDS patients. Nature **36**: 575-577.

Autran, B., C.M. Mayaud, M. Raphael. 1988. Evidence for a cytotoxic T-lymphocyte alveolitis in human immunodeficiency virus-infected patients. AIDS **2**: 179-183.

Barré-Sinoussi, F., J.C. Cherman, F. Rey, T. Nugeyre, S. Charmaret, J. Gruest, C. Dauguet, C. Axlen-Blin, F. Vezinet-Brun, C. Rouzioux, W. Rozenbaum and L. Montagnier. 1983. Isolation of a T-lymphotropic retrovirus from a patient at risk for acquired immune deficiency syndrome (AIDS). Science **220**: 868-71.

Blumberg, R.S., T. Paradis, R. Byington, W. Henle, M.S. Hirsch, and R.T. Schooley. 1987a. Effects of Human Immunodeficiency Virus on the cellular immune response to Epstein-Barr virus in homosexual men: characterization of the cytotoxic response and lymphokine production. J.Inf.Dis. **155**: 877-890.

Blumberg, R.S., T.J. Paradis, D. Crawford, R.E. Byington, M.S. Hirsch, R.T. Schooley. 1987b. Effects of human immunodeficiency virus on the cytotoxic response to Epstein-Barr virus transformed B lymphocytes. AIDS Res.Hum Retroviruses **3**: 303-315.

Cann, A.J., J.A. Zack, A.S. Go, S.J. Arrigo, Y. Koyanagi, P.L. Green, Y. Koyanagi, S. Pang, and I.S.Y. Chen. 1990. Human Immunodeficiency Virus type 1 T-Cell tropism is determined by events prior to provirus formation. J. Virology **64**: 4735-4742.

Castro, B.A., C. D. Weiss, L. D. Wiviott, and J. A. Levy. 1988. Optimal conditions for recovery of the Human Immunodeficiency Virus from peripheral blood mononuclear cells. J. Cl. Microb. **26**: 2371-2376.

Chakrabarti, L., M. Robert-Guroff, F. Wong-Staal, R.C. Gallo and B. Moss. 1986. Expression of the HTLV-III envelope gene by a recombinant vaccinia. Nature **320**: 535-537.

Chehimi, J., S. Bandyopadhay, K. Prakash, B. Perussia, N.F. Hassan, H. Kawashima, D. Campbell, J. Kornbluth and S.E. Starr. 1991. In vitro infection of Natural Killer cells with different human immunodeficiency virus Type 1 isolates. J. Virology **65**: 1812-1822.

Chen, L., B. Tourvieille, G.F. Burns, F.H. Bach, D. Mathieu-Mahul, M. Sasportes and A. Bensussan. 1986. Interferon: a cytotoxic T lymphocyte differentiation signal. Eur. J. Immunol. **16:** 767-770.

Chenciner, N., F. Michel, G. Dadaglio, P. Langlade-Demoyen, A. Hoffenbach, A. Leroux, F. Garicia-Pons, G. Rautmann, B. Guy, J.M. Guillon, C. Mayaud, M. Girard, B. Autran, M.P. Kieney and F. Plata. 1989. Mulltiple subsets of HIV-specific cytotoxic T lymphocytes in humans and mice. Eur. J. Immunol. **19:** 1537-1544.

Cheng-Mayer, C., M. Quiroga, J.W. Tung, D. Dina, J.A. Levy. 1990. Viral determinants of Human Immunodificiency Virus Type 1 T-cell or macrophage tropism, cytopathogenicity, and CD4 antigen modulation. J. Virology **64:** 4390-4398.

Cheng-Mayer, C., D. Seto, M. Tateno, and J.A. Levy. 1988a. Biologic features of HIV that correlate with virulence in the host. Science **240:** 80-82.

Cheng-Mayer, C., J. Homsy, L.A. Evans, and J.A. Levy. 1988b. Identification of human immunodeficiency virus subtypes with distinct patterns of sensitivity to serum neutralization. Proc. Natl. Acad. Sci. USA **85:** 2815-2819.

Claverie, J.M., P. Kourilsky, P. Langlade-Demoyen. 1988. T-immunogenic peptides are constituted of rare sequence patterns: use in the identification of T epitopes in the human immunodeficiency virus *gag* protein. Eur. J. Immunol. **18:** 1547-1553.

Collman, R., N.F. Hassan, R. Walker, B. Godfrey, J. Cutilli, J.C. Hastings, H. Friedman, S.T. Douglas, and N. Nathanson. 1989. Infection of monocyte-derived macrophages with Human Immunodeficiency Virus Type 1 (HIV-1). J. Exp. Med. **170:** 1149-1163.

Coombs, R.W., A.C. Collier, J.P. Allain, B. Nikora, M. Leuther, G.F. Gjerset, L. Corey. 1989. Plasma viremia in human immunodeficiency virus infection. New Engl.J.Med. **321:** 1626-1631.

Cornetta, K. and W. French Anderson. 1989. Protamine sulfate as an effective alternative to polybrene in retroviral-mediated gene-transfer: implications for human gene therapy. J. Virol. Methods **23:** 187-194.

Cullen, B.R. and W.C. Greene. 1989. Regulatory pathways governing HIV-1 replication. Cell **58:** 423-426.

Dahl, K.E., T. Burrage, F. Jones, and G. Miller. 1990. Persistent nonproductive infection of Epstein-Barr virus-transformed human B lymphocytes by human immunodeficiency virus type 1. J. Virol. **64:** 1771-1783.

Dahl, K., K. Martin, and G. Miller.1987. Differences among Human Immunodeficiency virus strains in their capacities to induce cytolysis or persistent infection of a lymphoblastoid cell line immortalized by Epstein-Barr virus. J. Virol. **61**: 1602-1608.

Duc-Nguyen, H. 1968. Enhancing Effect of Diethylaminoethyl-Dextran on the focus-forming titer of murine sarcoma virus (Harvey Strain). J. Virol. **2**: 643-644.

Duh, E.J., W.J. Maury, T.M. Folks, A.S. Fauci, and A.B. Rabson. 1989. Tumor Necrosis Factor α activates human immunodeficiency virus type 1 through induction of nuclear factor binding to the NF-kB sites in the long terminal repeat. Proc. Natl. Acad. Sci. USA **86**: 5974-5978.

Fanger, M.W. L. Shen, R.F. Graziano, and P.M. Guyre. 1989. Cytotoxicity mediated by human Fc receptors for IgG. Immunol. Today.**10**: 92-99.

Farnet, C.M. and W.A. Haseltine. 1990. Integration of human immunodeficiency virus type 1 DNA *in vitro*. Proc.Natl.Acad.Sci.USA. **87**: 4164-4168.

Fleischer, B. 1988. Non-specific propagation of human antigen-dependent T lymphocyte clones. J. Immunol. Meth. **109**: 215-219.

Folks, T.M., K.A. Clouse, J. Justement, A. Rabson, E. Duh, J.H. Kehrl, and A.S. Fauci. 1989. Tumor Necrosis Factor α induces expression of human immunodeficiency virus in a chronically infected T-cell clone. Proc. Natl. Acad. Sci. USA **86**: 2365-2368.

Gottlieb, M.S., R. Schroff, M. Schanker, J.D. Weisman, T.F. Peng, R.A. Wolf, and A. Saxon. 1981. *Pneumocystis carinii* pneumonia and mucosal candidiasis in previously healthy homosexual men: Evidence of a new acquired immunodeficiency. N. Engl. J. Med. **305**: 1425-1430.

Green, W. C. 1991. The molecular biology of human immunodeficiency virus type 1 infection. The New Engl. J. of Medicine. **321**: 308-317.

Gregersen, J.P., S. Mehdi, A. Baur, and J. Hilfenhaus.1990. Antibody- and complement-mediated lysis of HIV-infected cells and inhibition of viral replication. J.Med. Virology. **30**: 287-293.

Gruters, R.A., F.G. Terpstra, R. De Jong, C.J. Van Noesel, R.A.W. Van Lier, and F. Miedema. 1990. Selective loss of T cell functions in different stages of HIV infection. Eur.J.Immunol. **20**: 1039-1044.

Guillon, J.M., B. Autran, M. Denis. 1988. HIV-related lymphocytic alveolitis. Chest **94**: 1264-1270.

Hammes, S.R., E.P. Dixon, M.H. Malim, B.R. Cullen, and W.C. Greene. 1989. Nef protein of human immunodeficiency virus type 1: Evidence against its role as a transcriptional inhibitor. Proc.Natl.Acad.Sci.USA. **86:** 9549-9553.

Hazan, V., D. Thomas, J. Alcami, F. Bachelerie, N. Israel, H. Yssel, J.L. Virelizier, and F. Arenzana-Seisdedos. 1990. Stimulation of a human T-cell clone with anti-CD3 or Tumor Necrosis Factor induces NF-kB translocation but not Human Immunodeficiency Virus 1 enhancer-dependent transcription. Proc.Natl.Acad.Sci.U.S.A. **87:** 7861-7865.

Ho, D.D., T.R. Rota, and M.S. Hirsch. 1986. Infection of human monocyte/macrophages by human T-lymphotropic virus type III. J. Clin. Invest. **77:** 1712-1715.

Hoffenbach, A., G. Langlade-Demoyen, E. Dadaglio, F. Wilmer, C. Michael, G.C. Maynar, B. Autran, F. Plata. 1989. Unusually high frequencies of HIV-specific cytotoxic T lymphocytes in humans. J. Immunol. **142:** 452-462.

Hoxie, J.A., D.M. Matthews, K.J. Callahan, D.L. Carrel, and R.A. Cooper. 1986. Transient modulation and internalization of T4 antigen induced by phorbol esters. J. Immunol. **137:** 1194-1201.

Hu, Sl., S.G. Kosowski, J.M. Dalrymple. 1986. Expression of AIDS virus envelope gene in recombinant vaccinia viruses. Nature **320:** 537-540.

Ikeuchi, K., S. Kim, R.A. Byrn, S.R. Goldring and J.E. Groopman. 1990. Infection of nonlymphoid cells by Human Immunodeficiency Virus Type 1 or Type 2. J. Virol. **64:** 4226-4231.

Joly, P., J.M. Guillon, C. Mayaud, F. Plata, I. Theodorou, M. Denis, P. Debre, and B. Autran. 1989. Cell-mediated suppression of HIV-specific cytotoxic T lymphocytes. J. Immunol. **143:** 2193-2201.

Kannagi, M., L.V. Chaufoux, C.I. Lord, N.L. Letvin. 1988. Suppression of simian immunodeficiency virus replication in vitro by CD8+ lymphocytes. J.Immunol. **140:** 2237-2242.

Kielian, M., and Helenius A. 1986. Entry of alphaviruses. In The Togaviridae and Flaviviridae. Edited by S. Schlesinger and M.J. Schelesinger. Plenum Press, New York. pp. 91-119.

Kiency, M.P., G. Rautmann, D. Schmitt. 1986. AIDS virus *env* protein expressed from a recombinant vaccinia virus. Bio Technology **4:** 790-795.

Kim, S., K. Ikeuchi, R. Byrn, J. Groopman, and D. Baltimore. 1989. Lack of a negative influence on viral growth by the *nef* gene of human immunodeficiency virus type 1. Proc.Natl.Acad.Sci.USA. **86:** 9544-9548.

Kowalski, M., L. Bergeron, R. Dorfman, W. Haseltine, and J. Sodroski. 1991. Attenuation of Human Immunodeficiency Virus Type 1 cytopathic effect by a mutation affecting the transmembrane envelope glycoprotein. J. Virol. **65:** 281-291.

Koyanagi, Y., W.A. O'Brien, J.W. Zhao, D.W. Golde, J.C. Gasson, and I.S.Y. Chen. 1988. Cytokines alter production of HIV-1 from primary mononuclear phagocytes. Science **241:** 1673-1675.

Lacoste, J., M. D'Addario, A. Roulston, M.A. Wainberg, and J. Hiscott. 1990. Cell-specific differences in activation of NF-kB regulatory elements of Human Immunodeficiency Virus and Beta Interferon promoter by Tumor Necrosis Factor. J. Virol. **64:** 4726-4734.

Langlade-Demoyen, P., F. Michel, A. Hoffenbach, E. Vilmer, G. Dadaglio, F. Garicia-Pons, C. Mayaud, B. Autran, S. Wain-Hobson, and F. Plata. 1988. Immune recognition of AIDS virus antigens by human and murine cytotoxic T lymphocytes. J.Immunol. **141:** 1949-1957.

Leonard, R., D. Zagury, I. Desportes, J. Bernard, J.F. Zagury, and R.C. Gallo. 1988. Cytopathic effect of human immunodeficiency virus in T4 cells is linked to the last stage of virus infection. Proc.Natl.Acad.Sci. USA **85:** 3570-3574.

Levy, J.A., J. Shimabukuro, T. McHugh, C. Casavant, D.P. Stites, and L.S. Oshiro. 1985. AIDS-associated retroviruses (ARV) can productively infect other cells besides human T helper cells. Virology **147:** 441-448.

Levy, J.A. 1990. Changing concepts in HIV infection: challenges for the 1990s. AIDS **4:** 1051-105

Ma, X., K. Sakai, E. Golub, and D.J. Volsky. 1990. Interaction of a noncytopathic Human Immunodeficiency Virus type 1 (HIV-1) with target cells: efficient virus entry followed by delayed expression of its RNA and protein. Virology **176:** 184-194.

McDougal, J.S., A. Mawle, S.P. Cort, J.K.A. Nicholson, G.D. Cross, J.A. Scheppler-Campbell, D. Hicks, and J. Sligh. 1985. Cellular tropism of the human retrovirus HTLV-III/LAV. I. Role of T cell activation and expression of the T4 antigen. J. Immunol. **135:** 3151:3162.

Maddon, P.J., J.S. McDougal, P.R. Clapham, A.G. Dalgleish, S. Jamal, R.A. Weiss, and R. Axel. 1988. HIV infection does not require endocytosis of its receptor, CD4. Cell **54:** 865-874.

Maes, R., W. Sedwick, and A. Vaheri. 1967. Interaction between DEAE-Dextran and nucleic acids. Biochim. Biophys. Acta **134**: 269-276.

Malkowsky, M., K. Philpott, A.G. Dalgleish, A. L. Mellor, S. Patterson, A.D.B. Webster, A.J. Edwards, and P.J. Maddon. 1988. Infection of B lymphocytes by HIV and their susceptibility to Cytotoxic T Lymphocytes. Eur. J. Immunol. **18:** 1315-1321.

Mann, D.L., S. Gartner, F.L. Sane, H. Buchow, and M. Popovic. 1990. HIV-1 transmission and function of virus-infected monocytes/macrophages. J. Immunol. **144:** 2152-2158.

Maraskovsky, E.,W.F. Chen, and K. Shortman 1989. IL-2 and IFN-χ are two necessary lymphokines in the development of cytolytic T cells. J. Immunol. **143:** 1210-1214.

Massari, F.E., G. Poli, S.M. Schnittman, M.C. Psallidopoulos. 1990. In vivo T lymphocyte origin of macrophage-tropic strains of HIV. J. Immunol. **144**: 4628-4632.

McClure, M.O., M. Marsh, and R.A. Weiss. 1988. Human immunodeficiency virus infection of CD4-bearing cells occurs by a pH-independent mechanism. EMBO J. **7**: 513-518.

McCutchan, J.H. and J.S. Pagano. 1968. Enhancement of the infectivity of Simian Virus 40 deoxyribonucleic acid with Diethylaminoethyl-Dextran. J. Natl. Cancer Inst. **41**: 351-356.

Mellors, J.W., B.P. Griffith, M.A. Ortiz, M.L. Landry, and J.L. Ryan. 1991. Tumor Necrosis Factor-α/Cachectin enhances Human Immunodeficiency Virus Type 1 replication in primary macrophages. J. Inf. Dis. **163:** 78-82.

Nishanian, P., K.R. Huskins, S. Stehn, R. Detels, and J.L. Fahey. 1990. A simple method for improved assay demonstrates that HIV p24 antigen is present as immune complexes in most sera from HIV-infected individuals. J.Infec.Dis. **162**: 21-28.

Nixon, D.F., A.R.M. Townsend, J.G. Elvin, C.R. Rizza, J. Gallwey, A.J. McMichael. 1988. HIV-1 gag-specific cytotoxic T lymphocytes defined with recombinant vaccinia virus and synthetic peptides. Nature **336**: 484-487.

Osborn, L., S. Kunkel, and G.J. Nabel. 1989. Tumor necrosis factor α and interleukin 1 stimulate the Human Immunodeficiency Virus enhancer by activation of the nuclear factor *k*B. Proc. Natl. Acad. Sci. USA **86**: 2336-2340,

O'Shea, S., M. Cordery, W.Y. Barrett, D.D. Richman, C. Bradbeer, and J.E. Banatvala. 1990. HIV excretion patterns and specific antibody responses in body fluids. J. Med.Virol. **31**: 291-296.

Pantaleo, G., S. Koenig, M. Baseler, H. Clifford Lane, and A.S. Fauci. 1990a. Defective clonogenic potential of CD8+ T lymphocytes in patients with AIDS: expansion in vivo of a nonclonogenic CD3+ CD8+ HLA-DR+ CD25- T cell population. J. Immunol. **144:** 1696-1704.

Pantaleo, G., A. De Maria, S. Koenig, L. Burini, B. Moss, M. Baseler, H. Clifford Lane, and A.S. Fauci. 1990b. CD8+ T lymphocytes of patients with AIDS maintain normal broad cytolytic function despite the loss of human immunodeficiency virus-specific cytotoxicity. Proc. Natl. Acad. Sci. U.S.A. **87:** 4818-4822.

Plata, F., B. Autran, L.P. Martins, S. Wain-Hobson, M. Raphael, C. Mayaud, M. Denis, J.M. Guillon, and P. Debre. 1987a. AIDS virus-specific cytotoxic T lymphocytes in lung disorders. Nature **328:** 348-51.

Plata, F. et al. 1987b. Cytotoxic T lymphocytes in HIV-induced disease: implications for therapy and vaccination. Immunodeficiency Rev. **1:** 227-246.

Plata, F. 1989. HIV-specific Cytotoxic T Lymphocytes. Research in Immunology **140:** 89-124.

Reiss, P., J.M.A. Lange, A. de Ronde, F. de Wolf, J. Dekker, C. Debouck, and J. Goudsmit. 1990. Speed of Progression to AIDS and degree of antibody response to accessory gene products of HIV-1. J. Med.Virol. **30:** 163-168.

Ruprecht, R.M., L.D. Bernard, R. Bronson, M.A. Gama Sosa, and S. Mullaney. 1991. Castanospermine vs. its 6-O-Butanoyl Analog: A comparison of toxicity and antiviral activity in vitro and in vivo. J. of Acq. Immune Def.Synd. **4:** 48-55.

Sattentau, Q.J. 1989. HIV infection and the immune system. Biochim. et Biophys. Acta **989:** 255-268.

Scadden, D.T., M. Zeira, A. Woon, Z. Wang, L. Schieve, K. Ikeuchi, B. Lim, and J.E. Groopman. 1990. Human immunodeficiency virus infection of human bone marrow stromal fibroblasts. Blood **76:** 317-322.

Scheurich, P., B. Thoma, U. Üger, P. Pfizenmaier. 1987. Immunoregulatory activity of recombinant human tumor necrosis factor (TNF)-α: Induction of TNF receptors on human T cells and TNF-α-mediated enhancement of T cell responses. J. Immunol. **138:** 1786-1790.

Schmitt, M.P., A.M. Steffan, J.L. Gendrault, D. Jaeck, C. Royer, C. Schweitzer, C. Beyer, C. Schmitt, A.M. Aubertin, and A. Kirn. 1990. Multiplication of Human Immunodeficiency Virus in primary cultures of human Kupffer cells - possible role of liver macrophage infection in the physiopathology of AIDS. Res. Virol. **141:** 143-152.

Schnittman, S.M., M.C. Psallidopoulos, H.C. Lane, L. Thompson, M. Baseler, F. Massari, C.H. Fox, N.P. Salzman, and A.S. Fauci. 1989. The reservoir for HIV-1 in human peripheral blood is a T cell that maintains expression of CD4. Science **245:** 305-308.

Sethi, K.K., H. Näher, I. Stroehmann. 1988. Phenotypic heterogeneity of cerebrospinal fluid-derived HIV-specific and HLA-restricted cytotoxic T-cell clones. Nature **335:** 178-181.

Stevenson, M., T.L. Stanwick, M.P. Dempsey and C.A. Lamonica. 1990. HIV-1 replication is controlled at the level of T cell activation and proviral integration. EMBO J. **9:** 1551-1560.

Sundqvist, V., J. Albert, E. Ohlsson, J. Hinkula, E.M. Fenyö, and B. Wahren. 1989. Human Immunodeficiency Virus type 1 p24 production and antigenic variation in tissue culture of isolates with various growth characteristics. J. Med. Virol. **29:** 170-175.

Takahashi, H., J. Cohen, A. Hosmalin, K.B. Cease, R. Houghten, J. Cornette, C. DeLisi, B. Moss, R.N. Germain, and J.A. Berzofsky. 1988. An immunodominant epitope of the human immunodeficiency virus envelope glycoprotein gp 160 recognized by class I MHC-restricted murine cytotoxic T lymphocytes. Proc. Natl. Acad. Sci. USA **85:** 3105-3109.

Tateno, M., F. Gonzalez-Scarano and J.A. Levy. 1989. Human Immunodeficiency Virus can infect CD4-negative human fibroblastoid cells. Proc.Natl.Acad.Sci.USA. **86:** 4287-4290.

Tersmette, M., R.A. Gruters, F.de Wolf, R.E.Y. de Goede, J.M.A. Lange, P.T.A. Schellekens, J. Goudsmit, H.G. Huisman, and F. Miedema. 1988. Differential syncytium inducing capacity of Human Immunodeficiency Virus isolates in patients with AIDS and AIDS-related complex. J. Virol. **62:** 2026-2032.

Tozzi, V., S. Britton, A. Ehrnst, R. Lenkei, and Ö. Strannegård. 1989. Persistent Productive HIV infection in EBV-transformed B Lymphocytes. J. Med. Vir. **27:** 19-24.

Tsubota, H., C.I. Lord, D.I. Watkins, C. Morimoto, H.L. Letvin. 1989a. A cytotoxic T lymphocyte inhibits acquired immunodeficiency syndrome virus replication in peripheral blood lymphocytes. J.Exp.Med. **169:** 1421-1434.

Tsubota, H., D.J. Ringler, M. Kannagi, N.W. King, K.R. Solomon, J.J. MacKey, D.G. Walsh, and N.L. Letvin. 1989b. CD8+ CD4- lymphocyte lines can harbor the AIDS virus *in vitro*. J. Immunol. **143:** 858-863.

Via, C.S., G.C. Tsokos, N. I. Stocks, M.Clerici, G. M. Shearer. 1990. Human Allogeneic Responses. Demonstration of three pathways of T helper cell activation. J. Immunol. **144:** 2524-2528.

Volsky, D.J., M.G. Pellegrino, G. Li, K.A. Logan, J.E. Aswell, N.P. Lawrence, and S.R. Decker. 1990. Titration of human immunodeficiency virus type 1 (HIV-1) and quantitative analysis of virus expression in vitro using liquid RNA-RNA hybridization. J. Virol. Meth. **28**: 257-272.

Vyakarnam, A., J. McKeatin, A. Meager, and P.C. Beverley. 1990. Tumour necrosis factors (α,ß) induced by HIV-1 in peripheral blood mononuclear cells potentiate virus replication. AIDS **4**: 21-27

Walker, B.D., S. Chakrabarti, B. Moss, T.J. Paradis, T. Flynn, A.G. Durno, R.S. Blumberg, J.C. Kaplan, M.S. Hirsch, and R.T. Schooley. 1987a. HIV-specific cytotoxic T lymphocytes in seropositive individuals. Nature. **328**: 345-48.

Walker, B.D., M. Kowalski, W.C. Goh. 1987b. Inhibition of human immunodeficiency virus syncytium formation and virus replication by castanospermine. Proc. Natl. Acad. Sci. USA. **84**: 8120-8124.

Walker, B.D., C. Flexner, T.J. Paradis, T.C. Fuller, M.S. Hirsch, R.T. Schooley, and B. Moss. 1988. HIV-1 reverse transcriptase is a target for cytotoxic T lymphocytes in infected individuals. Science. **240**: 64-66

Walker, B.D., C. Flexner, K. Birch-Limberger, L. Fisher, T.J. Paradis, A. Aldovini, R. Young, B. Moss, and R.T. Schooley. 1989. Long-term culture and fine specificity of human cytotoxic T-lymphocyte clones reactive with human immunodeficiency virus type I. Proc.Natl.Acad.Sci. USA **86**: 9514-9518.

Walker, C.M., D.J. Moody, D.P. Stites, J.A. Levy. 1986. CD8+ lymphocytes can control HIV infection in vitro by suppressing virus replication. Science **234**: 1563-1566.

Watson, A.J., J. Klaniecki, and C.V. Hanson. 1990. Psoralen/UV inactivation of HIV-1 infected cells for use in cytologic and immunologic procedures. AIDS Res. and Human Retroviruses **6**: 503-513.

Weinhold, K.J., T.J. Matthews, P.M. Ahearne, A.J. Langlois, H.K. Lyerly, D.S. Tyler, K.C. Stine, D.T. Durack and D.P. Bolognesi. April 1988. Cellular anti-gp120 cytolytic reactivities in HIV-1 seropositive individuals. The Lancet: 902-904.

Wong-Staal, F. 1990. Human Immunodeficiency viruses and their replication. In Virology. 2nd ed. Edited by B.N. Fields. Raven Press, New York. pp 1529-1543.

Yamada, O., T. Matsumoto, R. Sasoaka, and T. Kurimura. 1990. Variations in growth capacity of HIV in peripheral blood mononuclear cell preparations from different individuals. AIDS **4**: 35-40.

Young, J.W. and R.M. Steinman. 1987. Mononuclear phagocytes as targets for cytolytic T lymphocytes. J. Immunol. Meth. **100:** 99-105.