THE APPLICATION OF THE POLYMERASE CHAIN REACTION TO THE DETECTION AND CHARACTERIZATION OF HUMAN IMMUNODEFICIENCY VIRUS AND HEPATITIS B VIRUS NUCLEIC ACID.

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CONTENTS

ABBREVIATIONS

ADP	AIDS directed program
AIDS	acquired immunodeficiency syndrome
anti-HBc	antibody to HBcAg
anti-HBe	antibody to HBeAg
anti-HBs	antibody to HBsAg
bp	base pair
BSA	bovine serum albumin
cDNA	complementary DNA
CPE	cytopathic effect
CUVD	circular unintegrated viral DNA
ddw	deionised distilled water
dH_2O	doubly distilled water
DNA	deoxyribonucleic acid
dsDNA	double stranded DNA
dNTPs	deoxyribonucleotides
ELISA	enzyme linked immunosorbent assay
EM	electron microscope
EPICS	electronic programmable integrated cell sorter
FCS	foetal calf serum
FITC	fluorescein isothiocyanate
HBcAg	hepatitis B core antigen
HBe negative	negative for HBeAg and anti-HBe
HBeAg	hepatitis B e antigen
HBsAg	hepatitis B surface antigen
HIV	human immunodeficiency virus

HIV-1	human immunodeficiency virus type 1
HWB	heparinised whole blood
Ig	immunoglobulin
IgG	immunoglobulin class G
IgM	immunoglobulin class M
L-PCR	ligation mediated PCR
LTR	long terminal repeat
LUVD	linear unintegrated viral DNA
MRC	Medical Research Council
P	patient
PBMCs	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PCR-RD	PCR restriction digest
RIA	radioimmunoassay
RIPA	radioimmunoprecipitation assay
RNA	ribonucleic acid
RNP	ribonucleo-protein complex
RT-PCR	reverse transcribed PCR
s/n	supernate
Taq	DNA polymerase from the thermophilic bacterium *Thermus aquaticus*
UVD	unintegrated viral DNA

ABSTRACT

The polymerase chain reaction (PCR) is an *in vitro* amplification technique used to synthesize multiple copies of a nucleic acid sequence. The reaction is driven by *Taq* polymerase which copies the template sequence directed by 2 sequence-specific oligonucleotide primers. The technique results in the exponential increase of template sequence, permitting the detection of rare nucleic acids. This thesis considers the application of PCR to the detection and characterization of human immunodeficiency virus (HIV) nucleic acid and hepatitis B virus (HBV) DNA in clinical samples.

HIV-1 can infect a subset of T lymphocytes and establish a latent infection, resulting in very few cells in the peripheral blood harbouring viral nucleic acid. The sensitivity offered by PCR was, therefore, essential for the detection of HIV-1 nucleic acid in patient peripheral blood mononuclear cells (PBMCs). Primers were selected from the *pol* and *env* sequences for the detection of HIV-1 DNA, and amplifications of the envelope gene were used to demonstrate sequence variability.

HIV-1 DNA was detected in all 32 patients (59 of the 61 PBMC samples tested). Viral DNA sequences were detected in all nuclear extracts tested, 71% of cytoplasmic extracts, and in 50% of purified monocytic cells.

Primers were selected to differentiate between HIV-1 DNA structural forms. Covalently closed circular unintegrated viral DNA (CUVD) with 1 or 2 LTR sequences was detected by amplifying across the junction site, and linear unintegrated viral DNA (LUVD) was detected using a ligation-mediated PCR (L-PCR). CUVD was detected in 65% of patient samples and was associated with disease progression ($0.02>P>0.01$). The detection of LUVD was negative for all patient samples.

The hepatitis B virus replicates in hepatocytes and is released into the blood

of infected individuals. The infection is conventionally diagnosed by immunoassays detecting viral antigens and their corresponding antibodies in sera, and the presence of viral DNA is determined by hybridization assays. HBV DNA was detected in patient serum samples using 4 specific primers in a nested PCR. The primers were selected to hybridize to the S-gene sequence of HBV using the nucleotide consensus sequence for 5 HBsAg subtypes (*adrcg*, *adw*, *adyw*, *ayw*, and ayw_2).

The presence of HBeAg in serum is indicative of infectivity. The detection of HBV DNA by PCR was compared with HBe status in 115 patient samples. All HBeAg positive and 25% of HBeAg negative samples were positive for HBV DNA. To determine the source of the viral DNA in these samples, the DNA extraction method was applied to free virus, and IgG and IgM complexed virus to establish the association of viral DNA with free virus and HBeAg.

The HBsAg is a complex protein with a common determinant *a*, and 4 main sub-determinants *d*, *y*, *w*, and *r*. A total of 38 HBV DNA positive samples had been subtyped for the *d* and *y* determinants by radioimmunoprecipitation assay (RIPA). These samples were subtyped by PCR using a *y*-specific primer for the nested amplification. Twenty samples were subtyped *y* by both methods, 15 samples *d* by both methods, and 3 samples were *d* by RIPA and *y* by PCR.

A total of 9 samples (including the 3 disputed samples) were subtyped by a third method to determine their true subtype. The S-gene was amplified by PCR, and the resulting band was digested by *Sau* 3A restriction enzyme. Six samples were subtyped identically by each method, 2 of the 3 disputed samples subtyped *d* in agreement with RIPA, and 1 sample subtyped *y* in agreement with PCR.

The relevance and significance of these results are discussed.

PUBLICATIONS AND PRESENTATIONS

PUBLICATION.

Nicholson, W.J., Black, S.H., Simmonds, P., Chung, C-W., Aw, D., & Peutherer, J.F. (1992). Comparison of hepatitis B virus subtyping of *d/y* determinants by radioimmunoprecipitation assay and the polymerase chain reaction. *Journal of Medical Virology* **36** 21-27.

PRESENTATION.

The above work was presented at a Hepatitis workshop at the 119th Ordinary Meeting of The Society for General Microbiology, Edinburgh, 9-12 April 1991.

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DECLARATION

All of the investigations and procedures presented in this thesis were performed by the author unless otherwise stated.

The thesis was composed by the author.

INTRODUCTION

CHAPTER 1: THE POLYMERASE CHAIN REACTION.

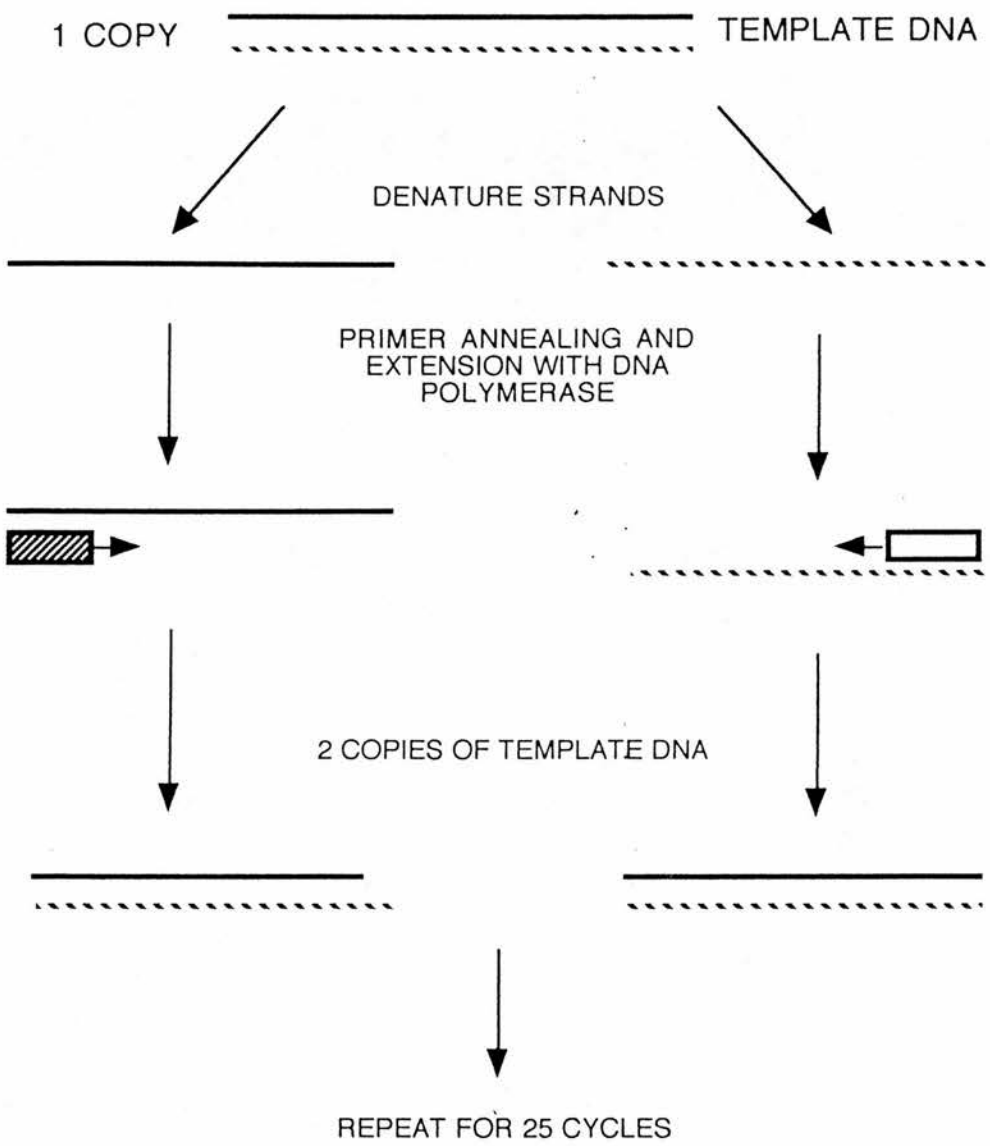
The polymerase chain reaction, invented by Kary Mullis in 1985, has rapidly become a useful analytical tool for the detection and characterization of nucleic acids (Saiki *et al.*, 1985; Mullis & Faloona, 1987; Saiki *et al.*, 1988). This is most apparent in the number of publications utilising the technique, rising from 3 in 1986 to 1700 articles in 1990 (Eeles *et al.*, 1992).

The reaction is an *in vitro* method of DNA synthesis in which a specific segment of DNA is replicated. The resulting rapid generation of millions of copies of target sequence, makes it possible to supply the large quantities of template DNA required for molecular analysis. The reaction consists of 3 segments per cycle: denaturing the target DNA, annealing of 2 specific primers to opposite strands of the flanking sequence of the target, and synthesis from the 3' ends of the primers in such a way that their extension products overlap. The products of each cycle are complementary to the primers, and so the target DNA concentration is doubled in each successive cycle. Repeated thermal cycling, therefore, results in an exponential accumulation of the specific DNA sequence (Figure 1).

Some knowledge of the flanking sequences of the target DNA is required for selection of specific primers. Optimally, the primers are 20-30 bases long, with a G+C content of 50-60%, and their sequences impart specificity to the reaction. Amplification with these primers can be followed by a secondary or "nested" amplification with internal primers. The nested primers amplify a small sequence internal to the primary amplification product, resulting in a more sensitive test with greater specificity imparted by the 4 primers.

The amplification products are detected by ethidium bromide visualisation in agarose gels (Chehab *et al.*, 1987; Kogan *et al.*, 1987; Orkin, 1987), oligomer restriction analysis (Kwok *et al.*, 1987; Duggan *et al.*, 1988), or by Southern blot hybridization (Mullis *et al.*, 1986: Larzul *et al.*, 1988; Thiers *et al.*, 1988).

Figure 1. **THE POLYMERASE CHAIN REACTION**



Repeated cycling of the denaturing, annealing and extension temperatures results in the doubling of the template DNA each time, corresponding to a theoretical exponential increase in the target sequence.

PCR has considerable advantages over existing techniques of nucleic acid detection. The reaction can be used to detect sequences varying in length from 25 to several thousand bases, and offers greater sensitivity, requiring only a single template for detection (Simmonds *et al.*, 1990a). The starting material can be DNA or cDNA derived from RNA templates, while the sample can be derived from any source (tissue and body fluids), and can be in any state as long as the target sequence is intact. Clinical samples, where the target sequence is a very minor component of the total nucleic acid extract (Simmonds *et al.*, 1990a), or paraffin embedded tissue and palaeontological material, where the nucleic acid may be of very poor quality, have been used (Shibata *et al.*, 1988; Pääbo, 1989).

1.1 DNA polymerase.

The original design of PCR utilised the Klenow fragment of the *E. coli* DNA polymerase I (Saiki *et al.*, 1985). The thermolability of this enzyme required fresh enzyme to be added after each denaturing step. Although a number of other enzymes were tested (Keohavong *et al.*, 1988), the most important modification to the reaction was the use of the thermostable DNA polymerase (Saiki *et al.*, 1988). The enzyme (*Taq* polymerase) was first isolated from the thermophilic eubacterium *Thermus aquaticus* YT-1 strain which grows at 75˚C (Chien *et al.*, 1976), and incorporates nucleotides at a rate of more than 60 per second at 70˚C and 24 per second at 55˚C (Gelfand & White, 1990). The enzyme lacks 5'-3' exonuclease activity and is, therefore, responsible for the introduction of point mutations in the amplification product (Chien *et al.*, 1976). The rate of incorrect incorporation is such that a 10^6-fold amplification of a 100bp dsDNA sequence would result in 0.8 point mutations (Keohavong & Thilly, 1989). Despite this rate being higher than with other polymerases (T4, modified T7, and Klenow fragment), the overall efficiency and thermostability

of *Taq* polymerase favours its use for PCR. The gene was, therefore, cloned into *E. coli*, resulting in the production of a standardized enzyme activity. This allowed the automation of PCR, making it a simple and rapid method of nucleic acid amplification.

1.2 Limitations of PCR.

PCR is the method of choice for almost all problems involving the detection of small quantities of nucleic acids because of its speed and sensitivity. The enormous amplification power of the reaction, however, is also its main disadvantage, resulting in false-positive and negative results.

1.2.1 False-positive amplifications.

There are 3 main sources of contaminating template DNA: positive samples handled in tandem by the medical staff at source, cloned material, and accumulating amplification products in the laboratory. It is, therefore, important that all personnel associated with the collection and processing of samples are aware of the need to avoid cross-contamination.

The main source of contamination is from the accumulation of PCR products in the laboratory. A PCR vial contains up to 10^{12} copies of template sequence, so that an aerosol droplet (10^{-6}ul) may contain 10^{5} templates (Persing, 1991). It is imperative to safeguard against this form of contaminant. Various measures can be taken to reduce the risk, including: the allocation of laboratory space to various stages of PCR work where appropriate; the use of absorbent disposable work surfaces; decontamination of surfaces with a weak acid solution (0.25M HCl); the preparation, aliquoting and storage of reaction mixtures in PCR-free areas; autoclaving of all materials and reagents not damaged by heat; and processing of numerous well characterized negative controls with samples in small batches.

Although contamination is controlled by good laboratory practice, it can

become a major problem in laboratories generating vast quantities of amplified material, resulting in the reporting of false results (Tidy & Farrell, 1989). In response to this, 2 methods have been developed which alter the amplification product preventing its ability to act as a template in the event of contamination (Longo *et al.*, 1990; Cimino *et al.*, 1991; Isaacs *et al.*, 1991).

1.2.2 False-negative amplifications.

There are 2 main sources of false-negative results: the amplification of non-specific sequences in preference to the target DNA, and inefficient amplification of target sequences.

PCR is a biphasic reaction with the initial stage screening the sample for the target sequences, and the subsequent cycles replicating the chosen sequence. The most likely by-products from inaccurate early amplifications arise from high primer:template ratios resulting in primers colliding with themselves forming primer-dimers, and from mis-priming with other sequences present, giving rise to non-specific bands (Li *et al.*, 1990). This is most apparent when the desired sequence is present at a low-copy-number in large quantities of non-specific DNA (eg. HIV-1 DNA in PBMC DNA extracts). Under these circumstances, it is very important that during the initial cycles the primers hybridize to and amplify specific sequences in preference to the non-specific cellular sequences. This can be achieved using high stringency thermal cycling and low primer concentration for the initial cycles (Ruano *et al.*, 1989).

The efficiency of PCR is dependent on many variables: denaturation temperature; annealing temperature of the primer/template duplex; extension rate and concentration of *Taq* polymerase; length and secondary structure of the target DNA sequence; and the actual and transition temperatures reached by samples (Williams & Anderson, 1992). These factors may vary with each

primer pair. Careful optimisation is, therefore, required to obtain maximum amplification efficiency.

The reaction theoretically results in the exponential amplification of the target DNA sequence. A simple relationship has been determined between the efficiency of amplification and the expected increase in the target sequence (Figure 2). From this, it is clear that a small decrease in the amplification efficiency results in a dramatic decrease in the amplification product concentration (Table 1).

In a typical PCR cycle, the samples are denatured at 94-95˚C for 1min, the primers are annealed at 40-60˚C for 1min, and DNA then synthesized at 60-72˚C for 1-3min.

The most critical steps are the denaturing and extension temperatures. Insufficient heating can prevent complete denaturation, reducing the available template concentration. A low re-annealing temperature confers low stringency and, therefore, permits mis-priming and subsequent amplification of non-specific sequences. If the temperature is too high, the annealing of primers is inhibited, again resulting in a lack of amplification of the target DNA. It is, therefore, important to optimise the cycling temperatures to maximise the stringency and amplification for each primer pair.

The reaction vials can also influence the amplification efficiency. The thickness of the tubes can affect the transfer of heat from the block to the sample producing a considerable temperature lag, resulting in samples not achieving the required temperature for the desired time (Williams & Anderson, 1992).

Although *Taq* is a thermostable polymerase, continual heating to temperatures of 95˚C results in a loss of activity. The enzyme half-life has been calculated to be 40min at 95˚C. However, even after 50 cycles of

Figure 2. Calculation of the amplification rate of the polymerase chain reaction (Clewley, 1989).

$$Y=(1+X)^n$$

Where, Y= extent of amplification in n cycles

X= mean efficiency per cycle

n= number of cycles.

For example, at an amplification efficiency of 95% (X) for 25 cycles (n),

$$Y=(1+0.95)^{25}$$

$$Y=17818260$$

Therefore, the target DNA sequence will have been amplified approximately $1.7x10^7$-fold after 25 cycles at an average cycle efficiency of 95%.

Table 1. Relative yields expected from 25 cycles of PCR at varying amplification efficiencies.

Amplification efficiency (X)	Extent of amplification for 25 cycles (Y)	Relative yield
100	33554432	1.00
95	17818260	0.53
90	9307649	0.28
85	4778518	0.14
80	2408866	0.07
50	25251	0.0008

denaturing at 94-95°C for 1min, more than 50% of the enzyme activity remains (Gelfand & White, 1990). Therefore, the loss of enzyme activity through thermal cycling is unlikely to be limiting. Enzyme activity can be restrictive when the template concentration exceeds the enzyme concentration. Under these conditions, the efficiency of the reaction is reduced, and DNA synthesis is aborted prematurely. The increased melting temperature of the template duplex in the later cycles often prevents complete denaturation preventing priming of DNA synthesis.

Although alterations to the reaction can overcome these problems, all amplifications reach a plateau concentration of target sequence at which point the reaction should be terminated. Further cycling after this results in the exponential amplification of non-specific bands until they too reach their plateau concentration. It is, therefore, important to optimise the cycle number to maximise specific amplification combined with minimum non-specific amplification.

1.3 Applications of PCR.

Numerous modifications to PCR have led to a variety of uses beyond the original application of generating large amounts of a specific DNA sequence.

The use of multiple sets of primers in a single reaction has led to the screening of environmental samples for bacteria (Bej *et al.*, 1990) and for multi-factorial genetic disorders (Chamberlain *et al.*, 1988). Degenerate primers have also been used to detect sequence similarities in virus families (Mack & Sninsky, 1988) while inverse-PCR permits the analysis of unknown sequences flanking a region of known sequence (Triglia *et al.*, 1988; Ochman *et al.*, 1990). Another modification, using an anchored-PCR, amplifies nucleic acid with variable termini (Loh *et al.*, 1989) and ligation-mediated PCRs can direct the synthesis of DNA from the end of an unknown sequence (Bukrinsky

et al., 1991).

PCR can also be used to incorporate restriction enzyme sites to facilitate cloning (Scharf *et al.*, 1986); direct sequencing of amplification products (Wrischnik *et al.*, 1987; Simmonds *et al.*, 1990b); analysis of point mutations (Almoguera *et al.*, 1988); and in the synthesis of gene fusion products (Horton *et al.*, 1989).

The sensitivity, specificity, and versatility of PCR has resulted in its application to numerous scientific challenges. It has been used in the fields of forensic medicine for DNA fingerprinting and paternity claims (Higuchi *et al.*, 1988; Li *et al.*, 1988), in phylogenetic and evolutionary studies (Bruns *et al.*, 1989; Pääbo *et al.*, 1989), and in oncology (McMahon *et al.*, 1987; Lee *et al.*, 1988; Neri *et al.*, 1988). It has also been applied to the identification of genetic disorders such as sickle cell anaemia (Saiki *et al.*, 1985), ß-thalassaemias (Wong *et al.*, 1987; Cai *et al.*, 1988) and Duchenne muscular dystrophy (Chamberlain *et al.*, 1988), and is expected to play a major role in the human genome project (Rose, 1990).

PCR has probably been most useful in its application to the detection of infectious agents. The reaction has been used to supplement existing technologies, particularly where samples are of insufficient size, or when existing methods are time consuming and difficult. An example of this is the use of PCR to detect DNA sequences of *Mycobacterium tuberculosis* in samples to confirm the clinical diagnosis. PCR is used to replace the conventional laboratory diagnosis by culture which can take up to 6 weeks to confirm (Pao *et al.*, 1990; Sjöbring *et al.*, 1990; de Wit *et al.*, 1990).

PCR has also been applied to the detection of numerous viruses which are difficult or impossible to culture such as HBV (Larzul *et al.*, 1988 & 1989; Kaneko *et al.*, 1989a & b; Liang *et al.*, 1989; Lo *et al.*, 1989; Ulrich *et al.*, 1989; Zeldis *et al.*, 1989), or like HIV, which is present in such small quantities

that it is difficult to detect (Kwok *et al.*, 1987; Byrne *et al.*, 1988; Hart *et al.*, 1988; Laure *et al.*, 1988; Murakawa *et al.*, 1988; Taylor, 1988).

Arguably the most reported application of PCR to date has been in the detection and characterization of the human retrovirus HIV. PCR was developed shortly after the discovery of HIV, and has proved to be very useful in the detection of HIV DNA: (a) prior to seroconversion (Loche & Mach, 1988; Imagawa *et al.*, 1989; Wolinsky *et al.*, 1989); (b) in newborn infants with maternal antibody (Laure *et al.*, 1988; de Rossi *et al.*, 1988), and (c) in the confirmation of infection in individuals with indeterminate antibody status (Farzadegan *et al.*, 1988).

CHAPTER 2: HUMAN IMMUNODEFICIENCY VIRUS TYPE 1 (HIV-1).

2.1 HIV-1 and AIDS.

The aetiological agent of AIDS is the human immunodeficiency virus type-1 (HIV-1). It was first isolated from patients presenting with unusual opportunistic infections and depleted $CD4^+$ cell counts indicative of a severe immunodeficiency (Gottlieb *et al.*, 1981; Masur *et al.*, 1981; Friedman-Kien *et al.*, 1982).

The infectious agent was isolated in T lymphocytes from a lymph node biopsy of a patient with AIDS related lymphadenopathy, was reverse transcriptase dependent, and was observed budding from the cell membrane in a manner characteristic of retroviruses (Barré-Sinoussi *et al.*, 1983; Popovic *et al.*, 1984; Gallo *et al.*, 1984; Klatzmann *et al.*, 1984a; Levy *et al.*, 1984). The retrovirus subsequently isolated from these patients was named HTLV-III (Popovic *et al.*, 1984), LAV (Barré-Sinoussi *et al.*, 1983), or ARV (Levy *et al.*, 1984). It is a member of the *Lentiviridae*, is the aetiological agent of human AIDS, and is now universally known as the human immunodeficiency virus type-1 (Coffin, 1991).

HIV-1 was first detected through its ability to infect $CD4^+$ T lymphocytes, although other cell types such as monocytes/macrophages, Langerhan's cells and glial cells can be infected *in vitro* and *in vivo* and may play an important role in pathogenesis (Koenig *et al.*, 1986; Nicholson *et al.*, 1986; Gendelman *et al.*, 1988; Orenstein *et al.*, 1988; Rappersberger *et al.*, 1988; Kunsch *et al.*, 1989).

The first 3 cases of AIDS in Scotland were diagnosed in 1981 (2 male & 1 female). Cumulative data up to 30 September 1992 show a dramatic increase in infected individuals (1845; 1368 male, 471 female & 6 sex not known) with an additional 324 individuals with clinical AIDS (278 male & 46 female) and a

further 224 AIDS associated deaths (196 male & 28 female). This equates to a total of 2393 known HIV-1 infections in Scotland since 1981, of which 941 were attributable to Lothian (CDU, 1992; LRC, 1992). The World Health Organization figure for all reported AIDS cases up to July 1992 is 501 272 although this will be subject to under-reporting (WHO, 1992).

2.2 Screening for HIV-1 infections.

HIV-1 has had a profound impact on medicine, science, and society. The immense public awareness has forced the rapid dissemination of scientific information which has been both beneficial and central to the genesis of myths surrounding the infection. In the UK and USA, HIV was first diagnosed in male homosexual and drug using communities. Issues concerning HIV and AIDS have, therefore, touched on facets of modern society which were already subject to moralization through fear, prejudice, and ignorance. Despite HIV being primarily a heterosexual disease, the damaging effects of the epidemiology of infection in the developed world has led to the views of members of the public and scientific communities being coloured by their prejudices. One of the main consequences of this is the continued underestimation of the spread of HIV due to the lack of widespread screening. However, until the stigma and prejudices associated with the infection are dissipated, individuals are unlikely to be willing to be tested because of their fear of the repercussions.

The diagnosis of HIV is routinely based on a variety of immunoassays specific for viral antigens and their corresponding antibodies. The initial screening for antibodies to viral surface and core proteins is by RIA or ELISA, with confirmation by alternative immunoassays using different antigens and test formats. The Western blot using viral lysate or proteins prepared from cDNA has been widely used in this way (Davey & Lane, 1990).

2.3 Classification of HIV-1 infection.

The immunodeficiency associated with HIV infection gives rise to a broad spectrum of disorders resulting directly from the viral cytopathology or indirectly mediated by the host immune system and secondary infections.

To categorise these stages for both medical and scientific clarity, the Centers of Disease Control in Atlanta defined 4 main clinical stages (CDC groups I to IV; CDC, 1986). CDC group I defined the acute glandular fever-like illness associated with seroconversion which is observed in 10-15% of infections. The asymptomatic period which may last for months to years was classified CDC group II, with group III describing patients with unexplained persistent generalised lymphadenopathy (PGL). CDC group IV was further subdivided (A-E) to describe the spectrum of disorders and secondary infections associated with the immunodeficiency (Table 2). Subgroup A includes patients with HIV constitutional disease, with all symptoms attributable to the advanced HIV infection in the absence of secondary infections. The disease is defined by persistent fever (>1 month), weight loss of greater than 10% baseline, and chronic diarrhoea (>1 month). Subgroup B consists of patients with neurological disease such as dementia, myelopathy, or peripheral neuropathy which is the result of HIV invasion of the nervous system. Subgroup C is subdivided into 2 categories, and includes patients with clinical disease associated with secondary infections. Patients in subgroup D are classified by secondary cancers usually indicative of a defect in cell-mediated immunity, and subgroup E is an open category for conditions not previously defined. Patients can be classified into any number of CDC IV subgroups, and may range from moderately ill to severely immunodeficient and life threatening infections and disorders (CDC, 1986; Mildvan & Solomon, 1987).

Table 2. Centers for Disease Control classification of HIV infection (CDC, 1986).

CDC group	Definition
Group I	Acute infection
Group II	Asymptomatic infection
Group III	Persistent generalised lymphadenopathy
Group IV	Other HIV diseases:
Subgroup A	Constitutional disease
Subgroup B	Neurological disease
Subgroup C	Secondary infectious diseases
Category C-1	Specific secondary infectious diseases listed in the CDC surveillance definition for AIDS
Category C-2	Other specified secondary infectious diseases
Subgroup D	Secondary cancers
Subgroup E	Other conditions

The CDC have since revised the classification system to incorporate the significance of $CD4^+$ cell counts to the clinical condition and to include more clinical conditions (CDC, 1992).

2.4 Epidemiology and transmission.

The transmission of HIV is predominantly sexual, or by inoculation with contaminated materials. HIV has been successfully isolated from most body tissues and secretions including; blood, plasma, bone marrow, lymph nodes, neurological tissue, breast milk, semen, saliva, urine, cerebrospinal fluid, cervical secretions and tears (Groopman *et al.*, 1984; Ho *et al.*, 1984; Feorino *et al.*, 1985; Fujikawa *et al.*, 1985; Ho *et al.*, 1985; Salahuddin *et al.*, 1985; Thiry *et al.*, 1985; Zagury *et al.*, 1984; Vogt *et al.*, 1986; Wofsy *et al.*, 1986; Allain, 1987; Li *et al.*, 1992). Exposure to any of these tissues or secretions would, therefore, be considered an exposure to the virus, however, in practice, efficient transmission occurs mainly by sexual contact (both hetero- and homosexual), parenteral inoculation of infected blood or blood products, artificial insemination, transplantation of infected tissues, and from mother to offspring (Handsfield *et al.*, 1985; Redfield *et al.*, 1985; Stewart *et al.*, 1985; Allain, 1987; Mok *et al.*, 1987; Simonds *et al.*, 1992).

2.5 Classification of Retroviruses.

The *Retroviridae*, like other viral families, share common morphology, biochemistry, and physical properties. The viruses are characterised by their ability to reverse transcribe genomic RNA to DNA, a property resulting in the family name *retro*, (Latin; *backwards*).

They were originally classified into 3 subfamilies based on their ability to cause disease (Matthews, 1979). The *Oncoviridae* (from the Greek word *onkos* meaning tumour) were grouped for their ability to cause tumours; *Spumaviridae* (from the Latin *spuma* meaning to foam) formed vacuolated or

foamy cells *in vitro* but have so far not been associated with disease in animals or humans; and *Lentiviridae* (*lenti* being the Latin word for slow) which caused slowly progressive diseases in their hosts. The family has since been re-classified, and is now divided into 7 genera based on molecular analysis (Table 3; Coffin, 1991).

The *Lentiviridae*, of which HIV-1 is the human prototype, have the most complex genomic structure and cause immunodeficiencies, neurological disorders, and arthritic diseases in a number of mammalian hosts (Table 4).

2.6 Morphology of HIV-1.

HIV-1 is an enveloped lentivirus of diameter 80-120nm, with 72 envelope-glycoprotein projections of approximately 8-10nm (Collin *et al.*, 1991; Gelderblom, 1991). The virions have a density of 1.16-1.18g/cm^3 in sucrose gradients, and are readily disrupted with lipid solvents and detergents (Matthews, 1979). Viral particles are predominantly protein (60% weight) with cellular derived lipid (35%), cellular and viral carbohydrate (3.5%) with approximately 2% of the weight contributed by the RNA genome (Matthews, 1979). Thin sections of the virus reveal the presence of an outer lipid bilayer derived from the host cell envelope, with an inner membrane or shell, and a central nucleoid structure. The viral core is characteristically rod shaped, although EM sections demonstrate a variety of morphologies depending on the angle of the section (Gelderblom, 1991). The RNA genome associated with reverse transcriptase, protease and integrase activity is surrounded by the nucleocapsid protein forming the ribonucleo-protein complex (RNP). This is contained within the core and is surrounded by the matrix protein (Gelderblom, 1991). The core structure is attached at the narrow end to the matrix protein by a core-envelope-link protein (Höglund *et al.*, 1990) and surrounded by lateral bodies of irregular shape which may result from excess

Table 3. Classification of Retroviruses (Coffin, 1991).

Retrovirus genera
Mammalian type B oncovirus group
MLV-related viruses
(mammalian type C retrovirus group)
Type D retrovirus group
Avian type C retrovirus group
(ALV-related viruses)
Foamy virus group
HTLV-BLV group
Lentivirus group

MLV, murine leukaemia virus; ALV, avian leukosis virus; HTLV, human T cell lymphotropic virus; BLV, bovine leukaemia virus.

Table 4. Clinical manifestations of lentivirus infections (Coffin, 1991; Egberink, 1991)

Virus	Host	Disease
HIV-1 and 2	human	immunodeficiency, opportunistic infections, lymphadenopathy, neurological syndrome
SIV	monkey	immunodeficiency, neurological syndrome
Visna-maedi	sheep	progressive pneumonia, encephalomyelitis, generalised wasting
CAEV	goats	arthritis, encephalomyelitis, pneumonia
EIAV	horses	fever, weight loss, anaemia
FIV	cats	immunodeficiency, generalised lymphadenopathy, opportunistic infections, emaciation, neurological syndrome
BIV	cattle	lymphadenopathy, persistent lymphocytosis, wasting

HIV, human immunodeficiency virus; SIV, simian immunodeficiency virus; CAEV, caprine arthritis/encephalitis virus; EIAV, equine infectious anaemia virus; FIV, feline immunodeficiency virus; BIV, bovine immunodeficiency virus.

gag or regulatory proteins (Gelderblom, 1991). The viral structure is then enveloped by a cellular derived lipid bilayer with viral spikes composed of the transmembrane and surface glycoproteins (Leis *et al.*, 1988; Gelderblom, 1991). The protein nomenclature for retroviruses was standardized by Leis *et al.*, (1988) and is summarised in Table 5.

2.7 Genomic organisation of HIV-1.

The HIV-1 genome consists of linear positive-sense single-stranded RNA molecules that are capped and polyadenylated (approximately 9.2kb). Each viral particle contains 2 identical copies of RNA which are held together by hydrogen bonding (Matthews, 1979). Like all retroviruses, the HIV-1 genome contains 3 main protein coding genes, *gag*, *pol*, and *env*, flanked by regulatory sequences (R and U5 at the 5' end; U3 and R at the 3' end). During reverse transcription of the genome the regulatory sequences are duplicated resulting in the presence of the viral long terminal repeats (U3-R-U5) at both ends of the viral DNA (Schüpbach, 1989; Figure 3).

2.8 Replication of HIV-1.

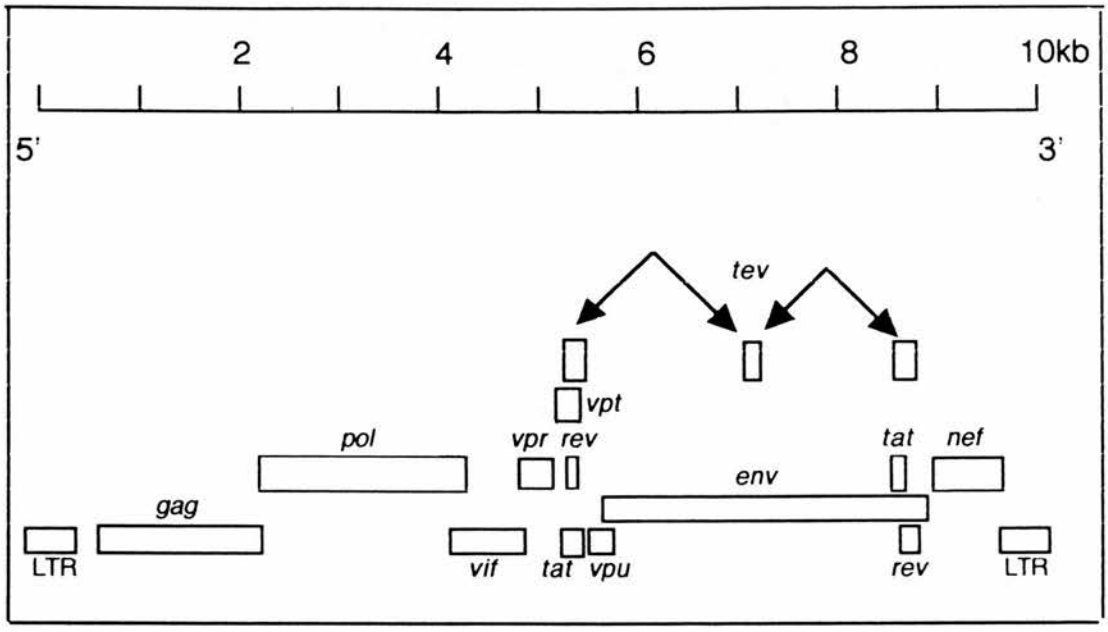
HIV, like all intracellular parasites, must attach and gain entry to susceptible cells to initiate replication. Two mechanisms of viral attachment have been elucidated from *in vitro* studies. Firstly, the virus tropism for T helper/inducer lymphocytes, monocytes, and macrophages expressing the CD4 differential antigen is mediated by an interaction with the viral surface glycoprotein gp120 (Dalgleish *et al.*, 1984; Klatzmann *et al.*, 1984a & b; Levy *et al.*, 1985; McDougal *et al.*, 1985; Deen *et al.*, 1988). The second mechanism involves the interaction of HIV specific antibodies with Fc receptors on the surface of some cells (Homsy *et al.*, 1989; Jouault *et al.*, 1989; Laurence *et al.*, 1990; McKeating *et al.*, 1990). The extent of viral attachment *in vivo* by either of these methods is, however, not known.

Table 5. Standardization of retroviral protein nomenclature using the example of HIV-1 proteins (Leis *et al.*, 1988).

Standard	Protein function	HIV-1 protein
MA	Matrix protein	p17
CA	Capsid protein	p24
NC	Nucleocapsid protein	p7
PR	Protease	p12
RT	Reverse transcriptase	p66
IN	Integration protein	p32
SU	Surface protein	gp120
TM	Transmembrane protein	gp41

p, protein
gp, glycoprotein

Figure 3. The genomic organisation of HIV-1



The figure depicts the coding exons for HIV-1 proteins. The structural proteins are coded for by *gag*, polymerase, integrase and proteinase enzymes by *pol* and the envelope glycoproteins by *env*. The other exons code for regulatory proteins active in the control of viral replication.

Once attached the virus must gain entry to the cell. The mode of penetration *in vivo* has yet to be proven, although 2 methods have been demonstrated *in vitro*. HIV can enter cells by direct fusion of the viral envelope with the cellular plasma membrane (Stein *et al.*, 1987; McClure *et al.*, 1988; Sinangil *et al.*, 1988) or by CD4 receptor-mediated uptake in clathrin coated pits (Pauza & Price, 1988; Sinangil *et al.*, 1988). It may be that both methods are utilised, and that the concentration effect observed *in vitro* also determines the mechanism of uptake *in vivo* (Goto *et al.*, 1988; Grewe *et al.*, 1990).

Aspects of the replication mechanism of retroviruses were determined by extensive research on a number of avian viruses, particularly avian sarcoma virus (ASV) of chickens, and spleen necrosis virus (SNV) of ducks.

Upon entry to the host cell the virion RNA is reverse transcribed to synthesize an anti-sense DNA copy in the cytoplasm of the cell. This is transcribed into dsDNA in parallel with the digestion of the template RNA, and is transported to the nucleus of the cell for integration (Varmus *et al.*, 1974a; Varmus & Shank, 1976). The replication mechanism duplicates the terminal sequences of the RNA genome, resulting in the formation of the long terminal repeats characteristic of the proviral DNA (Varmus & Swanstrom, 1982).

Although it is now widely accepted that retroviruses replicate via a DNA intermediate, it took almost a decade of careful experimentation to provide unequivocal evidence for the existence of a reverse transcriptase enzyme. Much of the initial work was carried out on Rous sarcoma virus (RSV) in which early experiments with inhibitors of DNA synthesis suggested that not only was DNA synthesis required for a productive infection, but the DNA was synthesized by viral components (Temin, 1963, 1967, 1968; Bader, 1965; Murray & Temin, 1970). The ultimate discovery which convinced most scientists of the proviral theory was the demonstration of RNA dependent DNA polymerase activity associated with RSV (Baltimore, 1970; Temin &

Mizutani, 1970). Despite opposition to the DNA provirus theory, Howard Temin was eventually rewarded with the Nobel prize in 1975, and his acceptance lecture was published in Science (Temin, 1976).

2.9 Integration of viral DNA.

Analysis of the structure of retroviral unintegrated DNA (UVD) revealed the presence of covalently closed circular forms in infected cells (Varmus *et al.*, 1974b; Kakefuda *et al.*, 1977; Shank *et al.*, 1978a & b). Two main forms of circular molecules were detected, differing by the presence of 1 or 2 LTR sequences (Shank *et al.*, 1978a & b, 1981; Hsu *et al.*, 1978; Yoshimura & Weinberg, 1979). These unintegrated circular forms demonstrated a variety of mutations with up to 50% expressing deletions and inversions, and some molecules, possibly resulting from autointegration, were of a greater size than the linear forms (Shoemaker *et al.*, 1980, 1981; Olsen *et al.*, 1990). The viral DNA was, therefore, present as relaxed circular (CUVD) and linear (LUVD) structures, and it was postulated that a circular form was the integration precursor as with bacteriophage lambda (Nash, 1975).

Evidence to support this theory came from the use of ethidium bromide which intercalates with DNA, thereby interfering with subsequent DNA interactions. The addition of ethidium bromide to cultures before infection was shown to inhibit integration of the provirus, and this was associated with a decline in CUVD (Guntaka *et al.*, 1975). Sequence analysis of the LTR-LTR junction of the 2LTR CUVD molecules showed a sequence similar to *att* in phage lambda, again supporting integration by homologous recombination (Panganiban & Temin, 1984).

In contrast to these findings, purification and separation of UVD prior to transformation assays, showed that the LUVD had a 5-10 fold higher specific infectivity than CUVD forms (Fritsch & Temin, 1977). Subsequent

development of a cell free *in vitro* integration system established that the linear DNA associated with viral protein was the integration intermediate (Brown *et al.*, 1987, 1989; Fujiwara & Mizuuchi, 1988; Lee & Coffin, 1991). The *in vitro* assays showed that the initial step in proviral integration was the removal of 2 bases from the 3' end of the linear molecule (Hsu *et al.*, 1978; Lee & Coffin 1990, 1991). The viral genome was then integrated into the target DNA duplicating the cellular sequence flanking the provirus. The length of sequence duplicated was shown to be characteristic of the virus; avian leukosis virus has a 6 base repeat (Lee & Coffin, 1990), murine leukaemia virus 4 bases (Fujiwara & Craigie, 1989), and HIV-1 a 5 base duplication (Vink *et al.*, 1990), and the protein required was coded for by the 3' sequence of the viral *pol* gene (Donehower & Varmus, 1984; Schwartzberg *et al.*, 1984; Quinn & Grandgenett, 1988; Vink *et al.*, 1990). Sequence analysis of the flanking cellular DNA revealed no apparent sequence specificity, although integration was associated with DNase 1-hypersensitivity sites (Vijaya *et al.*, 1986; Rohdewohld *et al.*, 1987), and replicative or transcriptionally active sites (Varmus *et al.*, 1977; Shih *et al.*, 1988; Scherdin *et al.*, 1990).

2.10 Protein products of *gag*, *pol*, and *env* genes.

Once integrated into the cellular genome, the provirus can be transcribed by cellular enzymes to synthesize viral components. The *gag* and *pol* genes are transcribed to give 2 products, the p55 *gag* precursor, and the p160 *gag-pol* precursor (Hammarskjöld & Rekosh, 1989). Both are proteolytically cleaved by the viral protease to produce 4 *gag* proteins; p17, the matrix protein; p24, the capsid protein; p7, the nucleocapsid protein,; and p9, a proline-rich protein of unknown function (Hammarskjöld & Rekosh, 1989; Oroszlan & Luftig, 1990; McClure & Dalgleish, 1992). The *pol* protein precursors are also cleaved by the viral protease to produce p12 (protease), p66/51 (reverse transcriptase)

and p32 (integrase) (Hammarskjöld & Rekosh, 1989; Oroszlan & Luftig, 1990; Vaishnav & Wong-Staal, 1991).

The *pol* gene is in a -1 reading frame relative to the *gag* gene, and so a ribosomal frameshift is required to allow expression of *pol* sequences. A short sequence at the *gag-pol* overlap directs this frameshift approximately 5% of the time. In this way, large amounts of structural *gag* proteins are synthesized, and relatively small quantities of catalytic *pol* proteins are made (Oroszlan & Luftig, 1990; Vaishnav & Wong-Staal, 1991).

The primary product of the *env* gene is the glycoprotein gp160 which is cleaved by cellular enzymes to form the transmembrane and surface glycoproteins gp41 and gp120 respectively. Although the glycoproteins remain non-covalently linked, their cleavage is mandatory for viral binding, entry, and infection of susceptible cells (Modrow *et al.*, 1987; McCune *et al.*, 1988; Willey *et al.*, 1988; Hart *et al.*, 1991).

2.11 Regulatory genes of HIV-1.

A number of potential regulatory genes have been determined for HIV-1. The genes identified to date include *tat*, *rev*, *nef*, *vif*, *vpu*, *vpr*, *vpt*, and *tev* or *tnv* (Figure 3).

The *tat* gene codes for a 14kDa nuclear protein from 2 coding exons and is essential for viral replication (Arya *et al.*, 1985; Sodroski *et al.*, 1985; Fisher *et al.*, 1986; Dayton *et al.*, 1986; Hauber *et al.*, 1987). The protein trans-activates expression of viral genes via a transactivating response region (TAR) situated in the LTR sequences (Rosen *et al.*, 1985; Hauber & Cullen, 1988). The exact mechanism of this activation is not known although *in vitro* studies have demonstrated effects at the transcriptional, post-transcriptional, and translational levels (Cullen, 1986; Feinberg *et al.*, 1986; Peterlin *et al.*, 1986; Wright *et al.*, 1986; Hauber *et al.*, 1987; Kao *et al.*, 1987; Muesing *et al.*,

1987).

The *rev* gene product, like *tat*, is a nuclear protein coded by 2 exons and is essential for viral replication (Cullen *et al.*, 1988; Feinberg *et al.*, 1986; Sodroski *et al.*, 1986b). The protein (19-20kDa) is phosphorylated and appears to act via a *cis*-acting sequence in *env* named *rev* responsive element (RRE). Activation results in the switch from early to late protein synthesis, thereby stimulating the production of structural proteins (Feinberg *et al.*, 1986; Kim *et al.*, 1989; Malim *et al.*, 1989).

The *nef* gene product is coded for by a single ORF at the 3' end of *env* continuing into the 3' LTR sequence and is not essential for viral replication (Allan *et al.*, 1985; Terwilliger *et al.*, 1986). The cytoplasmic protein (25-27kDa) is phosphorylated, and mutants replicate to a greater extent than wild type virus, demonstrating a negative regulatory role (Franchini *et al.*, 1986; Guy *et al.*, 1987; Niederman *et al.*, 1989).

The *tat*, *rev*, and *nef* regulatory genes are the most clearly defined, although other sequences have now been identified. The *vif* (virus infectivity factor) gene codes for a 23kDa protein expressed *in vivo* which appears to have an effect on the infectivity of the virus (Lee *et al.*, 1986; Sodroski *et al.*, 1986c; Fisher *et al.*, 1987). Mutants are characterised by a reduction in infectivity despite normal levels of intracellular viral protein and extracellular virus (Kan *et al.*, 1986; Sodroski *et al.*, 1986c; Strebel *et al.*, 1987). A 15-20kDa cytoplasmic protein is coded for by the *vpu* gene (Cohen *et al.*, 1988; Matsuda *et al.*, 1988; Strebel *et al.*, 1988). Mutational analysis demonstrated a role for the gene product in the export of virus particles, with an associated decrease in intracellular viral protein accumulation, and subsequent syncytium formation and cell death (Strebel *et al.*, 1988; Terwilliger *et al.*, 1989; Klimkait *et al.*, 1990). *Vpu* mutants expressed levels of viral protein equivalent to the wild type and were observed undergoing intracellular budding, suggesting a

possible role in assembly and/or budding of progeny virus (Klimkait *et al.*, 1990).

Unlike all other regulatory genes, the product of the *vpr* gene is associated with the virion. The protein (15kDa) is present in multiple copies, and is thought to play a role in the early stimulation of viral replication and subsequent CPE (Ogawa *et al.*, 1989; Cohen *et al.*, 1990). The *tev* or *tnv* protein (28kDa) has both *tat* and *rev* activities and is known to be present early in the replication cycle although a regulatory role has yet to be determined (Benko *et al.*, 1990; Salfeld *et al.*, 1990). A final highly conserved ORF has been demonstrated and named *vpt* although no expression has been established *in vitro* (Cohen *et al.*, 1990).

In addition to the regulatory proteins so far described for HIV, non-coding sequences have been detailed which are important for viral replication. A packaging sequence has been demonstrated between the 5' LTR and *gag* gene (Lever *et al.*, 1989; Clavel & Orenstein, 1990) and binding sites have been determined for the cellular transcriptional activators Sp1 and NF-kB (Jones *et al.*, 1986; Nabel & Baltimore, 1987).

2.12 Cytopathogenicity.

To understand the mechanism by which cell death occurred in retroviral infections, a number of groups attempted to determine the proviral copy number, and infectivity in susceptible cells. It had been observed that cells infected with SNV or ALV resulted in a biphasic infection, with an initial acute phase associated with cell death. The cells surviving this cytopathic phase appeared to be chronically infected, resulting in little or no cell death. Hybridization and transformation studies demonstrated quantitative and qualitative differences in the viral DNA content of the 2 populations of cells. The acute phase infection resulted in multiple provirus integration sites, of

which only a fraction were infectious, in contrast to the chronically infected cells which contained only one infectious integrated provirus. Since multiple integrations were associated with the cell pathology, it was proposed that they impaired the function of cellular genes, and over produced viral products leading to cell death (Battula & Temin, 1978; Keshet & Temin, 1978).

Further analysis of viral DNA in infected cells demonstrated that the accumulation of UVD correlated with the observed cytopathic effects (Keshet & Temin, 1979; Temin *et al.*, 1979). Quantitation of viral DNA during the transition from the acute to chronic stages of infection demonstrated a 70 to 80-fold decrease in UVD, and a 7 to 8-fold decrease in integrated DNA (Keshet & Temin, 1979; Weller *et al.*, 1980).

The accumulation of UVD was efficiently blocked by the addition of neutralising antibody to the culture (Weller *et al.*, 1980; Temin, 1988), which led to the theory that the build up of UVD resulted from a second round of infection, or superinfection. Superinfection was thus prevented in chronically infected cells by the establishment of viral interference, a property believed to be related to the envelope of the virus (Temin *et al.*, 1979; Weller & Temin, 1981; Chen & Temin, 1982). However, quantitation of UVD and virus production failed to establish a relationship suggesting that UVD was transcriptionally defective, and/or that virus production was solely directed by the integrated provirus (Keshet & Temin, 1979; Temin *et al.*, 1979; Weller & Temin, 1981; Chen & Temin, 1982).

Although a role for UVD in the retroviral life cycle has yet to be established, a relationship has been demonstrated with the clinical outcome in some retroviral infections. UVD is associated with osteopetrosis in chickens infected with ALV (Robinson & Miles, 1985), but more importantly it has been implicated in the cytopathogenicity observed in cats suffering from feline AIDS (Mullins *et al.*, 1986; Hoover *et al.*, 1987).

2.13 Detection and characterization of HIV-1 nucleic acid.

HIV-1 DNA was initially detected by hybridization assays. Dot blot hybridization was able to detect viral DNA in 1 infected cell in 900 negative cells, and *in situ* hybridization 1 in 50000 cells (Pezzella *et al.*, 1989; Singer *et al.*, 1989). This was found to be insufficient sensitivity for HIV-1 DNA detection in some patients (Simmonds *et al.*, 1990a). The polymerase chain reaction, developed in parallel to early HIV research, has rapidly been adopted by researchers as the only available method with sufficient sensitivity for HIV-1 DNA detection in clinical samples from patients at all stages of infection.

HIV-1 DNA was first detected by PCR in 1987 using 20-25 cycles of amplification followed by hybridization (Kwok *et al.*, 1987). Although relatively insensitive, this led the way in the rapid optimisation of PCR for the detection of single copy DNA templates (Simmonds *et al.*, 1990a). HIV-1 DNA was detected in samples both positive and negative for virus isolation (Ou *et al.*, 1988) and was rapidly confirmed to be more sensitive than virus isolation as it was possible to detect HIV-1 DNA in up to 100% of seropositive patients who were negative by virus culture (Psallidopoulos *et al.*, 1989; Schneweis *et al.*, 1989; Stoeckl *et al.*, 1989). PCR has also been invaluable in the diagnosis of infection in infants born to HIV positive mothers where maternal antibodies prevent conventional diagnosis, and small sample volumes make virus isolation techniques difficult (de Rossi *et al.*, 1988; Laure *et al.*, 1988). It has also been applied to the diagnosis of infected individuals up to 42 months prior to seroconversion (Loche & Mach, 1988; Imagawa *et al.*, 1989; Wolinsky *et al.*, 1989), and in confirmation of infections in previously seropositive patients who have lost HIV specific antibodies (Farzadegan *et al.*, 1988). PCR is, therefore, a more rapid and sensitive technique than virus isolation and hybridization assays for the direct detection of HIV-1 in all groups of infected patients.

The direct detection of HIV-1 DNA by PCR in PBMCs is most probably due to the presence of integrated proviral DNA in susceptible cells, and is highly indicative of infection. It is, however, possible that the amplification bands observed arise from the detection of UVD as observed in other retroviral infections (Varmus *et al.*, 1974a & b; Kakefuda *et al.*, 1977; Shank *et al.*, 1978a & b; Robinson & Miles, 1985; Mullins *et al.*, 1986; Hoover *et al.*, 1987). To determine the forms of viral DNA present in clinical samples it was decided to modify PCR to detect HIV-1 DNA and to differentiate between different structural forms present.

The detection of HIV-1 DNA alone is not indicative of a productive infection. To demonstrate active viral replication, total RNA was extracted from patient samples and assayed for by reverse transcription and amplification of cDNA (RT-PCR). HIV-1 RNA was similarly detected from whole virus using gp120/160 specific antibody coated beads for extraction of infectious particles.

CHAPTER 3: THE HEPATITIS B VIRUS (HBV).

3.1 HBV and hepatitis B.

The hepatitis B virus (HBV) is the aetiological agent for serum hepatitis, first described in the early 20th century (Propert, 1938). It was differentiated from other agents responsible for hepatitis by studies in human volunteers showing different routes of transmission (Neefe, 1946), incubation periods (Paul *et al.*, 1945), and a lack of cross immunity (Havens, 1945). The discovery of the Australia antigen by Blumberg in 1964, and its subsequent association with hepatitis (Blumberg, 1964; Blumberg, *et al.*, 1967; Bayer *et al.*, 1968; Okachi & Murahani, 1968; Prince, 1968; Gerin *et al.*, 1969; Barker *et al.*, 1973), led to the detection of the viral particle (Dane *et al.*, 1970). The Australia antigen, now known as the hepatitis B surface antigen (HBsAg), was first identified in the form of 20nm virus-like spherical and tubular particles. These are non-infectious, containing no genetic information, and are composed solely of HBsAg and host-derived lipid (Shih & Gerin, 1977; Aggerbeck & Peterson, 1985). They are very abundant in the blood of infected individuals (50-300ug HBsAg/ml of serum), and elicit a strong antibody response which confers immunity to reinfection (Ganem & Varmus, 1987). The role of these particles in HBV infection is not known, although it is speculated that they adsorb neutralising antibody, facilitating the free movement of infectious virions. This theory is supported by the development of arthritis due to immune complexes formed in the joints of acutely infected individuals (Gocke, 1975).

HBV was thought to be unique from all other virus groups due to its unusual liver tropism, genome structure, and proposed replication strategy. The discovery of hepatotropic viruses in woodchucks (Summers *et al.*, 1978), ground squirrels (Marion *et al.*, 1980) and domestic ducks (Mason *et al.*, 1980) with similar morphology and molecular biology, led to the formal

recognition of a new virus family called the Hepadnaviridae (Gust *et al.*, 1986). The term hepadna virus was first used by Robinson, (1980) to combine the cell tropism of the virus (hepa) with their unusual (DNA) genomes for hepatitis viruses.

The lack of an *in vitro* culture system for HBV has retarded the understanding of its molecular biology, however, comparative studies of other hepadnaviruses in their appropriate hosts has allowed considerable advances to be made.

3.2 Clinical spectrum.

Hepatitis B is highly contagious and is transmitted vertically and horizontally in body fluids such as blood, saliva, semen, vaginal secretions, and breast milk. Currently, the main modes of transmission are sexual, vertical transmission at birth, and the sharing of needles by drug users. The rate of transmission has been reduced, largely due to educating those at risk, the screening of blood donations, and the use of passive and active immunization. Hepatitis B immunoglobulin is given to protect exposed individuals, while the plasma derived and now the recombinant yeast subunit vaccines (HBsAg) are offered in developed countries to sub-populations of the community who are professionally or socially at risk (Teo, 1992).

Most adult HBV infections are self-limited and 90-95% resolve completely within 6 months of the onset of symptoms. Such individuals may experience clinical features ranging from an asymptomatic infection to an explosive and life threatening form known as fulminant hepatitis. A typical acute infection has 4 distinct phases: 1) the incubation period of 30-180 days; 2) the pre-icteric phase of 3-10 days with symptoms of malaise and weakness, followed by anorexia, intermittent nausea, vomiting, and a dull abdominal pain; 3) the icteric phase usually lasting 1-3 weeks characterised by the presence of

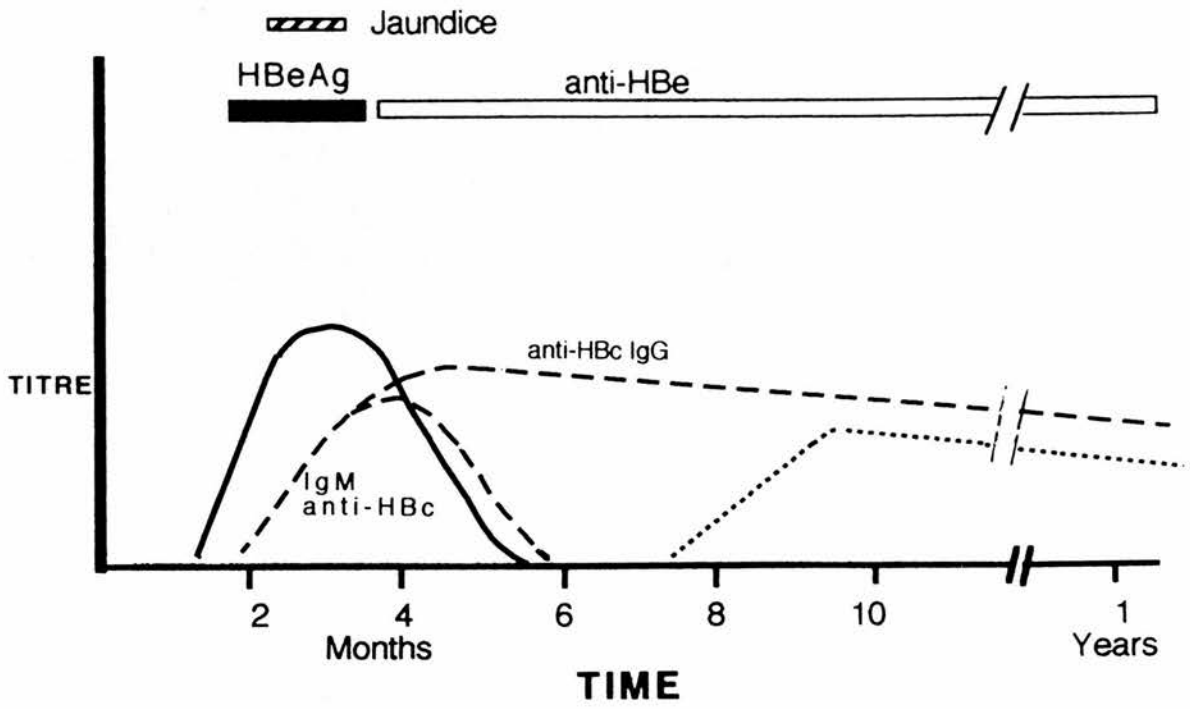
jaundice, pale stools, and dark urine; and 4) the convalescence phase (Koff, 1978; Jungle & Deinhardt, 1985).

The disease is diagnosed by laboratory detection of elevated bilirubin and amino transferases (indicating liver cell damage), and the detection of HBsAg and anti-HBc in the serum (Hoofnagle, 1990). The typical chronology of infection with HBV is represented in Figure 4. The first diagnostic indicator of infection is the detection of HBsAg in patients' serum. The antigen is present prior to symptoms, with a maximum titre corresponding to the height of liver damage. The HBeAg is a product of the core gene, is associated with viral replication and usually appears shortly after the detection of HBsAg. The detection of IgM anti-HBc antibodies is transient, and is a clear indicator of the acute phase of the infection. Seroconversion to anti-HBe marks the resolution of the infection, which is later confirmed by the disappearance of HBsAg and the subsequent appearance and increase in titre of anti-HBs neutralising antibodies.

A distinguishing feature of HBV is its ability to establish a chronic infection. Up to 10% of acute infections, particularly infections of the old and young, progress to a chronic state. This is characterised by the persistence of HBsAg and abnormal liver function for 6 months or more. Two categories of chronicity have been defined: chronic persistent hepatitis (CPH), and chronic active hepatitis (CAH). Both forms are clinically similar, but CAH, unlike CPH, is associated with a high risk of developing liver disease such as cirrhosis and hepatocellular carcinoma (Hirschman, 1990).

The carrier state is, therefore, a very important development of the infection, establishing a large reservoir of infection, and increasing the risk of developing chronic liver diseases.

Figure 4. The chronology of events in a patient with acute Hepatitis B infection.



HBsAg ---- anti-HBc

HBeAg anti-HBe

Jaundice ..... anti-HBs

3.3 Morphology of HBV.

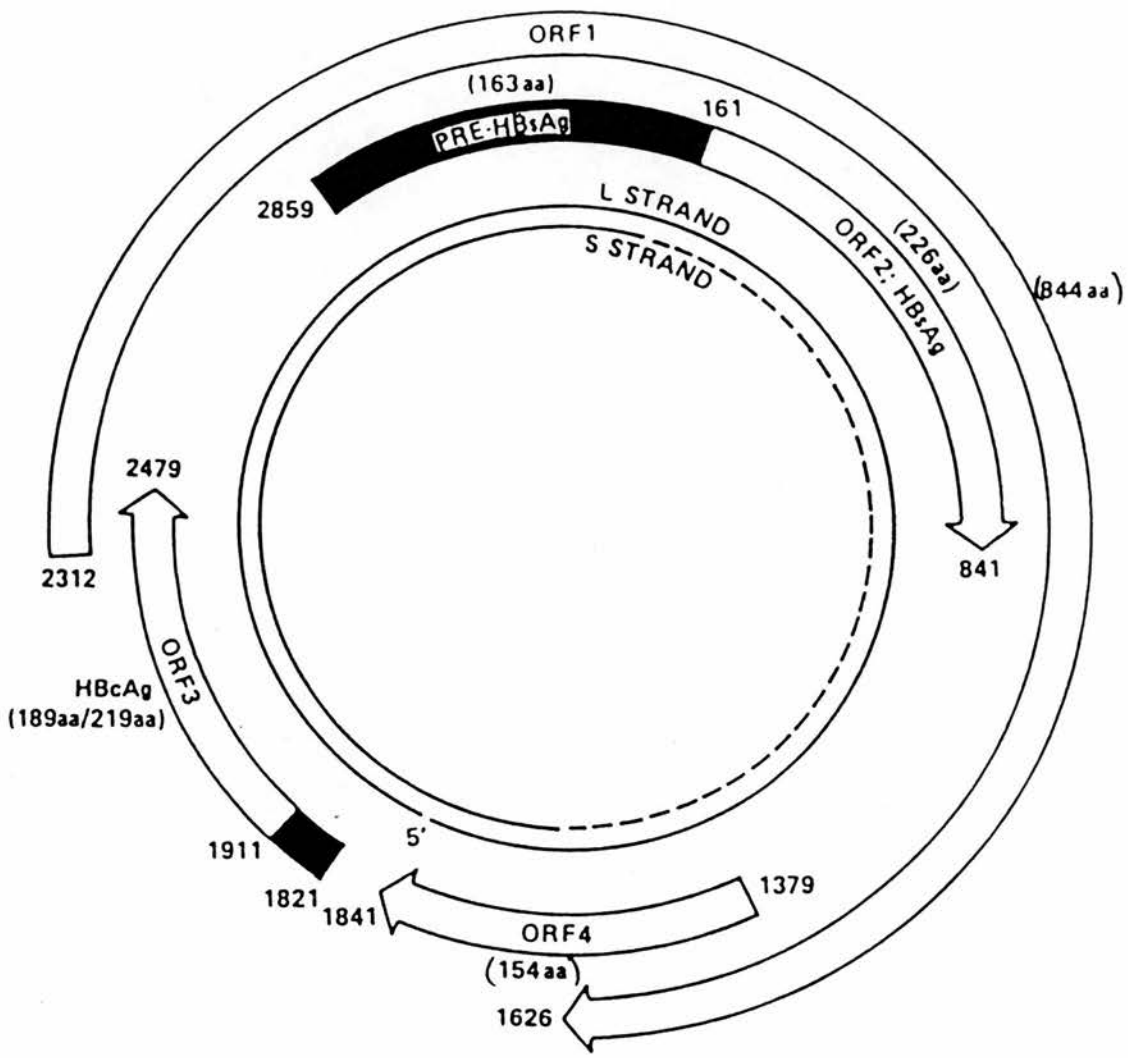
Hepadna viruses have a distinctive morphology with an electron-dense spherical inner core (27nm diameter) surrounded by an outer shell (Dane *et al.*, 1970). The outer shell, or envelope, is composed of cellular derived lipids and carbohydrates with a viral protein HBsAg (Dane *et al.*, 1970). Treatment of the virion with non-ionic detergents revealed an antigen distinct from HBsAg called the core antigen (HBcAg). HBcAg forms the inner core or nucleocapsid structure, and is only found as part of the virion in the blood of infected individuals (Almeida *et al.*, 1971). Disruption of the nucleocapsid leads to the release of a third HBV protein, the hepatitis B e antigen (HBeAg), which is found in a non-particulate form in the blood (Magnius & Espmark, 1972; Takahashi *et al.*, 1979).

The viral genome, contained within the core, is a circular molecule of partially double stranded DNA (Robinson *et al.*, 1974), and is associated with DNA polymerase and protein kinase activity (Hirschman *et al.*, 1971; Kaplan *et al.*, 1973; Robinson & Greenman, 1974; Albin & Robinson, 1980).

3.4 HBV genomic structure.

The HBV DNA genome is unique in that the small circular molecule is only partially double stranded (Summers *et al.*, 1975; Sattler and Robinson, 1979; Figure 5). The long (L) minus strand (3128 bases) contains all the coding capacity, and is held in a circular form by the variable length (1700-2800 bases) short (S) strand (Marion & Robinson, 1983). The L strand has 4 open reading frames (ORF), termed P, S, C, and X, and the S strand has no ORF of any considerable length. ORF P overlaps the other 3 coding sequences, and encodes the viral polymerase (Tiollais *et al.*, 1985). ORF S codes for 3 forms of the HBsAg, and is divided into the pre-S1, pre-S2, and S-gene regions (Galibert *et al.*, 1979; Valenzuela *et al.*, 1979).

Figure 5. Physical map of HBV DNA genome



The genome is represented by the lines labelled L and S strands (long and short strands respectively). The dotted line depicts the incomplete region of the S strand which is synthesized during replication by the virion polymerase. This region varies in length in different hepadnaviruses. The arrows represent open reading frames (ORF 1 to 4) of the L strand. The nucleotide positions are given with amino acid lengths in brackets. ORF1 codes for the polymerase enzyme, ORF2 for HBsAg, and ORF3 for HBcAg. The function of ORF4 has still to be determined. This figure was adapted from Marion & Robinson, (1983).

The major or S protein is coded for by the S-gene, and is the main protein present in each of the 3 forms of HBsAg containing particles (Cattaneo *et al.*, 1983). The middle protein initiates at the second start codon at pre-S2, and is mainly present in the spherical particles (Stibbe & Gerlich, 1983). The large protein is coded for by pre-S1, pre-S2, and the S-gene, and is found predominantly in filaments and virus particles (Heermann *et al.*, 1984). ORF C codes for the HBc and HBe antigens, and like the S region has a pre-C coding sequence (Pasek *et al.*, 1979). The function of the X-gene remains to be determined, although there is evidence to suggest a possible role in the *trans*-activation of the virus (Spandau & Lee, 1988).

3.5 Replication of HBV.

Upon entry to the cell, the viral nucleocapsid is transported to the nucleus of the cell where the genome is converted to covalently closed circular DNA by the viral DNA polymerase (Summers *et al.*, 1975; Hruska *et al.*, 1977; Landers *et al.*, 1977). The genome is then transcribed into 5 main species (2.4kb, 2.1kb, 0.9kb, and 2 at approximately 3.5kb). Two (2.1kb and 2.4kb) transcripts are translated to produce the 3 HBsAg polypeptides (Cattaneo *et al.*, 1983; Standring *et al.*, 1984), and the 0.9kb transcript codes for the X gene product (Gough, 1983; Araki *et al.*, 1989). The two longest transcripts exceed the genomic length due to terminal redundancy, and differ by the presence or absence of the pre-core translation initiation site (Büscher *et al.*, 1985; Möröy *et al.*, 1985; Enders *et al.*, 1987). The shorter transcript is the mRNA for synthesis of core and polymerase proteins, and also acts as the template for reverse transcription to produce the viral genome (Gowans *et al.*, 1981; Summers & Mason, 1982). The longer transcript is the mRNA for the synthesis of HBeAg (Nassal *et al.*, 1990; Yaginuma *et al.*, 1987). The genomic RNA template is assembled with viral polymerase and HBcAg to form the

nucleocapsid. Once encapsidated, reverse transcription of the RNA template is initiated, generating the long minus strand which then acts as a template for the shorter plus strand synthesis (Summers & Mason, 1982; Lien *et al.*, 1986; Seeger *et al.*, 1986). The lipid and HBsAg envelope can be acquired any time after core assembly, irrespective of the replicative state of the viral genome (Miller *et al.*, 1984).

3.6 The hepatitis B viral e and surface antigens.

The hepatitis viral antigens were initially identified and characterised by immunodiffusion techniques. When these tests were carried out in agar gels, it was possible to distinguish separate antigen-antibody reactions represented by precipitin lines, and to determine the relationship between antigens. For a review of the methodology see Ouchterlony, (1958).

3.6.1 The hepatitis B e antigen.

Competitive radioimmunoassays have now been developed for the determination of HBe status of patient sera. The assays detect HBeAg and its corresponding antibodies, and samples negative for both are classified HBe negative. The antigen is found free in infectious sera, and its presence has been analysed in relation to the patient's sex, serum liver enzyme concentrations, age, HBsAg titre, cirrhosis, and specific HBsAg subtypes (Nielsen *et al.*, 1974; Couroucé-Pauty & Plançon, 1978; Miller *et al.*, 1978; Nath *et al.*, 1978; Barr *et al.*, 1979; Sasaki *et al.*, 1979; Aldershvile *et al.*, 1980; Realdi *et al.*, 1980). The only consistent association established is with active viral replication and, therefore, infectivity (Magnius *et al.*, 1975b). In support of this, HBeAg positive sera used for transmission studies in chimpanzees were found to be 10^8-fold more infectious than anti-HBe positive sera (Shikata *et al.*, 1977), and HBeAg has also been associated with a higher rate of transmission from mother to child (Okada *et al.*, 1976) and in needle-

stick injuries (Alter *et al.*, 1976). The correlation of HBeAg with infectivity was further supported by its association with the detection of HBV DNA polymerase in serum samples (Alter *et al.*, 1976; Imai *et al.*, 1976; Cappel *et al.*, 1977; Tong *et al.*, 1977; Alberti *et al.*, 1979).

3.6.2 The hepatitis B surface antigen.

HBsAg is a complex molecule of protein, lipid, and carbohydrate found in 3 forms in the sera of patients infected with HBV (Steiner *et al.*, 1974; Dreesman *et al.*, 1975). The antigen is a component of the viral envelope, but also forms spherical particles of diameter 22nm, and filamentous structures of variable length (diameter 22nm), both of which are non-infectious and found in abundance in sera of infected individuals (Bayer *et al.*, 1968). There are 3 forms of the HBsAg, the major, middle, and large surface antigen (Cattaneo *et al.*, 1983; Stibbe & Gerlich, 1983; Heerman *et al.*, 1984). The major protein is found in all 3 forms in the blood, whereas the middle protein is mainly found in the spherical particles, and the large protein in filamentous particles and virions.

All HBsAg molecules contain a group specific determinant (*a*), and one of each pair of determinants *d/y* and *w/r* which are usually mutually exclusive (Levene & Blumberg, 1969; Purcell *et al.*, 1969; Raunio *et al.*, 1970; Le Bouvier, 1971; Greenberg & Gocke, 1971; Kim & Tilles, 1971; Bancroft *et al.*, 1972; Le Bouvier *et al.*, 1972; Gordon *et al.*, 1972; Holland *et al.*, 1972). This was apparent from the diffusion techniques, as spurs occurred indicating partial identity between test antigens. Further determinants (*g* and *q*) and subdeterminants (w_1-w_4) were later defined (Soulier & Couroucé-Pauty, 1973; Magnius *et al.*, 1975a; Shorey, 1976).

Once the subtypes had been determined, more rapid assays were sought for their detection. The complement fixation test was developed offering a more

sensitive and rapid subtyping method (Purcell *et al.*, 1969; Schmidt & Lennette, 1970), however, it was the production of antibodies to HBsAg (Millman *et al.*, 1970), and in particular high affinity monoclonal antibodies (Wands & Zurawski Jr., 1981) which led to the use of highly sensitive and specific radioimmunoassays (Shorey, 1976; Hoofnagle *et al.*, 1977; Burrell *et al.*, 1978; Fang *et al.*, 1978; Ukkonen & Koistinen, 1979).

The advent of molecular techniques, such as the cloning and sequencing of HBV DNA, permitted sequence analysis of the amino acid and base composition of the antigen and its gene (Maxam & Gilbert, 1977; Sanger *et al.*, 1977; Burrell *et al.*, 1979; Charnay *et al.*, 1979; Galibert *et al.*, 1979). This led to the discovery that the *d* and *y* subtypes were both associated with amino acid 122 (Okamoto *et al.*, 1986), and that *w* and *r* subtypes were defined in the hydrophilic region from amino acid 110 to 160 (Takeshima *et al.*, 1985). Comparisons of known subtypic sequences of HBsAg and site directed mutagenesis determined that nucleotide 365 of amino acid 122 determined the *d/y* subtype, and nucleotide 479 of amino acid 160 determined the *w/r* subtype (Peterson *et al.*, 1984; Okamoto *et al.*, 1987a, 1987b). In both cases, the nucleotide was either G (*y* and *r*) or A (*d* and *w*) coding for arginine or lysine which determined the subtype. Later studies suggested that although amino acids 122 and 160 were critical to the subtype determinants, other residues may be involved in conforming these determinants (Okamoto *et al.*, 1989a; Ashton-Rickhardt & Murray, 1989).

Although no clinical significance has been demonstrated, the 9 subtypes (*ayw*$_1$, *ayw*$_2$, *ayw*$_3$, *ayw*$_4$, *ayr*, *adw*$_2$, *adw*$_4$, *adrq-*, and *adrq+*) have distinct geographical distributions (Couroucé-Pauty *et al.*, 1983) and have been used to trace sources of infection (Mayumi & Nakajima, 1973) and ancestral migration (Yamashita *et al.*, 1975). In addition to these well documented subtypes, atypical subtypes such as *adyw, adyr, adwr, aywr, adywr, ad, ar,*

and *aw* have been described (Nordenfelt & Le Bouvier, 1973/74; Mazzur *et al.*, 1975; Robinson, 1980; Tachibana *et al.*, 1989).

3.7 Detection and subtyping of HBsAg DNA.

HBV DNA has been detected in serum samples by hybridization assays. Viral DNA was detected in 91-100% of HBeAg positive samples, 5-100% of HBe negative samples, and up to 58% of anti-HBe antibody positive samples (Weller *et al.*, 1982; Hadziyannis *et al.*, 1983; Lieberman *et al.*, 1983; Scotto *et al.*, 1983; Karayiannis *et al.*, 1985; Bonino *et al.*, 1986; Carloni *et al.*, 1987; Cheng *et al.*, 1989). Similarly, the application of PCR to HBV DNA detection in serum demonstrated a 100% association with HBeAg, and up to 89% with anti-HBe antibody positive samples (Larzul *et al.*, 1988, 1989; Kaneko *et al.*, 1989a, 1989b; Liang *et al.*, 1989; Okamoto *et al.*, 1989b; Sumazaki *et al.*, 1989; Ulrich *et al.*, 1989; Zeldis *et al.*, 1989). It was, therefore, decided to apply PCR to the detection of HBV DNA in a cohort of hepatitis B patients, and to determine the association with HBe status.

A method to selectively extract viral DNA from whole virus and immunoglobulin complexed virus was developed using antibody coated polystyrene beads. The aim of this was to determine the form of viral DNA present in the serum of patients at different clinical stages (free virus, immunoglobulin complexed virus, or naked viral DNA), and to establish a relationship with HBe status and infectivity of the patient.

The subtyping of HBsAg has been based on immunoassays due to the abundance of HBsAg present in serum samples throughout the infection. However, due to the increased sensitivity of HBV DNA detection offered by PCR, it was decided to develop a method of PCR subtyping using primers specific for the S-gene DNA sequence.

MATERIALS AND METHODS

AIMS OF THE THESIS.

The polymerase chain reaction (PCR) is the most sensitive method for the detection of specific nucleic acid sequences. In view of this, it was decided to apply the technique to the detection and characterization of human immunodeficiency virus and human hepatitis B virus nucleic acids in clinical samples. Areas of research included:

Human immunodeficiency virus.

1) Detection of HIV DNA in patient peripheral blood mononuclear cells (PBMCs) by nested PCR using *pol* and *env* specific primers.
2) Characterization of the structural forms of unintegrated viral DNA (UVD) detected in cell lines and clinical samples.
3) Detection and characterization of viral DNA in PBMC subsets and in nuclear and cytoplasmic extracts.
4) Detection of viral RNA sequences from infectious virus using anti-gp120 antibody coated polystyrene beads.

Hepatitis B virus.

1) Detection of HBV DNA in patient sera and correlation to HBe status.
2) Subtyping of HBsAg determinants *d* and *y* by subtype specific amplification of the S-gene by PCR, and restriction digest of subtype common PCR products, and comparisons of these DNA subtyping methods with conventional HBsAg subtyping by RIPA.
3) Detection of HBV DNA in sera after antibody coated polystyrene bead extractions of whole virus and IgG and IgM complexed virus.

CHAPTER 4: MATERIALS AND METHODS.

4.1 SOURCES OF PATIENT SAMPLES.

4.1.1 HBsAg and anti-HBs positive sera.

Sera submitted to the Hepatitis Reference Laboratory (HRL, Department of Medical Microbiology, The University of Edinburgh) were tested by standard radioimmunoassays for HBsAg (Blood Products Laboratory, Elstree), anti-HBs (Hepatitis Reference Laboratory (HRL), Edinburgh), and HBeAg/anti-HBe (Middlesex Hospital). The 115 samples studied (P35-P149) were selected to include a range of HBV serological markers: 58 HBsAg/HBeAg positive; 6 HBsAg positive, HBeAg/anti-HBe negative; 25 HBsAg/anti-HBe positive; 11 anti-HBs positive, HBeAg/anti-HBe negative; 10 anti-HBs/anti-HBe positive; 3 HBsAg/anti-HBs negative, HBeAg/anti-HBe negative; and 2 HBsAg/anti-HBs negative, anti-HBe positive (Appendix I).

4.1.2 HBV negative sera.

Samples from 10 low-risk seronegative individuals were used as negative controls. These samples were supplied by co-workers at HRL, who had submitted sera for assessment of their response to the HBV vaccine.

4.1.3 HIV-1 positive and negative heparinised whole blood samples.

Heparinised whole blood (HWB) samples were supplied by Dr A. McMillan and colleagues, from patients attending the Genito-Urinary Medicine out-patient clinic, Royal Infirmary of Edinburgh. Samples (20ml) were collected by venipuncture, and supplied in 25ml universals containing 300 units of heparin (approximately 12U/ml of blood, Leo Laboratories Ltd.). Total lymphocyte and $CD4^+$ cell counts were supplied if available.

A total of 61 samples from 34 patients were received over a period of 31 months from July 1989 to February 1992. Thirty-two patients were known to

be HIV-1 positive, and 2 were partners of HIV-1 positive patients, and were included as high risk negative controls. Patients were aged between 21 and 54 years; 27 male and 7 female.

Cells from HIV-1 negative blood donors were supplied as leucocyte concentrates on a weekly basis (Scottish Blood Transfusion Service).

4.2 VIRUS ISOLATION.

4.2.1 Separation of PBMCs from HWB samples.

Approximately 20ml of heparinised blood was layered onto an equal volume of Lympho-paque (Nyegaard Diagnostica), and centrifuged at 450g for 30min. After separation, the cells were washed 3 times in RPMI (Gibco BRL), counted in a haemocytometer, and processed for culture and PCR analysis. The plasma was tested for p24 antigen, and plasma and cells not used immediately were stored at -70°C. Cells for storage were counted and resuspended in freezing medium (heat inactivated foetal calf serum (40min at 56°C) supplemented with 8% DMSO, Sigma).

4.2.2 Cell counting.

A small volume of cells was diluted in white cell diluting fluid (0.01% gentian violet, 1% acetic acid in dH_2O) to give approximately 50 to 100 cells per large square on the Neubauer haemocytometer. Cells were counted in the centre and 4 corner squares, and the total number divided by 5 to give an average cell count per square. This was multiplied by 10^4 to calculate the number of cells per millilitre of suspension. The number of cells per ml of undiluted suspension was calculated as follows;

$$\text{No. of cells per ml of undiluted cell suspension} = \frac{10^4 \, n \, y}{5}$$

Where, n is the total cell count, and y the dilution factor.

4.2.3 HIV-1 p24 Ag detection.

Patient plasma samples and culture supernates were analysed for p24 viral antigen by an enzyme immunoassay (Coulter HIV p24 antigen assay), and confirmed by neutralization (Coulter HIV-1 p24 antigen neutralization kit), according to the manufacturers instructions.

4.2.4 Complete medium for virus isolation.

RPMI 1640 (041-02402) was supplemented with 10% heat inactivated foetal calf serum, 100IU/ml penicillin, 100ug/ml streptomycin, 1mM Sodium pyruvate, 2mM L-glutamine, 1.25ug/ml amphotericin-B, and 1IU/ml of recombinant interleukin-2 (IL-2, NIBSC). All materials were purchased from Gibco BRL unless otherwise stated.

4.2.5 Virus isolation from PBMCs.

Approximately half the cells recovered from the HWB samples were used for virus isolation ($3x10^6$ - 10^7 cells). Cells were resuspended in complete medium and stimulated on day 1 with phytohaemagglutinin (90ug of PHA/ml, Wellcome). Cells ($2x10^6$) from HIV negative donors prepared in the same way 3 to 7 days earlier were added giving a final volume of 10ml in T25 flasks (Costar). Cultures were maintained at 37°C in 5% CO_2 for 28 days. On days 7, 14, 21 and 28 approximately 1ml of supernate was removed for p24 Ag analysis, $1x10^6$ stimulated donor cells were added, and the culture volume replenished with fresh complete medium to a final volume of 10ml.

4.2.6 Virus isolation from plasma.

Plasma was cultured by the method of Ho *et al.*, (1989). Briefly, plasma (equivalent to 1 in 3 and 1 in 6 final dilutions) was incubated with $2x10^6$ PHA/IL-2 stimulated cells from HIV negative blood donors in complete RPMI to a final volume of 3ml in 24 well plates (Costar). After 24h incubation at 37°C

in 5% CO_2, the cells were pelleted and resuspended in 3ml of complete RPMI. Half the culture medium was removed and stored on a weekly basis for p24 Ag detection, and the culture was replenished to 3ml with complete RPMI. No additional donor cells were added.

4.2.7 Small volume virus isolation from PBMCs.

After separation, the cells were washed 3 times in RPMI then cocultivated in complete RPMI with $2x10^6$ stimulated donor cells in a final volume of 1.5ml in 24 well plates. The cultures were stimulated with 5IU/ml of IL-2, assayed for p24 Ag every 3-4 days, and replenished with complete medium containing 1IU/ml of IL-2. No additional donor cells were added.

4.2.8 Virus isolation from HWB.

Whole blood (to give a final dilution of 1 in 3 and 1 in 6) was cocultivated with 3-7 day PHA/IL-2 stimulated HIV negative donor PBMCs ($2x10^6$) in a final volume of 3ml of complete medium containing 20IU/ml of IL-2 in 24 well plates for 28 days. Plates were incubated at 37˚C in 5% CO_2 for 24h. The cells were washed twice with RPMI, and resuspended in a total volume of 1.5ml. Supernate was removed every 3-4 days for p24 Ag analysis, and the medium replaced (containing 1IU/ml of IL-2). Stimulated donor cells ($5x10^5$) were added every 7 days.

4.3 VIRUS TITRATION AND INOCULATION OF CELL CULTURES.

4.3.1 Cell lines and virus stocks.

Two viral strains and ten cell lines were used: HIV-1_{RF}, a viral isolate from Haiti; and HIV-1_{IIIB}, an isolate from USA; Hut78, human cutaneous T-cell lymphoma; U937, human monocyte-like cell; C8166, human T-lymphoblastoid cell line; MT4, HTLV-1 transformed human T-cell; HeLa T8+, human epithelial-like cell; Sup-T1, non-Hodgkins T-cell lymphoma; LC5, HIV

susceptible clone of human embryonic lung fibroblasts; U138MG, human glioblastoma cell; THP1, human monocytic leukaemic cell line; HB1, human B cell line (James *et al.*, 1990). All cell lines and viral strains were supplied by the MRC AIDS directed programme unless otherwise stated (Appendix II). Uninfected blood donor PBMCs were obtained from the Scottish Blood Transfusion Service.

4.3.2 Titration of HIV-1 virus inoculum.

Supernate from HIV-1 infected cells was titrated by assaying 5 replicates of each dilution (1 in 10 to 10^7) in C8166 cells in 96 well plates (Costar). The plates were incubated at 37°C in 5% CO_2, and scored for the presence of syncytia over 7 days. The 50%-tissue culture infective dose ($TCID_{50}$) was calculated by the method of Reed and Muench as described in Appendix III.

4.3.3 DNase I treatment of viral supernates.

Viral supernates were treated with 50U/ml of DNase I (Gibco, BRL) in RPMI 1640 with 5mM $MgCl_2$ for 60min at room temperature and filtered prior to use (0.45um, Costar).

4.3.4 Inoculation of cells with HIV-1.

The HIV-1 supernate was diluted to $4x10^4$ $TCID_{50}$/ml in complete RPMI without FCS. Cells ($5x10^6$) were resuspended in 2ml of viral supernate (m.o.i. of 0.008), and incubated for 1h at 37°C in 5% CO_2. The cells were washed three times in RPMI and resuspended in 5ml of complete medium (serum-starved cells were incubated in complete RPMI without FCS for 3 days prior to inoculation, and throughout the experiment). At each time point, $2x10^5$ cells were removed, pelleted and resuspended in lysis buffer for DNA extraction.

4.3.5 Inoculation and fixation of cells for electron microscopy studies.

Viral supernate ($HIV\text{-}1_{RF}$) was titrated and used to inoculate C8166 cells

(m.o.i. of 1) at 4°C, to allow viral attachment without penetration. Cells ($2x10^6$) were removed at time 0 and the culture was transferred to an incubator (37°C in 5% CO_2). Cells were further harvested at times 5, 10, 15, 20, 25, 30, 40, 50 and 60min, and days 3, 4, 5, 7 and 8.

Harvested cells were pelleted, washed in cacodylate buffer (100mM Sodium cacodylate, pH7.3), and resuspended in 2.5% glutaraldehyde (EM grade) in cacodylate buffer for 5h at room temperature. Cells were washed twice in buffer, and fixed for 1h at room temperature in 2% osmium tetroxide in cacodylate buffer. The cells were washed, pelleted, and embedded in 2% noble agar for processing. The embedded pellet was dehydrated in 50%, 70%, and 95% alcohol for 15min each, and twice in absolute alcohol for 20min. The pellet was cleaned in 2 changes of epoxy propane for 15min, and left overnight in a 1:1 mixture of epoxy propane and araldite (araldite CY 212, used as described in the manufacturers protocol). The pellet was incubated twice with fresh araldite for 1h, embedded in araldite in a beam capsule, and polymerised at 60°C for 48h. Ultrathin sections of about 50nm were cut on an Ultratome (LKB) and collected on gold grids of 600 mesh. The sections were dried at room temperature, and stained in the dark for 30min with saturated aqueous uranyl acetate. The grids were washed in buffer, and counter stained in a CO_2 free atmosphere for 5min with Reynolds lead citrate. The grids were washed thoroughly in buffer, dried, and examined in a Hitachi 12A transmission electron microscope. All reagents were purchased from Agar Scientific (Essex).

4.4 SEPARATION AND ANALYSIS OF CELLULAR SUBSETS.

4.4.1 Dynabeads.

Dynabeads (Dynal (UK) Ltd.) are microspheres of uniform size (4.5um) with identical physical and chemical properties. Each microsphere is coated with a

polystyrene shell, allowing covalent binding of biomolecules (eg. antibodies) resulting in a highly specific binding capacity. The core of each particle contains maghemite, permitting the isolation of microspheres bound to target cells by a magnetic particle concentrator (MPC, Dynal). Antibody coated Dynabeads can, therefore, be utilised to purify cell subsets which express unique surface antigens.

M-450 tosylactivated Dynabeads were coated with anti-CD14 antibodies for monocyte/macrophage cell isolation and M-450 Dynabeads, pre-coated with antibodies to CD19, CD8, and CD4 antigens, were used for the separation of B cells, T-cytotoxic/suppressor cells, and T-helper/inducer cells respectively.

4.4.2 Covalent binding of anti-CD14 antibodies to tosylactivated Dynabeads.

M-450 tosylactivated Dynabeads were thoroughly resuspended and an aliquot removed. The microspheres were collected by applying the MPC for 2-4min, and the supernate was removed. The microspheres were resuspended in ddw, and the process repeated several times. The washed Dynabeads were finally resuspended in a volume of ddw equal to that of the original aliquot. The monocyte-specific antibody (anti-CD14, SAPU, Law Hospital, Carluke) was dissolved in 0.05M borate buffer (pH9.5) to a concentration of 150ug/ml, and an equal volume of microspheres in suspension was added, giving an estimated antibody to bead ratio of 5:1 (as described by the manufacturer). The mix was incubated overnight at 22°C for 22h with end-over-end rotation, the microspheres were collected with the MPC, and the supernate discarded. The pellet was washed at room temperature in 5ml of 0.01M PBS containing 0.1% BSA, 3 times for 10min, once for 30min, and overnight at 4°C. The Dynabeads were collected and resuspended in 0.1% BSA in PBS at a concentration of $4x10^8$ Dynabeads/ml, and stored at 4°C.

4.4.3 PBMC subset separation by antibody coated Dynabeads.

Antibody coated Dynabeads were mixed with PBMCs, at an estimated ratio of 1.5:1, for 30min at 2-8°C to reduce the non-specific binding, and periodically observed under the light microscope for rosette formation. The number of cells present in each cellular subset was estimated from the normal distribution of PBMCs (70% T cells (CD4:CD8 ratio 2:1); 10% B cells; and 20% monocytic cells, Roitt *et al.*, 1987).

The bound cells were separated by applying the MPC for 2-5min, and the process repeated until the cells had been incubated with Dynabeads coated with each of the antibody specificities. The cells were isolated sequentially by Dynabeads coated with antibodies to CD19, CD14, CD8 and CD4 antigens. The separated cells were washed twice with PGN (PBS (pH7.2), containing 5% normal mouse serum (Serotec), and 20mM glucose), resuspended in 200ul of PGN, and split into 5 equal fractions for analysis.

4.4.4 Labelling cells for flow cytometric analysis.

Anti-CD4, anti-CD8, anti-CD14, and anti-CD19 fluorescein isothiocyanate conjugated antibodies (Serotec) were used for fluorescein-activated cell sorting analysis of the cell fractions.

Samples were processed in 5 equal fractions, one of which was incubated with each of the 4 FITC-conjugated antibodies (10ug), and the fifth fraction was kept as a negative control. The reaction vials were mixed and incubated on ice in the dark for 30-60min, and the pellets washed twice with 1ml of PGB (PBS pH7.2, containing 20mM glucose and 0.5% BSA). The samples were transferred to a 0.75ml eppendorf tube, pelleted and resuspended in 300ul of buffered paraformaldehyde (47mM Sodium cacodylate (pH7.2) and 1% (w/v) paraformaldehyde) for flow cytometric analysis. (This was adapted from the method of Serotec).

4.4.5 Analysis by flow cytometry.

The samples were analysed within 24h of fixing using an EPICS "C" flow cytometer (Coulter Electronics, Luton, UK) fitted with a 5 watt laser and utilising a power output of 300mW at 488nm. The cells were passed under pressure through the laser beam, and the light scattered, reflected, and emitted by the cells was detected by various photosensors and analysed by the computer to generate 3 pieces of data for each event.

The small angle deflected light (forward light scatter) was an indicator of cell size, and large angle deflected light (90° light scatter) was representative of the granularity of the cells. Cells were also analysed using FITC-conjugated antibodies specific to surface markers. In this way, the cells were separated by size and granularity into lymphocyte and monocyte sub-populations, and further analysed for subset specific CD antigens using FITC-conjugated antibodies to CD4, 8, 14 and 19 antigens (Moscicki *et al.*, 1985; Mason *et al.*, 1987).

At least 1000 events were analysed for each sample, and the results expressed as a percentage of events showing fluorescence greater than the background level.

4.4.6 Separation of monocytic cells by adherence.

Patients' cells were cultured in T25 flasks in complete medium with 20% FCS at 37°C and 5% CO_2 with no cell mitogens. On day 3, the flask was tapped repeatedly and non-adherent cells removed. Adherent cells were washed 3 times with pre-warmed RPMI, removed carefully using a cell scraper (Costar), and stored in freezing medium at -70°C for PCR analysis.

4.5 NUCLEIC ACID EXTRACTIONS.

4.5.1 Total DNA extractions from sera.

Sera were extracted and amplified in batches of 10. Each batch, numbered randomly, consisted of 5 test samples and 5 controls. Three controls were from low-risk seronegative donors, and 2 controls were HBV DNA positive samples. The positive controls (*d* and *y* subtypes) were patient samples which were found to subtype consistently by RIPA and PCR in preliminary studies.

Sera (200ul) were incubated for 45min on ice with proteinase K (Sigma) at 100ug/ml in an equal volume of lysis buffer (200mM NaCl, 100mM EDTA, 100mM Tris, pH8.0). N-lauroylsarcosine was added to a final concentration of 1% (w/v), and samples were incubated at 65°C for 3h. After phenol/chloroform extraction, the aqueous phase was precipitated with 2 volumes of ethanol and 0.1 volume of 4M potassium acetate (KAc) at -20°C for a minimum of 45min. The precipitate was pelleted in a microfuge, washed and resuspended in 100ul of ddw (each 10ul aliquot was equivalent to 20ul of neat serum).

4.5.2 Total DNA extraction from cells infected with HIV_{RF}.

Cells were extracted from in batches of 10 consisting of cells from each culture (uninfected C8166 and PBMC cells, HIV-1_{RF} infected C8166 and PBMC cells, and HIV-1_{RF} infected C8166 cells in serum-starved medium) for 2 complete time points. Each batch, therefore, contained 2 negative cell controls for each time point. Cells ($2x10^5$) were resuspended in lysis buffer (100mM NaCl, 50mM EDTA, 50mM Tris, pH8.0) with 100ug/ml of proteinase K (Sigma), and 1% (w/v) N-lauroylsarcosine and incubated at 60°C for 3h. DNA was repeatedly extracted by a mixture of phenol, chloroform and isoamyl alcohol (50:49:1, Sigma) until the interphase was clear. The DNA was precipitated in 4M KAc (0.1 volume), and pure ethanol (2 volumes) at -20°C for a minimum of 45min. The DNA was pelleted and washed in ethanol, then

resuspended in a small volume of ddw (100ul) for PCR analysis (each 10ul aliquot contained DNA from $2x10^4$ cells).

4.5.3 Total DNA extraction from cell lines infected with HIV_{IIIB}.

Cell lines infected with HIV-1_{IIIB} were extracted by a rapid method described by Collin *et al.*, (1991). Briefly, cells ($2x10^5$) were removed from the culture at each time point and pelleted. The pellet was resuspended in 100ul of RPMI and an equal volume of buffer (100mM KCl, 20mM Tris-HCl, 500ug/ml proteinase K and 0.1% (v/v) NP-40), and incubated at 60°C for 2h. The reaction was terminated by inactivating the enzyme at 95°C for 15min. No further purification was carried out, and samples were stored at -70°C prior to amplification. Negative cells were included in each batch to control for cross-contamination. Each 10ul aliquot was equivalent to 10^4 cells.

4.5.4 Total DNA extraction from patient PBMCs.

Cell samples were routinely extracted as described for cell lines infected with HIV_{RF} with the exception that patient cells were extracted in batches of 10: 5 test samples and 5 negative controls. No positive controls were included in standard extractions to minimise the possibility of contamination. The samples were numbered so that each was adjacent to 2 negative controls, and batches were processed in numerical order at all times. Most of the samples tested were from known HIV positive individuals, and so completely negative batches were unlikely. Batches that did not yield a positive result were repeated with the inclusion of a positive control. Positive control samples were obtained from cell lines infected with HIV-1_{RF} or HIV-1_{IIIB} which had previously been shown to be positive by PCR. The number of cells extracted varied from patient to patient. However, the cell equivalents of each amplification aliquot is supplied, when available, in Appendix IV.

4.5.5 Nuclear and cytoplasmic DNA extraction.

The method of Ellison *et al.*, (1990) was modified for the extraction of nuclear and cytoplasmic DNA. The cells ($1x10^6$) were washed in buffer A (10mM Tris; pH7.4, 150mM KCl, 5mM $MgCl_2$, 1mM dithiothreitol, 20ug aprotinin per ml) and pelleted at 175g (benchtop GC centrifuge, Beckman) for 15min. Cells were resuspended in 1ml of buffer A with 0.5% (v/v) Triton X-100, incubated at 4°C for 30min, and pelleted at 1000g for 10min. The nuclear pellet was washed in buffer A, and both the cytoplasmic and nuclear fractions were extracted as described for cell line studies (4.5.2), and resuspended in 100ul of ddw (each 10ul aliquot equating to $1x10^5$ cells).

4.5.6 Quantitation of total extracted DNA.

Extracted DNA was quantified by measuring the ultraviolet absorbance at 260nm and 280nm. Samples were diluted 1 in 100 in ddw, and absorbance measured in a spectrophotometer (CE 292, Cecil). The ratio of optical densities (260/280) gave an indication of the sample purity, pure DNA giving a ratio of 1.8. The concentration of DNA was calculated from the equation: True A_{260} x D x 50 = DNA concentration (ug/ml), where A_{260} = absorbance at 260nm, True A_{260} = (A_{260}-A_{280}) x 2, D = the dilution factor (=100) and 50 refers to the dsDNA concentration at A_{260} of 1.

4.5.7 Extraction of total RNA.

This method was kindly supplied by Dr Peter Balfe (Department of Medical Microbiology, UCMSM, London). All plastics to be used were pre-treated with chloroform to remove RNases, and where appropriate, buffers were treated with 0.1% diethyl pyrocarbonate (DEPC) for 12h at 37°C, and autoclaved for 30min at 121°C.

PBMCs were washed with PBS (Mg^{2+}, Ca^{2+} free), and resuspended in 1 volume of RNAzol solution (4M guanidium thiocyanate, 25mM sodium citrate,

1% (w/v) N-lauroylsarcosine, and 0.72% (v/v) beta-mercaptoethanol), 1 volume of water saturated phenol (Sigma), and 0.1 volume of 2M sodium acetate (pH4). A total volume of 2ml was used for extracts of 10^6 cells, and 1ml for each antibody coated bead extraction. Chloroform was added to a final volume of 10% (v/v), the sample was inverted, incubated on ice for 15min, and centrifuged for 15min (MSE microfuge, Scotlab). The upper phase containing the RNA was transferred to a clean vial and precipitated with an equal volume of propan-2-ol for 45min at -20°C. The sample was pelleted for 15min, washed twice with ice-cold 75% ethanol, and dried under vacuum for a maximum of 10min. The RNA pellet was resuspended in 21ul of DEPC treated ddw in preparation for cDNA synthesis. One HIV negative control PBMC sample was included per batch of 10 samples.

4.5.8 Antibody coated bead capture extraction of viral nucleic acids.

Samples were incubated at 37°C for 2h, or overnight at room temperature, with antibody coated beads and 10U of DNase I. The beads were washed (distilled H_2O) a minimum of 5 times, and transferred to eppendorf vials for nucleic acid extraction.

Bead extracts for HBV DNA were carried out on 200ul of sera, and were extracted in lysis buffer (200mM NaCl, 100mM EDTA, and 100mM Tris, pH8.0) for 3h at 60°C as described in 4.5.2.

Bead extracts of HIV-1 RNA were from 300ul of sample, and were processed as described in 4.5.7.

4.6 THE POLYMERASE CHAIN REACTION.

All batches were amplified for 25 cycles on a water cooled heating block (PHC-1 or PHC-2, Techne, Scotlab). Each cycle consisted of denaturation of the samples at 95°C for 36s, primer annealing at 50°C for 42s, and DNA synthesis at 65°C for 3min. The final extension of cycle 25 was held for a total

of 5min to ensure completion of all strands. All subsequent reactions were for a further 25 cycles as above, utilising 2ul of the previous amplification product. False-positive or negative results obtained in any controls resulted in the batch being discarded and the amplification repeated on fresh aliquots.

4.6.1 Amplification buffer for HBsAg DNA detection.

This method was adapted from the method described by Simmonds *et al.* (1990a). An aliquot (10ul) of extracted DNA was amplified in PCR buffer (67mM Tris, 16.7mM ammonium sulphate, 6.7mM magnesium chloride, 10mM ß-mercaptoethanol) with 75nM of each primer, 45uM of each dNTP (Boehringer Mannheim), 1 unit of *Taq* polymerase (Perkin Elmer Cetus, supplied by MRC ADP), 170ug/ml of BSA (DNase free, Pharmacia), and 40ul of paraffin layered on top. Batches were amplified as described (4.6).

4.6.2 Amplification buffer for HBV DNA detection after antibody coated bead extractions.

An aliquot (10ul) of the extracted DNA was diluted in amplification buffer (50mM KCl, 10mM Tris-HCl 15mM $MgCl_2$, and 0.1% Triton X-100, pH8.8, Promega) with 75nM of each outer primer, 36uM of each dNTP, 170ug of BSA (DNase free, Pharmacia), and 1 unit of *Taq* polymerase (Promega). Samples were amplified as described (4.6).

4.6.3 Amplification buffer for HIV-1 DNA detection by PCR.

Samples were processed in batches as described in the methods for DNA extraction. An aliquot (10ul, or $2x10^4$ cell equivalents) of each extract was transferred to 90ul of PCR buffer (50mM KCl, 10mM Tris-HCl (pH8.8 at 25˚C), 1.5mM $MgCl_2$, and 0.1% Triton X-100, Promega Corporation), containing 75nM of the upstream and downstream primers, 45uM of each dNTP (Boehringer Mannheim), 1 unit of *Taq* polymerase (Promega Corporation),

170ug/ml of BSA (Pharmacia), and layered with paraffin. The samples were amplified as described (4.6).

4.6.4 Source of DNA oligonucleotide primers.

All oligonucleotides used in this study were synthesized by Oswel DNA service (Department of Chemistry, The University of Edinburgh) and purified by high-performance liquid chromatography. Their sequences, coordinates and sources are described in detail in Appendix V, and the method of calculating their concentrations is given in Appendix III.

4.6.5 Primer pairings for HBsAg DNA detection.

The primary amplification of HBV DNA was with primers 058C/869C resulting in a band of 548bp, and the nested amplification with 057C/870C (235bp).

4.6.6 Primer pairings for HIV-1 DNA detection.

HIV-1 DNA was detected in cell lines by primers specific for the *pol* gene. The primary amplification with primers 160H/161H yielded a band of 244bp, and the nested reaction used 002C/003C yielding a 128bp product. A second set of primers was used for detection of the *env* gene, and for studies of the variability within the envelope region. The primary amplification was with primers 401C/404C (438bp), and the nested cycles with 402C/403C (317bp).

4.6.7 Analysis of PCR products by agarose gel electrophoresis.

Amplification products were analysed on agarose gels (Sigma) against 0.5ug DNA VI molecular weight marker (Boehringer Mannheim) in 0.5 x TBE containing ethidium bromide (50ng/ml). The samples were loaded in tracking dye (6 X buffer; 0.25% bromophenol blue, 0.25% xylene cyanol FF, and 30% glycerol in water), and electrophoresed at a constant voltage of 150V (Midi-gel apparatus, Northumbria Biologicals Limited, and Bio-rad 200V power supply). HIV-1 PCR products were analysed on 1.5% gels, and HBV PCR products on

2% gels.

4.6.8 Reverse transcription of RNA extracts and amplification of cDNA (RT-PCR).

cDNA was synthesized from RNA extracts using a thermostable r*Tth* reverse transcriptase RNA PCR kit (N808-0069, Perkin Elmer Cetus). The kit contained materials for the synthesis and amplification of cDNA in a single reaction vial (RT-PCR). The thermostable enzyme contained both RNA and DNA dependent synthesizing activities, enhanced by the use of manganese and magnesium chelating ions respectively.

Each RNA extract was split into 3 equal fractions (7ul), 2 fractions were diluted in 17ul of RT mix (10mM Tris-HCl (pH8.3), 90mM potassium chloride, 1mM manganese chloride, 200uM of each dNTP, 5 units of reverse transcriptase enzyme, and 0.75uM of the upstream primer) and overlaid with mineral oil. One vial contained primer 160H to reverse transcribe the *pol* gene sequence, and one vial contained the *env* primer (401C). The third vial was not reverse transcribed, and was used as an amplification control for contaminating viral DNA. All samples, including DNA controls, were incubated at 70˚C for 15min on a heating block (PHC-1 or PHC-2, Techne, Scotlab). The reaction solution was then diluted further with PCR mixture to a final volume of 100ul (4% (v/v) glycerol, 8mM Tris-HCl (pH8.3), 0.6mM EGTA, 0.04% (v/v) Tween-20, 2mM magnesium chloride, and 0.15uM of the downstream primer). The primers utilised for the amplification reaction were 161H for the *pol* sequences, and 404C for the *env* sequences (both *pol* primers were added to the DNA control vial). The amplification was for 2min at 95˚C for 1 cycle, 1min at 95˚C and 1min at 60˚C for 35 cycles, and 7min at 60˚C for 1 cycle. The amplification products were then amplified with the appropriate nested primers, and the products electrophoresed as described in 4.6.6 and 4.6.7.

Each batch of samples amplified included 1 HIV negative control (included in the extraction), and 1 non-reverse transcribed control for each patient sample.

4.6.9 The synthesis of a *pol* specific biotinylated oligonucleotide by PCR.

The polymerase chain reaction was used to generate HIV-1 specific *pol* sequences labelled with biotin. The reaction mixture was as described in 4.6.1 with the exception that 75% of the dATP in the nested amplification was biotinylated (Biotin-14-dATP, BRL). The HIV-1 non-infectious clone BH10 (Hahn *et al.*, 1984) was linearised with *Sst* 1 and utilised as the template sequence, and primers 160H/161H were used for both amplification reactions for 25 cycles.

4.6.10 Preliminary amplifications to detect 1 and 2LTR CUVD.

One and 2LTR CUVD were initially detected using a standard nested PCR utilising a variety of primer pairs from *gag*, *env*, and the LTR sequences. The primary amplification was with 782K/783K (bands of 996bp and 1630bp for 1 and 2 LTR CUVD respectively), and the nested amplification with primers 780K/781K for 1 and 2 LTR CUVD (277bp for 1 and 2 LTR forms), and 779K/781K for 2LTR CUVD (447bp).

4.6.11 Optimisation of 2LTR CUVD detection.

To enhance the detection of viral sequences over the non-specific sequences amplified with the 2LTR CUVD specific primers, samples were amplified with 3 sets of primers.

The primary step was with primers 782K/783K (resulting in bands of 996bp and 1630bp for 1 and 2LTR CUVD respectively), followed by a secondary amplification using 1 primary and 1 nested primer (779K/782K (396 or 1030bp) or 781K/783K(413 or 1047bp)) with a tertiary amplification with

primers 779K/781K (447bp).

These amplification protocols were applied to PBMC DNA extracts from patient samples P1, P4b, P13, P20c, and P25b. All samples were amplified and analysed as described in 4.6.3 and 4.6.7.

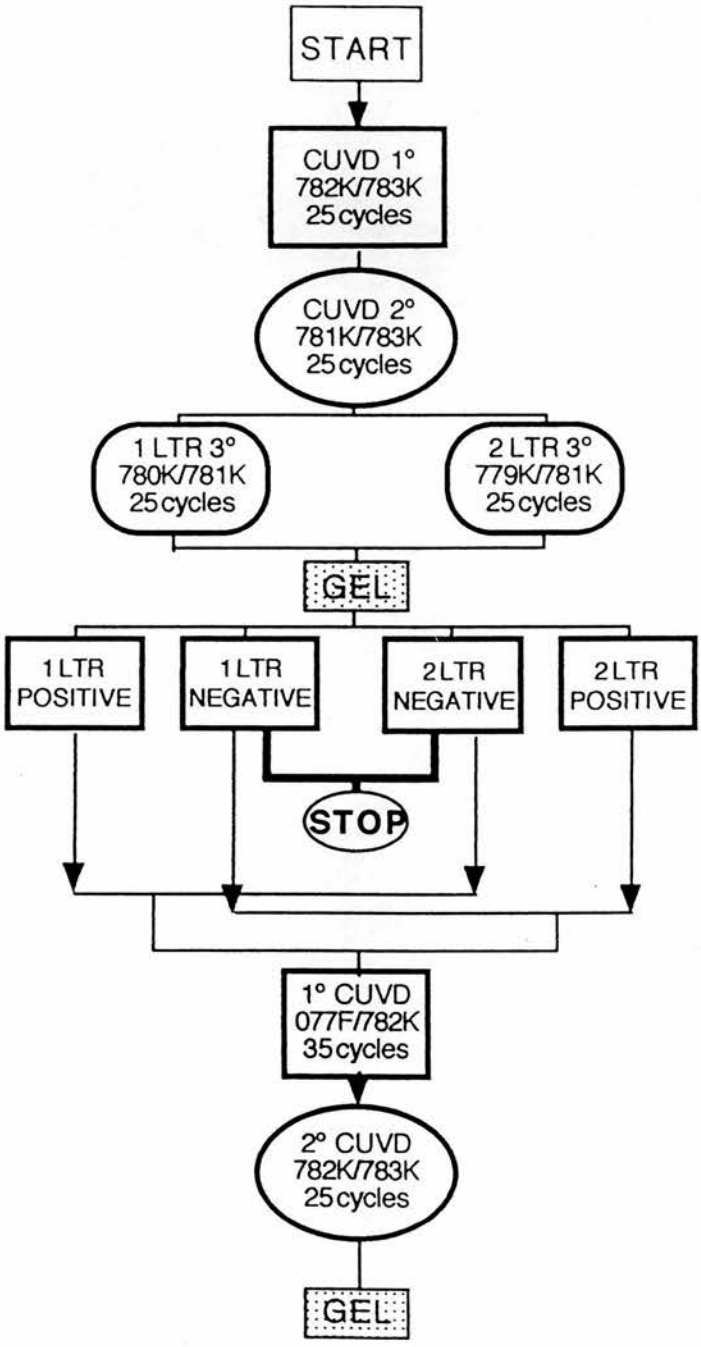
4.6.12 Simultaneous detection of 1 and 2LTR CUVD for confirmation.

Samples negative for one CUVD form and positive for the other were amplified with primers from the *gag* and *env* sequences which were able to detect both 1 and 2LTR CUVD forms simultaneously. The primary amplification with primers 077F/782K resulted in bands of 1933bp and 2567bp for 1 and 2LTR forms respectively. An aliquot was then amplified for 35 cycles with the nested primers 782K/783K, resulting in bands of 996bp and 1630bp (for 1 and 2LTR CUVD forms respectively).

4.6.13 Optimum primers for the detection of 1 and 2LTR CUVD.

The final sequence of primers utilised for optimum detection of 1 and 2LTR CUVD forms in cellular extracts by PCR is presented as a flow diagram in Figure 6. Due to the increase in sensitivity obtained for 2LTR CUVD detection using a tertiary amplification, samples for 1LTR CUVD detection were also tertiary amplified. All samples were, therefore, initially amplified with the primary and secondary primers (782K/783K and 781K/783K respectively), with further amplifications with 780K/781K for 1LTR CUVD detection (276bp), and 779K/781K for 2LTR CUVD detection (447bp). For confirmation of these results, samples were amplified for 25 cycles with primers 077F/782K (1933 and 2567bp for 1 and 2 LTR CUVD respectively), and 35 cycles with primers 782K/783K. Unlike the product from the tertiary amplification for 1LTR CUVD, the amplification product from these primers was able to distinguish between 1 and 2LTR forms. The nested amplification with non-LTR primers was, therefore, essential for the unequivocal detection of 1LTR CUVD forms.

Figure 6. Flow chart of amplification steps for 1 and 2 LTR circular unintegrated viral DNA (CUVD) detection



The flow diagram depicts each step involved in the detection of CUVD forms in the patient PBMC extracts. Amplifications are designated primary (1°), secondary (2°), and tertiary (3°). CUVD amplifications are for both 1 and 2 LTR forms of CUVD. Amplifications for 1 or 2 LTR CUVD sequences only, are listed as 1 LTR or 2 LTR respectively. After tertiary amplifications, products were analysed by gel electrophoresis (gel), and depending on the results, were either stopped or re-amplified with alternative primers specific for both 1 and 2 LTR CUVD forms.

4.6.14 Determination of the minimum oligonucleotide length for priming of DNA synthesis.

Three sets of amplifications were attempted to try and determine the minimum length requirement for an oligonucleotide to initiate DNA synthesis. Small sequences of 3-9 bases were selected to hybridize to the nested *pol* primer (002C) sequence (Figure 7a). They were chosen to have a guanosine base at the 3' end to reduce the amplification efficiency resulting from mispriming events (Figure 7b, Newton *et al.*, 1989; Kwok *et al.*, 1990). The oligonucleotides were used to amplify pooled DNA extracted from patient samples 11b, 16 and 19a (see Appendix IV for patient details). The initial experiment used the primers 160H/161H for the primary amplification, and 556-559L/003C for the nested amplification. The other 2 experiments utilised a primary amplification with 556-559L/161H, and a nested amplification with 002C/003C or 559L/003C (Figure 7c).

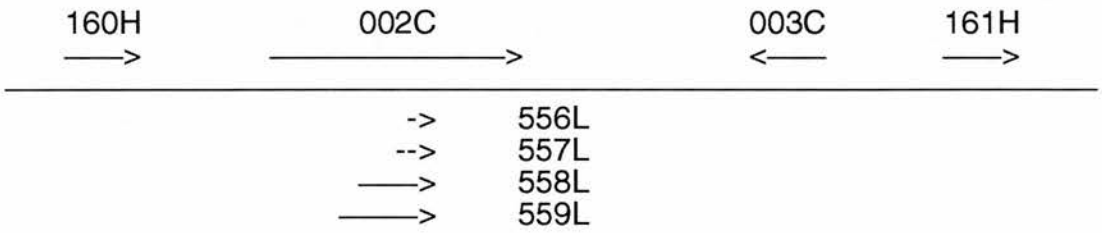
For each experiment the PCR was as described previously (4.6.3) with the exception that primers were added to individual reaction vials and not to the overall PCR mix. In this way, it was possible to control for contamination in the general PCR mixture, and to utilise the primers 160H/161H and 002C/003C as positive controls for the detection of viral DNA.

4.6.15 Ligation-PCR for the detection of LUVD.

Samples (20ul, corresponding to $4x10^4$ cells) were diluted in ligation buffer to give a final concentration of 50mM HEPES, 20mM $MgCl_2$, 3mM DTT, 0.1mM ATP, 10% (v/v) DMSO, 10ug/ml BSA, and 75nM of ligation sequence (021N) in a total volume of 30ul. The mixture was covered with paraffin, and incubated at 95°C for 10min to denature the DNA. T4 RNA ligase (1 unit, Gibco BRL) was added to each tube, and incubated for 1h at 4°C. The reaction was terminated by destroying the enzyme at 95°C for 5min. The

Figure 7. The base composition and relative positions of the primers and oligonucleotides used to determine the minimum sequence requirement for priming of DNA synthesis.

a) Schematic representation of *pol* primer orientations.



b) Base composition of oligonucleotides 556L, 557L, 558L, and 559L, and their relative positions to primer 002C.

002C	5' GGAGGAAATGAACAAGTAGATAAATT 3'
556L	TAG
557L	AGTAG
558L	CAAGTAG
559L	AACAAGTAG

The oligonucleotides were chosen with a 3' guanosine base to reduce the efficiency of amplification resulting from mis-priming events (Newton *et al.*, 1989; Kwok *et al.*, 1990). The primers were utilised in 3 experiments designed to determine the minimum sequence requirement for priming DNA synthesis.

c) Primer combinations utilised.

Experiment number	**Primary primers**	**Secondary primers**
1	160H/161H	556L-559L/003C
2	556L-559L/161H	002C/003C
3	556L-559L/161H	559L/003C

For full details of these primers see Appendix IV.

samples were amplified by PCR in a total volume of 100ul as described (4.6.3). The primary amplification was with primers 022N/782K (931bp), secondary PCR with 022N/586N (392bp), and tertiary PCR with 022N/781K (348bp). The secondary and tertiary amplifications utilised a 2ul aliquot of the preceding amplification product, and the results were analysed by gel electrophoresis (4.6.7).

4.7 HYBRIDIZATION ASSAYS.

4.7.1 Loading of samples for dot blot hybridization.

Membranes were cut to size, and pre-wet in ddw (nitrocellulose, Schleicher and Schull) or 0.1% Triton X-100 (nylon, Amersham). The samples (denatured at 95˚C for 10min) were loaded using a Hybri-dot manifold (Gibco, BRL), and a minimum of 500ul of ddw was passed through each well to wash the sample onto the membrane. Membranes were removed from the manifold with the vacuum still pulling to prevent spreading of the samples due to back-wash. They were then denatured (1.5M Sodium chloride, 0.5M Sodium hydroxide for both nitrocellulose and nylon membranes) and neutralised (1.5M Sodium chloride, 0.5M Tris (pH8.0) for nitrocellulose, and 1.5M Sodium chloride, 0.5M Tris (pH7.2), 1mM EDTA for nylon membranes) for 1min each, and fixed. Control wells were included for each membrane. Standard controls included ddw, salmon testes DNA, and biotinylated DNA. Nested PCR products of negative controls were also included as appropriate.

4.7.2 Hybridization conditions for nylon membranes.

The method of hybridization used was a modification of the Amersham protocol for radioisotopic probes. Membranes were incubated overnight at 42˚C in a rotary hybridization oven (Hybaid), with 10ml of pre-hybridization buffer (45% deionised formamide, 6 x SSC (20 x SSC; 3M Sodium chloride

(pH7.0), 300mM Sodium citrate), 5 x Denhardt's (100 x Denhardt's; 2% (w/v) BSA, 2% (w/v) Ficoll, 2% (w/v) polyvinylpyrrolidine), 0.5% SDS, and 20ug/ml of sonicated salmon testes DNA (Sigma)). Hybridization was at 42°C for 4h in 10ml of buffer (as above) with 600ng/ml of biotinylated single stranded DNA probe (581NBio, see Figure 8 for the sequence and coordinates of this probe). Post-hybridization washes consisted of 2 washes in 2 x SSC/0.1% SDS for 3min, and 2 in 0.2 x SSC/0.1% SDS for 3min at room temperature. The temperature was raised to 50°C for 2 washes in 0.16 x SSC/0.1% SDS for 15min, with a final rinse at room temperature in 2 x SSC/0.1% SDS. Membranes were finally baked at 80°C for 10min, to reduce background noise.

4.7.3 Hybridization conditions for nitrocellulose membranes.

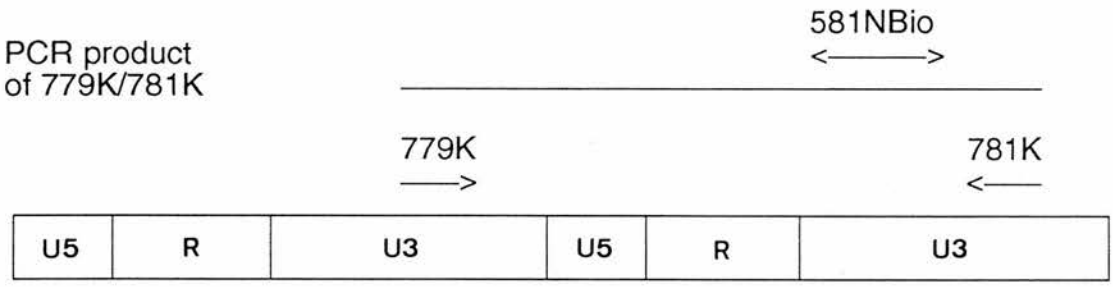
Nitrocellulose membranes were pre-hybridized for 2 hours at 42°C in 45% deionised formamide, 5 x SSC, 5 x Denhardt's, 25mM Sodium dihydrogen phosphate (pH6.5), and 100ug/ml of salmon testes DNA. The hybridization reaction was at 42°C for 4 hours in 5ml of 10% (w/v) dextran sulphate, 45% deionised formamide, 0.02% Denhardt's, 5 x SSC, 23mM sodium dihydrogen sulphate (pH6.5), 100ug/ml of salmon sperm DNA, and 600ng/ml of probe. Post-hybridization washes were as described for nylon membranes.

4.7.4 Colour reaction for the detection of biotinylated DNA.

Following the BRL DNA detection system instruction manual, filters were blocked and incubated as directed to detect biotinylated DNA. The membranes were washed in buffer 1 (0.1M Tris (pH7.5), 0.1M NaCl, 2mM $MgCl_2$, 0.05% (v/v) Triton X-100) for 1min, blocked at 65°C for 60min (42°C for 20min for nitrocellulose membranes) in fresh pre-warmed buffer 2 (3% (w/v) BSA in buffer 1), and incubated at room temperature with Streptavidin (2ug/ml of buffer 1) for 10min. Membranes were washed with 20 to 40-fold

Figure 8. Orientation, sequence and coordinates of the biotinylated probe 581NBio.

a) Schematic representation of the sequence specificity of the HIV-1 LTR specific probe (581NBio).



b) Sequence and coordinates of 581NBio.

5' GGCGTGGCCTGGGCGGGACTGGGGGAGTGGCGAGCCCTCAG 3'

The biotinylated oligonucleotide (sense) specific for the U3 sequence of the viral LTRs at position -36 to -75 and 9041 to 9080 (based on the published sequence of HIV-1 by Ratner *et al.*, 1985) (synthesized by Oswel DNA services).

greater volume of buffer 1 than employed in the Streptavidin step, and gently agitated for 3min. This step was repeated twice. Further incubation with Biotin-alkaline phosphatase (1ug/ml of buffer 1) at room temperature for 10min was followed by two 3min washes with buffer 1, and similarly with buffer 3 (0.1M Tris (pH9.5), 0.1M NaCl, 50mM $MgCl_2$). Colour development was in the dark, at room temperature, with freshly prepared dye solution (330ug/ml of NBT in 7.5ml of buffer 3, mixed gently, then add 165ug/ml of BCIP). The reaction was terminated by washing membranes in 20mM Tris (pH 7.5)-5mM EDTA, and baking for 10-20min at 80°C (1-2min at 80°C for nitrocellulose).

4.7.5 Comparison of UV fixation of DNA to nylon, against baking of nylon and nitrocellulose membranes.

Dilutions (neat, 1 in 10, 1 in 100, and 1 in 200) of a nested *pol* PCR sample were denatured and spotted onto pre-wet membranes (2 nylon and 1 nitrocellulose). The samples were denatured and neutralised as described (4.7.3). The nitrocellulose and one nylon membrane were baked at 80°C for 2h under vacuum, while the second nylon membrane was kept moist, wrapped in clingfilm, and placed DNA side down on a Transilluminator (TM-36, 302nm peak wavelength, Ultra-violet Products Inc., Cambridge) for 5min to covalently fix the DNA. The membranes were then processed and developed as described using the PCR generated *pol* specific biotinylated probe (4.7.4 to 4.7.6).

4.7.6 Optimisation of UV fixation of DNA to nylon membranes.

Biotinylated DNA (20pg of 581NBio; Figure 8) was loaded onto a nylon membrane in an array of 6 pairs of dots. The membrane was kept moist and covered in clingfilm for UV fixation. To vary the time each pair of DNA dots was exposed to the UV irradiation, aluminium foil was used to block the

samples from the light source. The membrane was placed directly onto the UV light source, and each pair of dots exposed to 10, 20, 30, 40, 50 or 60s of UV irradiation. The membrane was cut into 2 identical strips, and one strip was boiled in ddw for 1min to remove any non-covalently bound DNA. The strips were developed together, and the results compared.

4.7.7 Detection of LTR sequences using a biotinylated probe specific for the U3 region.

To ensure that the probe was specific and sensitive, 2 LTR CUVD specific PCR positive DNA was spotted directly onto nylon as described (4.7.3). The samples were from the cell line studies, using the laboratory RF strain of HIV-1 (C8166 cells infected with HIV-1_{RF} in complete RPMI from days 3, 4, 5, and 7). After denaturation and neutralisation, the membrane was fixed by UV light, pre-hybridized at 42°C overnight, hybridized for 4h with 400ng/ml of 581NBio probe, and developed.

4.7.8 Southern transfer of DNA to nylon membranes from agarose gels.

The biotinylated oligonucleotide (581NBio) was selected to demonstrate the transfer of DNA to nylon membranes. Diluted samples of 581NBio (1.29ug, 967.5ng, 645ng, 322.5ng, 129ng, and 64.5ng per well) were electrophoresed on a 1.5% agarose gel against a biotinylated ladder (20ng and 40ng of biotinylated DNA marker VI, Boehringer Mannheim), and the resulting bands were transferred to a nylon membrane using a semi-dry multi gel electroblotter (Ancos, Denmark). Filter paper was soaked in transfer buffer (0.15M tri-sodium citrate dihydrate, pH 7) and placed on the anode plate. The membrane was cut to size, pre-wet in 0.1% Triton X-100, washed in transfer buffer, and placed on top of the filter paper. The gel was washed 3 times in transfer buffer, and layered on top of the membrane. Further layers of buffer soaked filter paper were added, and the cathode plate attached. A transfer

current of 0.8mA/cm^2 was applied. The gel was transferred for 1 hour, and viewed to observe the efficiency of band transfer. The membrane was UV fixed, and developed for the direct detection of biotinylated DNA.

4.7.9 Southern blot hybridization of 2LTR CUVD nested amplification products with the biotinylated probe 581NBio.

Nested 2 LTR CUVD amplifications (15ul of P4b, P20c, P1, and P13 amplification products) were separated on a 1.5% agarose gel against a biotinylated ladder (DNA marker VI biotinylated, Boehringer Mannheim), and transferred onto a nylon membrane at 0.8mA/cm^2 for 5h. Membranes were further processed as described for dot blot hybridization on nylon membranes.

4.8 HBsAg SUBTYPING METHODS.

4.8.1 HBsAg subtyping by radioimmunoprecipitation assay.

Samples were subtyped by RIPA by Mr S.H. Black (Virology Reference Laboratory, Department of Medical Microbiology, The University of Edinburgh) by the method described by Nicholson *et al.* (1992).

4.8.2 HBsAg DNA subtyping by PCR.

The primary amplified samples for HBsAg DNA detection (amplified for 25 cycles with primers 058C/869C) were further amplified with 3 sets of nested primers for HBsAg DNA subtyping. Primer pairings 869C/907E and 869C/394J were selected to detect subtype *y* DNA, and 869C/029H for subtype *d* (each amplification resulting in a band of 350bp). The 3' mismatches of the HBsAg DNA subtyping primers conferring specificity are depicted in Figure 9. The samples were amplified and electrophoresed as described for HBsAg DNA detection.

Figure 9. Position of subtype specific primers of the HBsAg gene from codon 117 to 130.

Subtype					
adr				g	t
adw	t				
adyw		t	c	g	
adw2	t				
ayw				c g g	t a
Consensus sequence	AGCACGGGAC		CATGC**AAA**AC		CTGCACGACT CCTGCTCAAG
Primer	907E			**GCC**TG	GACGTACTGA TGACGAGTTC
	029H			**TTT**TG	GACGTGCTGA GGACGAGTTC
	394J			**CC**TG	GACGTACTGA TGACGAGTTC

The consensus sequence of the above 5 HBV subtypes is given in uppercase, and their subtypic variation in lowercase. The primers selected to amplify the *d* and *y* subtypes are given (907E, 029H, and 394J reading 3' to 5') demonstrating their 3' mismatches with codon 122 (in bold).

4.8.3 *Sau* 3A digest of PCR products for confirmatory subtyping (PCR-RD).

To generate sufficient HBsAg DNA to visualise the restriction digest products on an agarose gel, an aliquot (2ul) of primary amplified product was re-amplified with the outside primer pair 058C/869C (548bp). The amplification product was restricted with 2.5 units of *Sau* 3A (Boehringer Mannheim) at 37°C for 1.5h, and the restriction product (15ul) was electrophoresed on a 2% agarose gel at a constant voltage of 150V.

4.9 ANTIBODY COATED BEAD EXTRACTIONS OF VIRAL NUCLEIC ACID.

4.9.1 Bradford's Coomassie Brilliant Blue protein concentration assay.

The assay was used to determine the protein concentration of the recombinant gp120 and anti-HIV antibodies supplied by the MRC ADP (ADP603, 335, 361, 362, 403, and 604, Appendix II). A standard protein (BSA DNase free, Pharmacia) was diluted in ddw to give a range of concentrations from 1mg to 100ng/ml. Protein dilutions (0.1ml) were added to 5ml of Bradford's reagent (0.01% (w/v) Coomassie Brilliant Blue G (Sigma), 4.7% (w/v) ethanol, and 8.5% (w/v) phosphoric acid) in a conical plastic centrifuge tube. The tubes were inverted and left for 2-60min before measuring the absorbance at 595nm (visible range). The spectrophotometer (SP 30V, Pye Unicam) was set against 5ml of reagent mixed with 0.1ml of ddw. The protein concentrations were plotted against their optical densities to give a standard curve for determination of the test protein concentrations. This was based on the method of Bradford, (1976).

4.9.2 Antibody coating of beads.

Polystyrene etched beads (6.5mm diameter, Northumbria Biologicals Ltd.) were freshly coated with antibody for each experiment. The beads were

incubated for 2h at room temperature with antibody diluted in carbonate-bicarbonate buffer (4mM anhydrous sodium carbonate, 46mM sodium bicarbonate, pH9.2) and air dried prior to incubation with samples.

4.9.3 Optimisation of antibody dilutions for HBV bead extractions.

Beads were coated with antibodies to HBsAg (anti-HBs horse antibody, Wellcome), and human IgM and IgG (anti-human IgM and IgG sheep antibodies, Scottish Antibody Production Unit, Law Hospital, Carluke). They were initially coated with serial dilutions of each antibody to determine the optimum antibody concentration (actual antibody concentrations were not supplied).

Anti-HBs coated beads (diluted 1 in 10 to 1 in 100) were incubated with 200ul of HBsAg (1 and 4ng/ml, BPL, Elstree) and negative sera for 2h at 37°C. The beads were washed (dH_2O) and incubated for a further 2h at 37°C with 200ul of iodinated anti-HBs antibody (30000cpm/200ul, BPL, Elstree). They were washed a final time, and gamma irradiation was counted over 60s (NE1612 turbo, Nuclear Enterprises).

Further dilutions of antibody (1 in 150 to 1 in 600) were used to coat fresh beads for direct detection with iodinated HBsAg (12000cpm/200ul, supplied by Mr P. McCulloch, Department of Medical Microbiology, The University of Edinburgh).

Beads coated with anti-human IgM and IgG antibodies were similarly assessed using iodinated human anti-HBc (66700cpm/200ul, supplied by Mr P. McCulloch).

4.9.4 Blocking of beads to prevent non-specific binding.

To demonstrate the specificity of the antibodies used for coating the beads, a panel of 5 sera (from patients 68, 71, 73, 146, and 147, Appendix I) were incubated with beads coated with: anti-HBs antibodies, anti-human IgG

antibodies, anti-human IgM antibodies, 5% BSA (Fraction V, Sigma) in carbonate/bicarbonate buffer, carbonate/bicarbonate buffer, and uncoated beads. Sera from patients 68, 146, and 147 had previously been shown to be HBV DNA positive after selective extraction for whole virus and IgM and IgG complexed virus. Sera from patient 71 had tested positive for IgM complexed virus, and patient 73 for free virus only. HBV DNA negative serum was included in each experiment.

The dried beads were incubated with test and control sera, and the resultant DNA extracts prepared and amplified as described (4.5.2 and 4.6.2 respectively).

4.9.5 Iodination of recombinant gp120 (rgp120).

Recombinant gp120 protein (rgp120) was iodinated by a modified method of Burrell *et. al.*, (1973). Ten microlitres of chloramine-T (0.25% (w/v) chloramine-T, 0.1M Tris-HCl (pH7.2), 0.85% (w/v) sodium chloride, 0.1% (w/v) sodium azide) was drawn into a glass pasteur pipette, with approximately 2ug of recombinant gp120 (ADP 604, Appendix II) separated by an air gap. The antigen and chloramine-T were carefully layered onto 500uCi of sodium iodide (125 Iodine, Amersham) in a 0.5ml eppendorf tube (Alpha Laboratories). The solution was mixed gently for 30-60s behind lead shields, and the reaction terminated by the addition of 10ul of sodium metabisulphite (0.6% (w/v) sodium metabisulphite, 0.1M Tris-HCl (pH7.2), 0.85% (w/v) sodium chloride, 0.1% (w/v) sodium azide) with gentle agitation as before. The reaction was left standing for 5min, diluted with 200ul of 0.2% potassium iodide in RIP buffer (PBS with 0.5% BSA and 0.1% Sodium azide) and carefully layered onto a 10ml Sephadex G-100 column (Pharmacia). The column was plugged with glass wool, washed with 20ml of Dulbecco solution, and equilibrated with a similar volume of 0.2% potassium iodide in RIP buffer prior to use.

Once loaded onto the column, the iodinated protein was washed through with excess 0.2% potassium iodide in RIP buffer. Fractions of approximately 1ml were collected, and 10ul aliquots were removed, diluted 1 in 10, and analysed for gamma irradiation over 60s (Gamma counter, NE1612 turbo, Nuclear Enterprises).

4.9.6 Optimization of HIV-1 specific antibody coating of polystyrene beads.

To determine the optimum antibody concentration, beads were coated with antibodies ADP335, 361, 362, or 403 at varying dilutions (1 in 50, 1 in 100, 1 in 200, and 1 in 400). The beads were then incubated with iodinated rgp120 to determine the specificity and efficiency of the antibody coating. Coated beads were incubated in triplicate, with 2 dilutions of labelled rgp120 (200ul of 20000 or 50000cpm) for 2h at 37°C. The beads were washed and analysed for gamma irradiation over a period of 60s, and the results expressed as a percentage of the labelled protein available.

Beads were coated with further dilutions of antibodies ADP335 and 403 (1 in 400, 1 in 500, 1 in 600, 1 in 800, 1 in 1000). Beads were coated in duplicate, with half the beads being counter blocked with BSA (5% BSA solution in carbonate/bicarbonate buffer) after antibody coating.

4.9.7 Extraction of HIV-1 whole virus from patient PBMC cocultures using beads coated with antibodies specific to the surface glycoprotein (gp120).

Polystyrene beads were coated with antibodies ADP335 (1 in 400 dilution), ADP361 (1 in 50 dilution), ADP362 (1 in 50 dilution), and ADP403 (1 in 500 dilution). The antibody coated beads were each incubated with culture supernate (300ul) from two p24 antigen positive patient PBMC cocultures (P11b and P20b). The incubation was overnight at room temperature, with

DNase I (10 units) and 5mM $MgCl_2$. The beads were washed and transferred to chloroform pre-treated eppendorf tubes for RNA extraction (4.5.8). The RNA extracts were reverse transcribed and amplified for *pol* and *env* sequences as described (4.6.8).

4.9.8 Quantification of whole virus capture and RNA extraction with anti-gp120 antibody coated beads.

To quantify the sensitivity of the whole virus capture reaction, dilutions of titrated viral supernate from HIV-1_{RF} infected C8166 cells were incubated with beads coated with antibody ADP361 (1 in 50 and 1 in 500 dilutions). The viral supernate ($TCID_{50}$ of $10^{7.17}$/ml) was diluted (10 fold dilutions), and aliquots (300ul) were incubated overnight at room temperature with a bead at each antibody dilution with DNase I (10 units) and 5mM $MgCl_2$. RPMI and supernate from uninfected C8166 cells were included as negative controls. The beads were processed as described previously (4.9.7), with the exception that the entire extract was amplified for *pol* sequences only.

RESULTS

CHAPTER 5: VIRUS ISOLATION.

In this study, virus was initially isolated from patient PBMCs to demonstrate infection by direct virus detection. Later isolation was attempted from small volumes of plasma and heparinised whole blood (HWB) to minimise the sample volume utilised for each isolation, and to maximise the number of cells recovered for PCR analysis.

5.1 Virus isolation from PBMCs, plasma, and heparinised whole blood (HWB).

Virus isolation was attempted in 41 samples from 29 patients. A single sample was received from 19 patients, and 2 or 3 samples from the remaining 10 patients. The average time between multiple samples was 6 months (range 1-10 months). Virus isolation from PBMCs, plasma, or heparinised whole blood was demonstrated by the presence of p24 Ag in the culture supernate over 28 days, and was positive in 22 of the 41 samples (Table 6). A sample from P2 was included as a high risk negative control, and numerous low risk negative controls were included (data not shown). All negative controls were isolation negative throughout.

Isolation from patient PBMCs was successful in 20 out of 34 samples (59%). The 14 culture negative samples were from 13 patients receiving no antiviral therapy (7 from CDC group II and 6 from CDC group III) and 1 patient who had received AZT for 2 months (CDC group IV).

Plasma isolation was successful in 3 out of 8 seropositive samples tested (38%). The 3 positive samples (P6, P15, & P21b) were from patients in CDC groups II, IV, and III respectively, who were not receiving anti-viral therapy. Samples P1, P4b, P13, and P25b were from patients receiving AZT treatment at the time of sampling, and were plasma culture negative.

Table 6. Virus isolation results from patient PBMCs, plasma, and heparinised whole blood (HWB).

Sample No.	PBMC	HWB	Plasma	Positive by any method
P1	nd	-	-	no
P2b*	-	-	-	no
P3a	+	nd	nd	yes
P3b	-	nd	nd	no
P4a	-	nd	nd	no
P4b	nd	-	-	no
P5	-	nd	nd	no
P6	nd	1 (a & b)	1 (b & c)	yes
P7a	+	nd	nd	yes
P8a	+	nd	nd	yes
P8b	+	nd	nd	yes
P8c	+	nd	nd	yes
P10	-	nd	nd	no
P11b	1	nd	nd	yes
P13	nd	-	-	no
P14a	+	nd	nd	yes
P14b	+	nd	nd	yes
P15	nd	nd	1 (b)	yes
P16	-	nd	nd	no
P17a	+	nd	nd	yes
P18a	+	nd	nd	yes
P19a	1	nd	nd	yes
P20a	-	nd	nd	no
P20b	1	nd	nd	yes
P20c	nd	-	-	no
P21a	+	nd	nd	yes
P21b	1 (s & T)	nd	1 (b & c)	yes
P22a	+	nd	nd	yes
P23a	+	nd	nd	yes
P23b	-	nd	nd	no
P24b	-	nd	nd	no
P25a	-	nd	nd	no
P25b	nd	-	-	no
P27a	-	nd	nd	no
P27b	-	nd	nd	no
P28a	+	nd	nd	yes
P28d	+	nd	nd	yes
P29b	-	nd	nd	no
P30b	+	nd	nd	yes
P31	-	nd	nd	no
P32a	-	nd	nd	no
P34b	+	nd	nd	yes

Letters represent the dilution of sample, or type of culture vessel: (a) 1 in 1.5 dilution, (b) 1 in 3 dilution, (c) 1 in 6 dilution, (s) for 24 well plate. All PBMC cultures were in T_{25} (T) flasks unless otherwise stated. +, positive culture; -, negative culture; and numbers refer to the week at which the culture was first positive. *, Patient 2 was included as a high risk HIV negative control. Low risk negative controls were isolation negative throughout (data not shown).

The final sample (P20c) was culture negative, CDC group III, and was from a patient receiving no anti-viral therapy.

HWB isolation was successful in 1 out of 6 seropositive samples (17%). The successful isolation was from P6, a patient receiving no AZT therapy in CDC group II. Four of the negative results (P1, P4b, P13, & P25b) were from patients on AZT therapy (CDC groups II & III), and 1 from a patient in CDC group III receiving no anti-viral therapy (P20c).

5.1.1 Comparison of the 3 isolation methods.

The overall rate of isolation by any method for the 41 seropositive samples cultured was 54% (22/41). Comparison of the efficiencies of the 3 methods was difficult as the techniques were not quantified, and the 7 samples tested by more than 1 method gave identical results.

Sample P2b was a high risk seronegative control and was negative by each of the 3 methods. P4b, P13, P20c, and P25b were each negative and P6 was positive by both HWB and plasma culture, and virus was isolated in both PBMCs and plasma of P21b.

Although the PBMC coculture method was the most successful in this study, it is possible that the low isolation rates obtained for HWB and plasma isolations were biased by the small number of samples tested.

5.1.2 Comparison of virus isolation with plasma p24 antigen detection, $CD4^+$ cell counts, AZT treatment, and CDC group.

Virus isolation was compared with $CD4^+$ cell counts (33 samples), plasma p24 antigen detection (41 samples), use of AZT therapy (41 samples), and CDC group (41 samples). The complete data is in Appendix IV, and the results tabulated (Tables 7-10) and analysed by the Chi-square test (Appendix III).

Table 7. Comparison of virus isolation result with detection of plasma p24 antigen.

	p24 antigen		
Virus isolation	**positive**	**negative**	**Total**
positive	7	15	22
negative	3	16	19
Total	10	31	41

Chi-square = 1.420, Degree of freedom = 1, and 0.50>P>0.10.

Table 8. Comparison of virus isolation and AZT treatment.

	AZT treatment		
Virus isolation	**positive**	**negative**	**Total**
positive	9	13	22
negative	5	14	19
Total	14	27	41

Chi-square = 0.966, degree of freedom = 1, and 0.50>P>0.10.

The results of Tables 7 and 8 were analysed by the Chi-square test using Yates correction (Appendix III). Virus isolation results were based on a single positive isolation from PBMCs, plasma, or HWB.

Table 9. Comparison of virus isolation and $CD4^+$ cell counts per millilitre of blood.

	$CD4^+$ cell count		
Virus isolation	**>200**	**200**	**Total**
positive	7	9	16
negative	12	5	17
Total	19	14	33

Chi-square = 2.431, degree of freedom = 1, and 0.50>P>0.10.

Table 10. Comparison of virus isolation from patient PBMCs with CDC groups.

	CDC group			
Virus isolation	**II**	**III**	**IV**	**Total**
positive	3	9	10	22
negative	8	10	1	19
Total	11	19	11	41

Chi-square = 9.524, degree of freedom = 2, and 0.01>P>0.001.

The results of Tables 9 and 10 were analysed by the Chi-square test using Yates correction (Appendix III). Virus isolation results were based on a single positive isolation from PBMCs, plasma, or HWB.

Statistical analysis showed that there was no correlation between virus isolation and detectable plasma p24 antigen detection ($0.50>P>0.10$), $CD4^+$ cell counts ($0.50>P>0.10$), or AZT therapy ($0.50>P>0.10$). There was, however, a significant association with CDC group ($0.01>P>0.001$), demonstrating an increase in isolation rate from patients with advanced disease.

5.1.3 Detection of viral DNA sequences by PCR in culture negative samples.

To prove that negative cultures contained HIV DNA, it was decided to extract DNA from the cells and amplify for viral sequences. Total cellular DNA was extracted from p24 antigen negative PBMC cocultures of 6 seropositive patients, and amplified for *pol* and *env* sequences. PBMCs of cocultures of 3 isolation positive samples (P11b, P19a, and P20b), a high risk seronegative sample (P2b), and numerous low risk donor PBMCs were included as controls.

The DNA extracts from control cocultures gave the expected amplification results throughout. Each of the 6 seropositive coculture extracts was positive for *pol* and/or *env* sequences by PCR despite the patient cells being diluted by uninfected donor cells (Table 11). The results demonstrate the presence of infected cells in the sample, suggesting that the coculture method used was inefficient in stimulating infected cells to produce new virions.

Table 11. HIV-1 DNA nested amplification results of cocultivated cells.

Patient No.	Amplification gene		VI result
	pol	*env*	
*P2b	-	-	-
P4a	+	-	-
P5	+	+	-
P10	+	+	-
P11b	+	+	+
P16	+	+	-
P19a	+	+	+
P20b	+	+	+
P25a	+	-	-
P31	+	+	-

* , high risk seronegative control patient; VI, combined results of PBMC, plasma, and heparinised whole blood virus isolation; +, positive: -, negative. PCR results were based on nested amplifications of the polymerase and envelope genes. Cultured PBMCs from low risk donors were included as negative controls, and were PCR negative throughout (data not included).

CHAPTER 6: ELECTRON MICROSCOPY STUDIES OF HIV-1 (RF) VIRAL UPTAKE AND RELEASE IN C8166 CELLS.

This work was carried out in the Electron microscopy (EM) suite, (Department of Medical Microbiology, The University of Edinburgh), under the careful supervision of Mr Derek Notman and Mrs Oonagh Gray. Samples were supplied to the EM suite fixed in 2.5% glutaraldehyde in cacodylate buffer, where they were processed and sectioned by Mrs Gray. Mr Notman supervised all viewing sessions, and both were involved in the development of electron micrographs of the material viewed.

6.1 Electron microscopy studies of viral uptake and release.

This section was designed to observe the mechanism of viral entry into susceptible cells. The C8166 cells were inoculated at 4°C to allow attachment without penetration. They were then transferred to an incubator, harvested at sequential time points, and positively stained for EM studies.

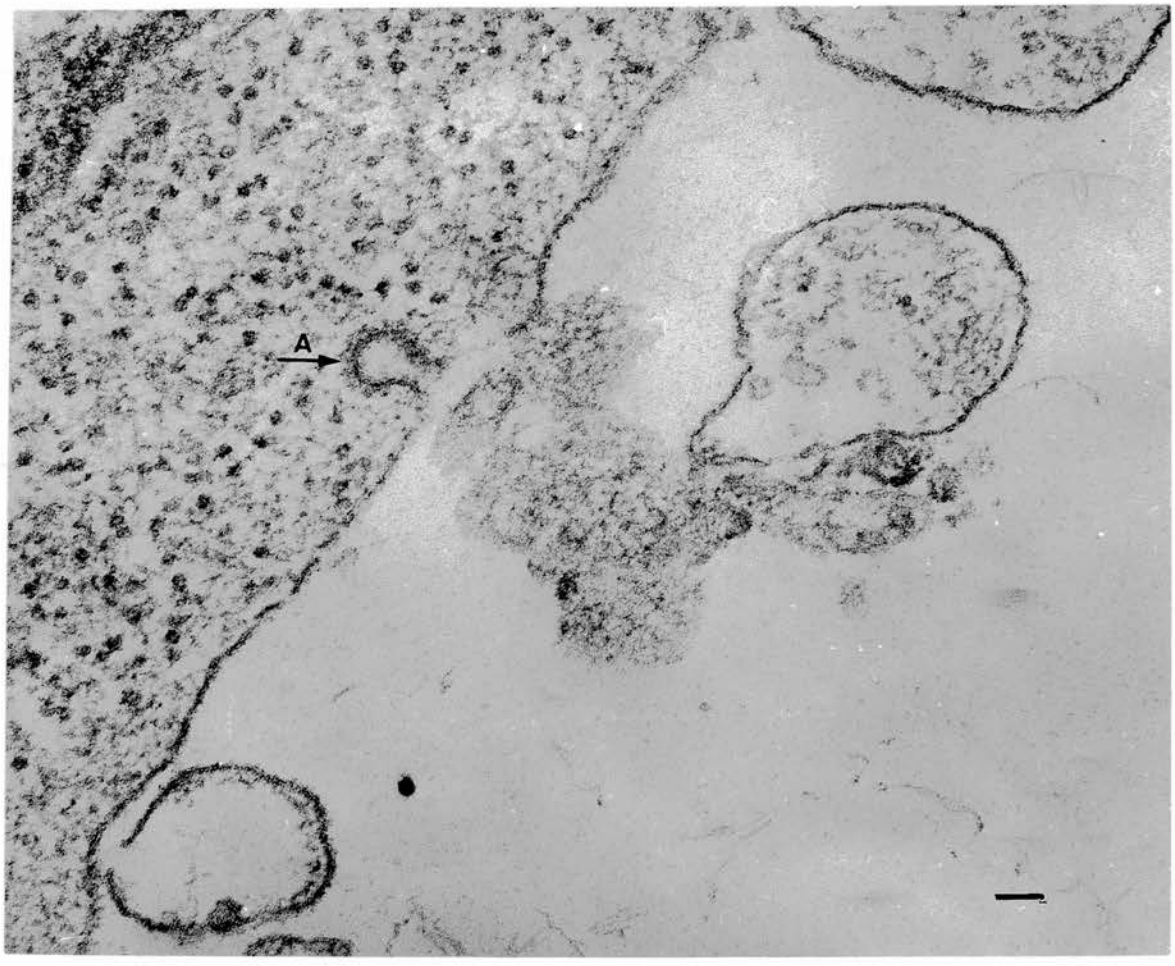
6.1.1 Viral entry into C8166 cells.

HIV-1_{RF} infected cells were harvested at time points 0, 5, 10, 15, 20, 25, 30, 40, 50, and 60min, and viewed for evidence of viral penetration. Sections of infected and uninfected cell pellets showed evidence of clathrin coated pits (Figure 10), and internal structures were observed in some intracellular vesicles of infected cells, although at no time point were these identified as viral particles (Figure 11). There was, therefore, no evidence of viral uptake by endocytosis.

Although not quantified, it was noted that the presence of virus decreased rapidly after incubation of the cells at 37°C, suggesting that viral uptake was very rapid.

Figure 10.

HIV-1_{RF} infected C8166 cell, harvested 25min post infection. Magnification 60K bar=100nm.
A Arrow indicates the formation of a clathrin coated pit for uptake and transport into the cell.



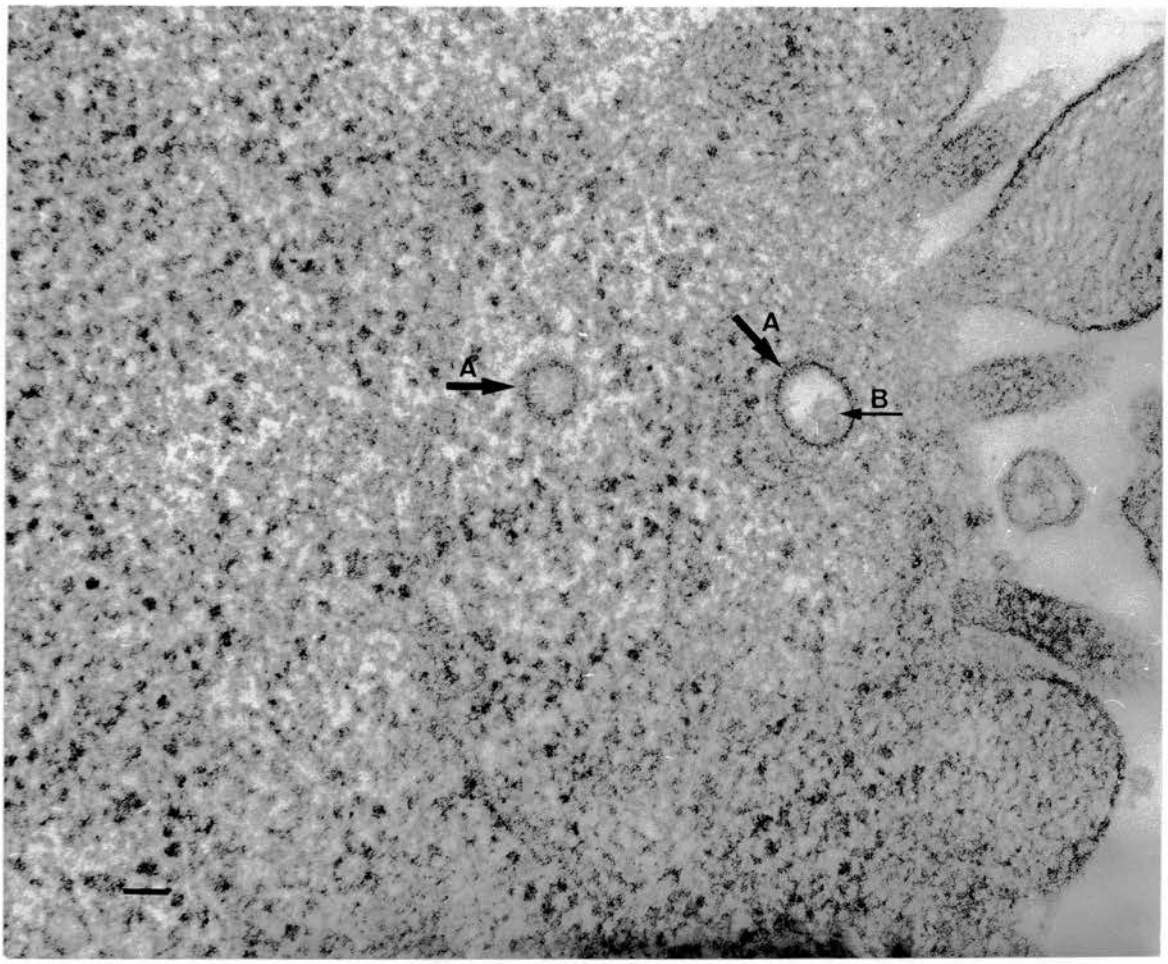


Figure 11.

HIV-1_{RF} infected C8166 cell, harvested at 25min, magnification 60K, bar=100nm.
A Two intracellular inclusions, probably clathrin coated vesicles, **B** unidentified inclusion within vesicle (approximately 45nm diameter).

6.1.2 Viral budding from C8166 cells.

Cells were infected 3 days prior to sampling to allow viral replication, thereby increasing the viral load. Virus was observed budding from the majority of sectioned cells. The budding process was characterised by an initial thickening and arching of the cell surface membrane (Figure 12), progressing to the formation of a horseshoe-like structure (Figure 13). The latter stages of the budding process completed the envelope structure releasing the immature virion from the cell membrane (Figure 14). The characteristic core structure formed upon release from the host cell, and was first observed in free virus surrounding infected cells (Figure 15).

Figure 12.

HIV-1_{RF} budding from C8166 infected cell, day 8 at 45K magnification, bar=100nm.
A Initial thickening of the cell membrane, **B** thickened cell membrane arching outwards to form the viral envelope.

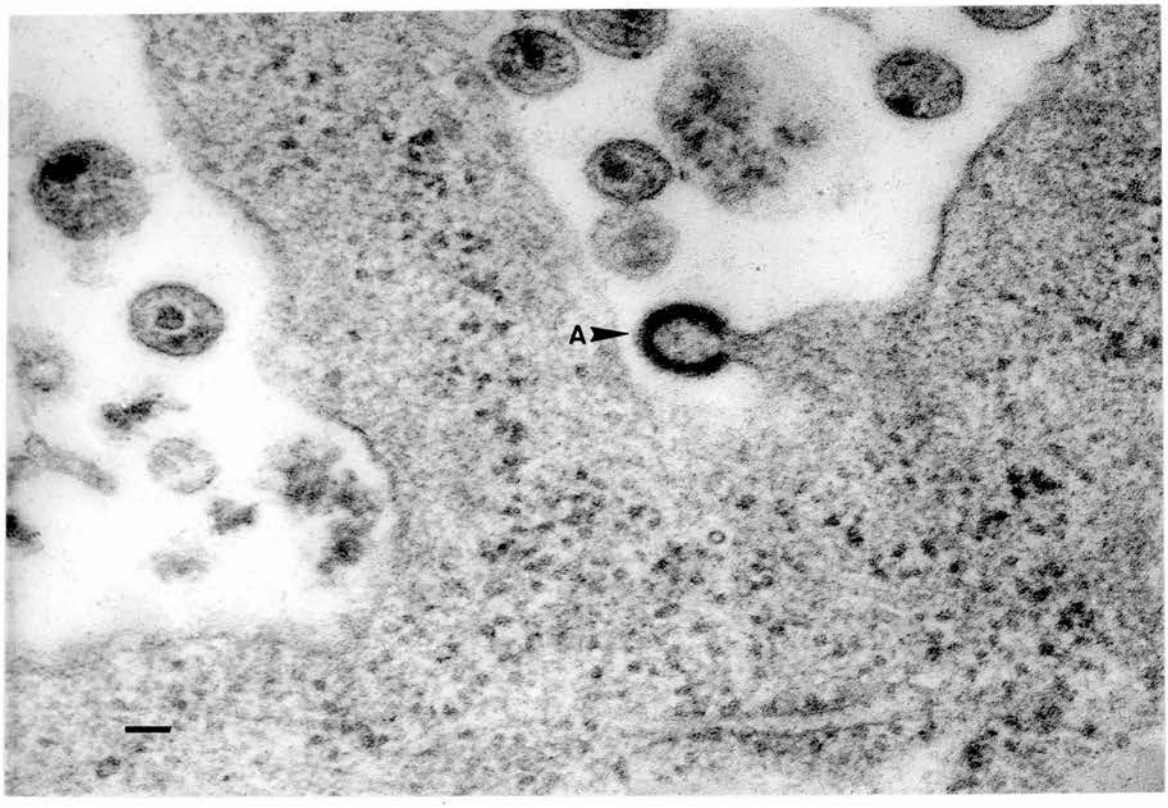
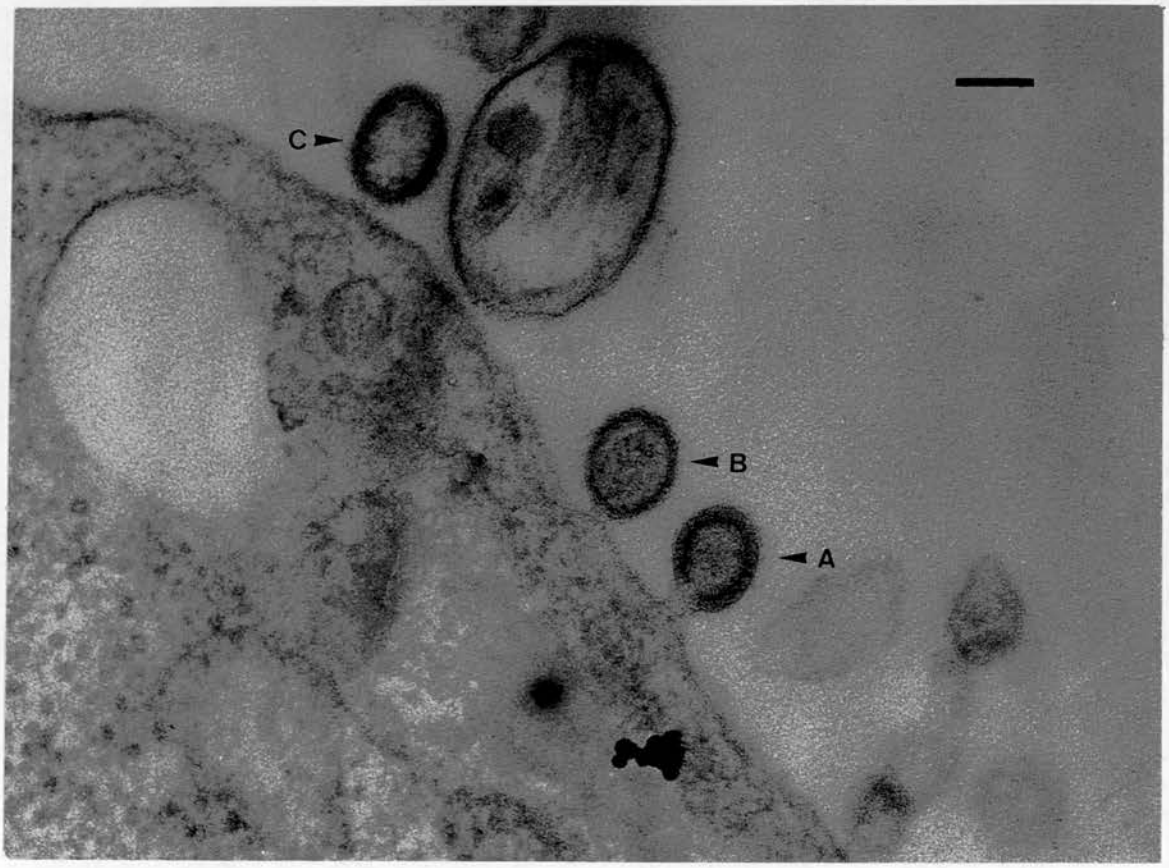


Figure 13.

HIV-1_{RF} infected C8166 cell, day 8. Magnification 75K bar =100nm.
A Budding HIV-1 particle demonstrating the characteristic horseshoe-like structure. Mature free virus can also be seen around the infected cell.

Figure 14.

Budding HIV-1_{RF} from C8166 infected cell, day 8. Magnification 90K, bar =100nm.
The electron micrograph demonstrates the latter stages of the HIV-1 budding process.
A Horseshoe-like budding structure nearing completion, **B** completion of the viral envelope immediately prior to release from the cell, **C** complete release of new immature virion from the cell membrane.



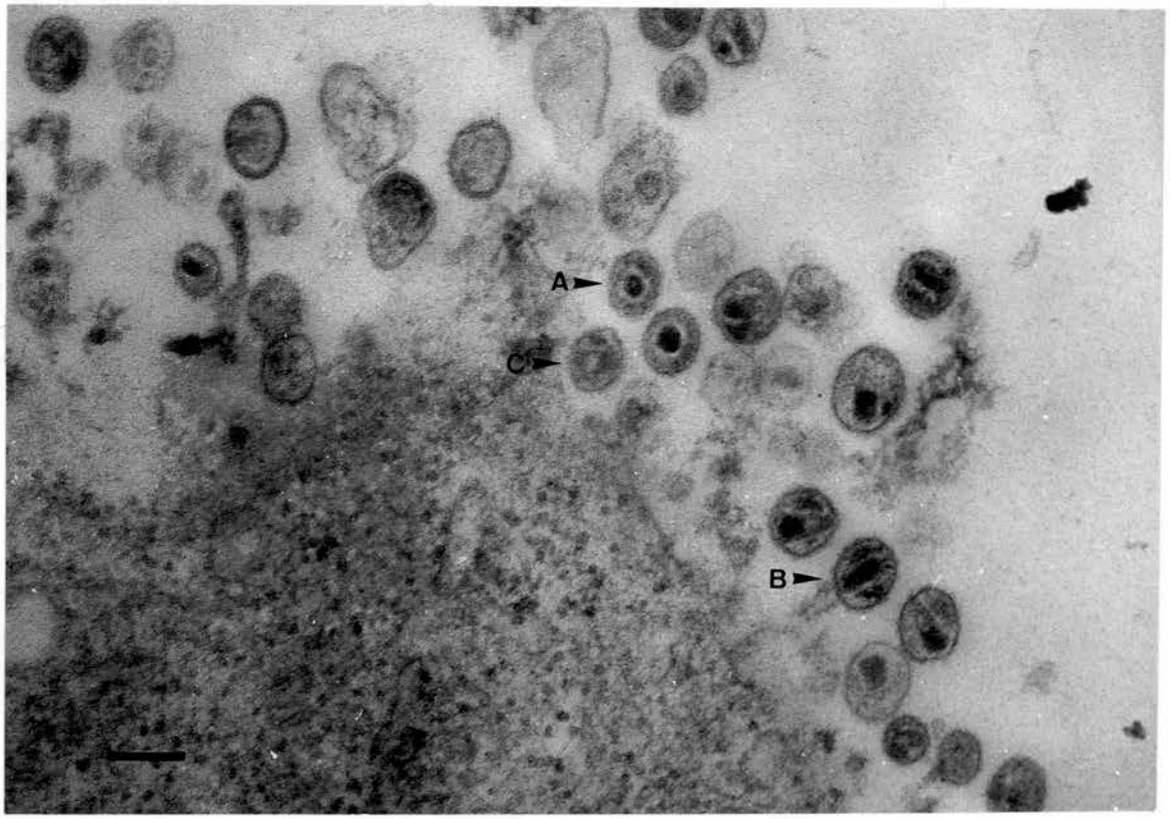


Figure 15.

New virions released from C8166 HIV-1_{RF} infected cell, day 8. Magnification 60K, bar =100nm.
New virions, demonstrating the characteristic core structures formed after completion of the viral budding process from the host cell.
A Cross-section of core, **B** longitudinal section of core, **C** immature core structure of newly released virion.

CHAPTER 7: DETECTION AND CHARACTERIZATION OF HIV-1 DNA BY PCR.

The methods used to characterize HIV-1 DNA in laboratory cell lines and patient PBMCs, were developed in collaboration with Drs. D.W-J. Aw and A.J. Shepherd. The data on infection of cell lines with HIV-1_{IIIB} was supplied by Drs. Aw and Shepherd, and is included with their kind permission.

To differentiate between HIV-1 DNA forms by PCR requires that the primers hybridize to, or amplify across, a unique sequence. Primers for the coding sequences (*gag*, *pol*, and *env*) are, therefore, type common primers amplifying all forms of viral DNA identically (Figure 16).

The CUVD forms differ from other viral DNA forms as they are covalently closed circular molecules. This produces junction sites which can be exploited by PCR to allow the differential detection of CUVD (Figure 17a).

The LUVD form does not have a unique sequence, but is unique as the viral LTR sequences are not flanked by viral or cellular DNA (unlike unintegrated circular and integrated linear viral DNA, Figure 17b). The integrated provirus is flanked by cellular sequences (Figure 17c) and is exclusively in the high molecular weight fraction of cellular DNA extractions.

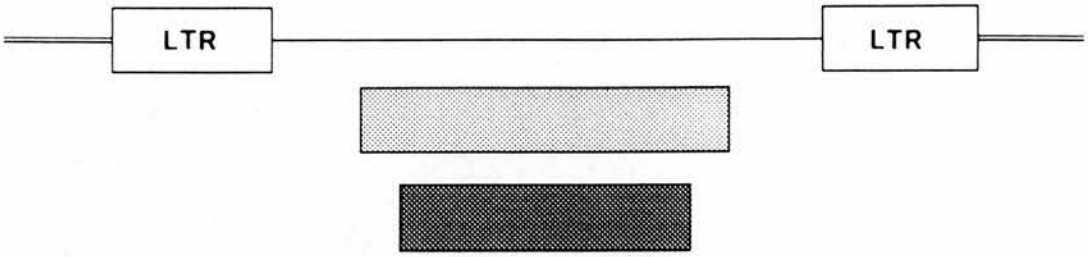
The aim of this chapter was to exploit these differences to develop PCR as a method of detecting and characterizing HIV-1 DNA forms in cell extracts.

7.1. Development of dot blot and Southern hybridization assays.

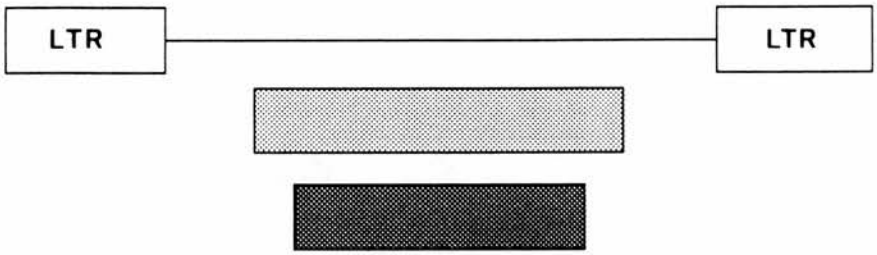
Southern hybridization assays were required during the development of PCR as a method of detecting CUVD forms. This section describes the development and optimisation of the hybridization assays used for the detection of DNA sequences by a biotinylated oligonucleotide probe on nylon membranes.

Figure 16. Detection of HIV-1 DNA using primers specific for *gag*, *pol*, and *env* sequences.

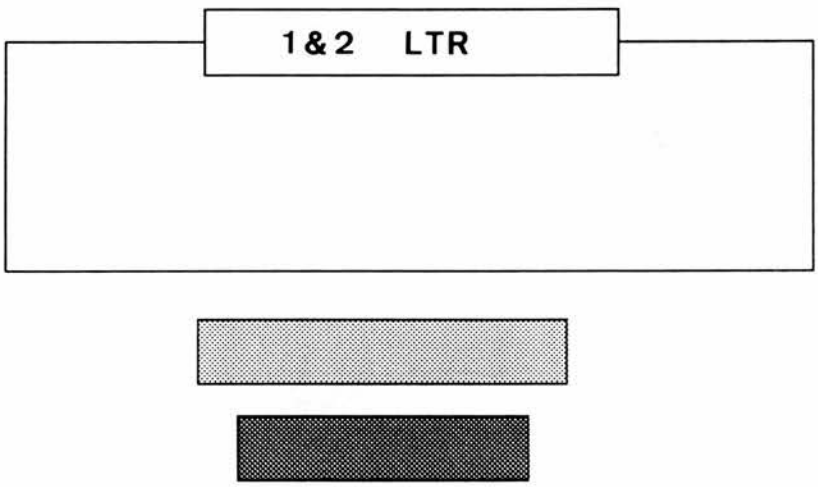
a) Linear integrated provirus.



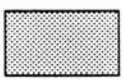
b) Linear unintegrated viral DNA.



c) Circular unintegrated viral DNA (1 and 2 LTR forms).



Key:



Primary amplification

Secondary amplification

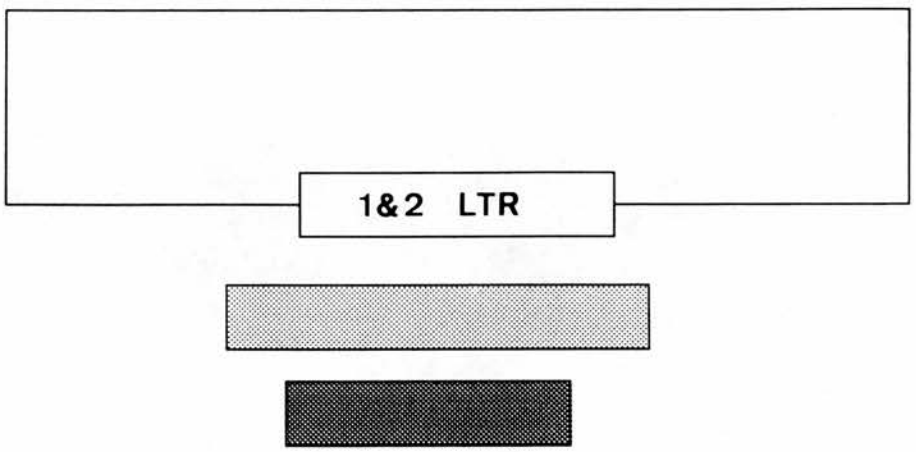
viral DNA

cellular DNA

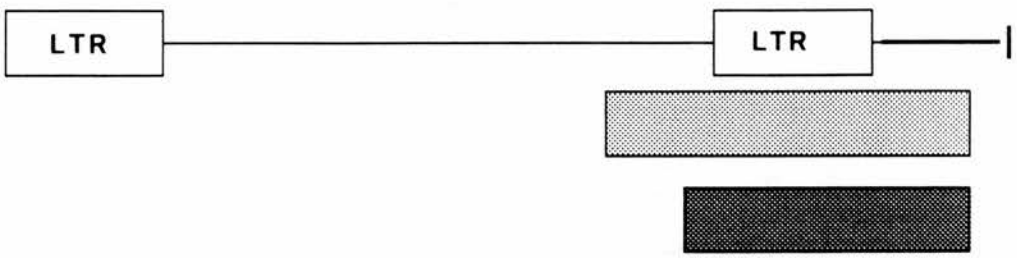
long terminal repeat

Figure 17. Characterization of HIV-1 DNA forms by PCR.

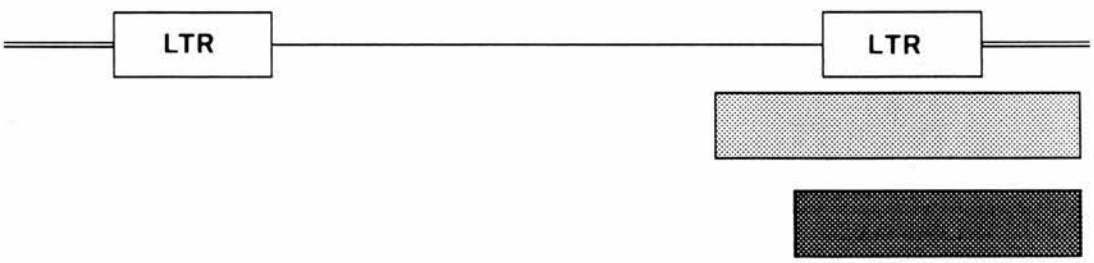
a) Circular unintegrated viral DNA (1 and 2 LTR forms) with unique junction sequences.



b) Linear unintegrated viral DNA. Exploitation of the blunt ends using a ligation mediated nested PCR.



c) Linear integrated proviral DNA flanked by cellular sequences.



Key:

Primary amplification

Secondary amplification

viral DNA

cellular DNA

long terminal repeat

7.1.1 Comparison of the detection of DNA sequences fixed by UV light to nylon membranes with baking to nylon and nitrocellulose membranes.

Fixation of samples on both nitrocellulose and nylon membranes by baking at 80°C is non-covalent, and so during the hybridization reaction samples may leach off the membrane, effectively reducing the sensitivity of the assay. Alternatively, DNA can be covalently bound to nylon membranes by exposure to ultra-violet (UV) light (Church and Gilbert, 1984). It was, therefore, decided to compare the sensitivity of detection of DNA sequences baked on nitrocellulose membranes against DNA baked and UV bound to nylon membranes.

Dilutions (1 in 10, 1 in 100, and 1 in 200) of a nested *pol* amplification product were spotted onto nylon and nitrocellulose membranes and fixed by UV light for 5min, or baking at 80°C for 2h. The samples were probed using a PCR generated biotinylated *pol* sequence, and the results of the colour reaction compared.

Although the background levels were considerably lower on nitrocellulose membranes, the most sensitive method of detection was with UV fixed samples on nylon membranes. Both nitrocellulose and nylon baked membranes detected the PCR product diluted 1 in 10, however, the UV fixed nylon membrane was also positive for the 1 in 100 diluted sample. All membranes were negative for the sample diluted 1 in 200. In addition to the sensitivity of detection, the rate and intensity of the signal obtained with UV fixation was greater than with baking. It was, therefore, decided to develop the method of UV fixation of DNA to nylon membranes in preference to baking of nitrocellulose or nylon membranes.

7.1.2 Optimisation of UV fixation of DNA to nylon membranes.

It was proposed that UV fixation of DNA to nylon membranes would increase

with time until maximum fixation had occurred, and a plateau effect would be observed. To demonstrate this, biotinylated DNA was loaded onto 2 identical nylon membranes. The dots were exposed for varying times to UV light, and one membrane was placed in boiling water prior to development of the colour reaction.

The resulting intensities of positive dots on each membrane were similar. The boiled membrane was positive for samples exposed to UV light for 20s or more, whereas the non-boiled strip was positive for all exposures (10 to 60 seconds). This showed that the fixation of DNA onto nylon membranes was not only covalent, but was also very rapid. In future experiments, samples were routinely exposed to UV light for 2min to ensure maximum fixation to the membrane.

7.1.3 Detection of LTR sequences by dot blot hybridization on PCR amplified extracts of C8166 cells infected with HIV-1_{RF}.

The HIV-1 specific probe (581NBio) was selected to hybridize to the U3 sequence of the viral LTR. To determine its specificity and sensitivity, the probe was used to detect PCR products from amplifications of the LTR sequences. Samples were used that had been amplified by the 2 LTR CUVD specific primers (782K/783K and 779K/781K) which synthesized a band of 447bp from the two U3 sequences flanking the 2LTR junction (Figure 8).

The 2 samples positive for 2 LTR CUVD by PCR (C8166 day 2 & MT4 day 4) were also positive by dot blot hybridization assay. The amplified sample of C8166 cells from 4 hours was clearly negative as expected, however the MT4 24h sample was indeterminate. Owing to the background noise and to the indeterminate nature of the result for MT4 24h sample, it was decided to increase the probe concentration from 400ng to 600ng/ml of hybridization solution.

7.1.4 Optimisation of DNA transfer by the semi-dry gel electroblotter.

Dilutions of biotinylated DNA (1.29ug to 64.5ng of 581NBio) were used to observe the efficiency of DNA transfer using the electroblotter. After 1h transfer, the agarose gel was viewed under UV light, demonstrating that the bands representing 129ng and 64.5ng of DNA had transferred completely, whereas the bands of higher concentrations had only transferred in part. The membrane was developed, and on initiating the colour reaction, all bands were visible within 30min. Subsequent studies varying the time of transfer showed that a minimum of 5h was required for the total transfer of DNA when 15ul of PCR product was electrophoresed (data not shown).

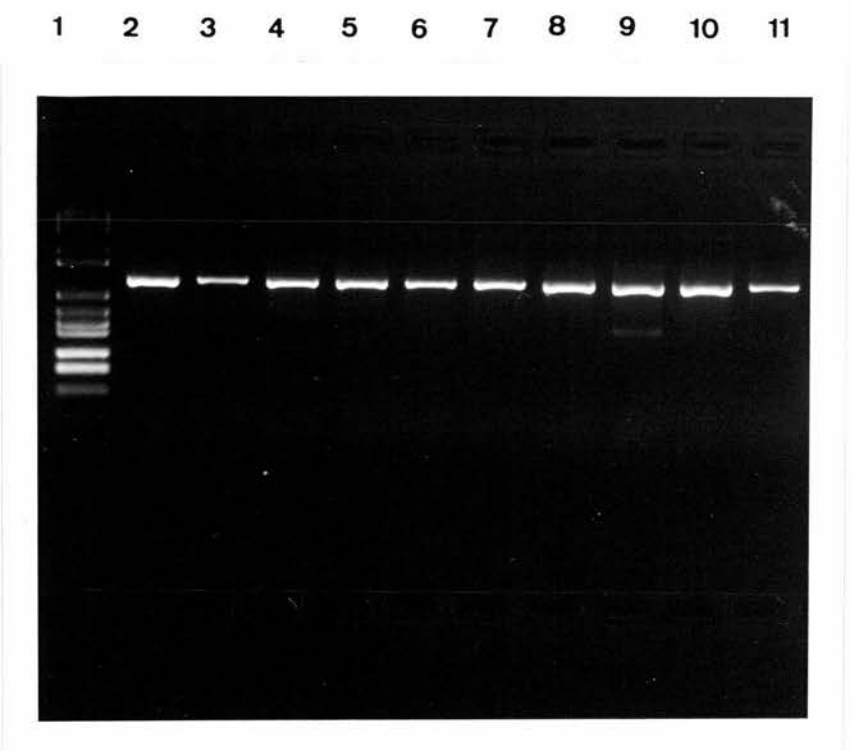
7.2 Optimisation of CUVD detection by PCR.

7.2.1 Detection of 2LTR CUVD by a nested PCR.

Patient samples were amplified with the nested PCR primers for 2LTR CUVD detection (782K/783K and 779K/781K). Preliminary amplification batches resulted in bands in all wells indicative of contamination, and so these batches were discarded and repeated. One batch (P3b, P11b, P16, P19a, and P31) demonstrated a second band which led to the realisation that the apparent contaminating band was heavier than the expected specific band of 447bp (Figure 18). To show that the faint band was the specific amplification product, and that the additional heavy band observed in each well resulted from aberrant hybridization of LTR primers, it was decided to transfer the bands by a Southern blot, and probe with an LTR specific biotinylated oligonucleotide.

Figure 18. Detection of 2LTR CUVD by nested PCR.

DNA extracts were amplified with the 2LTR CUVD specific primers 782K/783K and 779K/781K (447bp) and electrophoresed on a 1.5% agarose gel. A non-specific band of approximately 800bp was detected in all wells, with the predicted band of 447bp detected in sample P16 (lane 9).



Lane 1, DNA VI molecular weight marker (2176, 1766, 1230, 1033, 653, 517, 453, 394, 298, 234/220, 154); **lanes 2, 4, 6, 8, & 10**, negative controls; **lane 3**, P31; **lane 5**, P11b; **lane 7**, P19a **lane 9**, P16; **lane 11**, P3b

7.2.2 Detection of the U3 region of HIV-1 by Southern blot hybridization of 2LTR CUVD nested PCR products.

2LTR CUVD amplification products of 4 patient samples were transferred onto nylon and probed for a U3 specific sequence. One sample (P13) was PCR positive for 2LTR sequences, and 3 samples were PCR negative for 2LTR sequences (P1, P4b, and P20c) as determined by gel electrophoresis. The samples were transferred onto nylon with a biotinylated ladder to assist in the analysis of band lengths after the colour development (Figure 19).

As expected, sample P13 was positive for the 2LTR CUVD specific band (447bp). A heavier band (less than 1000bp) which cannot be excluded as the non-specific band observed in all samples was also detected by the LTR specific probe (Lane 3, Figure 19). However, the band was only visible in the 2LTR CUVD PCR positive sample (all samples and controls were positive for the non-specific band) and so may represent the primary amplification product for 1LTR CUVD forms (996bp). In support of this argument, sample P13 was later shown to be 1 and 2LTR CUVD positive by PCR (Appendix IV).

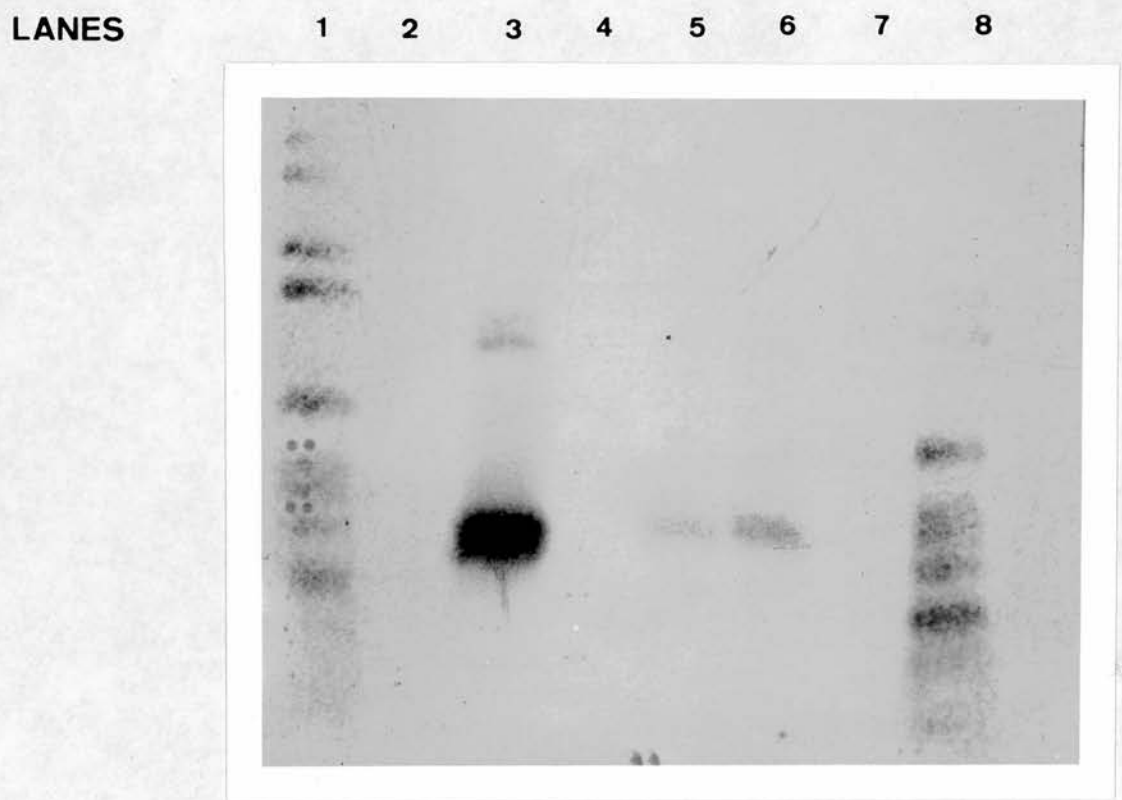
Of the 3 PCR negative samples, P1 was negative as expected, but P4b and P20c were both hybridization positive for the 2LTR CUVD band (Lanes 4, 5 and 6 respectively, Figure 19). Since PCR is the most sensitive method available for the detection of DNA, the positive results obtained for samples P4b and P20c were unexpected. This suggests that PCR did not amplify the samples sufficiently for detection by agarose gel electrophoresis, possibly due to the competition for materials arising from the amplification of the non-specific band.

The hybridization results showed that the non-specific band was not due to HIV-1 LTR sequences, and so it was hypothesized that the band resulted from aberrant priming of cellular sequences, and that it was competing for resources such as primers, *Taq* polymerase, and dNTP's.

Figure 19. Southern blot hybridization of 2 LTR CUVD nested PCR amplified samples.

Samples were amplified by the 2LTR CUVD specific primers (782K/783K and 779K/781K), electrophoresed on a 1.5% gel, and transferred to a nylon membrane. The samples were probed by the biotinylated oligonucleotide (581NBio) specific for the LTR U3 sequence (Figure 8).

The 2LTR CUVD specific band (447bp) was detected in lanes 3, 5, and 6, and an additional band (approximately 800bp) was detected in lane 3.



Lanes 1 & 8, biotinylated DNA VI, molecular weight marker (2176, 1766, 1230, 1033, 653, 517, 453, 394, 298, 234/220, 154); **lane 2, & 7**, negative controls; **lane 3**, P13; **lane 4**, P1; **lane 5**, P4b; **lane 6**, P20c.

The primers utilised in the 2 step amplification were from the U3, *gag* and *env* sequences. The primary primers (*gag* and *env* specific) had previously been utilised by other groups, and so the nested primers from the U3 LTR sequence were thought to be the more likely source of the non-specific priming. Alternative LTR primers were tested (585N/586N, Appendix V) but gave similar results with strong non-specific background bands (data not shown).

Sequence analysis of the viral LTRs has demonstrated some homology with T-cell growth factor and gamma interferon sequences (Starcich *et al.*, 1985). These sequences would be present in all samples and negative controls, and could explain the presence of the additional band.

Amplification aliquots from patient samples contained DNA from $7x10^3$ to $1x10^6$ cells, of which approximately 70% were T cells ($5x10^3$ to $7x10^5$). As a result of the large number and diploid nature of human cells present, the concentration of cellular sequences competing for materials was far in excess of viral sequences, which may have been present at a frequency as low as 1 copy of dsDNA per 80000 cells (Simmonds *et al.*, 1990a). This would explain why PCR, the most sensitive method available for DNA detection, was failing to detect viral DNA sequences in patient PBMC extracts.

It was thought that the reaction could be biased towards viral amplification by increasing the initial amplification with viral-only specific primers prior to using LTR specific sequences. To avoid using the same set of primers for 2 amplification reactions, the secondary amplification was designed to utilise a primary reaction primer with a nested primer. In this way, it was hoped to generate sufficient viral sequences to have a higher viral than cellular template concentration for the tertiary amplification with the original nested primers.

7.2.3 Tertiary 2LTR CUVD amplifications.

Aliquots of patient DNA extracts (P1, P4b, P13, P20c, and P25b) were tertiary amplified with 2 different sets of primers (3 pairs per set), to compare their sensitivity for the detection of 2LTR CUVD forms. All samples were amplified in duplicate, and the results were compared with the standard nested amplification (Figure 20).

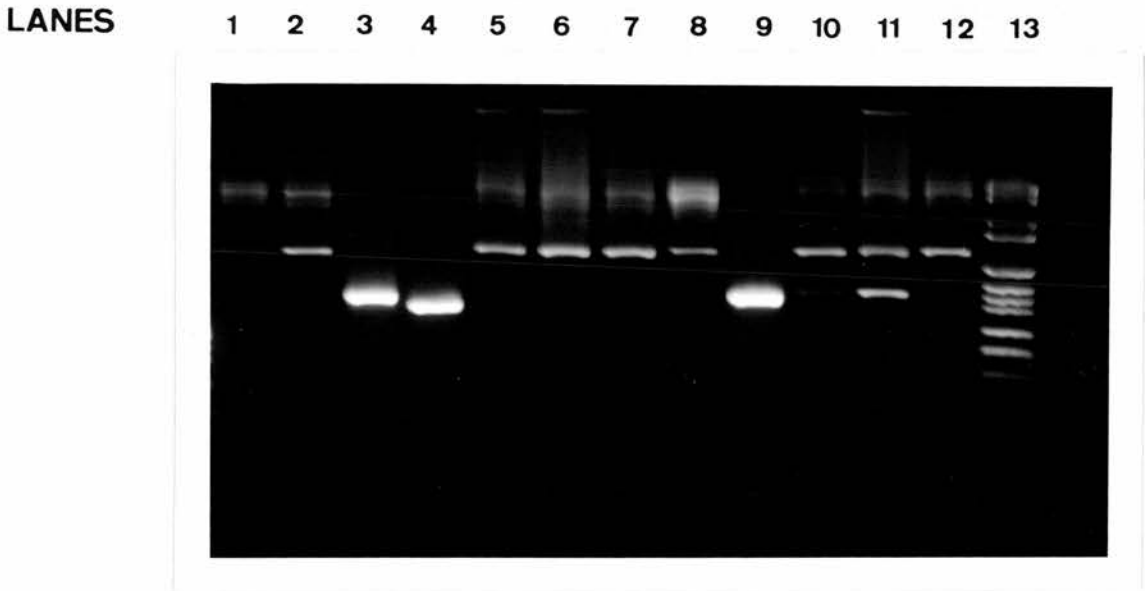
The nested PCR method only detected 2LTR CUVD in the P20c aliquots amplified (Gel b, Figure 20). The tertiary amplification with secondary primers 779K/782K detected 2LTR CUVD sequences in samples P4b, P13, and P20c, although the non-specific bands were still quite prominent (Gel a, Figure 20). However, using the secondary primers (781K/783K), 2LTR CUVD forms were detected in samples P4b, P13, and P20c (as with 779K/782K secondary primers), and the specific band was of a higher intensity than the non-specific bands (lane 10 of gel a and c, Figure 20). Therefore, the use of the secondary primer pair 781K/783K, prior to the original "nested" amplification with primers 779K/781K, appeared to bias the amplification reaction towards viral sequences, largely overcoming the aberrant amplification of cellular sequences in PBMC DNA extracts. This method was subsequently adopted for all 2LTR CUVD amplifications.

7.2.4 Comparison of the sensitivity of secondary and tertiary amplifications.

To determine if a nested amplification with the secondary primers 781K/783K was sufficient for 2LTR CUVD detection, extracts of cell lines infected with HIV-1_{IIIB} were amplified with primers 782K/783K, 781K/783K, and 779K/781K, and the secondary and tertiary amplification products were compared (Table 12).

Figure 20a. Comparison of the standard nested amplification for the detection of 2LTR CUVD with the tertiary amplification protocols.

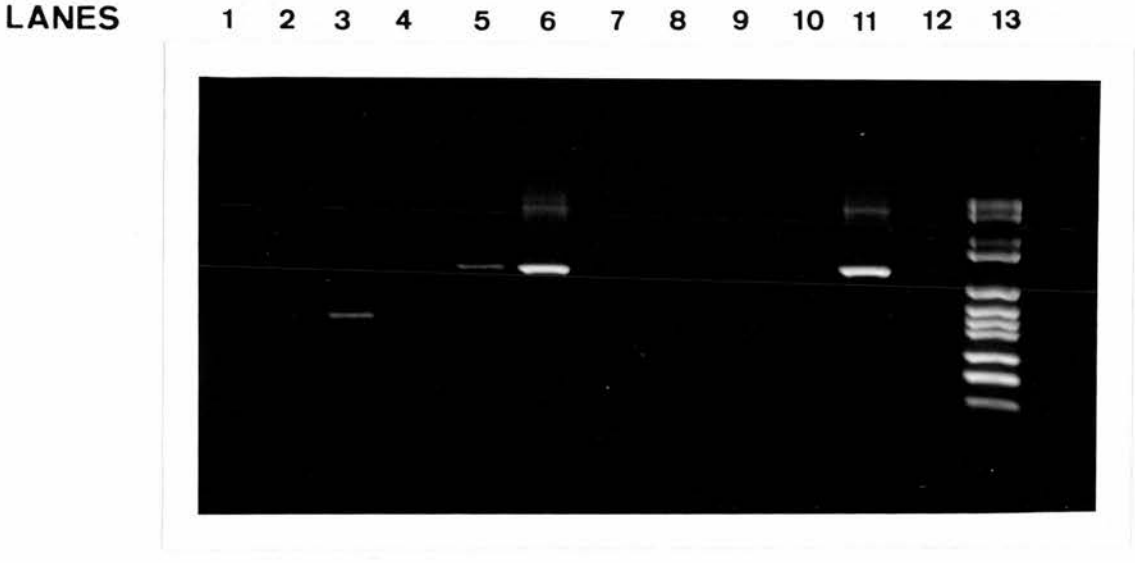
Gel a: Primary amplification 782K/783K, secondary amplification 779K/782K, tertiary amplification 779K/781K (447bp).



Lanes 1 & 7, negative controls; **lane 2 & 8**, P25b; **lane 3 & 9**, P20c; **lane 4 & 10**, P13; **lane 5 & 11**, P4b; **lane 6 & 12**, P1; **lane 13** DNA VI molecular weight marker (2176, 1766, 1230, 1033, 653, 517, 453, 394, 298, 234/220, 154). The non-specific band observed in all amplifications was approximately 800bp, and the 2LTR CUVD specific band was 447bp.

Figure 20b. Comparison of the standard nested amplification for the detection of 2LTR CUVD with the tertiary amplification protocols.

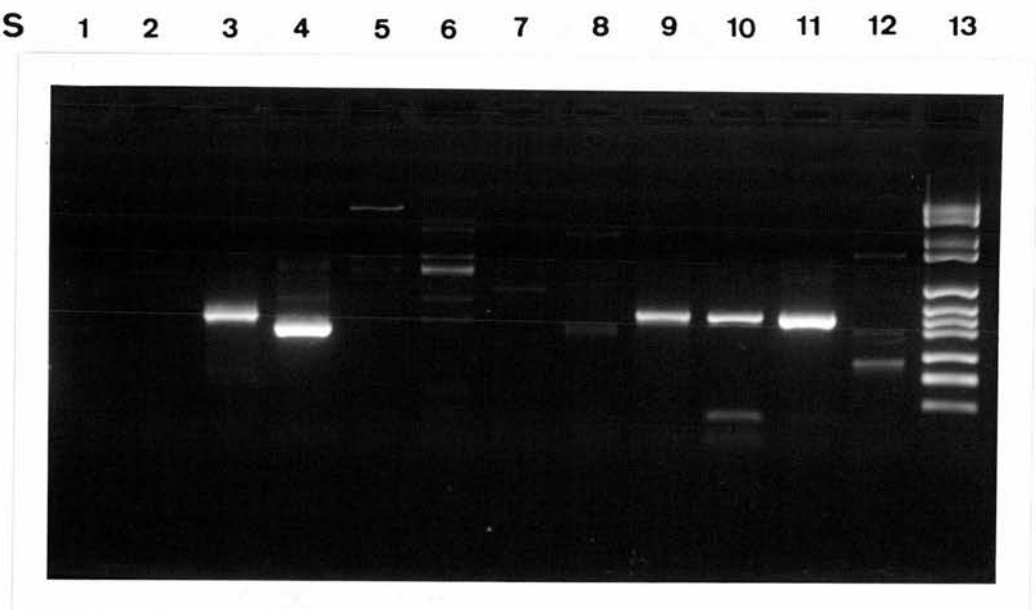
Gel b: Primary amplification 782K/783K, secondary amplification 779K/781K.



Lanes 1 & 7, negative controls; **lane 2 & 8**, P25b; **lane 3 & 9**, P20c; **lane 4 & 10**, P13; **lane 5 & 11**, P4b; **lane 6 & 12**, P1; **lane 13** DNA VI molecular weight marker (2176, 1766, 1230, 1033, 653, 517, 453, 394, 298, 234/220, 154). The non-specific band observed in all amplifications was approximately 800bp, and the 2LTR CUVD specific band was 447bp.

Figure 20c. Comparison of the standard nested amplification for the detection of 2LTR CUVD with the tertiary amplification protocols.

Gel c: Primary amplification 782K/783K, secondary amplification 781K/783K, tertiary amplification 779K/781K.



Lanes 1 & 7, negative controls; **lane 2 & 8**, P25b; **lane 3 & 9**, P20c; **lane 4 & 10**, P13; **lane 5 & 11**, P4b; **lane 6 & 12**, P1; **lane 13** DNA VI molecular weight marker (2176, 1766, 1230, 1033, 653, 517, 453, 394, 298, 234/220, 154). The non-specific band observed in all amplifications was approximately 800bp, and the 2LTR CUVD specific band was 447bp.

Table 12. Comparison of secondary and tertiary amplifications for 2LTR CUVD detection in MT4 and C8166 cell lines infected with HIV-1_{IIIB}.

	hours						days						
	0	1	2	4	8	12	1	2	3	4	5	8	12
MT4													
(2° amp)	-	-	-	-	-	-	-	+	+	+	+	+	+
(3° amp)	-	-	-	-	-	+	+	+	+	+	+	+	+
C8166													
(2° amp)	-	-	-	-	-	-	+	+	+	+	+	+	+
(3° amp)	-	-	-	+	+	+	+	+	+	+	+	+	+

Samples were tertiary amplified with 2LTR CUVD specific primers 782K/783K, 781K/783K, and 779K/781K. 2° amp, secondary amplification; 3° amp, tertiary amplification; -, negative; +, positive. Details of the cell lines are given in Appendix II.

The tertiary amplification gave more intense specific bands with fewer non-specific bands, increasing the sensitivity of detection by 2 to 3 time points. This confirmed that the tertiary amplification was more sensitive and specific for 2LTR CUVD detection in both cell line and patient PBMC DNA extracts.

7.2.5 Simultaneous detection of 1 and 2LTR CUVD forms in patient PBMC DNA extracts.

Multiple aliquots of sample extracts from patients 13 and 20 (P13 and P20c) were amplified for 1 and 2LTR CUVD forms using primers from the *gag* and *env* sequences (077F/783K and 782K/783K). Bands of 996bp and 1630bp were expected for the detection of 1 and 2LTR CUVD forms respectively, and both forms were detected simultaneously (Figure 21).

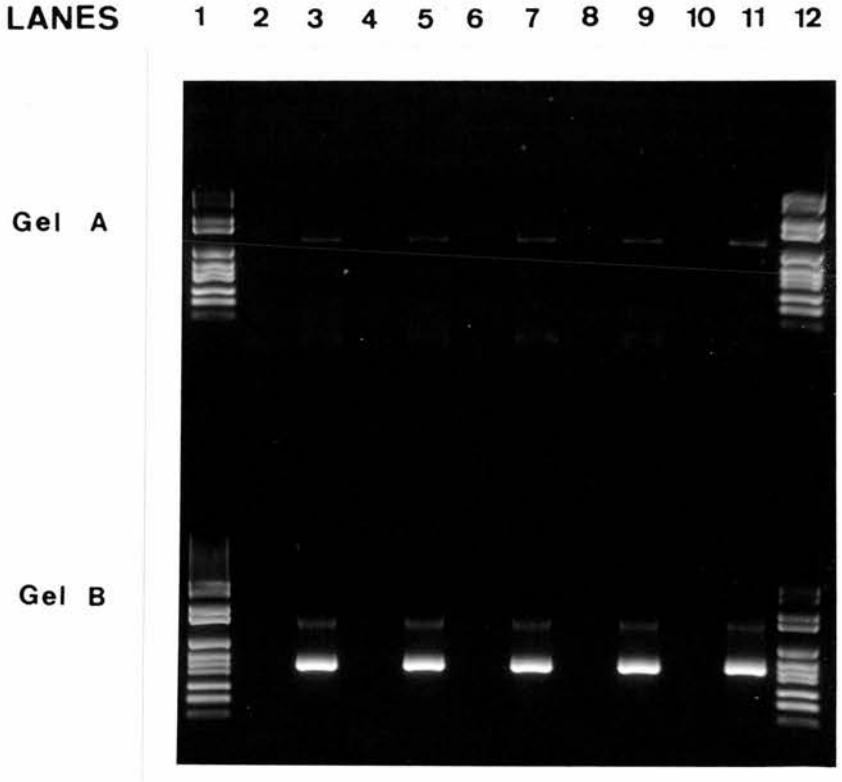
Both bands were detected in extracts of PBMCs from patients 13 and 20, however, in each case 1 form repeatedly amplified more efficiently for each patient. Extracts from P13 resulted in a clearer 2LTR band, while P20c extracts had a dominant 1LTR amplification band. Had both samples resulted in the same predominant band, it could have been concluded that the amplification reaction was more efficient for this form. This may have been due to the primers, or, for 1LTR forms, the fact that the amplification sequence was considerably smaller. However, as the 2 samples differed, this suggests a possible link to the actual proportions of each form present in the PBMCs of the infected individual. If this is true, it may be possible to relate the detection and form of CUVD to the clinical disease or cytopathology of HIV infection.

7.3 Optimisation of the detection of LUVD.

It was proposed to detect LUVD by ligating a unique sequence to the blunt LTR end, and to prime DNA amplification from this site (Figure 22). To determine whether or not the proposed primer 022N for the amplification of ligated LUVD could prime DNA synthesis without the presence of the ligation

Figure 21. Simultaneous detection of 1 and 2 LTR CUVD forms by PCR.

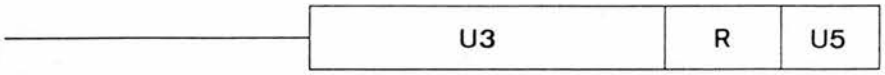
Patient PBMC extracts were amplified in replicate by *gag* and *env* primers (077F/782K, and 782K/783K) resulting in 1 and 2LTR CUVD specific bands of 996bp and 1630bp respectively.
Gel a: nested amplification results for P13 extracts.
Gel b: nested amplification results for P20c extracts.



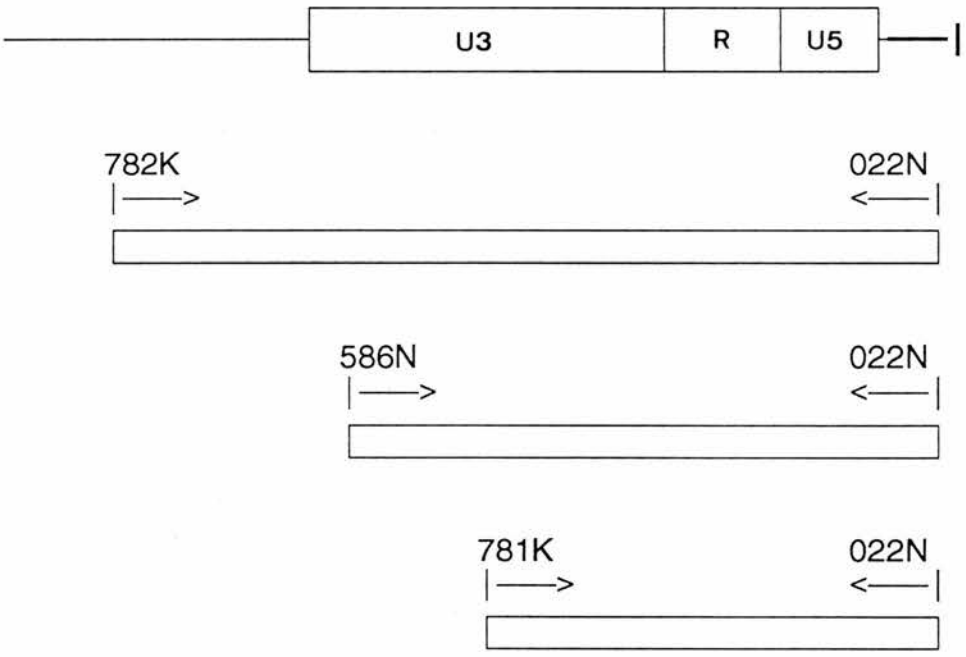
Lanes 1 & 12, DNA VI molecular weight marker ((2176, 1766, 1230, 1033, 653, 517, 453, 394, 298, 234/220, 154); **lanes 2, 4, 6, 8, & 10**, negative controls; **lanes 3, 5, 7, & 9**. Both 1 and 2 LTR CUVD specific bands were detected in both patients. The 2 LTR CUVD form was more dominant in P13 amplifications (Gel a), and the 1 LTR CUVD band in P20c amplifications (Gel b).

Figure 22. Detection of linear unintegtrated viral DNA.

a) Schematic representation of the 5' LTR of HIV-1.



b) Ligation of oligonucleotide (021N) to the viral LTR. Followed by primary amplification of viral LTR from the ligation sequence (021N) with primers 782K/022N, secondary amplification with primers 586N/022N, and tertiary amplification with primers 781K/022N.



Key:

amplification products

primers

sequence, small oligonucleotides (3, 5, 7 & 9 bases long, Figure 7) were analysed for their ability to prime DNA synthesis in the *pol* gene. Throughout the 3 amplification protocols tested, the positive and negative controls included gave the expected results.

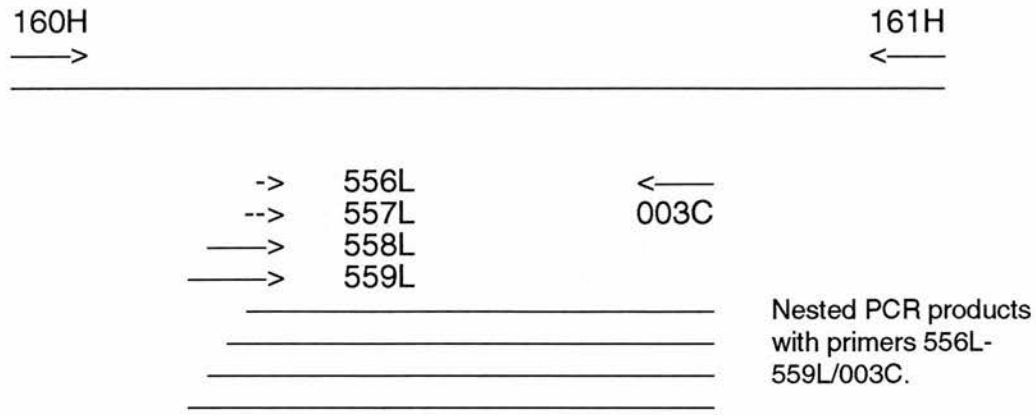
The first experiment, utilising primer pairs 160H/161H and 556-559L/003C, was to determine the ability of the short oligonucleotides to prime DNA synthesis (Figure 23a). The primary amplification with primers 160H/161H supplied a high concentration of template DNA with only 1 correct priming site for each oligonucleotide. Despite this, all test amplifications were negative for the expected bands of 112, 114, 116, and 118bp for primers 556L, 557L, 558L, and 559L respectively. From this it was concluded that an oligonucleotide of 9 bases or less was insufficient to prime DNA synthesis for efficient amplification by PCR.

The second experiment (primary primers 556-559L/161H and secondary primers 002C/003C) was designed to observe the effects of a primer overhanging the 3' end of the template DNA (Figure 23b). This was to mimic the possible priming event of primer 022N on a viral LTR sequence without the attachment of the ligation sequence (021N, Figure 22). All samples tested in this way were negative for the expected band of 128bp. Since the primers 002C/003C had previously been demonstrated to amplify *pol* sequences efficiently, these results suggested that a 16 base hybridization with an overhanging tail of 11 bases was inefficient at priming DNA synthesis.

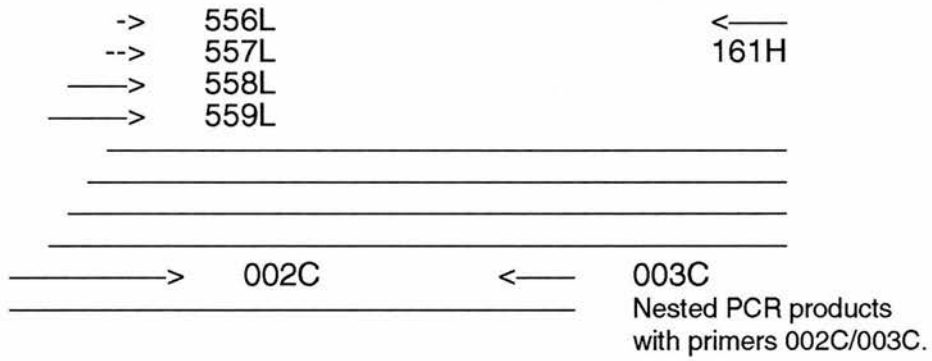
The final experiment evolved from the second in an attempt to identify the limits of an oligonucleotide's ability to prime when it has an overhanging tail. This experiment utilised the nested primer 559L (in conjunction with 003C) which hybridized by 3, 5, 7, or 9 bases to the primary amplified target DNA (primary primers 556-559L/161H) with an overhang of 6, 4, 2, or 0 bases respectively (Figure 23c). Although very similar to the previous experiment,

Figure 23. Determination of the minimum requirement for priming of DNA synthesis for PCR.

a) **Experiment 1.** Primary amplification with primers 160H/161H.



b) **Experiment 2.** Primary amplification with primers 556L-559L/161H.



c) **Experiment 3.** Primary amplification with primers 556-559L/160H and secondary amplification with primers 559L/003C.

the percentage of bases contributing to the overhanging tail was 0%, 22%, 44%, and 67%, whereas the previous experiment had overhangs of 38%, 46%, 54%, and 62%. All test results were negative, suggesting once more that an overlapping primer was not stable enough to initiate DNA synthesis.

Although these studies appeared to suggest that primers used for the amplification of ligated LUVD would be insufficient to initiate DNA synthesis without the ligation step, it was possible that the oligonucleotides 556-559L failed to prime DNA synthesis for other reasons. These may have included the occurence of interactions utilising the free primers in solution, or mis-priming in contaminating cellular DNA, both of which would alter the concentration of available sequences to prime DNA synthesis.

In view of this, preliminary ligation studies included the appropriate controls to demonstrate the requirement for the ligation sequence. Each sample was tested in triplicate. One vial contained all the necessary reagents while the other 2 vials lacked the ligation sequence or the ligase enzyme. All such controls were negative irrespective of the presence or absence of LUVD. Due to the small sample size available from many patients these controls were excluded from subsequent studies. HIV-1 negative controls were included throughout.

7.4 Cell lines infected with HIV-1_{IIIB}.

Ten cell lines were infected with HIV-1_{IIIB} to observe the timing and form of viral DNA synthesized in different cell types infected with the same viral strain.

7.4.1 Syncytium formation.

The formation of syncytia was only observed in T-cell derived lines (C8166, Hut78, MT4 and SupT1) infected with the IIIB isolate which led to the complete destruction of MT4 and C8166 cells by day 8. No syncytia were observed in non-T-cell derived lines (U937, THP1, HB1, LC5, U138MG, and

HeLa T8+). This suggests that the formation of syncytia was specific to the cell type and not to the virus strain, although the results obtained may reflect a combination of cellular and viral factors.

7.4.2 Overall viral DNA detection.

Cell lines derived from T lymphocytes were all HIV-1 DNA positive by 2h post-infection using the *pol* gene primers (Table 13). U937 and THP1 cell lines were also positive by 2h, whereas HB1 and LC5 lines were not positive until 8 and 12h post-infection respectively. The cell lines U138MG and HeLa T8+ failed to become infected and were HIV-1 DNA negative throughout the study (Table 14).

7.4.3 CUVD detection.

The overall detection of viral DNA in T-cell derived lines was within 2h of infection for each line. In contrast to this, the detection of either 1 or 2 LTR CUVD varied from between 2 and 12h, depending on the cell line. In both MT4 and SupT1 lines, the presence of 1 and 2 LTR forms appeared simultaneously whereas 2 LTR CUVD appeared before 1 LTR forms in C8166 and Hut78 cell lines. In all cell lines, once CUVD of either form had been detected it remained positive throughout the experiment (Table 13).

For non-T cell derived lines a similar pattern of positivity was observed. U937 cells were positive for 1 LTR CUVD 8 hours prior to the detection of 2 LTR forms, and THP1 and HB1 cells were first positive for both forms at the same time point (12h and day 3 respectively). LC5 cells became positive for viral DNA at 12h but did not produce detectable levels of CUVD during the experiment (Table 14).

Table 13. Detection of total viral DNA, and circular and linear unintegrated viral DNA, in T-cell derived lines infected with HIV-1_{IIIB}.

	hours						days						
	0	1	2	4	8	12	1	2	3	4	5	8	12
MT4													
HIV-1 DNA	-	+	+	+	+	+	+	+	+	+	+	nd	nd
1 LTR CUVD	-	-	-	-	-	+	+	+	+	+	+	nd	nd
2 LTR CUVD	-	-	-	-	-	+	+	+	+	+	+	nd	nd
Linear UVD	-	+	-	+	+	+	-	-	+	+	+	nd	nd
C8166													
HIV-1 DNA	-	-	+	+	+	+	+	+	+	+	+	nd	nd
1 LTR CUVD	-	-	-	+	+	+	+	+	+	+	+	nd	nd
2 LTR CUVD	-	-	+	+	+	+	+	+	+	+	+	nd	nd
Linear UVD	-	+	-	-	+	+	-	-	-	+	+	nd	nd
Hut78													
HIV-1 DNA	-	-	+	+	+	+	+	+	+	+	+	+	+
1 LTR CUVD	-	-	-	-	-	+	+	+	+	+	+	+	+
2 LTR CUVD	-	-	-	-	+	+	+	+	+	+	+	+	+
Linear UVD	-	-	+	-	+	+	-	-	-	+	+	+	+
SupT1													
HIV-1 DNA	-	+	+	+	+	+	+	+	+	+	+	+	+
1 LTR CUVD	-	-	-	+	+	+	+	+	+	+	+	+	+
2 LTR CUVD	-	-	-	+	+	+	+	+	+	+	+	+	+
Linear UVD	-	-	+	-	-	+	-	-	-	-	+	+	+

Details of the cell lines (in bold) are given in Appendix II. HIV-1 DNA, detection of viral DNA with *pol* primers (160H/161H and 002C/003C); 1 and 2 LTR CUVD, detection of circular unintegrated viral DNA with 1 and 2 LTR sequences respectively (for primer details see Table 12); Linear UVD, detection of linear unintegrated viral DNA after ligation. MT4 and C8166 cultures were destroyed by day 8. nd, not done.

Table 14. Detection of total viral DNA, and circular and linear unintegrated viral DNA in monocytic lines (U937, THP1, & U138MG), B cell line (HB1), lung fibroblastoid line (LC5), and epithelial line (HeLa T8+) inoculated with HIV-1_{IIIB}.

	hours						days						
	0	1	2	4	8	12	1	2	3	4	5	8	12
U937													
HIV-1 DNA	-	-	+	+	+	+	+	+	+	+	+	+	+
1 LTR CUVD	-	-	-	+	+	+	+	+	+	+	+	+	+
2 LTR CUVD	-	-	-	-	-	+	+	+	+	+	+	+	+
Linear UVD	-	-	-	-	-	-	-	-	-	-	+	+	+
THP1													
HIV-1 DNA	-	-	+	+	+	+	+	+	+	+	+	+	+
1 LTR CUVD	-	-	-	-	-	+	+	+	+	+	+	+	+
2 LTR CUVD	-	-	-	-	-	+	+	+	+	+	+	+	+
Linear UVD	-	-	-	+	+	-	-	-	-	-	-	+	+
HB1													
HIV-1 DNA	-	-	-	-	+	+	+	+	+	+	+	+	+
1 LTR CUVD	-	-	-	-	-	-	-	-	+	+	+	+	+
2 LTR CUVD	-	-	-	-	-	-	-	-	+	+	+	+	+
Linear UVD	-	-	-	-	-	-	-	-	-	+	+	+	+
LC5													
HIV-1 DNA	-	-	-	-	-	+	+	+	+	+	+	+	+
1 LTR CUVD	-	-	-	-	-	-	-	-	-	-	-	-	-
2 LTR CUVD	-	-	-	-	-	-	-	-	-	-	-	-	-
Linear UVD	-	-	-	-	-	-	-	-	-	-	-	-	-
U138MG													
HIV-1 DNA	-	-	-	-	-	-	-	-	-	-	-	-	-
HeLa T8+													
HIV-1 DNA	-	-	-	-	-	-	-	-	-	-	-	-	-

Details of the cell lines (in bold) are given in Appendix II. HIV-1 DNA, detection of viral DNA with *pol* primers; 1 and 2 LTR CUVD, circular unintegrated viral DNA with 1 and 2 LTR sequences respectively; Linear UVD, detection of linear unintegrated viral DNA after ligation. Control cultures of the non-susceptible (HeLa T8+) and uninfected (U138MG) cell lines were negative for HIV-1 DNA, CUVD, and LUVD at all time points.

7.4.4 LUVD detection.

All T-cell derived lines were positive for LUVD at various time points in the study. Unlike the CUVD results, the detection of LUVD was not constant after the initial detection, demonstrating a pattern of positive and negative time points (Table 13). In each cell line, the initial detection of LUVD lasted for 1 time point, and was followed by 1 or 2 time points of negativity before a second detection. The gap between the second and third detection was greater than between the first and second, and the length of time of detection also increased. If this pattern of appearance and disappearance represents rounds of infection involving the synthesis of LUVD for integration, then the increase in time of positivity may simply be due to the loss of synchrony of the culture.

Of the non-T-cell derived lines, the only lines to show a pattern of appearance and disappearance of LUVD was THP1. However, the pattern of detection was not similar to that of the T-cell lines, as the initial detection lasted for 2 time points, and the reappearance was more delayed. In contrast, HB1 and U937 were both negative for LUVD until day 4 and day 5 respectively, and once positive remained so. LC5 cells, as with CUVD detection, were negative throughout the experiment.

7.5 Cell lines infected with HIV-1_{RF}.

This section was designed to complement the study of HIV-1_{IIIB} in cell lines, to observe the differences in viral DNA synthesis attributable to the viral strain. In addition to this, cells were serum-starved to reduce cell growth and demonstrate the role of cellular factors in the synthesis of viral DNA. The viral strain was also used to infect donor PBMCs to compare the forms of viral DNA synthesized in C8166 cells with the cells susceptible to infection in the natural host.

7.5.1 Syncytium formation.

Syncytia were observed in C8166 cells infected with HIV-1_{RF} in complete medium but not in serum-starved cells or in uninfected cultures. No syncytia were observed in infected or uninfected donor PBMCs. This supports the data from IIIB infections indicating a cellular role in syncytium formation. The absence of syncytia in the serum-starved cells suggests that the state of the cells may be important for the life cycle of the virus.

7.5.2 Overall viral DNA detection.

HIV-1 DNA was not detectable in uninfected C8166 and PBMC cultures throughout the experiment. Viral DNA was first detected in the infected blood donor cells at 2h, but was not consistently positive until day 2. This was probably due to a small number of cells being infected resulting in some cell aliquots containing little or no viral DNA. Infected C8166 cells in complete medium were positive at 1h, and serum-starved cells at 3h (Table 15).

7.5.3 CUVD detection.

Infected C8166 cells in both complete and serum-starved medium were positive for both CUVD forms at the same time point although cells in complete medium were positive 1 day earlier than serum-starved cells (day 2 and day 3 respectively). The presence of CUVD in serum-starved cells (syncytia negative) rules out CUVD as the cause of these giant cells, although they may be a contributing factor. Human PBMCs, the host cells in human HIV infection, were not positive for CUVD forms until day 14. This again suggests that the cell type plays an important role in determining the timing and forms of viral DNA synthesized.

Table 15. HIV-1_{RF} DNA detection in infected C8166 cells (C8166/RF), serum-starved infected C8166 cells (C8166/SS), and infected blood donor lymphocytes (PBMCs/RF).

	hours							days						
	0	1	2	3	4	11	12	1	2	3	4	5	7	14
PBMCs/RF														
HIV-1 DNA	-	-	+	-	+	-	+	-	+	+	+	+	+	+
1 LTR CUVD	-	-	-	-	-	-	-	-	-	-	-	-	-	+
2 LTR CUVD	-	-	-	-	-	-	-	-	-	-	-	-	-	+
Linear UVD	-	-	-	-	-	-	-	-	-	-	-	-	-	-
C8166/RF														
HIV-1 DNA	-	-	+	+	+	+	+	+	+	+	+	+	+	+
1 LTR CUVD	-	-	-	-	-	-	-	-	+	+	+	+	+	+
2 LTR CUVD	-	-	-	-	-	-	-	-	+	+	+	+	+	+
Linear UVD	-	-	-	-	-	-	-	-	-	-	-	-	-	+
C8166/SS														
HIV-1 DNA	-	-	+	+	+	+	+	+	+	+	+	+	+	+
1 LTR CUVD	-	-	-	-	-	-	-	-	-	+	+	+	+	+
2 LTR CUVD	-	-	-	-	-	-	-	-	-	+	+	+	+	+
Linear UVD	-	-	-	-	-	-	-	-	-	-	-	-	-	-

PBMCs/RF, HIV-1_{RF} infected donor peripheral blood lymphocytes; C8166/RF, HIV-1_{RF} infected cells in complete medium; C8166/SS, HIV-1_{RF} infected cells in serum deficient medium; HIV-1 DNA, detection of viral DNA with *pol* primers; 1 and 2 LTR CUVD, circular unintegrated viral DNA with 1 and 2 LTR sequences respectively; Linear UVD, detection of linear unintegrated viral DNA via ligation step. Control cultures of uninfected C8166 and donor PBMCs were negative for HIV-1 DNA, CUVD, and LUVD at all time points (data not shown).

7.5.4 LUVD detection.

LUVD was only detected on day 14 in C8166 cells infected with HIV-1_{RF} in complete medium. This is in contrast to the results obtained with the IIIB strain of HIV, and may demonstrate differences attributable to the viral strain. However, it must also be remembered that the methods adopted for extracting total DNA differed, and so direct comparisons of the results must take account of this. Donor PBMCs and C8166 cells in serum-starved medium were negative for LUVD at each time point.

7.6 Detection and characterization of HIV-1 DNA in patient PBMCs.

The aim of this section was to detect HIV-1 DNA in PBMC extracts of seropositive patients, and to demonstrate the presence of different structural forms of UVD.

7.6.1 HIV DNA detection with *pol* and *env* primers.

HIV DNA was detected in patient cell samples using the *pol* and *env* primers. Where possible DNA was extracted from $2x10^5$ cells, and the resulting pellet was resuspended in ddw to give $2x10^4$ cell equivalents per amplification aliquot. The condition of some stored samples was such that DNA was extracted and amplified from a set volume. Extracted DNA was then quantified at a later date by absorbance at 260/280nm, and cell equivalents of DNA concentrations were calculated by the method described by Simmonds *et al.*, (1990a). Basically cells were assumed to be diploid, containing 6.6pg of DNA each. A DNA concentration of 1ug was, therefore, equivalent to $1.5x10^5$ PBMCs.

Samples were amplified from aliquots ranging from $7x10^3$ to $1x10^6$ cell equivalents. The result of this was that samples were not amplified from a fixed cell number, and so the HIV DNA load was not quantified (cell equivalents for each amplification result are given in Appendix IV).

All 32 seropositive patients were PCR positive for HIV-1 DNA sequences in PBMC extracts. However, only 59 of the 61 samples tested were positive for HIV-1 DNA (97%); 58 (95%) were *pol* positive, 56 (92%) *env* positive, and 55 (90%) were positive for both gene sequences. Four samples were negative for one gene sequence, and 2 samples were amplification negative by both *pol* and *env* primer pairs (Table 16).

Two samples (P24a and P25a) were PCR negative after nested amplifications with both *pol* and *env* primer pairs.

Patient 24 was asymptomatic, receiving no anti-virals, and had a $CD4^+$ count of 280/ml at the time of sampling. The amplification aliquot was equivalent to $1.2x10^5$ cells, and so was sufficient for detection of HIV DNA from PBMCs in 83% of cases described by Simmonds *et al.*, (1990a). It is possible that sample P24a had no infected cells in the amplification aliquots, as a subsequent sample from P24 (P24b, taken 3 months later) was found to be HIV DNA positive using the *pol* primers on an aliquot of $4x10^4$ cells. This suggests that although the sample size was possibly too small (the *env* amplification was negative), the number of infected cells may have increased during the 3 months, allowing HIV-1 DNA detection in a smaller aliquot than previously found to be negative in P24a.

Sample P25a was also negative for HIV DNA when an aliquot equivalent to $4x10^4$ cells was amplified. It seems likely that the sample size was too small for detection of viral sequences since a sample taken 9 months later (P25b) was positive for both *pol* and *env* sequences using an amplification aliquot of $2x10^5$ cell equivalents. During this time, the patient's $CD4^+$ cell count had decreased from 470 to 276 cells per ml of blood, and AZT therapy had been initiated (4 April 1991). This suggests a decline in the general health of the individual, associated with a marked decline in $CD4^+$ cell numbers. It is,

Table 16. HIV DNA detection of *pol* and *env* gene sequences in PBMCs of HIV positive patients.

Gene sequence amplified	Total No. of samples	Positive	Negative
pol	61	58 (95%)	3 (5%)
env	61	56 (92%)	5 (8%)
pol and *env*	61	55 (90%)	2 (3%)

A nested PCR was used for both *pol* and *env* detection. Samples were amplified with primers 160H/161H and 002C/003C for *pol* detection, and 401C/404C and 403C/404C for *env* detection. Details of the primers are given in Appendix IV.

therefore, possible that the proportion of infected cells had increased during this time, making detection by PCR easier.

Four samples were scored PCR positive on the basis of detection of viral sequences by only 1 set of primers (P23b, P24b, P32b, and P33b). Sample P33b was negative for *pol* sequences after amplification of DNA extracted from $2x10^5$ cells. The patient had been shown to be positive for both *pol* and *env* sequences 12 months previously with a similar amplification aliquot (P33a). During this time, no change had been observed in the patient's $CD4^+$ cell count or general health.

An aliquot of $5x10^4$ cell equivalents of P23b was insufficient for *env* detection although *pol* sequences were detected. A previous sample from the same patient 2.5 months earlier had been positive for both gene sequences in an aliquot of $4.5x10^5$ (P23a). P23a (unlike P23b) yielded virus in culture, suggesting that active viral replication may have been occurring *in vivo*. Also, during the 2.5 month period between the specimens, the patient had been receiving AZT therapy and so the total HIV DNA load in the patient's PBMCs may have been reduced in response to therapy, such as has been observed by Dickover *et al.*, (1992). In view of these facts, the amplification aliquot used for P23b may have been at the threshold of detection for PCR.

Sample P24b was negative for *env* sequences when $4x10^4$ cell equivalents were amplified. The patient had been tested 3 months earlier (P24a) and was found to be PCR negative (see above). P24a was PCR negative using $1.2x10^5$ cell equivalents, whereas P24b was *pol* positive using an aliquot of only $4x10^4$ cells. The patient was CDC group II on both sampling dates with similar $CD4^+$ cell counts (280 and 250). These results may be explained by an increased number of infected cells over the 3 months, or the aliquot amplified for P24b may, by chance, have contained 1 of the few circulating infected cells. The possibility of cross contamination from other samples is unlikely due

to the processing of samples between 2 negative donor cell controls. It is also highly unlikely that P24a is a false negative result, as fresh reaction mixtures were made for each batch. If any reagent had been omitted, the entire batch would have been negative, and amplification repeated. It therefore seems most likely that the samples contained very few infected cells and that only 1 amplification aliquot contained viral DNA.

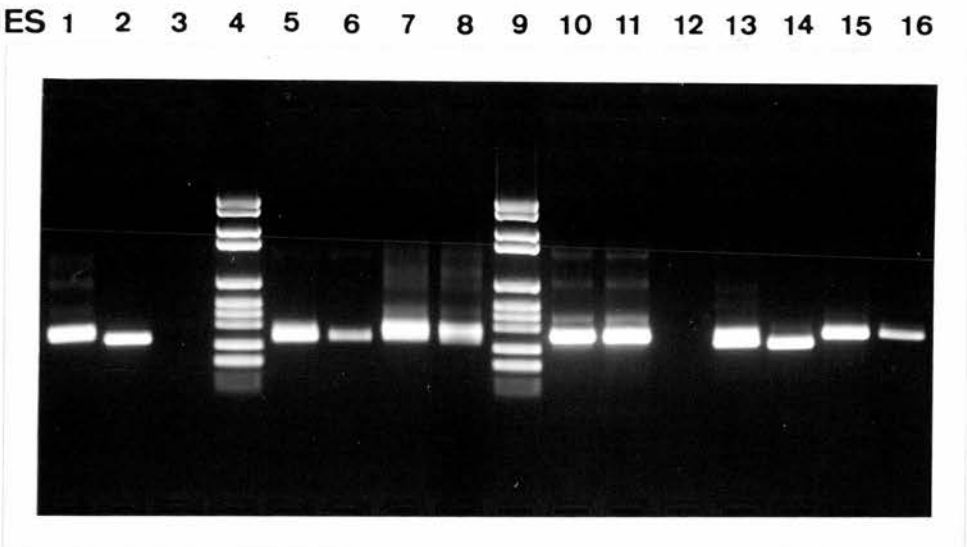
The final sample with only 1 positive result was sample P32b. The patient was from CDC group II, and had a high $CD4^+$ cell count of 620/ml. A large amplification aliquot of $3.75x10^5$ was positive for *pol* sequences only, whereas a previous sample (P32a) was positive for both *pol* and *env* at $2x10^5$ cells. Sample P32a had a considerably lower $CD4^+$ cell count of 470/ml in comparison with 620 cells/ml for P32b which, although unusual, may explain the negative *env* amplification result from such a large aliquot.

In general, positive *pol* amplifications would be expected in all aliquots containing HIV DNA as the gene is highly conserved in all HIV-1 isolates. In contrast, the sequence variation observed in the *env* gene would be expected to give rise to false-negative results due to poor amplification efficiencies arising from primer template mismatches (Newton *et al.*, 1989; Kwok *et al.*, 1990).

7.6.2 Length variation in the *env* gene.

Due to the sequence variation found in the *env* gene of viral isolates (Starcich *et al.*, 1986; Meyerhans *et al.*, 1989; Simmonds *et al.*, 1990a, 1990b), the band lengths obtained after *env* amplifications varied sufficiently to observe differences after agarose gel electrophoresis (approximately 30-50 base differences, Figure 24). Considerable band length variation was observed between patient samples (eg. the cytoplasmic amplification of P1 and the nuclear amplification of P4b, lanes 14 and 15 of Figure 24 respectively). In

Figure 24. Length variations observed for the *env* gene nested amplifications with primers 401C/402C, and 403C/404C (Appendix IV).



All extracts were from patient PBMCs unless otherwise stated.
Lane 1, P25b cytoplasmic extract; **lane 2**, P25b nuclear extract; **lane 3**, negative control; **lane 4**, DNA VI molecular weight marker (2176, 1766, 1230, 1033, 653, 517, 453, 394, 298, 234/220, 154); **lane 5**, P20c total DNA from adherent cells (separated by adhesion to plastic flask); **lane 6**, P20c total DNA extract; **lane 7**, P20c cytoplasmic extract; **lane 8**, P20c nuclear extract; **lane 9**, DNA VI molecular weight marker; **lane 10**, P13 cytoplasmic extract; **lane 11**, P13 nuclear extract; **lane 12**, negative control; **lane 13**, P4b cytoplasmic extract; **lane 14**, P4b nuclear extract; **lane 15**, P1 cytoplasmic extract; and **lane 16**, P1 nuclear etxract.

addition to this, length variation may be observed within a single patient sample (eg. P25b cytoplasmic and nuclear amplifications in lanes 1 and 2 respectively). Band lengths varied from 300-400 bases for all samples.

7.6.3 CUVD detection.

A total of 52 samples from patients were amplified for the detection of 1 and 2LTR CUVD. Thirty-four samples (65%) were positive for 1 or both forms (P8a and P26a were positive for 1 LTR CUVD only), and 18 samples (35%) were negative for both forms. The detection of CUVD was compared with CDC groups, virus isolation, p24 plasma Ag detection, $CD4^+$ cell counts, and the use of AZT treatment (Table 17).

7.6.4 LUVD detection.

Despite the tertiary amplification used for the detection of LUVD, all patient PBMC samples were tested negative.Both positive and negative controls were as expected, and so the absence of positivity in patient samples may be due to insufficient template. As with CUVD detection, the reaction utilised LTR specific primers which may have been used by cellular sequences, thereby reducing the efficiency of amplification. The detection of LUVD in cell line studies, where the infection had been synchronised, may have been due to the cells synthesizing linear viral DNA simultaneously, giving a higher concentration of sequences for detection than expected in unstimulated patient cells.

7.6.5 Detection and characterization of viral DNA in nuclear and cytoplasmic extracts of patient PBMCs.

Cells from 7 patients were extracted to separate their cytoplasmic and nuclear DNA. The fractions were amplified for total viral DNA and 1 and 2LTR CUVD detection (Table 18). Two samples had detectable HIV DNA in the nuclear

Table 17. Detection of CUVD in samples separated by CDC gro detection of p24 plasma antigen, isolation of virus, $CD4^+$ cell c ts, and use of AZT anti-viral therapy.

	CDC group				
	II	III	IV	po	neg.
CUVD pos.	6	10	18		7
CUVD neg.	6	10	2		7
Total	12	20	20	21	14

	p24 Ag		CD4+ counts		AZT treatment	
	pos.	neg.	*200	*200	Yes	No
CUVD pos.	11	22	9	13	19	15
CUVD neg.	1	17	11	6	4	14
Total	12	39	20	19	23	29

CDC groups, clinical groups as defined by the Centers for Disease Control (CDC, 1986); VI, virus isolation from patient PBMCs, plasma, and/or heparinised whole blood; p24 Ag, detection of p24 viral core antigen in the patients plasma; $CD4^+$ counts, number of cells per millilitre of blood expressing the CD4 differential antigen on their surface; *, less than or equal to; AZT therapy, patients receiving AZT therapy for a minimum of 3 weeks prior to venipuncture; pos., positive; neg., negative.

Table 18. HIV-1 CUVD detection in nuclear and cytoplasmic extracts from patient PBMCs.

Patient No.	Extract type	HIV-1 DNA	1 LTR CUVD	2 LTR CUVD
P1	N	+	-	-
	C	+	-	-
P4b	N	+	+	+
	C	+	-	-
P7d	N	+	+	+
	C	-	-	-
P13	N	+	+	+h
	C	+	+	+
P15	N	+	+	+
	C	-	-	-
P20c	N	+	+	+
	C	+	+	+
P25c	N	+	-	-
	C	+	-	-

Nuclear and cytoplasmic DNA was extracted from $1x10^6$ PBMCs. N, nuclear extract; C, cytoplasmic extract; HIV-1 DNA, detection of viral DNA with *pol* primers; +, positive; -, negative; +h, positive band present but approximately 400bp as opposed to the expected 447bp; CUVD, detection of circular unintegrated viral DNA with 1 or 2 LTR sequences using *gag*, *env*, and LTR specific primers.

fractions only, and 5 were positive for both nuclear and cytoplasmic extracts. Both samples positive for nuclear extracts only were positive for CUVD. Of the 5 samples with HIV DNA in both fractions, 1 had CUVD in the nuclear extract only, 2 were positive for CUVD in both fractions, and 1 sample had no detectable circular forms. The nuclear extract from P7d resulted in a distinct amplification product of approximately 600bp. Although this band is greater than the expected 2LTR CUVD product, it may result from an aberrant CUVD molecule which has additional sequences present. This has been proposed by Pauza and Galindo, (1989) for the generation of multi-meric CUVD forms. Analysis by dot or Southern blot hybridisation is required to confirm this.

7.7 Comparisons of CUVD detection with CDC groups, virus isolation, plasma p24 antigen detection, $CD4^+$ cell counts, and AZT therapy.

The detection of UVD forms was compared to markers of infection and disease progression to try and establish a role for these forms in the pathogenesis of HIV. Samples were only included in the comparison if they were positive for both *pol* and *env* sequences after a nested PCR.

7.7.1 CDC groups.

Of the 34 CUVD positive samples, 6 (18%) were CDC II, 10 (29%) CDC III, and 18 (53%) CDC group IV (Table 17). The increase in detection of CUVD forms with disease progression was more apparent when the results were expressed as a percentage of samples positive in each CDC group. Six out of 12 (50%) of CDC II, 10 out of 20 (50%) of CDC III, and 18 out of 20 (90%) of CDC IV samples were CUVD positive. These results were significant ($0.02>P>0.01$) indicating that the detection of CUVD is more likely in the later stages of disease (Table 19).

Table 19. Comparisons of CUVD detection with CDC groups, $CD4^+$ cell counts, viral p24 core antigen detection, AZT treatment, and virus isolation.

CUVD with	Number of samples	Chi-square	DF	Probability
CDC group	52	8.706	2	0.02>P>0.01
VI	35	0.402	1	P>0.50
p24 plasma Ag	51	3.337	1	0.10>P>0.05
$CD4^+$ (200)	39	1.325	1	0.50>P>0.10
AZT	52	5.406	1	0.05>P>0.02

CDC group, clinical definitions of patients as described by the Centers of Disease Control (CDC, 1986); VI, virus isolation from patient PBMCs, plasma, or heparinised whole blood; p24 Ag, detection of viral core antigen in patient plasma; $CD4^+$ cell counts, the number of cells per ml of whole blood expressing the Clusters of Differentiation antigen 4 (CD4) on their surface. Samples were separated into groups with more than 200 $CD4^+$ cells/ml, and those with 200 or less $CD4^+$ cells/ml (>200 and 200); AZT, patients receiving AZT therapy for a minimum of 3 weeks prior to venipuncture; DF, degree of freedom; and P, probability.

7.7.2 Virus isolation.

The virus isolation results of 35 samples from heparinised whole blood, plasma and PBMCs were compared to the detection of CUVD in patient PBMCs. Virus was isolated from 21 out of 35 samples (60%), 14 (67%) of which were also positive for CUVD. Isolation negative samples (14 or 40%) showed no difference in the presence or absence of CUVD (Table 17). Statistical analysis showed no significant correlation between detection of CUVD forms and the ability to isolate virus (Table 19).

7.7.3 p24 plasma Ag detection.

Fifty-one samples were compared. CUVD was detected in 11 out of 12 (92%) antigen positive samples, and 22 out of 39 (56%) antigen negative samples (Table 17). The majority of CUVD negative samples were also antigen negative (94%). The results were not statistically significant ($0.10>P>0.05$, Table 19).

7.7.4 $CD4^+$ cell count.

Comparisons were made of the relationship of CUVD detection and $CD4^+$ cell counts by grouping the results according to the $CD4^+$ counts of more than 200 and less than or equal to 200. Data were available for both CUVD detection and $CD4^+$ cell counts for 39 samples. The results for samples with $CD4^+$ cell counts greater than 200 showed that 9 out of 20 (45%) were positive, and 11 out of 20 (55%) negative for CUVD forms (Table 17). Thirteen out of 19 (68%) samples with counts less than or equal to 200 were positive for CUVD, and 6 out of 19 (32%) were negative. Of the 22 CUVD positive samples, 9 (41%) had counts greater than 200, and 13 (59%) had counts of less than or equal to 200. These results were not statistically significant (Table 19).

7.7.5 AZT treatment.

Fifty-two samples were compared, 23 of which were from patients who had been receiving AZT treatment for a minimum of 3 weeks. The levels of AZT treatment were not known, and consequently have not been taken into consideration. Thirty-four samples were CUVD positive, 19 (56%) on AZT treatment, and 15 (44%) receiving no known anti-retroviral treatment (Table 17). Of the 23 samples from patients receiving AZT, 19 (83%) were positive for CUVD, and 4 (17%) were negative. There were 29 samples obtained from patients on no therapy, 15 (52%) had CUVD forms, and 14 (48%) were negative. These results were statistically significant ($0.05>P>0.02$), demonstrating a relationship between patients on AZT therapy and the presence of CUVD (Table 19).

7.8 Comparison of viral markers, $CD4^+$ cell counts, and anti-viral therapy with CDC groups II, III, and IV.

Samples were categorized into their CDC groups, and analysed for detection of plasma p24 viral antigen, virus isolation, $CD4^+$ cell counts, and AZT therapy. The results are presented in Table 20, and the data were analysed by the Chi-square test, as described in Appendix III.

7.8.1 Virus isolation.

Virus was isolated from 22 out of 41 (54%) samples with a known CDC group status. Three (14%) of the positive cultures were from samples in CDC group II, 9 (41%) from CDC group III, and 10 (45%) from CDC group IV (Table 20). Virus was isolated from 3 out of 8 (38%) CDC II samples, 9 out of 20 (47%) CDC III samples, and 10 out of 11 (91%) CDC group IV samples. The data were statistically significant ($0.01>P>0.001$), demonstrating an increase in the success rate of virus isolation from patient PBMCs in the later stages of disease (Table 21).

Table 20. Comparison of CDC groups with viral markers, $CD4^+$ cell counts, and anti-viral therapy.

			$CD4^+$ count		
CDC group	**VI**	**p24 Ag**	**>200**	**200**	**AZT**
CDC II	3/11	2/17	13	4	1/17
CDC III	9/19	3/22	10	9	7/23
CDC IV	10/11	8/21	3	9	16/21
Total	22/41	13/60	26	22	24/61

The results are expressed as the number of positive samples out of the total number of samples for which data were available, with the exception of CD4 antigen positive cell counts which are expressed as the number of samples with counts greater than 200 and less than or equal to 200 cells per ml of whole blood. VI, samples from which virus was isolated from heparinised whole blood, plasma, or PBMCs; p24 Ag, samples positive for plasma p24 viral core antigen; AZT, patients receiving AZT for a minimum of 3 weeks prior to collection of the sample; CUVD, samples positive for 1 or 2 LTR circular viral DNA forms by PCR.

Table 21. Comparisons of CDC groups with $CD4^+$ cell counts, viral p24 core antigen detection, AZT treatment, and virus isolation.

CDC group with	Number of samples	Chi-square	DF	Probability
VI	41	9.524	2	0.01>P>0.001
p24 plasma Ag	60	5.158	2	0.10>P>0.05
$CD4^+$	48	7.534	2	0.05>P>0.02
AZT	61	20.694	2	0.001>P

The detection of CUVD was compared with; VI, virus isolation of virus from patient PBMCs, plasma, or heparinised whole blood: detection of p24 viral core antigen in the patients plasma (p24 plasma Ag); $CD4^+$, number of cells per ml of whole blood expressing the differential antigen 4 (cell count at a cut off value of >200/ 200); CDC groups II, III, and IV as defined by the Centers of Disease Control (CDC, 1986); and AZT, patients receiving AZT therapy for a minimum of 3 weeks prior to venipuncture. DF, degree of freedom; P, probability. The results were analysed by the Chi-square test with Yates correction for continuity for fourfold tables (Appendix III).

7.8.2 p24 plasma Ag detection.

Of the 60 patient samples included, 13 (22%) were p24 Ag positive of which 2 samples (15%) were from CDC group II, 3 (23%) from CDC III, and 8 (62%) from CDC IV (Table 20). While most Ag positive samples were from patients in CDC group IV, only 8 out of 21 (38%) of CDC group IV samples were Ag positive. This is still much higher than the other groups, with 2 out of 17 samples (12%) Ag positive in CDC group II, and 3 out of 22 (14%) in CDC group III. Statistical analysis showed no significant difference in antigen detection with disease stage ($0.10>P>0.05$; Table 21).

7.8.3 $CD4^+$ cell count.

The CDC grouping was known for 48 patients with $CD4^+$ cell counts: 17 were in CDC group II, 19 in CDC group III, and 12 in CDC group IV (Table 20). CDC II samples were predominantly of higher $CD4^+$ cell counts, 13 out of 17 (76%) had counts >200. CDC III samples were more evenly distributed, 10 out of 19 (53%) had a count >200, and CDC IV samples had consistently lower $CD4^+$ cell counts, 9 out of 12 (75%) less than or equal to 200. These results were statistically significant ($0.05>P>0.02$; Table 21) demonstrating a relationship between clinical progression and a loss of $CD4^+$ cells.

7.8.4 AZT treatment.

The CDC groupings and treatment with AZT were known for 61 patients. Twenty-four samples were from patients receiving treatment; 1 (4%) in CDC group II, 7 (29%) in CDC III, and 15 (67%) from CDC group IV (Table 20). When the samples were divided into CDC groups, 1 out of 17 (6%) CDC II samples, 7 out of 23 (30%) CDC III samples, and 16 out of 21 (76%) CDC IV samples were from patients receiving AZT treatment. The use of AZT treatment was significantly greater in the later stages of disease ($0.001>P$; Table 21).

7.8.5 Further analysis of data adjusted for sample size.

Statistical analysis is used to establish significant correlations, however, failure to do so does not prove the null hypothesis as it is quite possible that the data are insufficient to establish the relationship.

The results obtained for patient samples were insufficient for the standard Chi-square test which requires a minimum of 100 events. The data were, therefore, analysed using the Yates "correction for continuity" (Swinscow, 1991). The data which failed to establish a significant result were amplified by a factor of 2 or 3 to obtain in excess of 100 events (CUVD analysis with $CD4^+$ cell counts, p24 plasma antigen detection, and virus isolation; and CDC group with p24 plasma antigen detection). Three out of 4 sets of the adjusted data were significant when analysed, with the exception of virus isolation with the detection of CUVD (Tables 22 and 23). The adjusted data demonstrated a relationship of CUVD detection with lower $CD4^+$ cell counts ($0.02>P>0.01$); with the detection of p24 antigen in the plasma ($0.01>P>0.001$); but not with virus isolation ($0.10>P>0.05$). The data also confirmed a relationship of disease progression with the detection of p24 plasma antigen ($0.01>P>0.001$). Although not conclusive, the adjusted data gave an indication of the possible significance of the results had there been sufficient observations, with the same distribution of results, available for direct analysis by the Chi-square test.

Tables 22 and 23. Data from Tables 17 & 20 which were not significant were adjusted to contain in excess of 100 samples for each analysis. This was achieved by amplifying the data by a factor of 2 or 3. The results were then analysed as before (Appendix III) with the following results;

Table 22. Adjusted comparisons of CUVD detection with $CD4^+$ cell counts, viral p24 plasma antigen detection, and virus isolation.

CUVD with	Number of samples	Chi-square	DF	Probability
$CD4^+$ (200)	117	6.521	1	0.02>P>0.01
p24 plasma Ag	102	9.989	1	0.01>P>0.001
VI	105	2.917	1	0.10>P>0.05

Table 23. Adjusted comparisons of CDC groups with CUVD detection, $CD4^+$ cell counts, viral p24 core antigen detection, AZT treatment, and virus isolation.

CDC group with	Number of samples	Chi-square	DF	Probability
p24 plasma Ag	120	10.315	2	0.01>P>0.001

CHAPTER 8: DETECTION OF HIV-1 DNA IN PERIPHERAL BLOOD MONOCYTIC CELLS.

The aim of this section was to determine whether or not monocytic cells in the peripheral blood of HIV positive individuals were infected with the virus.

Initially, monocytic cells were isolated from PBMCs by their adherent properties to plastic. Adherent cells were collected and further analysed for viral DNA content by a nested polymerase chain reaction.

To improve the purity of these cell preparations, a highly specific cell separation technique was sought, based on the detection of surface antigens characteristic of cellular subsets. These antigens, known as clusters of differentiation or CD antigens, have been carefully characterised and used to describe cellular subsets (Bernard *et al.*, 1984). PBMCs can, therefore, be separated into cellular subsets by immunoselection using antibodies specific to a particular cell population. There are 2 forms of immunoselection; 1) negative selection, where a mixed population is depleted of a certain cell type and the remaining cells are further analysed, and 2) positive selection, where a specific sub-population is selected and removed for further studies. Irrespective of the method utilised, it is important that it is both rapid and gentle for the recovery of viable cells, that the selection is specific to obtain a high resolution, and that the method can be applied to both small and large samples.

There are 3 main methods available for immunoselection of PBMC subsets (Lea *et al.*, 1985). 1) Cell sorting using flow cytometry (FACS), which compares the size and granularity of each cell and sorts them accordingly. FACS analysis is well established although it is costly, requires considerable expertise, and is unsatisfactory for the analysis of infectious material due to the potential to form aerosols (Reinherz *et al.*, 1979; Moscicki *et al.*, 1985; Ohta *et al.*, 1986). 2) The separation of cells by incubation of a mixed cell

population in a flask coated with a subset specific antibody. This method, known as panning, has been successfully used to isolate cellular subsets with purities of up to 95% (Wysocki & Sato, 1978; Lum *et al.*, 1982; Nielsen *et al.*, 1989) although they are more frequently in the range of 50-70% (Mason *et al.*, 1987). Unfortunately, the technique is prone to non-specific binding of adherent cells, and often results in low yields (Basch *et al.*, 1983). 3) Cellular subsets may also be separated using specific antibodies bound to sheep red blood cells (SRBC). The SRBC bind to the cells expressing the specific antigen, forming large complexes or rosettes. These are readily removed by centrifugation, with a reported purity of >99% (Mason *et al.*, 1987). The technique is, unfortunately, not adaptable to the separation of large samples.

In view of these limitations, it was decided to apply a relatively new technique to the separation of patient PBMCs using immunomagnetic microspheres, or "Dynabeads". The beads offer a rapid, flexible and highly specific cell separation method which can be applied to the purification of any cell subset expressing a unique surface marker.

PBMCs were separated into T helper/inducer, T suppressor/cytotoxic cells, monocytic cells and B cells using specific monoclonal antibodies bound to commercially available magnetic microspheres (Dynabeads), and the purity of these cell preparations was assessed using fluorescence activated cell sorting.

8.1 Analysis of Dynabead sorted cells.

PBMC subsets can be distinguished on the basis of their surface antigens known as Clusters of Differentiation or CD antigens. By coating Dynabeads with monoclonal antibodies to CD antigens specific for certain cell subsets, it was proposed to remove T-cytotoxic/suppressor cells, T-helper/inducer cells, monocytic cells, and B cells by positive selection.

Dynabead-sorted cells were analysed by flow cytometry to demonstrate the specificity of the separation technique. The computer analysis of fluorescein labelled samples was carried out by Dr John Stewart (Department of Medical Microbiology, The University of Edinburgh) and was set to analyse the labelling of lymphocytic and monocytic cell populations.

8.1.1 Flow cytometric analysis of cells separated by antibody coated Dynabeads.

Uninfected PBMCs were separated by Dynabeads coated with antibodies to differential antigens 4, 8, 14, and 19. The purified cell fractions were analysed as two populations based on their size and granularity, and their percentage labelling with each FITC-conjugated antibody was determined (anti-CD4, CD8, CD14 and CD19).

The initial analysis of cells purified by anti-CD4 coated Dynabeads showed that lymphocytic cells stained with antibodies to CD4 (23.9%); CD8 (16.7%); CD14 (8%); and CD19 (1.2%): monocytic cells stained with antibodies to CD4 (8.7%); CD8 (6.4%); CD14 (16%); and CD19 (2.7%). Analysis of cells separated by Dynabeads coated with antibodies to CD8, CD14 and CD19 surface antigens gave similar patterns of low and non-specific labelling.

The labelling observed with antibodies to CD4, CD8 and CD19 in the monocytic cell fraction of anti-CD4 purified cells could have resulted from non-specific binding of the Fc portion of the antibody to the Fc receptor expressed on the surface of monocytic cells (Roitt *et al.*, 1987). However, the specific labelling of these cells with anti-CD14 was significantly greater than the labelling with any of the other conjugated antibodies, showing that monocytic cells had been isolated using the Dynabeads coated with anti-CD4 antibodies.

The cells analysed resulted in a strange pattern of specificity. Lymphocytes separated by anti-CD4 specific antibodies should only fluoresce with CD4

specific conjugated antibodies. Although fluorescence was highest with the CD4 specific conjugated antibody (23.9%), the anti-CD8 antibody labelling was relatively high as well (16.7%). Dynabeads are marketed as being coated with highly specific monoclonal antibodies, and are reported to permit the separation of very pure samples of peripheral mononuclear cell subsets (Gaudernack *et al.*, 1986). Since lymphocytic cells, and in particular $CD4^+$ lymphocytes, do not express Fc receptors on their cell surface, the high level of non-specific labelling detected is difficult to explain.

Once separated, the cells are permanently attached to the Dynabeads, and so it is possible that the Dynabeads interfered with the subsequent reactions. Firstly, the nature of the binding may result in the formation of clumps of beads and cells. This would block the delivery tube of the EPICS machine resulting in the absence of events to analyse (as seen with some fractions separated by anti-CD8, 14 and 19 separated cells, data not shown). Secondly, the Dynabeads may give rise to non-specific binding of conjugated antibodies if they were not sufficiently blocked prior to labelling.

8.1.2 Fluorescent antibody staining of negative blood donor cells.

Due to these results obtained with cells separated by Dynabeads, it was decided to test the labelling procedure on PBMCs from a normal blood donor. PBMCs from an HIV-1 negative donor were separated on Lympho-paque, and split into 5 equal fractions. Each fraction was incubated with 1 of the 4 conjugated antibodies (anti-CD4, anti-CD8, anti-CD14, and anti-CD19) and the final fraction used as a negative control. The labelled fractions were analysed for staining of monocytic and lymphocytic cells. Antibody coated Dynabeads were also incubated with each of the labelled antibodies to determine the extent of non-specific labelling associated with them.

The Dynabeads gave levels of fluorescence ranging from 1.2 to 1.4%, indicating a very small and consistent level above background for each antibody tested (Table 24).

The PBMCs were analysed for lymphocytic and monocytic cells as before. CD4 antigens were observed in 74% of monocytes, and 35% of lymphocytes; CD8 antigens on 81% of monocytes, and 18% of lymphocytes; CD14 antigens on 91% of monocytes, and 0.6% of lymphocytes; and CD19 antigens on 20% of monocytes, and 1% of lymphocytes (Table 24).

The total labelling of monocytic cells was greater than 100% (74% with anti-CD4; 81% with anti-CD8; 91% with anti-CD14; and 20% with anti-CD19). The results are, therefore, unlikely to be due to contaminating lymphocytic cells, but due to cross reactivity of monocytic cells with non-specific antibodies via Fc receptor molecules on the cell surface.

In contrast, the lymphocytic cells analysed gave an overall labelling of approximately 55% (35% with anti-CD4; 18% with anti-CD8; 0.6% with anti-CD14; and 1% with anti-CD19). Although the total CD4 and CD8 positive cells should have been approximately 90% (actual 53%), the ratio of CD4:CD8 positive cells (1.9:1) was in accordance with the expected ratio of 2:1. This is suggestive of inefficient labelling with the conjugated antibodies. Similarly, the percentage of cells labelled with B lymphocytic specific antibodies (anti-CD19) was considerably lower than the expected 10% (actual 1%). It is possible that some of the cells present in this fraction were not lymphocytes, however, they were also not $CD4^+$ monocytic cells (0.6% labelling) and so it is more likely that the cells were poorly labelled.

In general, the labelling reaction was as described by the manufacturer of the conjugated antibodies (Serotec). It is, therefore, possible that further optimisation of the method could have resulted in lower non-specific binding, and a higher specific interaction with cellular surface antigens.

Table 24. Staining of peripheral blood mononuclear cells (PBMCs) with fluorescein isothiocyanate conjugated antibodies to CD4, CD8, CD14, and CD19 surface antigens.

Antibody to surface antigen	Monocytes	Lymphocytes	Beads
CD4	74%	35%	1.3%
CD8	81%	18%	1.3%
CD14	91%	0.6%	1.2%
CD19	20%	1%	1.4%

Percentages refer to the fraction of each cell type which stained with each conjugated antibody specific for cellular surface antigens. CD4 is expressed on T-helper/inducer cells, monocytes, and macrophages; CD8 on T-cytotoxic/suppressor cells; CD14 on monocytes, macrophages, and some granulocytes; and CD19 on B-cells (Serotec).

8.2 HIV-1 DNA detection in monocytic cells by PCR.

Adherent cells were isolated from a total of 12 samples from 11 patients. Eleven samples were isolated by adherence to T25 flasks, and 1 sample was prepared by selective extraction of $CD14^+$ cells by magnetic Dynabeads coated with anti-CD14 antibodies. DNA from adherent cells was extracted and amplified for detection of HIV-1 DNA using *pol* and *env* primers. Six samples were positive for HIV-1 DNA and 6 were negative (Table 25). The cells were not quantified due to the small numbers of PBMCs used for the isolation.

Comparisons were made with CDC group, plasma antigen detection, virus isolation, CD4 cell count, and detection of CUVD. No patterns of association were observed, although this may be due to the small sample size available.

Interestingly, monocytic cells were separated from 2 consecutive samples from patient 20 (P20b and P20c). Sample P20b was separated by adherence, and sample P20c (received 9 months later) using anti-CD14 antibody coated Dynabeads. Monocytic cells from both samples were found to be positive for HIV-1 DNA after a nested amplification for the *pol* sequence.

Although both methods of isolating adherent cells suffer from problems of contamination with cells from other sub-populations, both samples from P20 were positive, lending support to the argument that HIV DNA is present in adherent/monocyte or macrophage cells in the peripheral blood (Gartner *et al.*, 1986).

Table 25. HIV-1 DNA detection in monocyte/adherent cells isolated from patient PBMC samples by adhesion to culture flasks or by specific binding to anti-CD14 antibody-coated Dynabeads. The PCR results were compared with other laboratory markers of infection.

		Markers				
Patient No.	**DNA**	**p24 Ag**	**CDC**	**AZT**	**VI**	**$CD4^+$**
P3b	+	-	II	no	-	280
P4a	+	+	II	no	-	180
P5	-	-	III	no	-	190
P10	+	-	III	no	-	468
P11b	-	-	III	yes	+	176
P16	-	-	III	no	-	360
P19a	-	-	III	no	+	560
P20b	+	-	III	no	+	350
P20c*	+	-	III	no	-	nd
P25a	+	-	III	no	-	470
P28c	-	nd	IV	no	nd	nd
P31	-	-	II	no	-	280

-, negative; +, positive; DNA, detection of HIV-1 DNA by PCR using a nested PCR with primers for the *pol* and *env* gene sequences (Samples were scored HIV DNA positive if either gene sequence was detected); p24 Ag, p24 plasma antigen detection; CDC, CDC group; AZT, use of antiviral therapy; VI, virus isolation; $CD4^+$, CD4 surface antigen positive cells per millilitre of blood; nd, not done; *, cells separated by anti-CD14 coated Dynabeads. All other samples were separated by adhesion to plastic flasks over a period of 3 days.

CHAPTER 9: DETECTION OF HIV-1 RNA.

Although the detection of HIV-1 viral DNA sequences is accepted as evidence of infection, it is not directly comparable to detection of free virus or viral activity. In contrast, the detection of HIV-1 RNA in clinical samples is indicative of replicative and free virus.

The initial aim of this study was to detect viral RNA sequences in clinical samples using a 2 step amplification (reverse transcription (RT)-PCR). This method was applied to RNA from whole virus extracts obtained using beads coated with antibodies to HIV-1 viral surface glycoproteins. In this way, it was hoped to correlate the detection of viral RNA to infectivity, and analyse its detection in relation to other markers of infection.

9.1 Optimisation of antibody coated bead extraction of HIV-1.

9.1.1 Determination of protein concentration by Bradford's Coomassie Blue assay.

The standard protein (BSA) concentrations (100ng to 1mg/ml) were plotted against their optical density value (OD at 595nm) to give a standard curve (Table 26 and Figure 25). The concentrations of test proteins (monoclonal antibodies ADP 335, 361 and 362; polyclonal antibodies ADP 403; and recombinant gp120 ADP 604, Appendix II) were determined from the standard curve by reading off the corresponding concentration for their optical densities (Table 27). The concentrations obtained in this way were used in later experiments.

9.1.2 Iodination of recombinant gp120 protein.

The iodinated protein was separated from free iodide by gel-filtration on a Sephadex G-100 column with excess buffer (0.2% potassium iodide in RIP). Fractions (1ml) were collected, diluted (1 in 10) and analysed for gamma

Table 26. Bradford's Coomassie Brilliant Blue Protein assay. Determination of the standard values using dilutions of BSA.

Conc./ml of BSA	1mg	100 g	10 g	1 g	100ng
Optical density	1.259	0.539	0.408	0.387	0.382

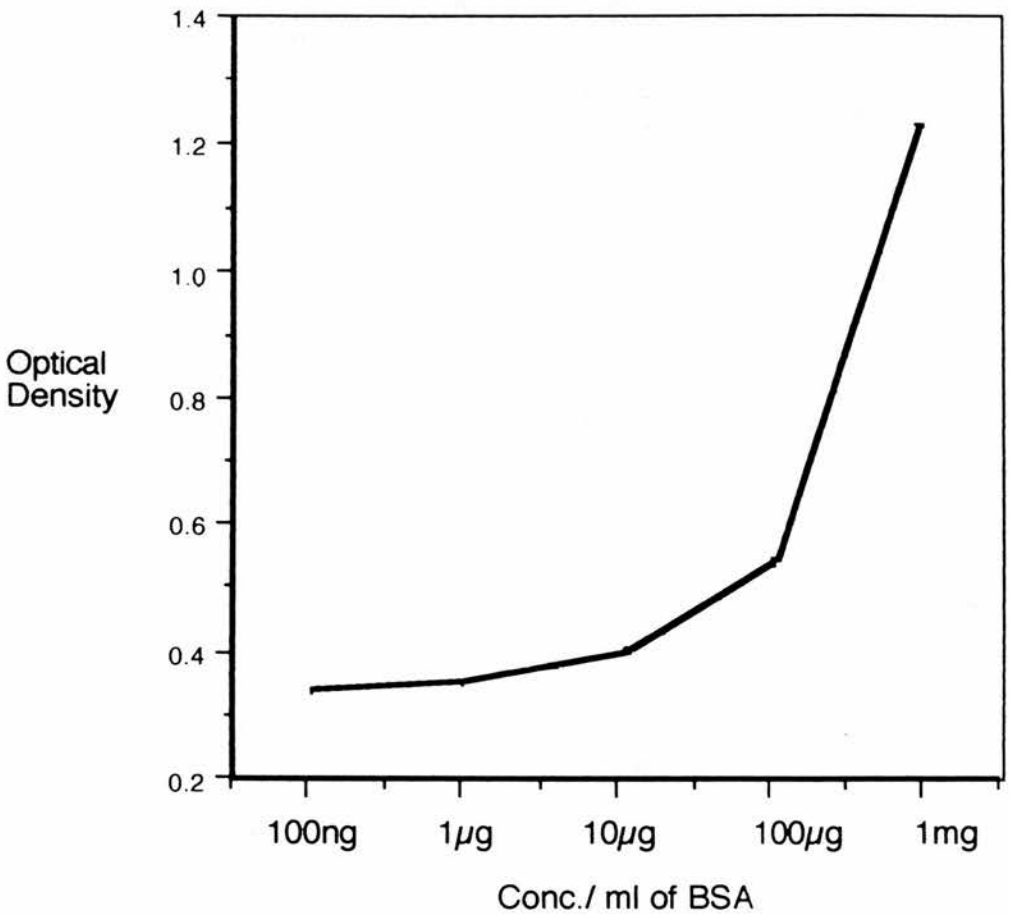
The standard protein (BSA) was diluted to give a range of concentrations from 100ng to 1mg/ml. The optical density was determined at 595nm by the method of Bradford, (1976) and plotted against the known protein concentration (Figure 25).

Table 27. Determination of the test protein concentrations from Bradford's Coomassie Brilliant Blue Protein assay standard curve (Figure 25).

MRC ADP No.	335	361	362	403	604
Optical density	0.773	0.887	0.915	0.953	0.612
Conc./ml	300 g	450 g	500 g	550 g	100 g

The concentration of each protein was determined by reading the corresponding concentration to their OD value at 595nm from the standard curve (Figure 25). Conc./ml, concentration per ml; MRC ADP No., Medical Research Council AIDS Directed Program number (Appendix II).

Figure 25. Bradford's Coomassie Brilliant Blue Protein assay. Determination of the standard value using dilutions of BSA.



The standard protein (BSA) was diluted to give a range of concentrations from 100ng to 1mg/ml. The optical density was determined at 595nm by the method of Bradford, (1976) and plotted against the known protein concentration.

irradiation over a period of 60 seconds. The results showed 2 peaks of activity, one corresponding to protein-bound Iodine, and the other to free Iodide (Figure 26).

Radioiodine interacts with proteins by substituting the side chains of tyrosine, and to a lesser extent histidine residues (Greenwood *et al.*, 1963; Bolton & Hunter, (1973); McConahey & Dixon, (1980)). From sequence data (kindly provided by Dr G. Clements of Celltech Research, Slough) the recombinant protein (gp120) was shown to contain 11 tyrosine and 7 histidine residues per molecule, representing 3.8% of the total amino acid content (Appendix VI). Assuming that all the available Iodide passed through the column, 57% was substituted into amino acid residues of rgp120 glycoprotein. However, assuming a loss of 5-10% of Iodide activity through adsorption to the column, up to 63% was incorporated (Table 28).

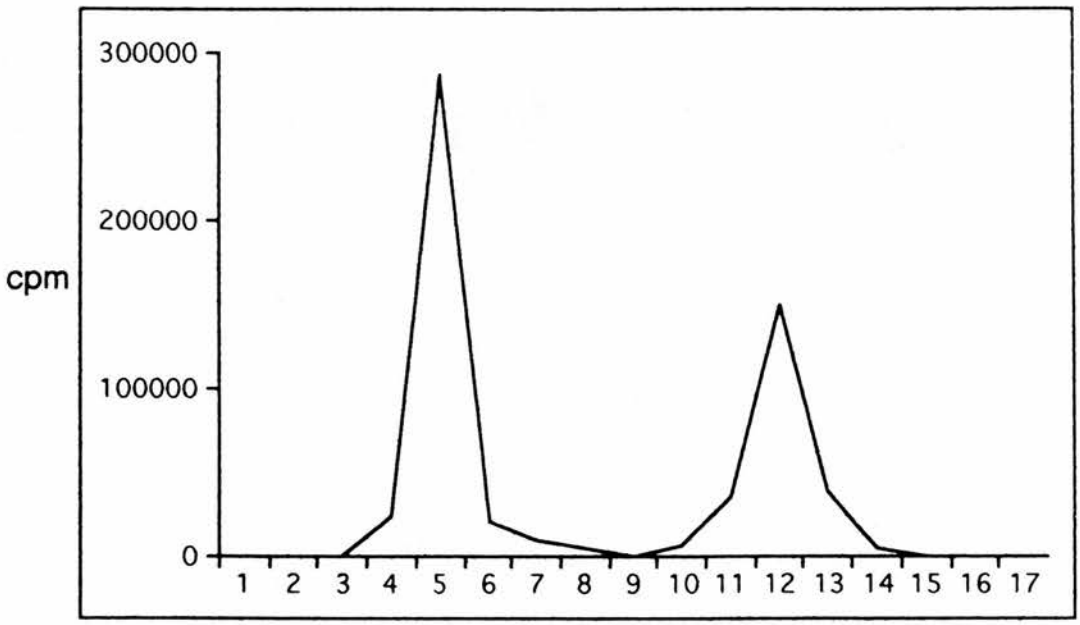
Dilutions of the peak fraction (fraction 5) were utilised for analysis of the specificity and sensitivity of the antibody coating procedure for polystyrene beads.

9.1.3 Optimisation of antibody coating of polystyrene beads.

Beads were coated at varying dilutions of antibodies, and their ability to bind iodinated recombinant gp120 protein was assessed in triplicate. The average return of counts at each antibody dilution was expressed as a percentage of the total counts available (Table 29).

The percentage return of counts ranged from 0.61% to 13.93%. Antibodies ADP335 and 403 gave very low returns with a maximum percentage return of 1.78 and 1.27 respectively. Their peak returns were however at the maximum dilution, and so it was decided to dilute these antibodies further (1 in 400, 1 in 500, 1 in 600, 1 in 800, and 1 in 1000). The use of BSA as a post-antibody coating blocking agent was also tested on these further dilutions (Table 30).

Figure 26. Separation of iodinated rgp120 glycoprotein from free iodine on Sephadex G-100.



The separation was carried out by gel-filtration of the iodination mixture containing ^{125}I-labelled rgp120 (2μg of approximately 140-160μCi/μg), sodium metabisulphite (60μg), cloramine-T (25μg), and approximately 185-215μCi of ^{125}I (iodine) diluted in 0.2% ptassium iodide in RIP buffer to a total volume of 250-300μl. The column was run with excess buffer (0.2% KI in RIP), and 1ml fractions were collected. The fractions were diluted 1 in 10, analysed for gamma irradiation over 60s, and the results plotted. The unretarded iodinated protein was eluted first (fractions 4-6), followed by a smaller peak of free iodine (fractions 11-13).

Table 28. Gamma irradiation activity of fractions of iodinated rgp120 collected from the Sephadex G-100 column.

Fraction	**1**	**2**	**3**	**4**	**5**
cpm	14	23	5	23492	286325
Fraction	**6**	**7**	**8**	**9**	**10**
cpm	21189	9447	4268	173	6276
Fraction	**11**	**12**	**13**	**14**	**15**
cpm	35071	150494	38460	5160	550
Fraction	**16**	**17**	**18**	**19**	**20**
cpm	204	147	92	127	94
Fraction	**21**	**22**	**23**	**24**	**25**
cpm	45	100	60	45	54
Fraction	**26**	**27**			
cpm	52	77			

Fraction, sequential fractions of 1ml collected from Sephadex G-100 column loaded with iodinated recombinant gp120 and washed through with 0.2% potassium iodide in RIP buffer; cpm, counts per minute. The cpm were plotted against the fraction number to see the peak counts responding to iodinated protein and free iodine (Figure 26). The percentage of free iodine incorporated into the recombinant protein was calculated from the total counts in the protein peak fractions (4-6) out of the total counts for the 27 fractions collected. From this it was calculated that 57-63% of the available iodine had been incorporated into the tyrosine and histidine residues of gp120.

Table 29. Determination of the specificity and efficiency of antibody coating of polystyrene beads with antibodies to HIV-1 surface glycoproteins, using iodinated recombinant gp120.

Dilution	Counts	ADP335	ADP361	ADP362	ADP403
1 in 50	20000	1.02%	7.19%	7.18%	0.98%
	50000	1.30%	13.93%	12.16%	0.61%
1 in 100	20000	0.84%	7.73%	4.91%	1.07%
	50000	0.86%	10.75%	8.47%	0.61%
1 in 200	20000	1.35%	5.45%	4.14%	0.98%
	50000	1.5%	8.46%	4.98%	1.00%
1 in 400	20000	1.78%	4.89%	3.48%	1.27%
	50000	1.70%	5.24%	4.73%	0.86%

Each incubation was carried out in triplicate and the results averaged. Iodinated rgp120 was used at 20000 and 50000cpm per incubation aliquot. Dilution, antibody dilution used to coat the beads; Counts, total counts of iodinated gp120 available to bind to antibody coated beads; %, the average counts returned as a percentage of the total counts available. ADP numbers refer to the MRC AIDS directed program (Appendix II).

Table 30. Further analysis of antibodies ADP335 and 403 for bead coating, with preliminary BSA blocking studies using recombinant iodinated gp120 label.

Dilution	Counts	ADP335	ADP335	ADP403	ADP403
1 in 400	20000	1.67%	**0.69%**	0.67%	**0.39%**
	50000	2.04%	**0.76%**	1.06%	**0.52%**
1 in 500	20000	1.81%	**0.58%**	1.14%	**0.48%**
	50000	1.78%	**0.68%**	1.12%	**0.39%**
1 in 600	20000	1.25%	**0.87%**	0.88%	**0.38%**
	50000	1.64%	**0.69%**	1.02%	**0.05%**
1 in 800	20000	1.70%	**0.54%**	0.88%	**0.40%**
	50000	1.50%	**0.57%**	1.24%	**0.40%**
1 in 1000	20000	1.26%	**0.38%**	0.94%	**0.29%**
	50000	1.18%	**0.48%**	1.40%	**0.44%**

Data in normal type was from the dilution studies, and data in bold from the BSA bead blocking experiment. Beads were blocked with a 5% solution of BSA in carbonate/bicarbonate buffer. Counts, total counts available; %, average counts returned as a percentage of the total counts available (dilutions were carried out in duplicate). ADP numbers refer to the MRC AIDS directed program (Appendix II).

No significant improvement was observed by further diluting the antibodies. This may be explained by the nature of the 2 antibodies with respect to the iodinated gp120 used to assay the coated beads. Antibody ADP335 was raised against a yeast recombinant gp160 glycoprotein, and so was likely to show stronger affinity for gp160 epitopes. The antibody ADP403, although raised against rgp120 (ADP604), was polyclonal and so anti-gp120 specific antibodies bound to the beads may be of a lower concentration than expected with monoclonal antibodies.

Antibodies ADP361 and 362 were both raised against the recombinant gp120 protein used to assay the bead coating efficiency. This may explain why the return of counts was significantly higher for these antibodies in comparison with beads coated with antibodies ADP335 and 403.

In addition to the specificities of the antibodies tested, the conformation of the recombinant glycoprotein may have been altered directly by the iodination procedure or indirectly by chemicals involved in the reaction thereby reducing its specificity for the antibodies (Greenwood *et al.*, 1963; Bolton & Hunter, 1973). Unfortunately, the amino acids associated with the binding of antibodies ADP335, 361, 362, and 403 were not known; if, however, tyrosine or histidine residues were involved, the binding capacity of the glycoprotein may have been altered by conformational changes induced by the iodination of their side chains.

The effects of the iodination process could have been monitored by assaying the iodinated glycoprotein in competition with the original (non-iodinated) glycoprotein in an anti-gp120 specific radioimmunoassay. In this way, the comparative specificities of the 2 glycoproteins could have been determined.

In view of these results, and in particular, the variation observed in *env* sequences from different patient isolates, it was decided to test all antibodies with patient samples. Beads for subsequent studies were coated with

antibodies at the following dilutions: ADP335, 1 in 400; ADP361, 1 in 50; ADP362, 1 in 50; and ADP403, 1 in 500.

The antibody coated beads blocked with a 5% BSA solution showed a dramatic reduction in binding capacity for rgp120 (Table 30). This may have been due to the BSA blocking binding sites of the antibodies, or to efficient blocking of non-specific binding of iodinated rgp120. Due to the low return of counts in these experiments it was decided to use antibody coated beads without BSA treatment for further studies.

9.2 HIV-1 RNA detection by RT-PCR.

9.2.1 HIV-1 RNA detection in patient samples.

Total RNA was extracted, reverse transcribed, and amplified for HIV-1 sequences in 8 samples from 7 patients using a reverse transcription-PCR kit. Six samples were of cells isolated by adherence from patient PBMCs, 1 sample of patient PBMCs, and 1 patient plasma sample. Samples were amplified for both *pol* and *env* sequences by a nested PCR, and the results tabulated (Table 31). Only 2 samples were PCR positive for viral sequences, 1 adherent sample (P4a) and the PBMC sample (P7). Both samples had viral markers indicative of viral activity (P4a was p24 plasma Ag positive, and P7 was positive for both 1 and 2LTR CUVD forms in PBMCs). All other samples were negative for viral RNA, p24 plasma Ag, and CUVD forms. All samples amplified without the reverse transcriptase step, and all HIV negative control samples were negative.

Unlike the detection of HIV-1 DNA, viral RNA is indicative of active viral replication and may not be detectable in all patient samples (eg. latently infected individuals, or those responding to chemotherapy). This may explain why only 2 out of 8 (25%) of samples were HIV-1 RNA positive. In addition to the lack of RNA in samples, the method of detection may require a threshold

Table 31. Detection of HIV-1 RNA in patient samples of adherent cells, plasma and PBMCs.

Patient No.	Sample type	HIV-1 RNA	p24 Ag	CUVD
P3b	Adherent cells	-	-	-
P4a	Adherent cells	+	+	nd
P7a	PBMC	+	-	+
P19a	Adherent cells	-	-	-
P20b	Plasma	-	-	nd
P20b	Adherent cells	-	-	nd
P25a	Adherent cells	-	-	-
P31	Adherent cells	-	-	-

RNA was extracted from all adherent cells isolated, from 1 x 10^6 PBMCs, and from 1ml of plasma. Extracts were scored HIV-1 RNA positive if either *pol* or *env* nested amplifications were positive. p24 Ag, detection of p24 viral core antigen in patients plasma; CUVD, detection of 1 and 2LTR circular unintegrated viral DNA in patient PBMCs; and nd, not done. Further details of patient samples can be found in Appendix V.
All HIV negative controls and non-reverse transcribed controls were negative (data not included).

level of RNA considerably higher than the required template concentration necessary for DNA PCR detection. The RT step is considerably less efficient than PCR, and so multiple copies of template RNA are required to synthesize a cDNA template for amplification. It is quite possible that the level of RNA in the patient samples was below this threshold, particularly in the adherent cell samples where the number of cells extracted from was not known. The RNA extracts were also split into 3 fractions (for *pol* and *env* amplifications, and for a viral DNA control) reducing the RNA concentration in each vial and making detection less likely.

9.2.2 Detection of whole virus RNA from patient PBMC coculture supernate.

The supernates from positive PBMC cocultures of 2 patients were incubated with antibody coated beads to assess the ability of the HIV-1 surface glycoprotein specific antibodies to bind free virus. The beads were coated with gp120 and gp160 specific antibodies ADP335, 361, 362, and 403 (Appendix II), and the RNA extracts were processed for the detection of *pol* and *env* sequences by RT-PCR (Table 32).

Samples P11b and P20b were both PCR negative for viral RNA after incubation with beads coated with antibodies ADP335, 362, and 403. However, after incubation with beads coated with antibody ADP361, both samples were positive for *pol* sequences, and sample P20b was also positive for *env* sequences, demonstrating the antibody's specificity for the viral surface gp120.

Antibody ADP361 was raised against the recombinant gp120 protein used to optimise the antibody coating procedure, and so it was decided to concentrate on developing a technique for extraction of whole virus from patient samples with beads coated with antibody ADP361.

Table 32. Detection of whole virus RNA in PBMC cocultures using an antibody coated polystyrene bead extraction method.

Patient sample	Antibody coating	amplification sequence *pol*	*env*
P11b	ADP335	-	-
P11b	ADP361	+	-
P11b	ADP362	-	-
P11b	ADP403	-	-
P20b	ADP335	-	-
P20b	ADP361	+	+
P20b	ADP362	-	-
P20b	ADP403	-	-

Details of the antibodies used are given in Appendix II. Antibodies were used to coat polystyrene beads at dilutions of 1 in 100 for ADP335, 1 in 50 for ADP361, 1 in 50 for ADP362, and 1 in 500 for ADP403. The beads were incubated with PBMC culture supernate to bind whole virus, extracted from in order to release viral RNA from bound virus, and the extracts were then reverse transcribed and amplified to detect *pol* and *env* specific viral sequences. +, positive; -, negative; antibody coating, specific antibody used to coat polystyrene beads.

9.2.3 Quantification of RNA detection by whole virus bead capture.

Anti-gp120 coated beads were used to capture whole virus from dilutions of titrated viral supernate to determine the efficiency of the viral capture and RT-PCR methods. The HIV-1_{RF} viral supernate ($TCID_{50}$ = $10^{7.17}$/ml, Appendix III) was assumed to contain in excess of 10^7 virions per ml (equating to more than $2x10^7$ RNA molecules per ml). The dilutions utilised represented a minimum of $6x10^2$ to $6x10^5$ RNA copies per extraction volume (300ul). The diluted aliquots of supernate were incubated with 2 dilutions of antibody coated beads (ADP361 diluted 1 in 50 and 1 in 500). RNA was extracted from the beads which had been incubated with the viral supernate, reverse transcribed, and amplified for *pol* specific sequences (Table 33).

Viral RNA was detected at dilutions of 1 in 10 and 1 in 10^2 after incubations with beads coated with antibody dilutions of 1 in 50 and 1 in 500 respectively. All other samples and controls (RPMI and uninfected C8166 cell culture supernate) were negative. The detection of viral RNA by RT-PCR after bead capture required a minimum of $6x10^4$ to $6x10^5$ RNA templates.

The requirement for such a high RNA template concentration correlates with the observation that the HIV-1 RNA concentration in patient samples may be below the level of detection for this method. Further optimisation of the technique may elicit an increase in sensitivity and, therefore, improve detection of virion associated RNA sequences in patient samples.

Table 33. Quantitation of whole virus capture by anti-gp120 antibody (ADP361) coated beads incubated with titrated HIV-1_{RF} viral supernate.

Supernate dilution	RNA templates	PCR result	
		1 in 50	1 in 500
1 in 10_2	$6x10^5$	+	-
1 in 10^2	$6x10^4$	-	+
1 in 10^3	$6x10^3$	-	-
1 in 10^4	$6x10^2$	-	-

Supernate dilution, dilution of viral supernate ($TCID_{50}$ of $10^{7.17}$/ml) incubated with antibody coated beads; RNA templates, number of genomic RNA templates for reverse transcription based on the assumption that the viral supernate contained approximately 10^7 viral particles per ml, and that each viral particle contains 2 copies of viral RNA; PCR result, detection of viral cDNA after reverse transcription of bead RNA extracts and amplification with the *pol* specific primers (PCR results are given for extracts using beads coated with antibody ADP361 (Appendix II) at dilutions of 1 in 50 and 1 in 500); +, positive; -, negative. Both RPMI and supernate of C8166 uninfected cells gave negative results throughout (data not shown).

CHAPTER 10: HEPATITIS B VIRUS.

The primary aim of this section was to compare the detection of viral antigens and their corresponding antibodies to the detection of viral DNA in clinical samples using the polymerase chain reaction. Samples which were HBV DNA positive, and had been HBsAg subtyped by Mr S.H. Black using a radioimmunoprecipitation assay (as detailed in materials and methods), were then subtyped using selective primers for the S-gene determinants. Finally, an antibody specific bead extraction method was developed to extract viral DNA selectively from free virus and IgG and IgM complexed virus. A table containing all the HBV data is given in Appendix I.

10.1 HBs and HBe status of patient serum samples.

Of the 115 patient serum samples included, 89 were HBsAg positive, 20 were positive for anti-HBs antibodies, and 6 were negative for both markers. Fifty-eight samples were positive for HBeAg, 37 for anti-HBe antibodies, and 20 were negative for both HBeAg and anti-HBe antibodies. The relationship between HBeAg and HBsAg was confirmed, as expected from the sequence of events presented in Figure 4. (Table 34). No statistical analysis was possible due to the fact that 2 cells of the table were equal to zero.

All control sera were HBe antigen and HBs antigen negative, but were positive for anti-HBs antibodies due to their vaccination history.

10.2 Detection of HBV DNA in patient serum using a nested PCR; comparisons with HBe and HBs markers.

HBV DNA was detected in 72 of the 115 serum samples tested (Table 35). Seventy were HBsAg positive and 2 were positive for anti-HBs antibodies. When compared with the HBe status of patients, 58 (100%) HBeAg positive, 12 (32%) anti-HBe positive, and 2 (10%) HBe negative samples were positive for HBV DNA by PCR.

Table 34. Comparison of HBs and HBe status of patient serum samples.

	HBe status			
HBs status	**Ag**	**neg**	**Ab**	**Total**
Ag	58	6	25	89
neg	0	3	3	5
Ab	0	11	9	21
Total	58	20	37	115

Ag, detection of viral antigen; Ab, detection of antibody specific to the viral antigen; neg, absence of viral antigen and its corresponding antibody.
From the data it is clear that the presence of HBeAg is closely associated with the presence of HBsAg, and not with its corresponding antibody. Statistical analysis was not possible due to at least one cell of the table equating to zero.

Table 35. Comparison of HBV DNA detection by a nested PCR, with HBs and HBe status in patient serum samples.

PCR result	HBe status			HBs status		
	Ag	neg	Ab	Ag	neg	Ab
Positive	58	2	12	70	0	2
Negative	0	18	25	19	6	18
Total	58	20	37	89	6	20

Ag, antigen positive; Ab, antibody positive; neg, no detectable antigen or corresponding antibody.
The detection of HBV DNA by PCR was compared to the HBs status of the patient. Unfortunately, statistical analysis was not possible as not all cells of the table were greater than zero. However, the data clearly shows that both HBe and HBs antigens are closely associated with the detection of HBV DNA in patients' sera.

Although statistical analysis of the data was not possible, the detection of HBV DNA was more closely associated with the presence of both HBe and HBs antigens than with their corresponding antibodies.

10.3 Subtyping of the *d* and *y* determinants of HBsAg.

A total of 38 samples had previously been subtyped for the *d* and *y* HBsAg determinants. These samples were then subtyped using a nested PCR, and 12 samples were further subtyped by restriction digest of the amplification product with the enzyme *Sau* 3A (PCR-RD). The results of the 3 methods were compared.

10.3.1 Development of the PCR subtyping method.

HBV DNA positive samples were subtyped for the *d* and *y* determinants using the *y* specific primers (907E and 394J), and the *d* specific primer (029H) with 807C as the pairing primer in each reaction.

Preliminary studies showed that the primers 029H and 394J amplified both *d* and *y* specific DNA efficiently (data not shown). The primer 029H had a T:C 3' mismatch, and 394J a C:A mismatch with the opposite subtype DNA, and were both able to amplify *d* and *y* subtypes of HBV DNA sufficiently for visualisation on agarose gels. These results were in agreement with the work by Kwok *et al.*, (1990) which showed T:C and C:A mismatches to be efficient at amplifying the target DNA. In contrast, Kwok *et al.*, (1990) demonstrated a G:A mismatch to be most effective at reducing the amplification efficiency of a primer. The *y* specific primer (907E) was selected with a 3' G:A mismatch with additional mismatches at the second and third bases. The primer pairing of 907E/870C was, therefore, able to distinguish between subtypes *d* and *y* by not amplifying *d* specific samples sufficiently for ethidium bromide visualisation on agarose gels. Due to the lack of a *d* specific primer, *d* subtyping was inferred on the basis of a negative result with the *y* specific

primer pair. Subtype *d* samples were often found to have visible primary amplification bands after the *y* specific nested amplification reaction. This was most probably due to the inefficiency of the nested primer pair permitting the primers transferred with the primary amplification product to continue amplifying the template DNA. In view of this, negative samples with the *y* specific primers were only accepted as true subtype *d* if the primary amplification band was visible indicating the presence of amplified viral DNA (Lane 3, Figure 27).

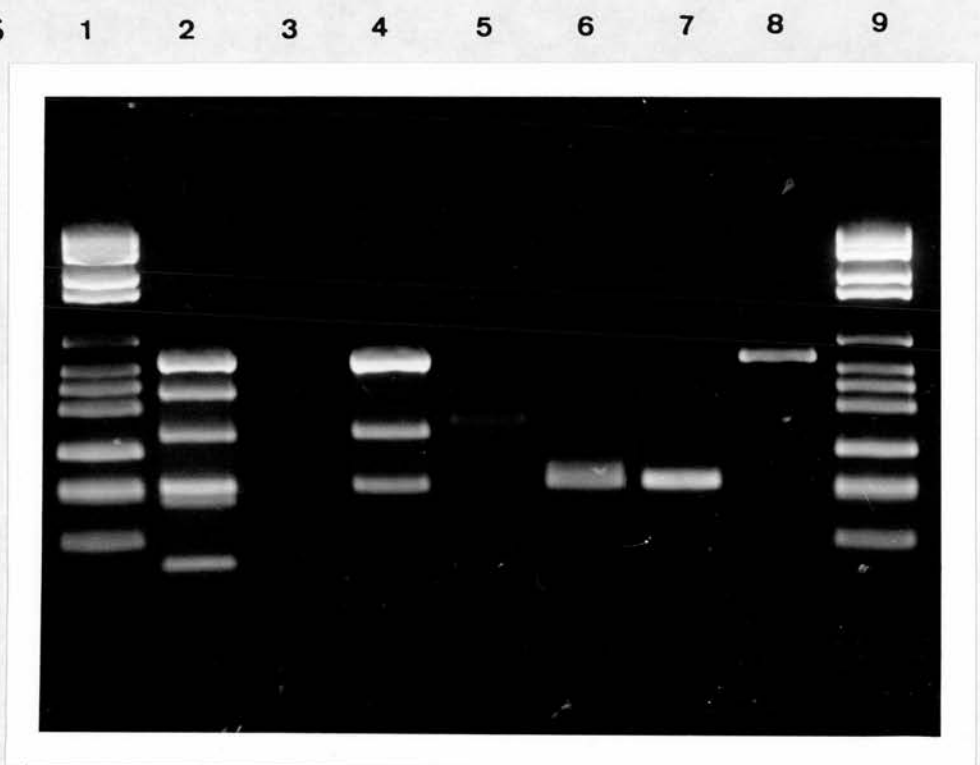
Due to these preliminary studies, all PCR subtyping reported used the nested primer pair 870C/907E for the detection of *y* specific DNA, and *d* subtyping was based on a negative result with these primers.

10.3.2 Comparison of HBsAg subtyping by RIPA and PCR.

A total of 38 HBV DNA positive samples had previously been subtyped by RIPA. Eighteen samples were subtyped *d* and 20 subtyped *y*. These samples were then subtyped by PCR using the *y* specific primer pair 907E/807C for the nested amplification step.

PCR subtyping of HBsAg was in agreement with 20 (100%) of the RIPA subtype *y* samples and 15 (83.3%) of the RIPA subtype *d* samples (3 samples (P65, P68, and P71) were subtyped *d* by RIPA, and *y* by PCR), giving an overall correlation of 92.1% for the 38 samples tested (Table 36). To resolve these discrepancies, it was decided to subtype the samples by a third and conclusive method. The samples were, therefore, amplified by PCR then digested by the restriction enzyme *Sau* 3A (PCR-RD).

Figure 27. Detection and subtyping of HBV DNA by PCR and *Sau* 3A digest.



Lanes 1 & 9, DNA VI molecular weight marker (2176, 1766, 1230, 1033, 653, 517, 453, 394, 298, 234/220, 154bp); **lane 2**, *Sau* 3A digest of P36 (subtype *d*, 548, 431, 321, 227, 204, 117bp); **lane 3**, subtype specific amplification of sample P36 a subtype *d* sample demonstrating the presence of the primary amplification band (548bp); **lane 4**, *Sau* 3A digest of sample P39 (subtype *y*, band lengths of 548, 321, and 227bp); **lane 5**, subtype specific amplification of sample P39 (band of 350bp); **lane 6**, nested PCR (235bp), with primary band showing; **lane 7**, nested PCR; **lane 8**, primary PCR band (548bp).

Table 36. Comparison of hepatitis B surface antigen subtyping by radioimmunoprecipitation assay (RIPA) and the polymerase chain reaction (PCR).

Total No. of samples	Subtyping by RIPA	Subtyping by PCR	
		d	*y*
18	***d***	15	3
20	***y***	0	20

The 3 samples which were subtype *d* by RIPA and subtype *y* by PCR were further analysed by restriction digest of the primary amplified band by *Sau* 3A (PCR-RD), and are presented in Table 37.

10.3.3 Subtyping of HBV DNA by *Sau* 3A restriction digest analysis of PCR products (PCR-RD).

Twelve samples were subtyped by each of the 3 methods (RIPA, PCR, and PCR-RD). Four of the samples (P36, P43, P46, and P60) consistently subtyped *d* by RIPA and PCR, and were included as subtype *d* controls. Samples P38, P44, P58, P59 and P73 were similarly included as subtype *y* controls, and P65, P68 and P71 were included as test samples.

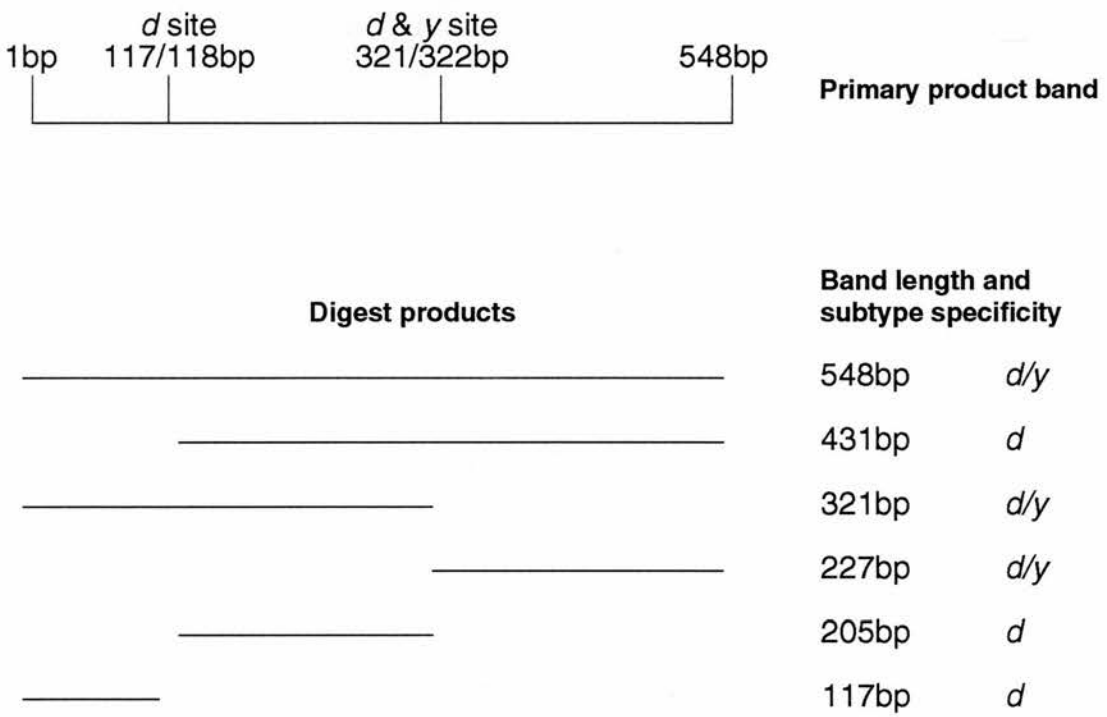
As predicted from the digest patterns obtained from the GenBank for *Sau* 3A, subtype *d* samples resulted in a total of 6 bands (117, 205, 227, 321, 431, and 548bp), and subtype *y* samples in 3 bands (227, 321, and 548bp) (Figure 27, Lanes 2 & 4 respectively). These restriction patterns result from 2 cuts for *d* specific samples, and 1 cut for *y* specific samples (Figure 28).

The nine control samples gave identical results with the 3 subtyping methods, confirming the predicted digest pattern of *Sau* 3A. Of the 3 samples with conflicting results, one confirmed the PCR result (P65, PCR-RD subtype *y*), while the other two samples (P68 and P71, both PCR-RD subtype *d*) confirmed the RIPA result (Table 37).

10.4 Selective extraction of free virus, and antibody complexed HBV using antibody coated polystyrene beads.

The method described for selective extraction of HBV DNA from serum was designed to differentiate between viral DNA associated with free infectious virus, immune complexed virus, and free viral DNA released from necrotic hepatocytes. Beads coated with anti-HBs antibodies were used to isolate free virus, while antibodies against human immunoglobulins G and M were used to isolate complexed virus. Due to the lack of a suitable extraction method for naked viral DNA, detection of this form of HBV DNA was dependent on detection of viral DNA by DNA extraction from sera in conjunction with

Figure 28. *Sau* 3A restriction digest patterns of the primary amplification band of HBsAg DNA (PCR-RD).



The double amplification of HBV DNA with the primary primers (058C/869C) resulted in the above band of 548bp. The concentration of template DNA was sufficiently high to prevent complete digestion by *Sau* 3A, and so the patterns represent a partial digest. The subtype *d* specific band resulted in a total of 6 bands (117, 205, 227, 321, 431, and 548bp), and *y* specific band in 3 bands (227, 321, and 548bp).

Table 37. Comparison of hepatitis B surface antigen subtyping by radioimmunoprecipitation assay (RIPA), the polymerase chain reaction (PCR), and *Sau* 3A restriction digest of PCR amplified HBsAg DNA (PCR-RD).

	Subtype determined by:		
Patient No.	**RIPA**	**PCR**	**PCR-RD**
P36	d	d	d
P43	d	d	d
P46	d	d	d
P60	d	d	d
P65	d	y	y
P68	d	y	d
P71	d	y	d
P73	y	y	y
P38	y	y	y
P44	y	y	y
P58	y	y	y
P59	y	y	y

PCR subtyping, HBsAg DNA was subtyped by a nested PCR which selectively amplified DNA of subtype *y* specificity; PCR-RD, *Sau* 3A restriction enzyme digest of HBsAg DNA amplified for 2x25 cycles with the primary amplification primer pair (058C/869C); RIPA subtyping, the use of subtype specific antibodies to detect conformational determinants of HBsAg (Nicholson *et al.*, 1992).

negative HBV DNA results from the antibody coated bead extractions described.

10.4.1 Optimisation of antibody coating of polystyrene beads.

The antibody to HBsAg was initially diluted 1 in 10 to 1 in 100, and incubated at each dilution with a negative control, 2 concentrations of HBsAg positive control, and detected by incubation with iodinated anti-HBs antibodies. The counts returned were very low, and the low level positive control was negative for all dilutions except 1 in 100 (Table 38).

The antibody was further diluted 1 in 150 to 1 in 600, incubated in duplicate with iodinated HBsAg (approximately 150,000cpm/ml), and the return of counts expressed as a percentage of the total available counts (Table 39). The peak return of counts from the combined results (Tables 38 and 39) indicated that an antibody dilution of 1 in 150 was optimum.

Anti-human IgG and IgM antibodies were similarly optimised. The antibodies were assayed directly using iodinated anti-HBc purified from a patient serum sample. The return of counts were also very low, but indicated that a dilution of 1 in 150 was optimum for anti-human IgG, and 1 in 500 for anti-human IgM.

10.4.2 Selective extraction of HBV DNA associated with free virus, and IgG and IgM complexed virus.

To determine the form of HBV DNA present in patient serum, 14 samples were selected which had tested positive for HBsAg DNA by PCR. Nine samples were positive for anti-HBe antibodies (P68, P69, P71, P73, P116, P117, P118, P148, and P149), 4 samples had detectable levels of HBeAg (P144, P145, P146, and P147), and sample P97 was negative for both viral markers. All samples were HBsAg positive except for P116 and P117 which had detectable levels of anti-HBs antibodies.

Table 38. Determination of the optimum antibody concentration for anti-HBs antibody coating of beads detected by incubation with 2 concentrations of HBsAg and subsequent detection with iodinated anti-HBs antibody.

Dilution of anti-HBs antibody	Sample	**Counts** returned	**Percentage of counts** returned	**Result**
1 in 10	N	51.724	0.2%	-
	P1	95.232	0.3%	+
	P2	56.750	0.2%	-
1 in 20	N	33.683	0.1%	-
	P1	183.239	0.6%	+
	P2	76.570	0.3%	+
1 in 40	N	37.207	0.1%	-
	P1	226.709	0.8%	+
	P2	70.731	0.2%	-
1 in 100	N	30.021	0.1%	-
	P1	406.979	1.4%	+
	P2	90.044	0.3%	+

N, negative serum control; P1, positive HBsAg control (1ng/ml); P2, positive HBsAg control (4ng/ml); +, positive; -, negative. All beads were incubated with a total of 30000cpm of iodinated anti-HBs antibody (HBsAg positive and negative controls, and iodinated anti-HBs antibody were supplied as part of a RIA kit for HBsAg detection, from BPL, Elstree). The cut off was determined by multiplying the negative mean by 2 (Cut off = 76.318).

Table 39. Determination of the optimum antibody concentration for bead coating with anti-HBs antibodies by direct detection with iodinated HBsAg.

Dilution	Counts returned*	Result
1 in 150	75%	+
1 in 200	57%	+
1 in 300	28%	+
1 in 400	54%	+
1 in 600	21%	+

* Two beads were incubated at each antibody dilution and the average counts returned calculated as a percentage of the total available counts. The cut off was calculated as described in Table 38; +, positive.

The 4 HBeAg positive samples each tested positive for free virus and IgM and IgG complexed virus. This was as expected considering the well documented association of HBeAg and infectivity. However, 2 samples (P68 and P148) with antibodies to HBeAg were also positive for each form of HBV DNA, suggesting that patients can still be infectious despite seroconversion from HBeAg to anti-HBe.

In total, 9 samples tested positive for whole virus (all HBsAg positive; 4 HBeAg and 5 anti-HBe antibody positive), 8 were IgM complexed virus positive (all HBsAg positive; 4 HBeAg and 4 anti-HBe antibody positive), and 7 were IgG complexed virus positive (all HBsAg positive; 4 HBeAg and 3 anti-HBe antibody positive). The 2 anti-HBs/DNA positive samples (P116 and P117) were negative for each form of virus, suggesting that their HBV DNA positivity may arise from naked viral DNA released from necrotic hepatocytes. The results are presented in Table 40.

An example of the results obtained in this way is given in Figure 29. HBV DNA was extracted from serum samples by incubation with anti-human IgM coated beads, and detected by PCR (Gel A, Figure 29). The results were then compared with HBV DNA detection in neat serum extracts (Gel B, Figure 29). Each patient sample was PCR positive for HBV DNA, but samples 69 and 73 were negative after the anti-human IgM bead extraction (Lanes 2 and 7 respectively, Figure 29). Numerous negative controls were included in each batch.

10.4.3 Determination of the specificity of antibody coated beads.

The polystyrene beads were designed to bind proteins, and so it was expected that beads insufficiently blocked would bind non-specifically, giving rise to false-positive results. To demonstrate this, HBV DNA positive sera from 5 patients (P68, P71, P73, P146, and P147) were incubated with blank beads

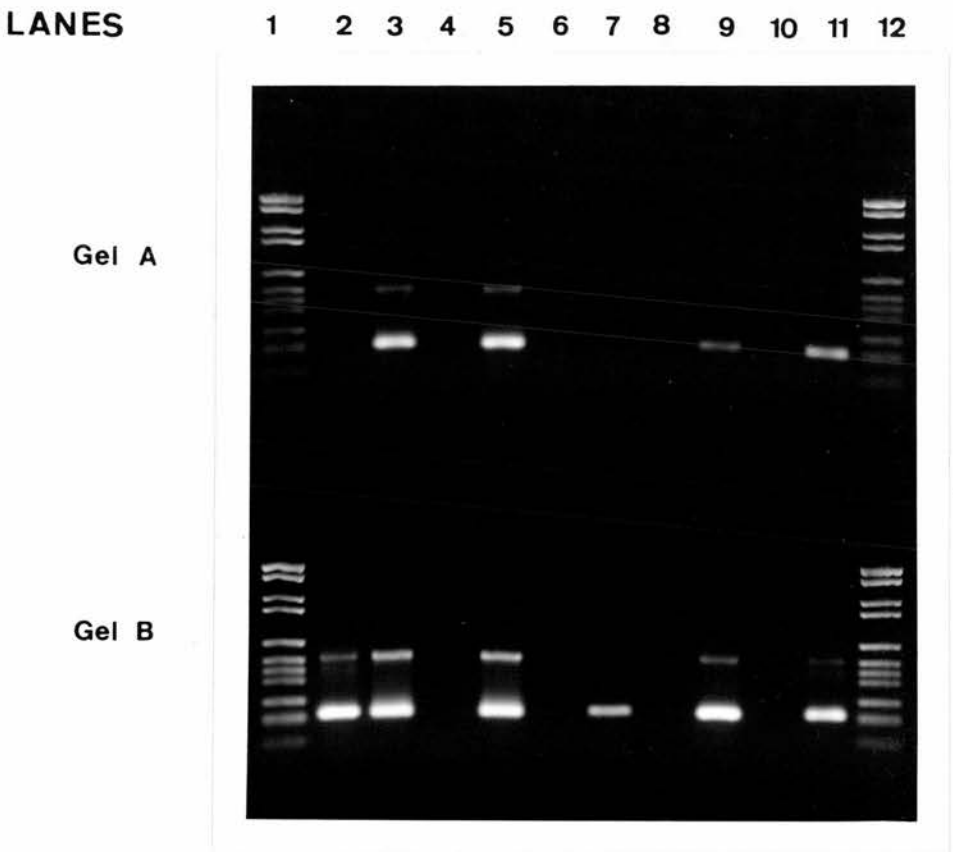
Table 40. HBV DNA amplification results for selective DNA extractions from patients sera using beads coated with antibodies to HBsAg, and human IgM and IgG antibodies.

	Beads coated with antibodies to			Viral markers in serum	
Sample	**HBsAg**	**human IgM**	**human IgG**	**HBe**	**HBs**
P144	+	+	+	Ag	Ag
P145	+	+	+	Ag	Ag
P146	+	+	+	Ag	Ag
P147	+	+	+	Ag	Ag
P97	-	-	-	neg	Ag
P148	+	+	+	Ab	Ag
P68	+	+	+	Ab	Ag
P69	+	-	+	Ab	Ag
P149	+	+	-	Ab	Ag
P71	-	+	-	Ab	Ag
P73	+	-	-	Ab	Ag
P118	-	-	-	Ab	Ag
P116	-	-	-	Ab	Ab
P117	-	-	-	Ab	Ab

+, positive; -, negative; HBe and HBs markers of infection; Ag, antigen positive sample; Ab, antibody positive; neg, no detectable antigen or antibody.

Figure 29. Detection of HBV DNA by extraction with anti-human IgM antibody coated beads.

Gel A: HBV PCR results of DNA extracted with anti-human IgM antibody coated beads.
Gel B: PCR results on total DNA extracted from serum samples.



Lanes 1 & 12, DNA VI molecular weight marker (2176, 1766, 1230, 1033, 653, 517, 453, 394, 298, 234/220, 154); **lanes 4, 6, 8, & 10**, negative controls; **lane 2**, P69; **lane 3**, P145, **lane 5**, P144, **lane 7**, P73; **lane 9**, P71; **lane 11**, P68.

and beads coated with: buffer, 5% BSA, and anti-HBs, anti-human IgG, and anti-human IgM antibodies. The amplification results of these extracts are presented in Table 41.

False-positive results arising from non-specific binding were demonstrated in 3 of the 5 samples tested (P68, P146, and P147). Although the false-positive results obtained with blank and buffer coated beads were expected, BSA is a known blocking agent (eg. hybridization assays) and so the false-positive results obtained with samples from P68, P146, and P147 raised doubts as to the specificity of the antibody coated extractions.

To determine the efficiency of the antibodies in blocking non-specific binding, beads were coated with 2 anti-HIV antibodies (ADP361 and ADP403, Appendix II), and incubated with sera from patient P68. The amplification results from these extracts were positive for HBV DNA, indicating that the antibody coating was not sufficient to block non-specific attachment of proteins.

Although all 5 serum samples tested were HBV DNA positive, samples from P71 and P73 were PCR negative after 5 of the 6 bead extracts. If it is assumed that the antibody coated beads were specific, a possible role of human IgG in the non-specific interactions observed with other sera might be suggested, as both P71 and P73 sera were PCR negative for HBV DNA after anti-human IgG bead extracts. However, if the antibody coatings are assumed to permit non-specific binding, the results obtained may relate to a concentration effect.

The non-specific binding observed clearly raises doubts over the specificity of the antibody coated bead extractions, however, repeated extractions of any patient's serum gave consistent results irrespective of the pattern of positivity (at least 10 results in Table 40 were carried out in duplicate or triplicate with identical results).

Table 41. Amplification results for HBV DNA extractions with blank beads, and beads coated with BSA, buffer, and anti-HBs, anti-human IgG, and anti-human IgM antibodies.

Patient No.	Bead coating					
	5% BSA	Buffer	Blank	anti-HBs	anti-IgM	anti-IgG
P68	+	+	+	+	+	+
P146	+	+	+	+	+	+
P147	+	+	+	+	+	+
P71	-	-	-	-	+	-
P73	-	-	-	+	-	-

The polystyrene beads were coated at room temperature in carbonate/bicarbonate buffer (pH 9.4), with 5% BSA (fraction V, Sigma), buffer only, anti-HBs (1 in 150 dilution), anti-human IgM (1 in 500 dilution), anti-human IgG (1 in 150 dilution), and blank (untreated).

DISCUSSION

DISCUSSION

The primary aim of this thesis was to develop the relatively new technique of nucleic acid amplification, known as the polymerase chain reaction, for the detection of human immunodeficiency virus and hepatitis B virus in clinical samples. Although this was already achieved for HIV (Saiki *et al.*, 1985), the application of PCR to HBV DNA detection was still to be described.

The study progressed to characterise the viral DNA of these viruses and , therefore, focused on qualitative analysis. No quantitative analysis was attempted due to the complexities involved. Primers were selected to determine the overall structure of HIV DNA, including unintegrated linear and covalently closed circular forms, and a *y*-specific primer was used to subtype the HBsAg DNA of HBV.

The data from these experiments are discussed in chapters 11 and 12.

CHAPTER 11: HUMAN IMMUNODEFICIENCY VIRUS .

11.1 HIV-1 isolation by cocultivation of patient PBMCs.

The laboratory diagnosis of HIV is based on the detection of antibodies to core and envelope proteins by ELISA, and confirmation by alternative ELISAs or Western blot (Davey & Lane, 1990). Although highly indicative of infection, the detection of viral-specific antibodies can be misleading. This is most apparent in babies born to seropositive mothers, where the passive transfer of maternal IgG antibodies gives rise to false-positive antibody results for the first 12-15 months of the infants life (Mok *et al.*, 1987; de Rossi *et al.*, 1988; Laure *et al.*, 1988). It has also been claimed that haemophiliacs may give false-positive results due to the presence of HIV specific antibodies arising from exposure to inactivated virus in blood products (Schneweis *et al.*, 1989). Clearly, the detection of antibodies in these 2 groups is insufficient for the laboratory diagnosis of infection. Also, immunoassays are inappropriate for the diagnosis of infected individuals in the window period prior to seroconversion, and in cases where loss of antibody has been reported (Borkowsky *et al.*, 1987; Imagawa *et al.*, 1989; Wolinsky *et al.*, 1989; Clark *et al.*, 1991; Daar *et al.*, 1991). In these situations, the direct detection of virus is important to confirm HIV infection.

11.1.1 Isolation from PBMCs.

Virus isolation from PBMCs is a method of direct viral detection from patient blood but requires considerable time, materials, and expertise (Davey & Lane, 1990; Coutleé *et al.*, 1991). The standard procedure is to cocultivate patient PBMCs with stimulated donor PBMCs. The culture supernate is then assayed for the presence of viral p24 antigen, and/or, reverse transcriptase activity over a period of 28 days (Ulrich *et al.*, 1988; Nicholson *et al.*, 1989; Stoeckl *et al.*, 1989). The efficiency of isolation is related to many factors, and so the technique requires careful optimisation. This is apparent in the range of published isolation rates from 25% to

100% (Ho *et al.*, 1989; Nielsen *et al.*, 1989).

HWB was processed on a Ficoll-hypaque gradient to recover PBMCs within hours of sampling. The cells were then cocultivated with 3-7 day old PHA stimulated donor PBMCs in the presence of IL-2. The cultures were assayed weekly for p24 antigen production, and all new positives were neutralised for confirmation. The isolation rate obtained in this way (59%), was comparable with other studies (Stoeckl *et al.*, 1989; Escaich *et al.*, 1991) although poor in relation to Ho *et al.*, (1989). A number of factors may have contributed to this. Firstly, cells were not separated on the Ficoll gradient exactly as recommended by the manufacturer. The heparinised whole blood was separated without prior dilution so as to facilitate the recovery of neat plasma for p24 antigen analysis. This may have contributed to a reduced lymphocyte recovery. Secondly, amphotericin-B was included in the culture medium to control fungal growth. This has been shown to bind to the steroid component of lipid envelopes and alter membrane permeability and function, resulting in a loss of infectivity for enveloped viruses including HIV (Schaffner *et al.*, 1986; Nielsen *et al.*, 1991). Thirdly, the cell mitogen phytohaemagglutinin (PHA) was used to stimulate both patient and donor cells (Davey & Lane, 1990). PHA is, however, toxic to cells and is generally removed from cultures after 1-3 days (Stoeckl *et al.*, 1989). Although PHA was removed from donor cell cultures prior to cocultivation, patient cells were cultured in its presence throughout. Each of these factors may have contributed to the sub-optimal isolation rate observed. Despite these considerations, the technique was confirmed by neutralisation in 20 out of 34 samples (59%).

Cocultivation of patient PBMCs was used to confirm the infection, and no attempt was made to quantify the viral load. Patients in the study were, however, confirmed HIV-seropositives on previous virus isolation, antibody and antigen detection and clinical presentation. It was, therefore, decided to stop virus isolation by cocultivation of patient PBMCs as it was an unnecessary usage of patient cells which could otherwise be utilised for PCR analysis.

11.1.2 HIV-1 isolation from plasma and heparinised whole blood (HWB).

More recently, isolation methods have been developed for small volumes of plasma (Coombs *et al.*, 1989; Ho *et al.*, 1989), and HWB (Bayliss *et al.*, 1989; Fiore *et al.*, 1990a & b). These methods were attractive due to the small volume of material utilised for each culture, and in particular to the minimal processing of blood required for isolation from HWB.

Plasma cultures were set up basically as described by Ho *et al.*, (1989) although plasma was not ultracentrifuged prior to culture. In addition to this, the exact IL-2 concentration utilised by Ho *et al.*, (1989) was not stated and so IL-2 stimulation was not necessarily identical. Despite the similarities of the 2 methods the isolation rates obtained differed considerably (38% in this study, and 100% in Ho *et al.*, (1989). Although the patients in both studies were from all stages of the disease, patients in the cohort of Ho *et al.*, (1989) were not receiving anti-viral therapy. In this study, 4 patients were receiving AZT treatment and 4 were not. The 3 patients from whom virus was isolated were not receiving anti-viral therapy. Although a very small study, the exclusion of patients on anti-viral therapy would improve the isolation rate to 75%. Coombs *et al.*, (1989) also isolated from plasma at all stages of infection with an overall success rate of 56%. As with Ho *et al.*, (1989), the study consisted of individuals not receiving AZT therapy. The main difference in the 2 studies, which may have contributed to the difference in isolation rates, was that Ho *et al.*, (1989) isolated virus in small volumes and Coombs *et al.*, (1989) in large volumes. Coombs *et al.*, (1989) were, therefore, diluting plasma 1 in 6 in comparison with Ho *et al.*, who diluted from 1 in 1.5 to 1 in 750. In this study, dilutions of 1 in 3 and 1 in 6 were used in small volumes. It is possible that plasma components are toxic to virus isolation and that a balance must be achieved between plasma concentration and virus titre. The isolation rate may, therefore, be enhanced by multiple dilutions and the use of small volumes which is reflected in the isolation rates of 56% (Coombs *et al.*, 1989), 75% (this study), and 100% (Ho *et al.*, 1989) for patients not receiving AZT therapy.

Similarly with HWB isolations, published studies either excluded patients on AZT therapy, or did not comment on its usage within their cohorts (Bayliss *et al.*, 1989; Fiore *et al.*, 1990a & b). Despite this, Fiore *et al.*, (1990b) demonstrated isolation from HWB to be more successful than PBMC cocultivation, particularly in asymptomatic patients. Although only 1 sample was positive for HWB isolation in this study, 4 of the other 5 samples were from patients receiving anti-viral therapy at the time of sampling. This may have contributed significantly to the isolation rate as the drug will have been present in the sample with potential to inhibit reverse transcription of the viral genome, and prevent viral replication. Interestingly, both plasma and HWB results were identical for the 6 samples compared.

Virus isolation techniques have progressed considerably from the initial attempts in the early 1980s (Barré-Sinoussi *et al.*, 1983; Gallo *et al.*, 1984; Klatzman *et al.*, 1984a; Levy *et al.*, 1984; Popovic *et al.*, 1984). Modifications to the method showed that isolation rates from patient PBMCs correlated to disease stage (Stoeckl *et al.*, 1989; Burke *et al.*, 1990; Davey & Lane, 1990; Ferre *et al.*, 1992). This is in agreement with the isolation rate obtained in this study, which showed a significant increase in isolation with disease stage ($0.01>P>0.001$, Table 10). Subsequent improvements in PBMC isolation techniques have shown that virus can be isolated from all groups of patients under the appropriate conditions (Ho *et al.*, 1989). A similar pattern has now emerged for plasma isolation, where lower isolation rates appear to reflect the clinical stage (Coombs *et al.*, 1989; Venet *et al.*, 1991), while Ho *et al.*, (1989) have again obtained maximum isolation rates at all stages of infection. It is, therefore, possible that infected individuals are continually viraemic, varying only in titre at critical stages. This would be in agreement with PBMC titre results obtained by Nicholson *et al.*, (1989), which showed viral titres to be greater in individuals who progressed clinically during the study, while non-progressors had lower and more stable titres.

The detection of p24 plasma antigen, although insensitive, is thought to be indicative

of viral activity and to correlate with disease stage (Goudsmit *et al.*, 1986; Paul *et al.*, 1987; Borkowsky *et al.*, 1989; Davey & Lane, 1990). When compared with published isolation rates, the antigen was detected in the plasma of 16% (Stoeckl *et al.*, 1989), 45% (Coombs *et al.*, 1989), and 74% (Ho *et al.*, 1989) of PBMC coculture positive patients which correlated with virus isolation rates of 45%, 97%, and 100% respectively. In this study, plasma p24 antigen was detected in 10 out of 41 cocultivated patient samples, 7 of which were isolation positive. This was not significant when analysed by the Chi-square test ($0.50>P>0.10$, Table 7). The apparently low detection rates of p24 Ag could be due to the insensitivity of the assay, and/or the presence of blocking antibody. The use of the newly developed immune complex dissociating p24 antigen kits may answer this. Alternatively, it is possible that antigen detection reflects the *in vivo* situation more accurately than patient PBMCs cocultured *in vitro*.

The ability to isolate virus from PBMCs of all groups of patients raises an interesting question about the state of the virus *in vivo*. The isolation of virus *in vitro* from asymptomatic patients with no other markers of replication may result from the successful stimulation of quiescent infected cells, as opposed to enhancement of existing replicating cells. To demonstrate that viral sequences were present in isolation negative samples, DNA extracts from 10 cocultures were amplified by PCR for HIV-1 DNA (Table 11). Three of the samples were culture positive and 7 culture negative (including a high risk seronegative control). The results indicated that the 9 seropositive samples were HIV-1 DNA positive, suggesting that infected cells had failed to be stimulated into viral production in the 6 isolation negative samples. A study by Mathez *et al.*, (1990) addressed this problem by irradiating cells prior to cocultivation. Gamma irradiation arrests cell division leading to cell death within 3 days. Such cells can continue RNA and protein synthesis, but cannot initiate synthesis. Therefore, cells actively producing virus *in vivo* will continue to do so *in vitro*, but quiescent cells will fail to be stimulated. Their comparative study revealed a

5 to 500-fold reduction in virus production from cells irradiated prior to coculture. The production of HIV in irradiated cells correlated to p24 plasma antigen levels ($P<0.001$), demonstrating a correlation with plasma antigen levels and active viral replication *in vivo*. The complications of this argument arise when plasma isolation results are compared. Ho *et al.*, (1989) isolated virus from 100% of plasma samples of which only 74% were also p24 Ag positive. Although Ho *et al.*, (1989) do not describe the vessels utilised in their study, the handling of small volumes for isolation is most readily achieved by the use of 24 well plates. The use of culture plates, however, removes the containment offered by individual flasks, and raises doubts over the possibility of cross-contamination. The incubation of such cultures results in considerable evaporation and inevitable condensation on the plate lid. It is, therefore, not possible to guarantee that during the sampling and feeding of such cultures that the condensation does not cross-contaminate the wells. This hazard requires the processing of large numbers of negative controls interspersed between patient samples, a critical step not described in publications of isolation results obtained in this way (Ho *et al.*, 1989; Fiore *et al.*, 1990a, 1990b).

The disparity in p24 Ag and plasma isolation rates observed by Ho *et al.*, (1989) may, alternatively, be due to the presence of anti-p24 antibodies blocking detection of the antigen. Combined analysis of plasma isolation rates, p24 antigen and antibody titres in a group of patients may resolve this problem.

In conclusion, the isolation of HIV from patient PBMCs does not reflect the replicative state of the virus *in vivo* as cells are stimulated *in vitro*. In contrast, plasma isolation results from the infection of susceptible donor cells by free virus produced *in vivo*, mirroring the *in vivo* replicative state. The isolation of virus from HWB combines both cell free virus and the stimulation of cell-associated virus and should, therefore, offer the most sensitive method. The use of small volumes of HWB not only offers 2 sources of virus, but also requires minimum handling of blood after collection making virus isolation less time consuming. The only apparent drawback of this method is

the potential inhibitory effects of toxic cellular components released from lysed cells. It may, therefore, be the isolation technique of the future for diagnosis, particularly when only small volumes of blood are available.

Despite the recent advances in virus isolation techniques, the conventional method remains a time-consuming and costly technique for HIV diagnosis requiring special laboratory conditions. Unless such methods as HWB and plasma isolation are streamlined and made cheaper, it is likely that alternative methods will be developed to overcome these problems for HIV confirmation.

11.2 HIV-1 infection of susceptible cell lines.

The use of established cell lines offers a pure population of susceptible cells for *in vitro* analysis of HIV-1 infection. Cell lines have been used to characterise tropism and growth patterns of clinical isolates (Schuitemaker *et al.*, 1992) and in studies of the life cycle of HIV-1 (Goto *et al.*, 1988, 1990; Gelderblom *et al.*, 1989; Robinson & Zinkus, 1990; Stevenson *et al.*, 1990a & b; Stieger *et al.*, 1991; Tang *et al.*, 1992).

In this study, 10 cell lines were infected with the well characterised Haitian and USA isolates of HIV-1 (RF and IIIB respectively). These infections were then used for electron microscopy studies and analysis of HIV-1 DNA synthesis.

11.2.1 Electron microscopy studies of HIV-1_{RF} viral uptake and release *in vitro*.

After random collision between a virus and a cell there are 2 main phases in the uptake of virus; firstly, recognition and attachment of the virus to susceptible cells and secondly, penetration and uncoating of the virus. HIV-1 recognises cells expressing the CD4 antigen and attaches via its surface glycoprotein gp120 (Dalgleish *et al.*, 1984; Klatzmann *et al.*, 1984; Lifson *et al.*, 1986). At least one other mechanism has been proposed in which HIV, complexed with specific antibodies, attaches via cell surface Fc receptors (Homsy *et al.*, 1989; Jouault *et al.*, 1989; Laurence *et al.*, 1990; McKeating *et al.*, 1990). Penetration of cells and uncoating of the virus have been demonstrated to occur *in vitro* by both the pH-independent

mechanism of direct fusion between viral envelope and cell plasma membrane (Stein *et al.*, 1987; Goto *et al.*, 1988; McClure *et al.*, 1988; Sinangil *et al.*, 1988; Grewe *et al.*, 1990), and by a pH-dependent mechanism of membrane fusion following receptor-mediated endocytosis (Goto *et al.*, 1988; Pauza & Price, 1988; Grewe *et al.*, 1990). Both methods are reported to occur within 10 minutes of incubation at 37°C, and it has been proposed that the surface glycoprotein gp41 plays a role in the fusion process (Kowalski *et al.*, 1987; Goto *et al.*, 1988; Grewe *et al.*, 1990).

It was decided to observe the early and late virus:cell interactions using ultra-thin stained sections of infected cells viewed by transmission electron microscopy.

C8166 cells were inoculated with HIV-1_{RF} at 4°C to allow recognition and attachment to occur. The temperature was then raised to 37°C to permit viral penetration, and cells were harvested over 60min to observe the budding process.

Pits and vesicles were observed on micrographs of the early cell sections (Figures 10 & 11). Although these resembled those reported by Goto *et al.*, (1988) and Grewe *et al.*, (1990), they were not associated with virus and were also present in cell controls. As the cells were growing in culture at the time of sampling, the pits and vesicles were most likely involved in nutrient transport (Goldstein *et al.*, 1979).

Due to the lack of evidence for receptor-mediated endocytosis, it may be assumed that the mechanism of uptake in C8166 cells infected with HIV-1_{RF} was by direct fusion at the cell surface. However, to synchronise the infection and achieve maximum viral attachment, the adsorption period was overnight at 4°C. Although this is thought to inhibit membrane mobility, HIV_{LAV} uptake, by direct fusion, has been reported at 4°C in a T lymphoblastic cell line (Stein *et al.*, 1987). Therefore, due to the long incubation at 4°C, and the processing time involved to fix the cells, it is possible that the majority of early virus:cell interactions had occurred and were consequently not observed.

The mechanism of lentiviral budding has been well studied and documented to the extent that it is used in the classification of retroviruses (Gelderblom *et al.*, 1989).

Electron micrographs of infected cells harvested on days 3 to 8 confirmed this process for HIV_{RF} infection of C8166 cells (Figures 12 to 15). Virus was seen to assemble at the cell surface. This was associated with a thickening of the cell membrane, progressing to a horseshoe-like protrusion which eventually closed releasing the virion. As with Gelderblom, (1991), Gelderblom *et al.*, (1989) and Katsumoto *et al.*, (1987), maturation of the cone-like core structure was observed after budding, however, this has been contradicted by studies of viral mutants which suggested that core maturation occurred prior to virion release (Goto *et al.*, 1990).

Cells were first viewed for budding virus on day 3, by which time the majority of cells showed stages of the budding process, suggesting that it started earlier. As cells were not viewed between 1 hour and day 3, the time at which budding commenced is unknown.

Direct comparisons of electron microscopy and viral DNA detection results for infected C8166 cells were hindered by the fact that the cells were inoculated at different temperatures. However, comparison established that viral penetration and uncoating had occurred within 2 hours of infection due to the detection of viral DNA. This supports studies which demonstrated viral penetration to be very rapid at 37°C (Goto *et al.*, 1988; Grewe *et al.*, 1990).

11.2.2 HIV-1 cytopathic effects (CPE).

Infection of susceptible cells with HIV-1 can lead to cell death by single cell lysis or cell fusion (Fauci *et al.*, 1988; Garry, 1989). Single cell lysis may result from excess budding of virus from the cell membrane, and cell fusion from the interaction of gp120 and CD4 molecules on the cell surface (Lifson *et al.*, 1986; Sodroski *et al.*, 1986a; Fisher *et al.*, 1988; Hussey *et al.*, 1988; Dedera & Ratner, 1991).

In this study, cells infected with HIV-1 RF and IIIB laboratory strains were observed daily for the production of syncytia. The initial observation in the IIIB study was that syncytium formation only occurred in T cell lines in agreement with Shepherd *et al.*,

(1992). This led to the total destruction of C8166 and MT4 cultures by day 8. In contrast, C8166 cells similarly infected with HIV-1_{RF} were not completely destroyed during the 14 day study, suggesting differences in the cytopathogenic properties of the viral strains which may be attributable to the envelope region associated with cellular tropism and syncytium-inducing (SI) capacity (Tersmette *et al.*, 1988; Cordonnier *et al.*, 1989; Liu *et al.*, 1990; Westervelt *et al.*, 1991). Of the 6 remaining syncytia-negative cell lines, 4 were later shown to be HIV-1 DNA positive indicating the establishment of an infection. These cells were, therefore, infected by HIV-1_{IIIB} but did not produce a visible CPE. This suggests that the virus itself is not solely responsible for syncytium formation, and indicates a potential role for cellular factors. Cells grown in serum-free medium are deprived of important growth factors and are generally retarded in their growth, although the degree to which growth is affected differs with the cell type. Some cells are completely arrested in the G_0 phase of cell division while others can adapt to divide normally (Chen & Temin, 1982; Yahi *et al.*, 1991). C8166 cells used in the study were grown in serum-free medium during the experiment and 3 days prior to inoculation. The effects on cell division were not determined; however, in contrast to C8166 cells grown in complete medium, serum-free cells did not form syncytia when infected with HIV-1_{RF}. This supports the earlier findings in HIV-1_{IIIB} infected cell lines (this study), and transfection studies using HXB-2 clones in SupT1 cells (de Jong *et al.*, 1992) which demonstrated that cellular factors may contribute to the cytopathology of the infection.

11.3 Viral DNA detection in cell lines.

Since the advent of PCR (Saiki *et al.*, 1985) the technique has rapidly become established as the most sensitive method of detecting HIV-1 infections. To ensure the detection of multiple isolates, the primers used in the reaction must hybridize to highly conserved sequences of the genome. The polymerase gene of HIV-1 is used in such studies (Laure *et al.*, 1988; Simmonds *et al.*, 1990a). We used 4 primers, in a

nested PCR, for the detection of *pol* sequences (Appendix IV). These primers had been extensively used by Simmonds *et al.*, (1990a) to establish their efficacy. The use of a nested PCR increased the sensitivity of the reaction, and improved its specificity by requiring the correct hybridization of 4 oligonucleotides to the target sequence. This method has been shown to detect single copy templates (Simmonds *et al.*, 1990a) and has since been applied to clinical quantification studies (Bell *et al.*, 1993). The *pol* primers were, therefore, applied to the detection of total HIV-1 DNA in sequential crude extracts of infected cell lines.

The detection of HIV-1 DNA by amplification of the *pol* gene detects all forms of viral DNA with an intact *pol* gene (Figure 16), and requires a minimum reverse transcription of 55% of the RNA genome. Therefore, the earliest time point at which this amplification was positive for each cell line probably relates to the initial reverse transcription of the genome, after uncoating, prior to completion of the LUVD molecule.

Of the 10 cell lines studies 7 were naturally susceptible to infection with HIV-1 (4 T cell lines & 3 monocytic lines) and 3 had been modified to become susceptible. The T lymphocyte and monocyte cell lines were all HIV-1 DNA positive for *pol* sequences 2h post-infection with HIV-1_{RF} and HIV-1_{IIIB}. The other 2 cell lines which became infected (HB1 and LC5) did not become DNA positive until 8 and 12h post infection respectively. Therefore, cells which were naturally susceptible to infection started to replicate virus more rapidly than cells which had been modified to become susceptible. This may reflect differences in the tropism of the viral strains for different cell lines, as cellular tropism of HIV-1, related to the *env* gene, has been associated with the rate and efficiency of the early stages of viral penetration (Kim *et al.*, 1990; Srivastava *et al.*, 1991).

The donor PBMCs infected *in vitro* with HIV-1_{RF} were first positive for HIV-1 DNA at 2h post-infection. This supports the theory of more rapid viral replication in naturally susceptible cells. However, HIV-1 DNA detection was not consistently positive in cell

aliquots until day 2 (Table 15). The percentage of susceptible cells present in donor PBMCs may be responsible for this result. In the PBMC culture, approximately 40-70% of the cells were lymphocytes of which only 60% are $CD4^+$. This gives a total of 24-42% of cells susceptible to infection by HIV-1, excluding $CD4^+$ monocytes. Therefore, with less than half the cells, and possibly as low as a quarter of the cells, present being susceptible to HIV-1, it will take longer for the virus to infect the equivalent number of cells observed in T cell line cultures. It is possible that during the initial harvested aliquots, very few donor PBMCs were infected, and consequently, not all cell aliquots removed for DNA extraction contained infected cells. This is supported by the findings of Kim *et al.*, (1989) where HIV-1 infection of H9 and $CD4^+$ enriched T helper cells showed that full length LUVD was detectable at 4 and 5h post-infection respectively. This showed that the purified cells were infected at a similar rate to the established cell line.

HIV-1 DNA was detected by 2h post-infection in RF infected C8166 cells grown in complete and serum-free medium, demonstrating that the initial steps of viral replication are independent of the state of the host cell. This is in agreement with *in vitro* studies with quiescent and stimulated T cells which showed that initiation of HIV-1 replication occurred at the same time in the 2 cell populations, but that replication was arrested prior to integration in quiescent cells (Stevenson *et al.*, 1990b; Zack *et al.*, 1990, 1992).

Comparison of HIV replication in quiescent and replicating cells may elicit mechanisms by which integration of the provirus can be prevented. This could prove to be advantageous in the development of therapeutic agents to prevent integration of UVD in quiescent T cells which are thought to be an inducible reservoir of infection.

11.3.1 Detection of linear unintegrated viral DNA (LUVD).

Upon entry to the host cell, HIV-1 initiates replication by reverse transcribing its RNA

genome to a double stranded DNA molecule. The detection of LUVD is, therefore, an early indicator of viral infection. The LUVD molecule has successfully been detected by Southern blot analysis (Pauza & Galindo, 1989; Pauza *et al.*, 1990; Robinson & Zinkus, 1990; Tang *et al.*, 1992; Titti *et al.*, 1992), however, this lacks the sensitivity offered by PCR.

To ensure that the DNA detected by PCR is LUVD requires the use of a unique characteristic of the template molecule. LUVD has no defining sequences or junction sites, but is unique in that the viral LTRs have free ends (Figure 16b). The molecule can, therefore, be detected by ligation of a small sequence and amplification from this sequence to an internal site giving a band of known length (Figure 17b).

This method was developed on early extracts from infected cell lines. Each time point was amplified in triplicate; one vial containing all reagents, a second vial lacking the ligase enzyme, and the third vial lacking the ligation sequence. In all samples, the 2 control vials were negative. Uninfected cell extracts were similarly amplified to ensure that cellular sequences could not give rise to false-positives. After extensive replicates of this work it was established that the ligation and amplification method was specific to the detection of HIV-1 LUVD.

The presence of unintegrated viral DNA (UVD) in non-human retroviral infections has been found to correlate with cytopathology *in vitro* and *in vivo* (Keshet & Temin, 1979; Temin *et al.*, 1979; Weller *et al.*, 1980; Robinson & Miles, 1985; Mullins *et al.*, 1986; Hoover *et al.*, 1987). There is now increasing evidence that these forms of viral DNA are present in HIV-1 infections *in vivo*. Both linear and circular UVD forms were first described in the tissue of AIDS and ARC patients (Shaw *et al.*, 1984). More recently, HIV UVD forms have been detected in patient samples from the brain and PBMCs, using PCR to amplify from or across unique sequences (Pang *et al.*, 1990; Bukrinsky *et al.*, 1991; Li *et al.*, 1991; Poznansky *et al.*, 1991; Dickover *et al.*, 1992; Juriaans *et al.*, 1992). As in other retroviral infections, the presence of UVD has been associated with secondary infections, or superinfections (Bergeron & Sodroski,

1992). However, as a maximum of 1% of PBMCs are thought to be infected with HIV, superinfection of these cells is statistically unlikely, although possible (Bukrinsky *et al.*, 1991).

To observe the effects of HIV-1 UVD forms on cytopathology, a variety of cell lines were infected with a known inoculum, and aliquots removed and analysed for detection and characterization of HIV-1 DNA forms. Four T-cell lines (C8166, Hut 78, MT4 and Sup-T1) and 6 non-T cell-lines (HB1, HeLa $T8^+$, LC5, THP1, U138MG and U937) were inoculated with HIV-1_{IIIB} to determine the role of cellular factors in the development of the infection. C8166 cells, in complete and serum-free medium, were also inoculated with HIV-1_{RF} to observe differences associated with the viral strain, and to demonstrate the importance of cellular activation in viral replication and cytopathology of HIV-1 infection of T cells. HIV seronegative donor PBMCs were infected with HIV-1_{RF} for comparison with C8166 cells, and also in view of the interest in applying the techniques to seropositive patient samples.

The detection of LUVD, particularly in T-cell derived lines, gave an interesting pattern of detection. The primers used for LUVD detection were specific to the *env* termini of the viral genome. This end of the genome is copied first (Varmus & Swanstrom, 1982), and so as little as 10% of the viral genome was required to be reverse transcribed for a positive detection by the ligation mediated-PCR. In view of this, positive results by this method did not relate to the completion of LUVD synthesis, but gave an indication that the process had started.

The application of this method to sequential extracts of HIV-1_{IIIB} infected cell lines detected viral DNA sequences within hours of infection. In all infected cell lines, except C8166, LUVD was detected before or at the same time point as total viral DNA. Although neither method of detection required a completed HIV DNA strand, the *pol* primers required 55%, and the ligation mediated-PCR approximately 10% of the genome to be reverse transcribed. Therefore, LUVD was expected to be detected before total HIV DNA. The reversal of this in IIIB infected C8166 cells was

most probably due to the efficiencies of the 2 amplification reactions. PCR is a highly specific and sensitive detection method, however, the detection of LUVD is limited by the ligation reaction which is non-specific and consequently inefficient. The detection of total HIV DNA is, therefore, more sensitive than the ligation dependent-PCR for LUVD detection.

The appearance and disappearance of detectable LUVD in T cell lines (Table 13) may have been attributable to a concentration effect taking the level below that required for the ligation mediated-PCR. Alternatively, the structure of the linear molecule may be rapidly altered upon completion, preventing detection by this method. Firstly, the free ends may be modified in preparation for integration. This is known to involve the removal of 2bp from either end (Pauza *et al.*, 1990; Vink *et al.*, 1990; Whitcomb *et al.*, 1990; Hong *et al.*, 1991) and would prevent hybridization of the ligation and viral specific primer. Secondly, the LUVD may be rapidly circularized in the absence of integration, preventing ligation and subsequent detection. A third possibility is that the appearance and disappearance of LUVD represents sequential rounds of viral infection. This is, however, highly unlikely due to the small time scale involved. For example, LUVD was first detected 1h post-infection in MT4 cells. The following time-point at 2h was negative, however, LUVD was again detected at 4h. This would suggest that HIV-1 had infected and replicated in MT4 cells with a life cycle of less than 4 hours. The pattern was also not due to sequential infections of cells with input virus as the cells were washed after the initial 1h incubation. The pattern of detection in non-T cell lines was much more suggestive of a low efficiency of detection (Table 14). Only THP1 cells were positive for LUVD on day 1, however, THP1, U937 and HB1 cells were all strongly positive later when progeny virions had been produced and were then able to infect more cells.

As expected, the overall data suggested that ligation mediated-PCR was less sensitive than the standard nested PCR used for total HIV DNA detection. However, the results from T cell lines also indicated that the initial LUVD molecule is rapidly

modified upon completion, resulting in the failure to detect it by our ligation mediated-PCR method. It is unlikely that this is due to instability of the molecule, as dsDNA is known to be relatively resistant to cellular nucleases (Nandi & Banerjee, 1993).

The detection of LUVD forms in HIV-1_{RF} infected cells differed considerably from the HIV-1_{IIIB} study. Only C8166 cells in complete medium (on day 14) were positive for LUVD. Samples from all other time points were negative despite the detection of LUVD in HIV-1_{IIIB} infected cell extracts used as positive controls. The concentration of LUVD present in the 2 studies may have differed considerably. This could have been due to the viral strain, or to the DNA extraction method used. Cells infected with HIV-1_{IIIB} were crudely extracted prior to amplification so that PCR aliquots contained cell debris and culture medium. Cells infected by HIV-1_{RF} were digested by proteinase K and N-lauroylsarcosine prior to repeated phenol:chloroform extractions and alcohol precipitation. Therefore, the RF extractions were considerably purer than those of IIIB, although both the extraction and precipitation steps will have led to the loss of DNA. It is possible that by purifying the DNA extract, LUVD has been lost, effectively resulting in false-negative results. In view of this, the apparent differences in detection of LUVD may be due to the viral strain, the extraction method, or a combination of both.

The primer used to amplify across the ligation and viral sequence junction was specific to the envelope LTR of the proviral copy of HIV-1. As this end of the genome is copied first, its detection represents the initiation of reverse transcription. As the ligation sequence was known to be non-specific, it was decided to amplify the *gag*-LTR terminus of the genome in a similar reaction, in an attempt to ascertain the time point at which LUVD synthesis was completed. The LUVD molecule is known to lose 2bp from either end prior to integration (Bushman *et al.*, 1990; Kulkosky *et al.*, 1990; Pauza, 1990; Whitcomb *et al.*, 1990; Hong *et al.*, 1991; Lee & Coffin, 1991). It was, therefore, decided to use 2 primers for the detection of full-length LUVD and the integration precursor with 2 bases deleted. This method was repeatedly applied to

extraction aliquots from HIV-1_{IIIB} infected cell lines. Despite the detection of early synthesis of LUVD in many of these extracts, the full-length and integration precursor linear molecules were not detected. Since the method was never shown to detect completed LUVD it is possible that it failed to detect template present in the extracts tested. However, the method was a direct copy of that used to detect *env*-LTR sequences and should, therefore, have worked similarly. If it is assumed that the method could detect LUVD, the results indicate that the completed form of LUVD may be very unstable. This is in keeping with the pattern of detection of LUVD synthesis observed in T cell lines, where it was proposed that the molecule was rapidly integrated or circularized upon completion.

Zack *et al.*, (1990) showed that many transcripts in quiescent cells did not complete synthesis. The observation may reflect the instability of full-length transcripts and explain why our method failed to detect these forms. However, the failure to detect these forms in stimulated cells favours the theory that the confirmation of these forms is rapidly changed, effectively escaping our method of detection.

The use of a variety of primer pairings may have been more useful in monitoring the rate of LUVD synthesis. This was used by Zack *et al.*, (1990) to measure the extent of reverse transcription in quiescent T cells. The primers were selected to amplify progressively later transcript sequences, based on the replication model described by Varmus and Swanstrom, (1984). The use of such primers would have increased the sensitivity of detection of LUVD forms by negating the need for the ligation sequence, and would have given more information as to the rate of progression of DNA synthesis. This development was, however, not possible due to both time and financial constraints.

11.3.2 The detection of circular unintegrated viral DNA forms (CUVD).

Early studies on the life cycle of retroviruses proposed that the integration precursor was a covalently closed circular DNA copy of the RNA genome (Varmus *et al.*,

1974a & b; Guntaka *et al.*, 1975; Kakefuda *et al.*, 1977; Shank *et al.*, 1978a & b). It is now known that the linear reverse transcript is the precursor to integration (Brown *et al.*, 1987, 1989; Fujiwara & Mizuuchi, 1988; Lee & Coffin, 1991) and so an alternative role for CUVD was sought.

Analysis of avian retroviral infections elicited a biphasic infection *in vitro*. The acute phase was associated with cell lysis followed by chronicity, with little or no cell death (Battula & Temin, 1978; Keshet & Temin, 1978). Further analysis of these infections associated the lytic phase with the presence of UVD (Keshet & Temin, 1979; Temin *et al.*, 1979; Weller *et al.*, 1980). However, despite extensive research of UVD forms, no direct role in the retroviral life cycle has been determined.

More recently, a link with clinical disease has been demonstrated *in vivo*. UVD has been correlated with osteopetrosis in chickens infected with ALV (Robinson & Miles, 1985) but more importantly, it has been implicated in the cytopathogenicity observed in cats suffering from feline AIDS (Mullins *et al.*, 1986; Hoover *et al.*, 1987). It was, therefore, decided to develop a method to detect CUVD in infected cells, to determine if these viral DNA forms play a role in HIV-1 infections *in vivo*.

The presence of CUVD in HIV-1 infections *in vitro* was initially described using Southern blotting techniques (Pauza & Galindo, 1989; Masuda & Harada, 1990; Pauza *et al.*, 1990; Robinson & Zinkus, 1990). Due to the sensitivity known to be required for *in vivo* HIV DNA detection (Simmonds *et al.*, 1990a; Ferre *et al.*, 1992; Wood *et al.*, 1993) it was decided to develop PCR as a method of detecting both 1 and 2LTR CUVD forms.

HIV-1 has 2 main forms of CUVD which are thought to be products of the linear transcript. The smaller molecule contains 1 LTR sequence and is formed by recombination of the 2 LTR sequences, while the larger form contains 2 LTR sequences and is the product of autointegration (Farnet & Haseltine, 1991). A minor population of circular forms include defective genomes with internal deletions and insertions, rearrangements and inversions (Li *et al.*, 1991; Juriaans *et al.*, 1992).

The detection of CUVD by PCR was first described in 1990 (Pang *et al.*, 1990; Pauza, 1990). Primers were used which amplified across the junction sequence of the circular molecules. Although both studies were able to detect CUVD neither study was able to discriminate fully between 1 and 2LTR forms.

In an attempt to develop a rapid and highly specific method of HIV-1 CUVD detection, it was decided to select pairs of primers which were not only specific to CUVD, but were also able to discriminate between 1 and 2LTR forms. The development of this method is described in detail in section 7.2.

The detection of 1 and 2LTR forms was complicated by a number of issues. The primers selected to amplify both 1 and 2LTR forms were largely specific to LTR sequences. Upon amplification, it was found that these oligonucleotides were priming cellular in preference to viral sequences, resulting in non-specific amplification bands (Figure 18). This was due to the universal presence of control sequences in the viral LTRs and cellular nucleic acid (Starcich *et al.*, 1985). The successful amplification of cellular sequences may have been due to the abundance of cellular DNA present in comparison to viral DNA and/or to the resistance of covalently closed circular DNA to denaturation (Pang *et al.*, 1990; Linz *et al.*, 1990). The amplification specificity was directed towards viral DNA by increasing the amplification to 3 reactions of 25 cycles, and by using non-LTR specific primers in the first 2 reactions to bulk-up the concentration of viral sequences (Figures 20a, b & c). Although tertiary amplifications increase the risk of cross-contamination, samples were screened for 1 and 2LTR CUVD forms by this method. The reliability of the data generated was supported by the lack of contamination in numerous negative controls incorporated.

Despite the efficiency of the method for CUVD detection, the primer selection was only able to definitively detect 2LTR CUVD forms. The band observed with the 1LTR specific primers was also generated by 2LTR forms. To overcome this problem, DNA extracts were re-amplified with alternative primers which hybridized to the *gag* and *env* genes only. In this way, the primers were able to distinguish between 1 and 2

LTR forms by amplifying the entire LTR sequences of the form resulting in a significant band length difference for the 2 forms. As the primers detected both forms in the same reaction it was feared that the primers would preferentially amplify the shorter sequence. This was not found in practice, suggesting that the nested PCR may also be an indicator of the proportion of each form present in the given population (Figure 21).

The technique was initially applied to DNA extracts of cell lines infected with HIV-1 IIIB and RF to establish the sequence of events in HIV-1 DNA synthesis, and was then used to detect the prevalence of CUVD forms in clinical samples.

The detection of CUVD in both IIIB and RF studies was invariably after overall viral DNA detection, although the time delay differed with the cell line studied. The delay in T cell lines infected with HIV-1_{IIIB} ranged from 1 to 11 hours, whereas some non-T cell lines did not have detectable levels of CUVD until day 3, up to 64 hours after initial HIV-1 DNA detection. This is in agreement with Farnet & Haseltine, (1990) who showed that LUVD was detectable by 2h and that CUVD forms were not present until 10h post-infection. The appearance of 1 and 2 LTR CUVD did not follow any obvious pattern, but in all but 1 cell line (U937) they were detected within 1 time point of each other. A greater delay was observed in the detection of CUVD in RF infected cells, although both cultures (complete and serum-free medium) were positive within 1 day of each other. This suggests that viral activity was retarded in serum-free cells, although CUVD forms were synthesized. Careful analysis is required to ensure that serum starvation is sufficient to arrest cells completely. This was not carried out, and so although slowed down, it is possible that C8166 cells maintain cellular activity despite these restrictions. However, the results show that the cells were sufficiently affected, by serum-starvation, to prevent the formation of syncytia. An interesting addition to this study would have been to change to complete medium during the study to see if the ability to form syncytia was returned.

In all cell lines, once CUVD forms had been detected they remained present

throughout the experiment. CUVD was, therefore, either synthesized continuously or was stable in the cell cultures.

Donor PBMCs were negative for both forms of CUVD until day 14 when, as with all cultures of RF, both 1 and 2LTR forms were detected simultaneously. The delay in detection may be due to the small number of infected cells and, therefore, excludes a direct comparison with C8166 infected cells. A more appropriate comparison would have been C8166 cells with a purified $CD4^+$ T helper lymphocytes population (Nielsen *et al.*, 1991).

The appearance of CUVD forms has been associated with cytopathic effects in other retroviral infections (Keshet & Temin, 1979; Temin *et al.*, 1979; Weller *et al.*, 1980). The accumulation of CUVD was efficiently blocked by the addition of neutralising antibody, demonstrating that the build up of CUVD forms was due to secondary or super-infections (Temin *et al.*, 1979; Weller & Temin, 1981; Chen & Temin, 1982; Robinson & Zinkus, 1990). The results with cell lines in this study are not in agreement with the published data, as detection of CUVD did not always correlate with syncytium formation. Also, cells were infected at a low multiplicity of infection for 1 hour and were then washed to remove free virus. It is unlikely, due to the ratio of virus:cells and to the time of exposure, that cells would have been infected by more than one virion. The presence of CUVD, therefore, appears to be attributable to single infections of susceptible cells. Further experimentation using anti-CD4 antibodies to block cell-to-cell and free virus infection may have supported this observation. The discrepancies with published studies on other retroviruses may be due to the insensitivity of their detection methods. Previously, CUVD forms have been detected by Southern hybridization requiring substantial copy numbers for detection (Keshet & Temin, 1979; Temin *et al.*, 1979; Weller *et al.*, 1980). In this study, CUVD forms were detected by sequential amplification with 3 sets of primers, each round potentially increasing the template concentration by more than 10^5 (Ou *et al.*, 1988; Saiki *et al.*, 1988).

Whether CUVD forms are the product of single or multiple infection events, the source of viral DNA is of interest. Upon entry to susceptible cells the virus initiates reverse transcription of its genome which results in the degradation of the RNA template (Varmus & Swanstrom, 1982) Therefore, for each virion, a maximum of 2 copies of LUVD can be synthesized. Since CUVD exists as 2 forms (1 & 2LTR), and is most probably present as multiple copies per cell, infected cells must produce viral DNA by some other mechanism. This could be mediated by cellular or viral DNA polymerase activity, using LUVD or the integrated provirus as a template.

Viral DNA synthesis from the provirus would be in agreement with Farnet & Haseltine, (1990), who showed that CUVD was only detected in the nucleus of infected cells. However, contradictory to this, CUVD has been detected prior to integration in some cell line infections (Gowda *et al.*, 1989; Kim *et al.*, 1989). These studies were based on Southern blot analysis of fractionated DNA extracts and may disagree due to the difference in copy number and not, therefore, accurately reflect the sequential synthesis of HIV-1 DNA forms.

The infection of resting T cells *in vitro* has contributed to the determination of the source of CUVD forms. Such infections have been shown to result in partial reverse transcripts and/or complete viral DNA forms which were unable to integrate (Gowda *et al.*, 1989; Stevenson *et al.*, 1990b; Zack *et al.*, 1990). Although LUVD synthesis was not affected, the accumulation of CUVD was inhibited (Li & Burrell, 1992). The lack of CUVD production in the presence of LUVD synthesis suggests a role for cellular factors for which TNF-alpha has already been implicated *in vitro* (Kitamura *et al.*, 1993).

However, UVD is seen to accumulate if integration is prevented, suggesting that viral DNA can be further synthesized from the initial linear DNA reverse transcript (Stevenson *et al.*, 1990b; Tang *et al.*, 1992). This accumulation has been associated with single cell lysis and the production of p24 antigen, indicating that the LUVD form can act as a template for transcription (Pauza *et al.*, 1990; Stevenson *et al.*, 1990a;

Tang *et al.*, 1992). The unequivocal detection of integrated proviral DNA may have helped determine the source of CUVD. However, largely due to the non-specific integration of the DNA, a satisfactory method of detecting this form by PCR has not been found.

This study was able to demonstrate the presence of at least 3 UVD forms in HIV-1 infection of a variety of cell lines. Although these forms were not quantified, the copy number is known to vary among retroviruses and with respect to the host cell. SNV infection of chicken cells leads to the production of up to 200 copies of UVD per cell in contrast to infections in rat cells which results in approximately 2 copies per cell (Keshet & Temin, 1979). Similarly, *in vitro* infection with HIV-1 produces 20 copies of UVD in H9 cells, 50-100 copies in Hut78 cells and 80 copies per cell in C8166 cells (Kim *et al.*, 1989; Robinson & Zinkus, 1990; Li & Burrell, 1992). The differences observed in detection of viral DNA forms suggests that the virus alone is not responsible for the formation of UVD forms, or for the overall timing of the viral life cycle. It would appear that the cell type plays an important role in these events, a role which may be determined by cellular factors or by the interaction of cellular and viral factors.

The variation observed may also reflect differences in the life cycles of the 2 viral strains (IIIB and RF). Isolates have been shown to have different growth characteristics determined by both the viral *env* sequence and the host cell (Tersmette *et al.*, 1988; de Jong *et al.*, 1992; Kuiken *et al.*, 1992; Fouchier *et al.*, 1992; Andeweg *et al.*, 1993). The IIIB isolate of HIV-1 may be significantly more adapted for rapid growth than the RF strain, resulting in a higher yield of viral DNA. This is supported by the detection of CUVD within 2h of HIV-1_{IIIB} infection, in contrast to RF which did not produce CUVD until day 3. Also, some C8166 cells infected with RF were still viable on day 14, whereas IIIB infected cells died between days 5 to 8, again suggesting that IIIB is a much more aggressive and cytopathic virus.

Using isolates from clinical samples, HIV-1 strains from patients with more advanced

disease have shown more rapid and cytopathic infections in a wider range of cell lines (Cheng-Mayer *et al.*, 1988). These growth properties have been labelled syncytium inducing (SI) and non-syncytium inducing (NSI) and have been shown to be a phenotypic characteristic of the viral isolate (Tersmette *et al.*, 1988). Mutational analysis of viral strains has attributed some of these characteristics to the *vif* gene, as mutants cause slow and non-cytopathic infections (Sakai *et al.*, 1991). More recent studies have shown that sequence variation in the V1-V2 and V3 hypervariable domains of the envelope gene can induce the SI phenotype in transfection studies (Andeweg *et al.*, 1992; Fouchier *et al.*, 1992; Kuiken *et al.*, 1992).

Two of the susceptible non-T cell lines gave interesting HIV-1 DNA patterns. Firstly, the glioblastoma cell line (U138MG) could not be infected with the IIIB strain of HIV-1, demonstrating that not all viral isolates can infect all HIV-1 susceptible cell lines. Gartner *et al.*, (1986) also found the IIIB strain to be 10000-fold less able to infect macrophage lines than T cell lines. Secondly, the embryonic lung fibroblast line (LC5), although infected by HIV-1, failed to produce detectable levels of UVD throughout the experiment. LC5 cells, therefore, appear to demonstrate true latency in this experiment. An important continuation of this work would be to repeat the study with other viral isolates, and look for patterns of UVD which may reflect viral or cellular control factors. In this way, it may be possible to obtain more cell lines which, like LC5, appear to become infected but are able to control the infection. It is possible that careful analysis of the biochemistry of such cell lines could reveal modulating factors which could be adopted therapeutically.

The progression of this work should include quantification studies to determine whether or not these forms coexist in individual cells or are mutually exclusive forms which are all derived from LUVD. If the latter was true, the induction of CUVD formation may prove to be of therapeutic value in the prevention of proviral integration. The build up of CUVD has been associated with TNF-alpha induction

(Kitamura *et al.*, 1993), however, in line with other retroviral infections it is more likely that these viral DNA forms are cytopathogenic (Weller *et al.*, 1980; Weller & Temin, 1981; Chen & Temin, 1982; Mullins *et al.*, 1986; Hoover *et al.*, 1987).

However, despite some associations with persistent infections *in vivo* (Rasty *et al.*, 1990), the accumulation of UVD has largely been correlated to cytopathology in HIV and other retroviral infections (Keshet & Temin, 1979; Temin *et al.*, 1979; Pauza *et al.*, 1990; Bergeron & Sodroski, 1992). It was, therefore, postulated that infection of quiescent T cells *in vivo* may lead to the accumulation of UVD as observed *in vitro* (Stevenson *et al.*, 1990b). A possible consequence of this is that all T cell stimulants may potentially induce HIV DNA integration and viral production leading to an increase in the number of infected cells and HIV-associated cell death.

In view of this, it was decided to determine the frequency of detection of CUVD forms in patient PBMC samples, and to attempt to correlate these findings with clinical markers of HIV infection.

11.4 Detection of HIV DNA in patient PBMC samples.

All patient samples were initially tested for the presence of HIV *pol* and *env* DNA sequences using a nested PCR. In this section, PCR was initially developed to confirm HIV-1 infection in seropositive patients diagnosed by ELISA and Western blot. The technique allowed the rapid detection of viral DNA from PBMCs demonstrating the presence of a product of viral replication.

The *pol* gene is highly conserved among HIV clinical isolates and is, therefore, the preferred sequence for amplification to detect DNA in the majority of patient samples. In contrast, the *env* gene contains variable regions which have been sequenced and characterized using PCR (Hahn *et al.*, 1986; Starcich *et al.*, 1986; Saag *et al.*, 1988; Simmonds *et al.*, 1990b; Wolfs *et al.*, 1990). The primers used for *pol* and *env* detection were those of Simmonds *et al.*, (1990a).

HIV-1 DNA sequences were detected by *pol* and/or *env* amplification in all 32

seropositive patients, although 2 of the 61 samples from these patients gave a negative result. The most plausible explanation for this is the absence of template in the amplification aliquot. Amplifications were carried out on DNA aliquots ranging from $7x10^3$ to $1x10^6$ cell equivalents. Published quantifications of HIV load indicate that the limit of detection may be as high as 4 to $8x10^4$ cells, it is possible, therefore, that some of the aliquots used in this study were inadequate (Simmonds *et al.*, 1990a; Srugo *et al.*, 1991).

11.4.1 Observation of variation in the *env* gene.

Analysis of sequence variation in the envelope gene was not studied, however, after amplification with *env*-specific primers, band length variations were detected by agarose gel electrophoresis. The band length varied by a minimum of 20-40 bases, and variation was observed between patients, in sequential samples from individual patients, and in nuclear and cytoplasmic extracts from single patient samples. This was in agreement with published data on the *env* gene, which show considerable sequence variation among and within patient samples (Meyerhans *et al.*, 1989; Balfe *et al.*, 1990; Nowak *et al.*, 1990; Pang *et al.*, 1991; Simmonds *et al.*, 1991). This variation is thought to contribute to cellular tropism (Cheng-Mayer *et al.*, 1990, 1991; Liu *et al.*, 1990; York-Higgins *et al.*, 1990; Cann *et al.*, 1992), the ability to infect cells in different biological compartments of the body such as the blood and cerebrospinal fluid (Liu *et al.*, 1990; Pang *et al.*, 1991; Steuler *et al.*, 1992), and in viral uptake and cytopathogenicity (Dalgleish *et al.*, 1984; Klatzmann *et al.*, 1984; Fauci, 1988; Garry, 1989). Some studies have shown a direct correlation between genetic diversity and more progressed disease, whilst others have not (McNearney *et al.*, 1990; Wolfs *et al.*, 1990).

Despite the extensive variation observed in the envelope gene, it is important to keep a perspective on the significance of these results. PCR amplification will occur if the primers bind, and cannot distinguish between infectious and non-infectious

sequences. To analyse significant variation would, therefore, require a purification step to isolate infectious sequences prior to amplification. This could be achieved by total RNA extraction of filtered plasma, or by antibody mediated extraction of whole virus RNA from clinical samples.

11.4.2 Detection of LUVD in seropositive patient PBMCs.

No linear UVD was detected in extracts of patient PBMCs. Since LUVD was not detected in all cell line studies, and in particular in the synchronised infection of donor PBMCs, the lack of detection *in vivo* was anticipated. Detection of LUVD *in vivo* is unlikely, due to the lack of synchronizations of the infection, and the small percentage of actively infected cells at any one time (Mathez *et al.*, 1990). It has been estimated that between 0.01 and 1% of patient PBMCs are infected with HIV (Psallidopoulos *et al.*, 1989; Schnittman *et al.*, 1989). Additionally, only a small fraction of the cells are actively producing virus at a given time (Harper *et al.*, 1986). The infected cells which are not transcriptionally active may be quiescent T cells which are thought to require activation for viral production (Gowda *et al.*, 1989; Stevenson *et al.*, 1990b; Tang *et al.*, 1992). However, the detection of LUVD might have been enhanced by increasing the amplification aliquot and by removing high molecular weight DNA which may have reduced the efficiency of the reaction. The lack of detection was, however, not due to technical difficulties as LUVD forms were detected, by this method, in cocultivated cells of clinical isolates and extracts of infected cell lines.

11.4.3 Detection of CUVD forms in seropositive patient PBMCs.

Due to the time constraints of this study, analysis of CUVD forms in clinical samples was cross-sectional. CUVD forms were detected in 34 of the 52 samples tested, relating to a total of 32 patients (15 positive for CUVD forms in sequential sample; 12 patients consistently negative; and 5 patients positive and negative in different PBMC samples). One patient (samples P12a & P12b) was positive for both 1 and 2

LTR forms over a period of 16 months. As found in infected cell lines *in vitro*, the apparent persistence of CUVD forms may be attributable to prolonged synthesis or to their stability in infected cells. One patient had detectable CUVD forms although the 2 LTR CUVD amplification resulted in a strong positive band (approximately 400bp) slightly smaller than the expected band of 447bp. Although further analysis was required, it was postulated that the band was due to aberrant forms of 2 LTR CUVD as described in published findings (Li *et al.*, 1991; Juriaans *et al.*, 1992).

Longitudinal analysis was possible in some patients where samples had been collected over a period of months. Sequential samples from 2 patients (P19 & P28) converted from CUVD negative to positive over 8 and 3 months respectively, with patient 28 still positive 5 months later. More interestingly, 2 patients lost detectable levels of CUVD. Patient 23 was positive in sample P23a, but was negative 2 months later (P23b). However, this may have been a false-negative result as the amplification aliquot used was negative for *env* sequences despite the previous sample (23a) being *env* positive. The second patient, (P17), was CUVD positive for 2 sequential samples 2 months apart (P17a & P17b) and was negative for the third sample (P17c) 2 months later. The loss of CUVD forms is contradictory to their continual synthesis and/or stability in PBMCs. Interestingly, the loss of CUVD forms was associated with a drop in $CD4^+$ cell counts from 240 to 100 cells per millilitre over the 4 months. The loss of CUVD was, therefore, associated with cell death as observed in retroviral infections of cell lines *in vitro* (Keshet & Temin, 1979; Temin *et al.*, 1979; Weller *et al.*, 1980).

Although this study has clearly demonstrated an association between CUVD forms and disease stage, an extensive longitudinal study is required to determine if these viral DNA forms are related to disease progression. The study would ideally follow patients over a period of 5-10 years, with sequential samples tested for viral DNA and RNA populations as well as surrogate markers of viral replication and disease progression. The study would also have to determine the effects of anti-retroviral

therapy. Statistical analysis of the data reported here showed a correlation of AZT therapy with the detection of CUVD forms. Published data on quantitative analysis of UVD in patients on antiviral therapy showed a considerable reduction in UVD copy number after therapy (Dickover *et al.*, 1992). Although no discrimination was made between linear and circular forms, this is contradictory to the findings of this study in which a significant correlation between CUVD forms and AZT therapy was found. This could be due to the common association of AZT therapy and CUVD forms with more advanced disease, and/or to the lack of quantitation of the circular forms in this study.

The decrease in UVD observed by Dickover *et al.*, (1992) was accompanied by an increase in integrated viral DNA. From other studies (Gowda *et al.*, 1989; Stevenson *et al.*, 1990b; Zack *et al.*, 1990; Tang *et al.*, 1992), these results suggest that the antiviral therapy may be stimulating infected quiescent T cells resulting in the integration of UVD transcripts. Due to the requirement of integration for retroviral replication, this is a highly undesirable action of an antiviral drug. Since most drugs act on the viral polymerase, the increase in integrated copies will facilitate the production of more infectious virus. This in turn may lead to infection of more susceptible cells, and enhance the emergence of drug resistant virions.

All patients analysed for CUVD forms had received AZT therapy. The average time of therapy for CUVD negative samples was 3 months (range 1-7) as opposed to CUVD positive samples which had an average of 9 months (range 1-23). It is, therefore, possible that fluctuations of CUVD levels did occur but were not detected due to the lack of quantitation of these results. *In vitro* studies have also shown AZT therapy to reduce the copy number of UVD in cells, although this was observed to be a transient effect which returned to near baseline levels by 3 days post treatment (Pauza *et al.*, 1990). Unfortunately, levels of integrated DNA were not monitored.

11.4.4 Viral DNA forms in nuclear and cytoplasmic extracts of patient PBMCs.

In trying to ascertain a role for CUVD in the life cycle of HIV-1 replication, it is important to determine the localization of these forms. Hybridization studies established that both 1 and 2 LTR forms were only present in nuclear extracts of cell lines (Kim *et al.*, 1989; Farnet & Haseltine, 1990). To investigate further, nuclear and cytoplasmic extracts were prepared from patient PBMCs to determine the localization of CUVD forms *in vivo*. The technique required gentle lysis of the cell membrane, leaving the nucleus intact to allow separation of the nuclear and cytoplasmic DNA extracts.

CUVD forms were detected in cytoplasmic extracts in 5 out of 7 patients tested, in direct contrast to the findings of Farnet & Haseltine, (1990). Although great care was taken to avoid disrupting nuclei prior to cytoplasmic separation, it was possible that nuclei were lysed releasing UVD into the cytoplasmic extract. Although this contamination would have been acceptable for hybridization analysis, it may have led to the false detection of HIV-1 sequences, by PCR, in cytoplasmic extracts.

Both nuclear and cytoplasmic extracts of some patients were positive for viral DNA but negative for CUVD forms. This could be due to the difference of amplification efficiencies of the *pol*-and CUVD-specific primers. The *pol* primers have been shown to be highly specific and efficient at amplifying viral sequences in both this study and by Simmonds *et al.*, (1990a). In contrast, the primers for CUVD detection have clearly been shown to be both viral and T-cell specific in this study, and so their amplification efficiency for viral CUVD detection will be reduced. It is, therefore, possible that CUVD forms were detected by the *pol*-specific primers and not by the CUVD-specific primers. This would account for the absence of CUVD forms in HIV DNA positive cytoplasmic extracts. Alternatively, the detection of viral DNA in the cytoplasm may have been due to the presence of LUVD. Similarly, the detection of viral DNA in nuclear extracts may have been due to the detection of linear or circular forms, but will also have included the detection of integrated provirus. However, due

to the lack of a discriminating method of integrated proviral detection, it was not possible to prove this.

11.5 STATISTICAL ANALYSIS OF DATA.

11.5.1 Correlation of the detection of CUVD forms with other markers of infection.

The detection of CUVD forms was analysed to correlate their presence with other viral markers and the patient's disease stage. The study was cross-sectional due to the small number of sequential samples from individual patients, and so the data could only be used to correlate markers to disease stages and not to disease progression which would have required a longitudinal study. Due to the slowly progressive nature of HIV infection, such a study would have required data collected over a period of 5-10 years which was clearly beyond the scope of this study.

The detection of CUVD was compared with virus isolation, p24 antigenaemia, total $CD4^+$ cell counts, AZT antiviral therapy, and the patients CDC grouping. Statistical analysis was by the Chi-square test, and the Yates "correction for continuity" was applied to compensate for the small number of samples (Swinscow, 1991).

The detection of CUVD forms was statistically significant in samples from patients receiving antiviral therapy, and from patients with more advanced disease. The interpretation of this data was difficult because of the known association of various markers with disease stage. For example, it seemed unlikely that patients receiving antiviral therapy would be more likely to have a build up of CUVD forms in the peripheral blood when the action of the drug is to terminate reverse transcription of the viral genome. In an attempt to clarify the situation, a similar comparison was made between viral markers and CDC groups. Significant associations were demonstrated with virus isolation, p24 antigenaemia, and the use of antiviral therapy. Therefore, the association of AZT therapy with CUVD detection may be attributable to the fact that both these factors correlate with more advanced stages of the

disease.

The lack of correlation between some markers may have been due to the small sample sizes analysed. Sufficient data could not be generated in the time available, and so it was decided to expand the data by multiplying by a factor of 2 or 3 to obtain a minimum of 100 results. The analysis of this data was not intended to be used in place of the genuine results, but merely as an indicator of the possible associations had the raw data been more extensive.

Once re-analysed, significant correlations were established between CUVD detection and $CD4^+$ cell counts of less than or equal to 200, and p24 antigenaemia. Similarly, a correlation was established between more advanced disease and p24 antigenaemia. Due to these associations with disease stage, it is difficult to determine if CUVD detection is correlated to other markers of infection, or if the associations observed are artifacts of the common association with a more advanced disease stage.

Expansion of this data on more clinical isolates is required to substantiate the results. However, from the data obtained, the significant association of CUVD forms and more advanced disease stage suggests a potential role for these DNA forms in the cytopathogenicity of HIV-1. This is in agreement with recent studies in feline AIDS, which have shown UVD forms to be associated with clinical disease (Mullins *et al.*, 1986; Hoover *et al.*, 1987).

11.6 DETECTION OF HIV-1 INFECTION IN PERIPHERAL BLOOD MONONUCLEAR CELL SUBSETS.

T lymphocytes are known to be the primary site of infection in PBMCs, HIV-1 infection of these cells alone does not explain the complex immunodeficiency observed in seropositive individuals with advanced clinical disease. To understand the pathology of the disease, and also for the development of therapeutic strategies, it is important to know which cell types are infected and subsequently affected by

HIV-1 infection.

HIV infects T helper/inducer cells via the CD4 surface marker, and so other cells expressing this antigen have been assayed *in vitro* for their susceptibility to infection. Monocyte/macrophage cells express a low copy number of CD4 antigens on their surface and so these adherent cells have been separated and inoculated with laboratory strains of HIV-1 (Wood *et al.*, 1983). *In vitro* studies showed that blood monocyte/macrophage cells were susceptible to HIV, demonstrating a less cytopathic and more persistent infection than seen in $CD4^+$ T cells (Gartner *et al.*, 1986: Ho *et al.*, 1986, 1992; Nicholson *et al.*, 1986; Salahuddin *et al.*, 1986; Koyanagi *et al.*, 1988; Kazazi *et al.*, 1989). Further *in vitro* studies have shown that it is possible to infect other antigen presenting cells such as alveolar and peritoneal macrophages (Salahuddin *et al.*, 1986; Olafsson *et al.*, 1991), brain glial cells (Cheng-Mayer *et al.*, 1987; Christofinis *et al.*, 1987; Dewhurst *et al.*, 1987; Kunsch *et al.*, 1989) and dendritic cells (Patterson & Knight, 1987).

Cells deficient in CD4 antigen have also been infected via uptake mechanisms involving Fc receptors and other undefined modes of entry. In this category are epithelial cells, lung fibroblasts, chondrocyte cells, synovial cells, foreskin fibroblasts (Ikeuchi *et al.*, 1990), $CD8^+$ T lymphocytes (de Maria *et al.*, 1991), B cells (James *et al.*, 1990) and natural killer cells (Chehimi *et al.*, 1991).

However, the ability to infect cells *in vitro* says little about the role they play in the infection *in vivo*. Studies on visna-maedi lentiviral infections in sheep have demonstrated viral tropism for monocyte and macrophage cells, and a source of persistence in precursor cells in the bone marrow (Gendelman *et al.*, 1985). This was an important development to the understanding of the mode of viral persistence in sheep, as differentiated macrophages were known to have a short life-span and were, therefore, unlikely candidates for the long term maintenance of infection (Gendelman *et al.*, 1985; Potts *et al.*, 1990; Spear *et al.*, 1990). FIV sequences have also been detected in bone marrow tissue in naturally and experimentally infected

cats (Beebe *et al.*, 1992). A similar role in persistence may be found for human macrophages in HIV-1 infections, as bone marrow progenitor cells have been infected with HIV-1 *in vitro* (Folks *et al.*, 1988; Steinberg *et al.*, 1991). It is important to examine if these cell subsets are infected *in vivo* to establish their role in the persistence and dissemination of the disease.

11.6.1 Separation of peripheral blood mononuclear cells into cellular subsets for PCR analysis.

Monocytes and macrophages are well documented as being targets for persistent HIV *in vivo* (Gartner *et al.*, 1986; Ho *et al.*, 1986; Koenig *et al.*, 1986; Nicholson *et al.*, 1986; Tschachler *et al.*, 1987). The viral tropism for cell subsets is determined by the surface glycoproteins encoded for by the *env* gene, and is associated with the rate and efficiency of the early stages of viral entry into the cell (Cordonnier *et al.*, 1989; Cheng-Mayer *et al.*, 1990, 1991; Kim *et al.*, 1990; Liu *et al.*, 1990; York-Higgins *et al.*, 1990; Hwang *et al.*, 1991; Srivastava *et al.*, 1991; Westervelt *et al.*, 1991). Viral isolates have been recovered which show selective tropism for $CD4^+$ lymphocytes and monocytes. Monocytic isolates generally replicate more slowly with little cell pathology, and T-cell tropic strains replicate rapidly and form syncytia (Tersmette *et al.*, 1988; von Briesen *et al.*, 1990; Liu *et al.*, 1990; Schuitemaker *et al.*, 1991, 1992). The monocytic, or non-syncytium inducing (NSI) isolates, are more frequently isolated from asymptomatic patients, and SI isolates are more often recovered from AIDS/ARC patients (Tersmette *et al.*, 1988, 1989; Schuitemaker *et al.*, 1991, 1992). In a study by Gendelman *et al.*, (1988) macrophage-tropic strains were isolated from most patients, and up to 90% of cells demonstrated infection as determined by RNA detection and electron microscopy. Despite this level of viral activity, budding was rarely seen from the cell membrane (Dahlberg *et al.*, 1981; Lairmore *et al.*, 1987; Orenstein *et al.*, 1988). HIV infection of monocytic cells was also observed to differ from lymphocytic cells in that viral surface glycoproteins were only observed in the

cytoplasm of monocytes, but were detected in the cytoplasm and on the cell surface of infected lymphocytes (Potts *et al.*, 1990). This supports the theory that lentiviral infections of monocytic cells can act as "Trojan horses", by avoiding immune surveillance thereby contributing to the persistence and dissemination of the infection (Gendelman *et al.*, 1985; Haase, 1986; Potts *et al.*, 1990).

To determine the extent of monocytic cell involvement in the *in vivo* infection, it was decided to separate monocytic cells from patient PBMCs, and to analyse their viral DNA content by PCR.

Preliminary studies separated the cells utilising the ability of monocytic cells to adhere to plastic. PBMCs separated from HWB on a Lympho-paque gradient were incubated for 3 days to allow adherence of cells. Although a short incubation of 45min would have been sufficient for attachment, cells were left for 3 days to allow non-monocytic cells to detach.

Monocytes from 11 patient PBMC samples were separated by this method. The samples represented patients from all stages of infection (3 from CDC II; 7 from CDC III; and 1 from CDC IV) although the number of samples tested was insufficient for detailed analysis. Samples from ten of the patients (all except P28) had been amplified by PCR for HIV-1 DNA sequences in PBMC DNA extracts, and 9 were positive for aliquots equating to 2-4x10^4 cells (Appendix IV). Patient 25 (sample P25a) was negative for HIV-1 DNA in an aliquot of 4x10^4 PBMCs, although a sample from the patient taken 9 months later was PCR positive (aliquot 2x10^5 cell equivalents).

HIV-1 DNA was detected in adherent cell extracts in 5 of these patients. This suggests that adherent cells of the peripheral blood are infected with HIV in some patients. However, the detection of HIV-1 DNA may have been due to the presence of contaminating $CD4^+$ cells. Published studies of cell separation by adherence alone claim purities in excess of 90% (Pennline *et al.*, 1981). Although this can be improved to >99% by the addition of 20-40% serum to the culture medium to reduce

B cell contamination, and by removing contaminating lymphocytes by complement mediated lysis, the separation is still based on non-specific selection (Gartner *et al.*, 1986; Spear *et al.*, 1990). HIV has been detected in patient peripheral blood monocyte/macrophage cells by isolation and by PCR detection of viral sequences (Ho *et al.*, 1986; Psallidopoulos *et al.*, 1989; Schnittman *et al.*, 1989; Spear *et al.*, 1990). In these studies, separation of the cells from PBMCs generally involved 2 purification steps. Adherence was most commonly used followed by complement mediated lysis of residual contaminating cells resulting in purities of 96% to 99% (Nicholson *et al.*, 1986; Gendelman *et al.*, 1988; Psallidopoulos *et al.*, 1989; Schnittman *et al.*, 1989; von Briesen *et al.*, 1990; Gendelman *et al.*, 1990; Spear *et al.*, 1990). Despite these claims of high purities, any contamination by $CD4^+$ T helper cells may have resulted in false detection of viral sequences.

To improve the purity of cell preparation, and to expand its application to other cell subsets, an alternative separation technique was sought, based on the detection of surface antigens characteristic of the cell subsets. To achieve this, sets of commercially available antibody-coated magnetic beads were used to capture monocytic cells as well as T helper/inducer, T cytotoxic/suppressor and B lymphocytes.

There were 2 main approaches available for the antibody-coated Dynabead separation of patient PBMCs. Firstly, cells could have been fractioned into equal aliquots and each incubated with a different specificity of Dynabead. However, this method reduced the number of each cell type available for separation, and would have resulted in $CD4^+$ monocytes being removed with T helper/inducer cells. Patient samples were, therefore, separated by the alternative method of sequential incubations with the 4 different antibody coated Dynabeads. Cells were initially incubated with anti-CD19 coated beads, as B cells were the least abundant cell type to be removed. $CD14^+$ cells were removed next, to reduce the contamination of the $CD4^+$ T cell subset with monocytes expressing the CD4 antigen and finally, CD8 and

CD4 positive cells were removed. The main drawback of this method was that any microspheres transferred with the remaining cells, could potentially contaminate the next cell separation.

The microspheres were mixed with cells at a ratio selected to obtain maximum purity by positive selection, and to reduce the possibility of complex rosette formation between the cells and beads. The formation of rosettes would have resulted in a high percentage of the target cells being removed, but would also have complicated further analysis of the cells by inhibiting interactions with cell surface markers and by physically blocking the delivery tip of the EPICS machine. However, this phenomenon may have been observed with samples isolated by anti-CD8, anti-CD14, and anti-CD19 coated beads.

The FACS analysis of the cells separated by Dynabeads proved very difficult to interpret. The general fluorescence patterns observed for sorted cells depicted mixed cell populations with low labelling efficiency. Analysis of the appropriate bead and cell controls showed that the magnetic microspheres did not contribute to the non-specific binding, but did reveal the labelling procedure to be inefficient. Unfortunately, due to time constraints, it was not possible to develop this method further.

However, from the data obtained from these preliminary studies, it was clear that existing single-step separation techniques were inadequate for further analysis by PCR. From the known sensitivity of PCR as a detection method, 100% purity is required. Although a simple and rapid method was desirable, the application of a 2 step separation technique was required to obtain a higher purity for PCR analysis. Monocytic cells could, therefore, be separated by adhesion over a short period of time followed by antibody specific binding to Dynabeads. Pure samples of $CD4^+$ and $CD8^+$ cells would be most readily achieved by separating T lymphocytes and depleting the sample of all undesired subsets by complement mediated lysis.

The antibody-coated Dynabeads have been used for the separation of cell subsets in published studies. Brinchmann *et al.*, (1988) claimed to have separated B and T cells

to a similar degree by FACS and antibody-coated Dynabeads. However, the purity of cells separated by the beads was not proven as the comparison was made on a quantitative basis. The cells attached to the beads were gently lysed and the released nuclei were subsequently counted and compared with the FACS data. In contrast, Gaudernack *et al.*, (1986) separated CD8 positive cells from PBMCs and demonstrated a purity of 95-97% of $CD8^+$ cells with 1-5% contamination with $CD4^+$ cells, and Folks *et al.*, (1989) separated $CD34^+$ myeloid progenitor cells from bone marrow with a purity of 99-100% as determined by immuno-staining and flow cytometry. The use of commercially available Dynabeads can be a highly specific and rapid method of cell separation. The purity of the product can be improved by careful handling and by the specificity of the coating antibody. The separation of cell subsets in this study was unsatisfactory due to the inability to demonstrate the purity of the sample. This may have been due to the use of an inappropriate method of analysis as the Dynabeads may have inhibited the attachment of labelled antibodies, and also changed the properties of the cells as analysed by the EPICS machine. However, despite the high purities achieved by other researchers (Gaudernack *et al.*, 1986; Folks *et al.*, 1989), the method was not fully suitable for PCR analysis.

Despite doubts over the purity of samples obtained by immunomagnetic selection, cell subsets were separated from PBMCs of patient 20 (sample P20c) using anti-CD14 antibody coated Dynabeads. The DNA extract from the purified cells was positive for HIV-1 DNA after PCR amplification, in agreement with the adhesion separated cell results for a sample from the same patient taken 9 months earlier (P20b). Although the purity of the cell preparations was not established, 6 of the 12 patient samples tested were PCR negative for HIV-1 DNA. These patients were all positive for viral sequences in PBMC aliquots and, therefore, demonstrate the selection of an HIV-1 negative cell subset. If the cell samples analysed are assumed to be pure, the results clearly show that HIV infects monocytic cells in some, but not all patients. This may relate to the pathology of the disease, to viral markers (eg. p24 plasma antigen, UVD), or to the clinical stage of the disease (CDC groups).

Unfortunately, analysis of these associations was not possible due to the small sample size.

Although cells were not quantified, it was clear that the total number of cells separated from some patients was small. The negative results could, therefore, have arisen from the absence of any infected cells due to inadequate sample size. The importance of aliquot size was further illustrated by the detection of HIV-1 DNA in the adherent cell extract of patient 25. This sample had previously been tested negative for viral DNA in an extract equivalent to 4×10^4 PBMCs. Although this could have been a quantitative effect, the patient may have harboured an NSI isolate which was only present in monocytic cells and was, therefore, below the level of detection in the PBMC extract.

In addition to the evidence of infection of monocytic cells obtained by PCR, the comparison of adherent cells from infected and uninfected individuals suggested that the cells were affected by HIV-1 infection. The cells from negative controls were observed to adhere rapidly and show a change in morphology over the following 3 days. Cells appeared to flatten and extend appendages over the surface of the plastic. In contrast, adherent cells from infected individuals showed no morphological changes with reduced adherent properties and a greater tendency to detach over time in agreement with Lioy *et al.*, (1993). These observations support the theory that monocytic/adherent cells are affected by HIV-1 infection and may themselves harbour the virus.

This area of research is currently undergoing expansion. PCR is being used to sequence the NSI and SI isolates, demonstrating a genetic basis to their tropism and cytopathogenicity (Fouchier *et al.*, 1992). The work is also moving towards the *in vivo* analysis of cell subsets which have been shown to support viral replication *in vitro*. This work may help explain the complex immunodeficiency observed in the later stages of HIV-1 infection, and may also open up areas for future targets of antiretroviral therapy.

11.7 Detection of viral RNA.

Although the detection of HIV DNA is indicative of an established infection, it does not correlate to the replicative state or infectious nature of a sample. A more informative method is where viral RNA is detected by PCR after a reverse transcriptase step (RT-PCR). This can be used to demonstrate the presence of free virus and/or transcriptionally active infected cells (Zhang *et al.*, 1991; Bagnarelli *et al.*, 1991a).

In this study, RNA was extracted from PBMCs, adherent cells and plasma from a small number of samples. In each extraction total RNA was recovered, in the presence of RNase free DNase, and reverse transcribed prior to amplification. The RNA may have been genomic and/or subgenomic transcripts. The samples used had all been stored at -70˚C over a period of months. The central aim was to establish a method of RNA detection, but also to show that viral activity was occurring in patient cells.

The plasma sample was included as a source of free virus. The patient was not selected for any reason other than plasma was available on the day of the experiment. Despite a positive virus isolation from PBMCs, the plasma sample was negative for HIV RNA sequences. Although the majority of patients are now thought to be viraemic (Ho *et al.*, 1989; Ottmann *et al.*, 1991) the concentration of RNA present in this sample may have been below the level of detection, particularly as the plasma was not ultracentrifuged prior to extraction.

PBMCs from sample 7a were isolation and HIV RNA positive. Although the patient was p24 antigen negative at the time of sampling, virus isolation, HIV RNA and CUVD detection are all indicative of viral activity.

RNA was extracted from a total of 6 adherent cell samples. Again these samples were not selected specifically for this experiment, and consequently only 1 sample

was from an antigenaemic patient. This sample was subsequently found to be the only HIV RNA positive adherent cell extract.

Due to the small number of samples tested, it was not possible to comment on the correlation of HIV-1 RNA detection with other viral markers. A larger study by Bagnarelli *et al.*, (1991a) detected HIV RNA in 23 out of 37 PBMC samples, but found no correlation with p24 antigenaemia. This is not particularly surprising as cellular sequences may represent subgenomic length transcripts and not relate to antigenaemia or viraemia. To detect HIV RNA from infectious virus would require an initial purification step to isolate whole virus. Although it is known that defective viral genomes can be enveloped, it was decided to develop a method of detecting infectious viral RNA by extracting total RNA from captured HIV-1 particles (Ottman *et al.*, 1991). This was achieved by coating polystyrene beads with anti-gp120 antibodies and incubating the beads with the patient samples.

Two PBMC coculture positive samples were used to develop this method. Supernates, containing the clinical isolates, were incubated with beads coated with 1 of 4 anti-gp120/160 specific antibodies. Both isolates were captured using the anti-gp120 specific monoclonal antibody (ADP361, Appendix II) and subsequently detected after RT-PCR. Further attempts to capture virus by this method were unsuccessful and so it was decided to use a viral supernatent of known $TCID_{50}$ to estimate the limit of detection. The method used was very crude but indicated that the technique required in excess of 6×10^4 RNA templates for a positive result.

Although efficiencies differ due to assay conditions and oligomers utilised, a quantitative RT-PCR study calculated the efficiency of the RT step to be 5% for a sequence of 400-500bp (Zhang *et al.*, 1991). In addition to the poor efficiency of the RT method, the capture step will have decreased the efficiency even further. The antibody used for this step was raised against $HIV\text{-}1_{IIIB}$ and may not, therefore, efficiently recognise other viral isolates. Alternative approaches to virion capture by immunoselection have utilised mixtures of monoclonal and polyclonal antibodies to

overcome this problem (personal communication, C. Loveday). The insensitivity of the RT-PCR method for whole virus capture may, therefore, be a combination of a low specificity antibody and an inefficient RT step. Considerable optimisation of both the virus capture and RT steps is required to validate this method of HIV genomic RNA detection.

Published methods of HIV RNA detection have largely concentrated on the detection of total plasma RNA which may be genomic or subgenomic transcripts released from lysed cells (Ottman *et al.*, 1991). Studies have detected viral RNA in the majority of patients from all clinical stages with little or no correlation to p24 antigenaemia and $CD4^+$ cell counts (Bagnarelli *et al.*, 1991b; Ottman *et al.*, 1991; Zhang *et al.*, 1991). However, RT-PCR of neat serum by Holodniy *et al.*, (1991) correlated HIV RNA detection with p24 antigenaemia and $CD4^+$ counts below 400/ml. The method differed from the others in that RNA was extracted from neat sera as opposed to an ultracentrifugation pellet. The difference in results may, therefore, be due to a concentration effect.

Although the capture assay was relatively unsuccessful, the capture of infectious particles is an important step for analysis of genomic RNA. The sensitivity of the assay may be improved by using smaller beads, a cocktail of specific antibodies, and/or by further optimising the antibody coating procedure. Alternatively, genomic RNA may be captured by poly T tails bound to a solid phase in individual vials or microtitre plates. The development of an assay to detect virion associated HIV RNA would be extremely useful in sequence analysis, particularly in determining the significant sequence variations; in the assessment of loss of sensitivity to therapeutic agents; and in the quantification of plasma viraemia.

CHAPTER 12: DETECTION OF HBV DNA IN HEPATITIS B SEROPOSITIVE SERA.

The initial aim of the HBV study was to apply PCR to the detection of HBV DNA sequences in the serum of a cohort of patients, and to correlate the results to the presence of HBeAg.

The hepatitis B e antigen is present during the early stages of infection, with seroconversion to anti-HBe positive being indicative of resolution of the acute infection (Figure 4). The antigen has been well characterised as a marker of viral replication, and has been closely associated with the detection of DNA polymerase activity and HBV DNA in the blood of infected individuals (Alberti *et al.*, 1979; Alter *et al.*, 1976; Cappel *et al.*, 1977; Imai *et al.*, 1976; Weller *et al.*, 1982; Hadziyannis *et al.*, 1983; Lieberman *et al.*, 1983; Scotto *et al.*, 1983; Karayiannis *et al.*, 1985; Bonino *et al.*, 1986; Carloni *et al.*, 1987; Cheng *et al.*, 1989).

The application of PCR to HBV DNA detection in sera has verified this association, demonstrating a 100% correlation of HBeAg with HBV DNA (Larzul *et al.*, 1988, 1989; Kaneko *et al.*, 1989a, 1989b; Liang *et al.*, 1989; Okamoto *et al.*, 1989b; Sumazaki *et al.*, 1989; Ulrich *et al.*, 1989; Zeldis *et al.*, 1989). While HBeAg is associated with the presence of hepatitis B virions and infectivity, the absence of detectable HBeAg does not exclude the presence of infectious viral particles. The increased sensitivity offered by PCR has shown that viral DNA sequences are present in the serum of patients with no detectable HBeAg (Kaneko *et al.*, 1989a & b; Liang *et al.*, 1989; Okamoto *et al.*, 1989b, 1990; Zeldis *et al.*, 1989). Similar results were obtained in this study using PCR to amplify a fragment of the S-gene. HBV DNA was detected in 100% of HBeAg positive sera, 10% of HBe negative sera and 32% of anti-HBe positive sera.

12.1 Determination of the potential infectivity of HBV DNA positive sera.

Determining the infectious status of patients is not only of scientific interest, but also

has implications in the control of transmission from infected individuals. The infectivity of patients is particularly important in those requiring surgical procedures, and in the management of HBsAg carriers. Hepatitis B carriers are not only an important source of both vertical and horizontal transmission, but are also at a greater risk of progressing to chronic liver disease and hepato-cellular carcinoma (Chen *et al.*, 1986). It may, therefore, be of use to detect any HBV DNA in the plasma, and in association with the patients HBe status determine the form of viral DNA present in an attempt to correlate it with disease progression.

The detection of viral sequences is largely assumed to correlate with the presence of whole virus in the patient's serum and, therefore, the detection of HBV DNA in HBe negative and anti-HBe positive sera suggested that these samples were also infectious. However, early data correlating the presence of HBeAg with HBV DNA also included infectivity studies where HBeAg positive sera were found to be approximately 10^8-fold more infectious than anti-HBe positive sera (Shikata *et al.*, 1977). Taking that information in conjunction with the data from this present study, where HBe negative sera were less likely to contain HBV DNA than anti-HBe positive sera, it was postulated that the viral sequences detected after seroconversion, from HBeAg positive to negative, do not correlate to whole free virus and, therefore, infectivity. In the absence of infectious free virus, the detection of viral sequences in HBeAg negative sera may be attributed to immune complexed virus or free viral DNA released from necrotic hepatocytes (Carman *et al.*, 1989; Liang *et al.*, 1989).

In this study, the forms of viral DNA present in clinical samples were determined by selective separation of whole virus and IgG- and IgM-complexed virus prior to DNA extraction. This was achieved by using polystyrene beads coated with anti-HBs, anti-human IgG, and anti-human IgM specific antibodies respectively. All incubations included DNase I to eliminate contamination by free viral DNA sequences. Due to the lack of a suitable method for extracting free DNA, its detection was based on a positive PCR result in the absence of all other forms of virus.

Initial amplifications after selective extraction of 14 HBV DNA positive samples showed that patients had a variety of viral forms in their sera (whole virus, and IgG & IgM complexed virus). All HBe and HBs antigen positive samples were positive for free and complexed virus. This was expected since the presence of both e and surface antigens correlated to the early stages of infection where viral replication is at its maximum (Figure 4). Seven samples had detectable levels of antibody to HBeAg, and a variety of viral forms were detected, although only 2 samples were positive for all 3 forms. Of the 4 samples with no detectable viral DNA by any of the extraction methods, 2 were HBsAg positive (1 anti-HBe antibody positive and 1 HBe negative) and 2 anti-HBs positive (both anti-HBe antibody positive). The viral DNA detected in these samples was not associated with whole virus and may, therefore, have been released from necrotic hepatocytes lysed after cessation of viral replication.

Although HBV seronegative controls were utilised throughout, these were monitors of the PCR method and did not control for the specificity of the antibody coating of the beads. To control for this, HBV seropositive sera were incubated with blank beads and beads coated with BSA and buffer. Samples which were positive for all 3 forms were also HBV DNA positive after extraction with the 3 control beads, while samples positive for only 1 form were negative after these extractions. HBV DNA positive samples incubated with blank and buffer coated beads were expected to result in non-specific detection of HBV DNA as the beads were designed to bind proteins non-specifically. The lack of detection in some virus positive samples may be due to a concentration effect. The non-specific binding of hepatitis B virions or immune complexes may have been restricted by an inappropriate pH (optimum binding pH9.2). Therefore, samples with fewer viral particles were less likely to bind non-specifically to the test beads. However, the results suggested that the coating process required further optimisation to eliminate the non-specific binding.

Although false negative PCR results did not appear to be a problem in this study, the

use of a single large diameter bead (6.5mm) offered a relatively small surface area available for antibody binding and subsequent particle recognition. This could present problems in samples with high HBsAg concentrations resulting from HBV DNA negative filamentous and spherical particles. This was overcome by Liang *et al.*, (1989) by coating Sepharose beads with an IgM HBsAg-specific monoclonal antibody. The antibody coated bead slurry was then incubated overnight with patient serum, and DNA was heat extracted prior to amplification. The use of small beads and a large antibody molecule (IgM) will also create a physical barrier against non-specific binding to the bead surface. This method proved highly sensitive, detecting <10 copies per 200ul of serum (Liang *et al.*, 1989), however, the specificity of the binding step was not tested.

In addition to non-specific binding of hepatitis B virus and immune complexes, beads coated with antibodies specific for human IgG and IgM could be saturated with antibodies unrelated to HBV. HBV DNA positive extractions were obtained using beads coated with anti-HBs, anti-human IgG and anti-human IgM specific antibodies. The main concern was that some of these detections were due to non-specific binding of free and complexed virus. In defence of the results, samples which were repeatedly extracted by this method gave consistent data. When compared with the patient HBV-specific antigen and antibody profile, the bead extraction and HBV specific PCR gave patterns of detection which correlated to the current understanding of HBV replication.

In conclusion, the method requires further optimisation, primarily by changing from large to smaller beads to enhance the specific binding of viral particles. The use of a panel of non-HBV specific antibodies to block HBV binding may also assist in optimising the coating process. Once operational, this method could be applied to a wide range of problems including the determination of patient infectivity by qualitative and quantitative analysis and detection of sequence variation in whole virus.

12.2 Comparison of hepatitis B surface antigen subtyping by RIPA, PCR and RD-PCR.

The subtyping of hepatitis B virus has conventionally been by characterization of the HBsAg phenotype. The protein has a tertiary structure determined, in part, by formation of disulphide cross-linking of cysteine residues in the region of the protein associated with the *d/y* and *w/r* subtypes (Gerin *et al.*, 1983; Takeshima *et al.*, 1985). These conformational differences were initially detected by agar gel diffusion techniques, but are now more commonly characterized by antibody specific immunoassays (Purcell *et al.*, 1969; Holland *et al.*, 1972; Hoofnagle *et al.*, 1977; Fang *et al.*, 1978; Ukkonen & Koistinen, 1979; Wands & Zurawski Jr., 1981; Usuda *et al.*, 1985; Ren *et al.*, 1988). Immunoassays are, however, highly dependent on the specificity of the antibodies used and have often given rise to indeterminate results, and/or compound subtypes expressing both *d* and *y* or *w* and *r* determinants (Nordenfelt & Le Bouvier, 1973-1974; Mazzur *et al.*, 1975; Couroucé-Pauty *et al.*, 1979; Paul *et al.*, 1986; Tachibana *et al.*, 1989). The recent application of site-directed mutagenesis has mapped the *d* and *y* determinants to codon 122 of the S-gene (Okamoto *et al.*, 1987a, 1989a).

The *d* and *y* subtypes differ at codon 122 of the S-gene (Okamoto *et al.*, 1987a, 1989a), where subtype *d* specific samples have a lysine residue (AAA) and subtype *y* samples an arginine residue (AGA, AGG or CGG). As a substantial number of the serum samples included in the HBV DNA detection study had previously been phenotyped for the *d* and *y* determinants by RIPA, it was decided to develop a comparative method of HBsAg subtyping using PCR.

The primary amplification product from the HBV DNA detection method was used as a source of viral DNA which spanned codon 122. To determine the subtype specificity of this product, primers were selected to hybridize to codon 122 at their 3' ends (Figure 9) on the basis that 3' primer-template mismatches would result in a loss of efficiency which could be exploited to amplify subtype *d* or *y* specific HBV

DNA only (Newton *et al.*, 1989; Kwok *et al.*, 1990; Cassol *et al.*, 1991; Larder *et al.*, 1991).

The results obtained in this way showed that different 3' mismatches amplified the *d* and *y* specific sequences at different efficiencies. Two primers (029H & 394J) were selected to hybridize to *d* and *y* specific subtypes respectively (Figure 9). Both primers had a 3' T mismatch with the opposing subtype, however, both primers were found to amplify both *d* and *y* specific sequences efficiently. This was in agreement with a study by Kwok *et al.*, (1990) which showed no reduction in amplification efficiency with 3' T:G and T:C mismatches. However, this was in direct contrast to a study which utilised a T mismatch at the 3' end of both primers to detect *d* specific HBV DNA (Norder *et al.*, 1990). Although the technique utilised in this study was similar to that of Norder *et al.*, (1990), the substantial difference in results may be attributable to variations in the methodologies. The main difference was that both primers of the pair used by Norder *et al.*, (1990) had 3' mismatches, whereas only 1 primer in the pair had a mismatch in this study. Since the differential amplification of sequences using primers with 3' end mismatches is dependent on a reduction in amplification efficiency and not to an absence of amplification, the use of 2 mismatched primers may have reduced the efficiency sufficiently to differentiate between subtypes. Also, nested PCR, as used in this study, is the most sensitive technique for the detection of nucleic acids, and so it is possible that despite the reduced efficiency, the amplification power was too great to discriminate between subtypes (Simmonds *et al.*, 1990a). Norder *et al.*, (1990) used a single PCR followed by hybridization to determine the HBsAg subtype. The additional loss of amplification power associated with the hybridization assay may also have contributed to the ability to differentiate between the 2 subtypes.

In keeping with the data published by Kwok *et al.*, (1989), it was decided to continue to subtype samples using the *y*-specific primer with a 3' G:A mismatch, and to base the *d*-specificity on HBV DNA positive samples which were amplification negative

using the *y*-specific primer pair. Using this method, 38 HBV DNA positive samples, which had previously been subtyped by RIPA, were further amplified with the *y*-specific primer pair. The 2 subtyping assays were in agreement in 92% of samples, with 3 giving conflicting PCR and RIPA results.

RIPA and PCR techniques differ considerably in their application and versatility in HBsAg subtyping. The application of RIPA requires the presence of large quantities of free circulating HBsAg protein in the blood of infected individuals, a characteristic of acute and chronic hepatitis B. However, the assay is not definitive as it is based on percentage blocking by both *d* and *y* specific antibodies which are likely to carry a degree of cross-reactivity due to the type common determinant *a*. Also, the assay requires the production of standard antibodies used to characterize the antigen, and so it is a costly and time consuming method of HBsAg phenotyping.

PCR subtyping is, in the first instance, dependent on the presence of viral DNA which is generally only present during the acute phase of the infection and is cleared prior to complete resolution of the infection. However, the method is extremely sensitive requiring less than 10 template sequences for detection. Subtyping by PCR is dependent on the comparative amplification efficiencies of template sequences differing by as little as a single base. It, therefore, requires careful monitoring to maintain an adequate differentiation. However, PCR can detect all forms of viral DNA with an intact target sequence and can, therefore, be applied to the detection and subtyping of cell-associated viral DNA (both episomal and integrated); free virus; immune-complexed virus; and viral sequences associated with hepato-cellular carcinoma. PCR is a more rapid, flexible and cheaper alternative to RIPA subtyping of HBsAg.

However, in view of the careful optimisation required for PCR subtyping using a *y*-specific oligonucleotide only, it was decided to develop an alternative method to compare with both PCR and RIPA HBsAg subtyping.

Alternative methods of HBV DNA subtyping have been published using a variety of

techniques. Yotsumoto *et al.*, (1990) used PCR to amplify the S-gene and sequenced the product for adenine bases only. Sequence analysis was in agreement with enzyme immunoassay data in 100% of samples despite the acknowledged risk of PCR associated misincorporation (Saiki *et al.*, 1988).

Other methods of typing by PCR have now been developed to compare viral genotypes as opposed to phenotypes. This concept was first described for HBV by Okamoto *et al.*, (1988) and was used to categorise the virus into 4 groups showing genetic relatedness. This analysis showed considerable variation in the pre-S region which was exploited by Shih *et al.*, (1991) and Norder *et al.*, (1992) demonstrating that some genotypes showed greater variation between 2 sequences of the same subtype than between different subtypes. Since some of the existing methodologies for HBsAg subtyping were found to be time consuming and in some cases contradictory, it was decided to persist with the use of PCR to try and offer a simple and rapid alternative.

This was achieved by amplifying the S-gene with the type-common primers used for detection, and digesting the amplification product with the restriction enzyme *Sau* 3A which distinguishes between *d* and *y* subtypes (PCR-RD). From restriction maps of 5 HBV subtypes (*adw, adyw, ayw, adw2* and *adrcg*), it was predicted that *d* subtype genomes would result in 3 bands (117, 204, 227bp) and *y* subtype 2 bands (227 and 321bp). When analysed by agarose gel electrophoresis, it was found that *d* subtype genomes resulted in 6 bands, and *y* subtype 3 bands. This was due to the incomplete digestion of template DNA generated by PCR. The predicted bands for a partial digest were 6 for *d* subtypes (117, 204, 227, 321, 431, and 548bp), and 3 for *y* subtypes (227, 321, and 548bp, Figure 28). The resulting bands were easily distinguished on agarose gels giving a reliable and rapid method of genotyping HBV DNA for the *d* and *y* determinants (Figure 27).

To determine the efficacy of PCR-RD for HBsAg subtyping, the method was evaluated on 8 samples for which PCR and RIPA subtyping was in agreement. The

PCR-RD results were in 100% agreement for the 4 *d*-specific and 4 *y*-specific subtypes (Table 37). The method was also used to determine the subtype of the 3 samples with conflicting RIPA and PCR subtypes. The samples were all subtype *d* by RIPA and *y* by PCR. The PCR results were most likely to be incorrect due to the lack of a *d*-specific detection method. It was unlikely that the reaction would incorrectly identify a *y*-specific sample, but conceivable that it could amplify a *d*-specific sequence sufficiently for detection. It was, therefore, not surprising to find that 2 of the 3 samples were subtyped *d* by the third method (Table 37). PCR-RD was, therefore, thought to be the most appropriate method of HBsAg subtyping. It offered a highly sensitive and flexible method which was also rapid and relatively inexpensive. The only apparent disadvantage of this method was in the detection of compound subtypes.

Although the *d* and *y* subtypes of HBsAg are generally mutually exclusive, mixed, or compound subtypes have been described (Le Bouvier *et al.*, 1972; Gordon *et al.*, 1972; Nordenfelt & Le Bouvier, 1973-74; Mazzur *et al.*, 1975; Gibson, 1976). Subtyping by PCR-RD will only detect *d*-specific sequences in a mixed population due to the lack of a unique *y*-specific band in the digest product. Similarly, due to the lack of a *d*-specific primer, the PCR method will fail to detect *d*-specific sequences in a mixed population. Although RIPA is capable of detecting both *d* and *y* subtypes in compound samples, it is still a less sensitive, more costly and time consuming method.

In view of these limitations, it is thought that PCR-RD is the method of choice for HBsAg subtyping, with the use of PCR subtyping as a back up for *y*-specific confirmation. This will still offer a very rapid and flexible method of HBsAg subtyping, which will be applicable to a number of sources of virus including fresh and frozen serum and tissue samples, and fixed tissue, particularly those associated with hepatocellular carcinoma. PCR methods of HBsAg are, therefore, very rapid and flexible alternatives to RIPA, with multiple research and clinical applications.

CONCLUSION

During this project, the polymerase chain reaction was successfully applied to the detection of HIV and HBV nucleic acid in clinical samples. The technique was then further applied to characterise the forms of nucleic acid present in these samples, requiring modifications to the standard method.

PCR was an essential tool for the detection of HIV-1 DNA in clinical samples due to the small number of infected cells present in the peripheral blood. Once detected, HIV DNA was characterised into linear and 1 & 2LTR circular unintegrated viral DNA forms (LUVD and CUVD). However, due to the non-specific integration of the proviral sequence, an acceptable PCR method of detection was not devised. This would be an immediate objective of any continuance of this project.

Whilst LUVD was not detected in clinical samples, the circular unintegrated forms were found to be significantly associated with a more advanced disease stage ($0.02>P>0.01$). It would, therefore, be of interest to expand this study, over time, to evaluate these forms of HIV-1 DNA as prognostic markers. A longitudinal study like this may also encompass the use of antiretroviral therapies and observe any effects on UVD production. This would require the quantification of these forms to monitor the load in relation to therapy.

The benefits of PCR to detect HBV DNA in sera of infected individuals was less apparent due to the abundance of HBV DNA which can be readily detected by hybridization assays. The increased sensitivity of PCR may, however, offer advantages for the analysis of viral activity in hepatitis B carriers. This group of patients are particularly at risk of developing liver disease including hepatocellular carcinoma. Careful monitoring of viral activity in these patients may be beneficial in understanding the mechanism of disease and also in determining the efficacy of antiviral therapies. PCR may, therefore, be used for optimum HBV DNA detection in

sera, and also to detect both viral DNA and RNA intermediates in infected hepatocytes.

Further to this, the use of such a sensitive and rapid technique as PCR to subtype HBsAg DNA may encourage researchers to determine if the antigen subtypes are clinically significant.

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APPENDICES

Appendix I. HBV sample data.

Table 42: HBV results of radioimmunoassays (RIA) for HBs and HBe status, HBV DNA detection by PCR, and HBsAg subtyping by radioimmunoprecipitation assay (RIPA), nested PCR of HBsAg DNA, and *Sau* 3A restriction digest of PCR amplified HBsAg DNA (PCR-RD).

Patient No.	HBs	HBe	PCR DNA	Subtype determined by:		
				RIPA	PCR	PCR-RD
P35	Ag	Ag	+	ay	ay	nd
P36	Ag	Ag	+	ad	ad	ad
P37	Ag	Ag	+	ad	ad	nd
P38	Ag	Ag	+	ay	ay	ay
P39	Ag	Ag	+	ay	ay	nd
P40	Ag	Ag	+	ay	ay	nd
P41	Ag	Ag	+	ay	ay	nd
P42	Ag	Ag	+	ay	ay	nd
P43	Ag	Ag	+	ad	ad	ad
P44	Ag	Ag	+	ay	ay	ay
P45	Ag	Ag	+	ay	ay	nd
P46	Ag	Ag	+	ad	ad	ad
P47	Ag	Ag	+	ay	ay	nd
P48	Ag	Ag	+	ay	ay	nd
P49	Ag	Ag	+	ay	ay	nd
P50	Ag	Ag	+	ay	ay	nd
P51	Ag	Ag	+	ay	ay	nd
P52	Ag	Ag	+	ay	ay	nd
P53	Ag	Ag	+	ad	ad	nd
P54	Ag	Ag	+	ad	ad	nd
P55	Ag	Ag	+	ay	ay	nd
P56	Ag	Ag	+	ad	ad	nd
P57	Ag	Ag	+	ad	ad	nd
P58	Ag	Ag	+	ay	ay	ay
P59	Ag	Ag	+	ay	ay	ay
P60	Ag	Ag	+	ad	ad	ad
P61	Ag	Ag	+	ad	ad	nd
P62	Ag	Ag	+	ay	ay	nd
P63	Ag	Ag	+	ad	ad	nd
P64	Ag	Ab	+	ay	ay	nd
P65	Ag	Ag	+	ad	ay	ay
P66	Ag	-	+	ad	ad	nd
P67	Ag	Ab	+	ad	ad	nd
P68	Ag	Ab	+	ad	ay	ad
P69	Ag	Ab	+	ad	ad	nd
P70	Ag	Ab	+	ad	ad	nd
P71	Ag	Ab	+	ad	ay	ad
P72	Ag	Ag	+	d/y	ay	nd
P73	Ag	Ab	+	ay	ay	ay
P74	Ag	Ag	+	d/y	ad	nd

Patient No.	HBs	HBe	PCR DNA	Subtype determined by:		
				RIPA	PCR	PCR-RD
P75	Ag	Ag	+	nd	nd	nd
P76	Ag	Ag	+	nd	nd	nd
P77	Ag	Ag	+	nd	nd	nd
P78	Ag	Ag	+	nd	nd	nd
P79	Ag	Ag	+	nd	nd	nd
P80	Ag	Ag	+	nd	nd	nd
P81	Ag	Ag	+	nd	nd	nd
P82	Ag	Ag	+	nd	nd	nd
P83	Ag	Ag	+	nd	nd	nd
P84	Ag	Ag	+	nd	nd	nd
P85	Ag	Ag	+	nd	nd	nd
P86	Ag	Ag	+	nd	nd	nd
P87	Ag	Ag	+	nd	nd	nd
P88	Ag	Ag	+	nd	nd	nd
P89	Ag	Ag	+	nd	nd	nd
P90	Ag	Ag	+	nd	nd	nd
P91	Ag	Ag	+	nd	nd	nd
P92	Ag	Ag	+	nd	nd	nd
P93	Ag	Ag	+	nd	nd	nd
P94	Ag	Ag	+	nd	nd	nd
P95	Ag	Ag	+	nd	nd	nd
P96	Ag	Ag	+	nd	nd	nd
P97	Ag	-	+	nd	nd	nd
P98	Ag	-	-	nd	nd	nd
P99	Ag	-	-	nd	nd	nd
P100	Ag	-	-	nd	nd	nd
P101	Ag	-	-	nd	nd	nd
P102	Ab	-	-	nd	nd	nd
P103	Ab	-	-	nd	nd	nd
P104	Ab	-	-	nd	nd	nd
P105	Ab	-	-	nd	nd	nd
P106	Ab	-	-	nd	nd	nd
P107	Ab	-	-	nd	nd	nd
P108	-	-	-	nd	nd	nd
P109	Ab	-	-	nd	nd	nd
P110	Ab	-	-	nd	nd	nd
P111	-	-	-	nd	nd	nd
P112	Ab	-	-	nd	nd	nd
P113	-	-	-	nd	nd	nd
P114	Ab	-	-	nd	nd	nd
P115	Ab	-	-	nd	nd	nd
P116	Ab	Ab	+	nd	nd	nd
P117	Ab	Ab	+	nd	nd	nd
P118	Ag	Ab	+	nd	nd	nd
P119	Ag	Ab	-	nd	nd	nd
P120	Ag	Ab	-	nd	nd	nd
P121	Ag	Ab	-	nd	nd	nd
P122	Ag	Ab	-	nd	nd	nd
P123	Ag	Ab	-	nd	nd	nd
P124	Ag	Ab	-	nd	nd	nd

Patient No.	HBs	HBe	PCR DNA	Subtype determined by: RIPA	PCR	PCR-RD
P125	Ag	Ab	-	nd	nd	nd
P126	Ag	Ab	-	nd	nd	nd
P127	Ag	Ab	-	nd	nd	nd
P128	Ag	Ab	-	nd	nd	nd
P129	Ag	Ab	-	nd	nd	nd
P130	Ag	Ab	-	nd	nd	nd
P131	Ag	Ab	-	nd	nd	nd
P132	Ag	Ab	-	nd	nd	nd
P133	Ag	Ab	-	nd	nd	nd
P134	-	Ab	-	nd	nd	nd
P135	Ab	Ab	-	nd	nd	nd
P136	Ab	Ab	-	nd	nd	nd
P137	-	Ab	-	nd	nd	nd
P138	Ab	Ab	-	nd	nd	nd
P139	Ab	Ab	-	nd	nd	nd
P140	Ab	Ab	-	nd	nd	nd
P141	-	Ab	-	nd	nd	nd
P142	Ab	Ab	-	nd	nd	nd
P143	Ab	Ab	-	nd	nd	nd
P144	Ag	Ag	+	nd	nd	nd
P145	Ag	Ag	+	nd	nd	nd
P146	Ag	Ag	+	nd	nd	nd
P147	Ag	Ag	+	nd	nd	nd
P148	Ag	Ab	+	nd	nd	nd
P149	Ag	Ab	+	nd	nd	nd

+, positive result; -, negative result; Ag, antigen positive; Ab, antibody positive; ay, HBsAg subtype *ay*; ad, HBsAg subtype *ad*; d/y, sample *ad* and *ay* HBsAg subtype on separate occassions; nd, not done. All PCR results represent amplification results of 10ul aliquots of extracted DNA equating to 20ul of neat serum.

Appendix II. Medical Research Council AIDS Directed Program (MRC ADP) reagents.

The repository reference number and source are given, with a description of the reagent and any relevant references to publications.

Repository reference:	ADP002
Source:	Mr P Clapham
Reagent:	**Hut78**; human cutaneous T-cell lymphoma
Reference:	Gazdar *et al.*, 1980; Mann *et al.*, 1989.
Repository reference:	ADP012
Reagent:	**U937**; human monocyte-like cell from a histiocytic lymphoma
Reference:	Sundstrom & Nilsson, 1976.
Repository reference:	ADP013
Reagent:	**C8166**; human T-lymphoblastoid cell line
Source:	Mr P. Chapman and Dr G. Farrar
Reference:	Salahuddin *et al.*, 1983.
Repository reference:	ADP026
Reagent:	**LC5**; HIV susceptible clone of human embryonic lung fibroblasts
Source:	Dr V Erfle
Reference:	Mellert *et al.*, 1990.
Repository reference:	ADP028
Reagent:	**U138MG**; human glioblastoma cell line
Source:	American Type Culture Collection
Reference:	Pontén & Macintyre, 1968.
Repository reference:	ADP031
Reagent:	**THP1**; monocytic leukaemic line
Reference:	Tsuchiya *et al.*, 1980.
Repository reference:	ADP101
Reagent:	**HIV-1_{IIIB}**
Source:	Drs R. Gallo & M. Popovic
Reference:	Popovic *et al.*, 1984.

Repository reference: ADP103
Reagent: **HIV-1_{RF}**
Cell line: Chronically infected H9 cells
Source: Drs R.C. Gallo and M. Popovic
Reference: Popovic *et al.*, 1984.

Repository reference: ADP016
Reagent: **MT4**; Human T-cell transformed by co-cultivating with leukaemia lymphocytes harbouring HTLV-1
Source: Mr P Clapham
Reference: Miyoshi *et al.*, 1982; Harada *et al.*, 1985.

Repository reference: ADP020
Reagent: **HeLa T8+**; Human epithelial-like
Source: NIAID AIDS Research and Reference Reagent Program
Reference: Maddon *et al.*, 1986.

Repository reference: ADP024
Reagent: **SupT1**; Non-Hodgkin, T-cell lymphoma
Source: NIAID AIDS Research and Reference Reagent Program
Reference: Smith *et al.*, 1984.

Repository reference: ADP335
Reagent: **Monoclonal antibody to gp160/gp120**
Host: Mouse (BALB/c)
Immunogen: Recombinant HIV-1 gp160 from yeast
Source: Dr C. Thiriart and C. Bruck
Reference: Thiriart *et al.*, (1989).

Repository reference: ADP361
Reagent: **Monoclonal antibody to HIV-1 gp120**
Host: Mouse (BALB/c)
Immunogen: Recombinant gp120 (CHO)
Source: Drs R.B. Ferns and R.S. Tedder

Repository reference: ADP362
Reagent: **Monoclonal antibody to HIV-1 gp120**
Immunogen: Recombinant gp120 (CHO)
Host: Mouse (BALB/c)
Source: Drs R.B. Ferns and R.S. Tedder

Repository reference:	ADP604
Reagent:	**HIV-1 recombinant gp120**
Expression system:	Chinese hamster ovary (CHO) cells
Source:	Celltech Ltd

Repository reference:	ADP923
Reagent:	**Interleukin-2**
Derivation:	Human recombinant DNA

Repository reference:	ADP403
Reagent:	**Antiserum to recombinant gp120 (CHO)**
Immunogen:	CHO derived recombinant gp120
Host:	Sheep
Source:	Dr M. Page

Appendix III. Methods of analysis.

1. Calculation of the 50% tissue culture infective dose ($TCID_{50}$) by the method of Reed and Muench (Johnson & Byington, 1990).

HIV-1 supernate was titrated in 5 replicates on C8166 susceptible cells and data was analysed from the greatest dilution in which each replicate contained no syncytia, to the greatest dilution in which each replicate contained syncytia. For the example of C8166 cells infected with HIV-1_{RF} virus, this includes virus dilutions of 1 in 10^5 to 1 in 10^7 (Table 43). The $TCID_{50}$ lies between these two dilutions, and so only data from that range of dilutions are included in the calculations.

Table 43. Results of the syncytia assay in C8166 cells infected with HIV-1_{RF}.

Dilution	CPE +	CPE -	Total infected	Total uninfected	Mortality ratio (%)
1 in 10^5	5	0	8	0	8/8 (100%)
1 in 10^6	3	2	3	2	3/5 (60%)
1 in 10^7	0	5	0	7	0/7 (0%)

Key: CPE +, number of wells at each dilution in which syncytia were observed; CPE -, wells at each dilution scored negative for syncytia formation. The total infected and uninfected columns represent the cumulative values of virus infected and uninfected wells respectively. The total infected column is added from the highest to the lowest dilution, and the total uninfected from the lowest to the highest dilution. The mortality ratio is calculated from the cumulative infected total divided by the cumulative uninfected total for each dilution.

From the calculated mortality ratio, the 50% endpoint dilution lies between 1 in 10^6 and 1 in 10^7 (60% & 0% respectively).

To obtain the interpolated value (x), the following formula was used:

$$x = \frac{\text{(\% mortality at dilution next above 50\%) - 50\%}}{\text{(\% mortality at dilution next above 50\%) - (\% mortality at dilution next below 50\%)}}$$

From the data in Table 43,

$$x = \frac{60\%-50\%}{60\%-0\%} = \frac{10\%}{60\%} = 0.17$$

The 50% endpoint dilution is 1 in $10^{6.17}$, and so the 50% titre is estimated at $10^{6.17}$. The $TCID_{50}$ per millilitre of viral supernate is therefore $10^{7.17}$ (100ul of diluted supernate was used for each well).

2. Method of calculating the molarity of oligonucleotides supplied by Oswel DNA services (Department of Chemistry, The University of Edinburgh).

Oligonucleotides were supplied in solution (1ml) with optical density readings at 264nm. Their molarities were then calculated from their OD readings on the basis of their molecular weight and extinction coefficient.

The molecular weight was calculated as follows:

M.Wt= [(251xnA)+(242xnC)+(267xnG)+(227xnT)
+(62x(n-1))+(54xn)+(17x(n-1))]

and the micromolar extinction coefficient (at 264nm) is calculated as below;

MEC = [(8.8xnT)+(7.3xnC)+(11.7nG)+(15.4xnA)]x0.9

Where, nA = number of Adenines; nC = number of Cytosines; nG = number of Guanines; nT = number of Thymines; and n = the total number of bases; 62 x (n-1) is the molecular weight of the phosphate groups; 54 x n allows for hydration of the DNA; and 17 x (n-1) allows for the cation on the phosphate groups (usually ammonium).

The extinction coefficient is multiplied by 0.9 as the base-base interactions in single-stranded DNA suppresses the absorbance at 264nm.

Example: For the primer 160H, the calculations are as follows:

Primer 160H 5' CATGGGTACCAGCACACAAAGG 3'

nA=8, nC=6, nG=6, and nT=2.

M.Wt= [(251xn8)+(242xn6)+(267xn6)+(227xn2)
+(62x(22-1))+(54x22)+(17x(22-1))]

M.Wt=8363g

so that the micromolar weight = 8.363mg

and

MEC = [(8.8xn2)+(7.3xn6)+(11.7n6)+(15.4xn8)]x0.9

= 229.32

To convert the OD units to milligrams

8.363g = 229.32 OD units

1mg = 229.31 8.363

= 27.42 OD units

The actual OD units supplied were 6OD/ml, and so the weight of oligonucleotide supplied

= 1mg x (6 27.42)

= 218.8ug/ml

The molarity = (218.8×10^{-3}) 8363

= 26.2uM

The primer concentration is therefore 26.2uM.

3. Statistical analysis of data by the Chi-square test.

Data was analysed by the Chi-square test, as described by Swinscow, (1991). For each set of data, the null hypothesis was that the groups had similar distributions. Two examples have been included, to demonstrate the methods of calculating Chi-square, degrees of freedom, and the probability of the results occurring in a 2 by 2 table (example 1) and a 3 by 2 table (example 2). Due to the small sample size in 2 by 2 tables, the Yates correction for continuity was applied.

Example 1. The null hypothesis is that the ability to isolate virus is similar in samples with or without CUVD forms.

Table 44. Comparison of detection of CUVD with virus isolation (VI).

	VI+	VI-	Total
CUVD+	14	7	21
CUVD-	7	7	14
Total	21	14	35

The table is in the basic format shown below:

	VI+	VI-	Total
CUVD+	a	b	a+b
CUVD-	c	d	c+d
Total	a+c	b+d	a+b+c+d

$$\text{Where Chi-square} = \frac{[(\text{lad-bcl})- (a+b+c+d)]\,(a+b+c+d)}{(a+b)(c+d)(b+d)(a+c)}$$

and, ll indicates that the smaller of the 2 numbers enclosed should be subtracted from the other.

From the calculation, the Chi square = 0.402

Degree of freedom = (No. of columns-1)x(No. of rows-1)

= (2-1)(2-1)

= 1

From the table of distribution of chi-square the result obtained at 1 degree of freedom has the probability of $P>0.50$. The result, therefore, does not show a significant correlation between the presence of CUVD and the ability to isolate virus, and so the null hypothesis was confirmed.

Example 2. The null hypothesis is that the distribution of CUVD forms is similar in CDC groups II, III, and IV.

Table 45. Detection of CUVD forms in samples categorised by CDC groups.

CDC group	CUVD +	CUVD -	Total
II	6	6	12
III	10	10	20
IV	18	2	20
Total	34	18	52

Calculation of expected numbers and chi-square.

Where, E = expected number and O = observed number.

e.g. for CUVD positive samples in CDC group II,

E = (11 52) x 34

and for CUVD negative samples in CDC group III,

E = (10 52) x 18

	E		O-E		(O-E) /E	
CDC group	CUVD+	CUVD-	CUVD+	CUVD-	CUVD+	CUVD-
II	7.85	4.51	-1.85	1.85	0.436	0.825
III	13.08	6.92	-3.08	3.08	0.725	1.371
IV	13.08	6.92	4.92	-4.92	1.851	3.498
Total	34	18	0	0	3.012	5.694

Chi-square = the sum of the 2 columns of (O-E) /E

= 3.012 + 5.694 = 8.706

Degree of freedom

= (No. of columns - 1) x (No. of rows - 1)

= (2 - 1) x (3 - 1)

= 2

From the chi-square tables, the probability at 2 degrees of freedom is $0.02>P>0.01$. The result is therefore significant, and so the null hypothesis is disproved. The results show that CUVD is more likely to be detected in the later stages of disease.

Appendix IV. HIV sample data.

Each patient has been allocated a number, with letters (a, b, etc) indicating multiple samples from any one patient. The date of sampling, current CDC group, $CD4^+$ cell count, and type of treatment, if any, has been detailed. The PCR results are given with cell equivalents of the amplification aliquot used. All samples are purified PBMCs unless otherwise stated.

Sample No.:	1	**Date of Sample:**	21.11.91
CDC group:	III	**$CD4^+$:**	260
VI:	negative	**plasma p24 Ag:**	negative
Treatment:	on AZT 10.91.		
Amp aliquot:	2×10^5		

DNA PCR results	***pol***	***env***	**1LTR**	**2LTR**	**LUVD**
	+	+	-	-	-

Sample No.:	2a	**Date of Sample:**	23.10.90.
CDC group:	not infected	**CD4+:**	nd
VI:	nd	**plasma p24 Ag:**	negative
Treatment:	none		
Amp aliquot:	1×10^5		

DNA PCR results	***pol***	***env***	**1LTR**	**2LTR**	**LUVD**
	-	-	-	-	-

Sample No.:	2b	**Date of Sample:**	17.02.92.
CDC group:	not infected	**CD4+:**	nd
VI:	negative	**plasma p24 Ag:**	negative
Treatment:	none		
Amp aliquot:	2×10^5		

DNA PCR results	***pol***	***env***	**1LTR**	**2LTR**	**LUVD**
	-	-	-	-	-

Sample No.:	3a	**Date of Sample:**	31.07.90.
CDC group:	II	**CD4$^+$:**	350
VI:	positive	**plasma p24 Ag:**	negative
Treatment:	none		
Amp aliquot:	6×10^5		

DNA PCR results	***pol***	***env***	**1LTR**	**2LTR**	**LUVD**
	+	+	-	-	nd

Sample No.:	3b	**Date of Sample:**	23.04.91.
CDC group:	II	**CD4$^+$:**	280
VI:	negative	**plasma p24 Ag:**	negative
Treatment:	none		
Amp aliquot:	*pol* & *env* 2×10^4; 1 & 2 LTR 4×10^4		

DNA PCR results	***pol***	***env***	**1LTR**	**2LTR**	**LUVD**
	+	+	-	-	nd

Sample No.:	4a	**Date of Sample:**	15.01.91.
CDC group:	II	**CD4$^+$:**	180
VI:	negative	**plasma p24 Ag:**	positive
Treatment:	none		
Amp aliquot:	2×10^4		

DNA PCR results	***pol***	***env***	**1LTR**	**2LTR**	**LUVD**
	+	+	nd	nd	nd

Sample No.:	4b	**Date of Sample:**	21.11.91.
CDC group:	II	**CD4$^+$:**	200
VI:	negative	**plasma p24 Ag:**	positive
Treatment:	on AZT 15.01.91.		
Amp aliquot:	2×10^4		

DNA PCR results	***pol***	***env***	**1LTR**	**2LTR**	**LUVD**
	+	+	+	+	nd

Sample No.:	5	**Date of Sample:**	15.01.91.
CDC group:	III	**CD4$^+$:**	190
VI:	negative	**plasma p24 Ag:**	negative
Treatment:	none		
Amp aliquot:	2×10^4		

DNA PCR results	***pol***	***env***	**1LTR**	**2LTR**	**LUVD**
	+	+	nd	nd	nd

Sample No.:	6	**Date of Sample:**	17.12.91.
CDC group:	II	**CD4$^+$:**	220
VI:	positive	**plasma p24 Ag:**	negative
Treatment:	none		
Amp aliquot:	2×10^4		

DNA PCR results	***pol***	***env***	**1LTR**	**2LTR**	**LUVD**
	+	+	+	+	nd

Sample No.:	7a	**Date of Sample:**	27.07.90.
CDC group:	IV	**CD4$^+$:**	50
VI:	positive	**plasma p24 Ag:**	negative
Treatment:	on AZT 02.90.		
Amp aliquot:	3×10^4		

DNA PCR results	***pol***	***env***	**1LTR**	**2LTR**	**LUVD**
	+	+	+	+	-

Sample No.:	7b	**Date of Sample:**	01.11.90.
CDC group:	IV	**CD4$^+$:**	30
VI:	nd	**plasma p24 Ag:**	negative
Treatment:	on AZT 02.90.		
Amp aliquot:	nq		

DNA PCR results	***pol***	***env***	**1LTR**	**2LTR**	**LUVD**
	+	+	+	+	nd

Sample No.:	7c	**Date of Sample:**	12.06.91.
CDC group:	IV	**CD4$^+$:**	7
VI:	nd	**plasma p24 Ag:**	negative
Treatment:	on AZT 02.90.		
Amp aliquot:	*pol* & *env* 1×10^5; 1 & 2 LTR 3.5×10^6		

DNA PCR results	***pol***	***env***	**1LTR**	**2LTR**	**LUVD**
	+	+	+	+	nd

Sample No.:	7d	**Date of Sample:**	01.07.91.
CDC group:	IV	**CD4$^+$:**	nd
VI:	nd	**plasma p24 Ag:**	negative
Treatment:	on AZT 02.90.		
Amp aliquot:	*pol* & *env* 2×10^4; 1 & 2 LTR 1×10^5		

DNA PCR results	***pol***	***env***	**1LTR**	**2LTR**	**LUVD**
	+	+	+	+	nd

Sample No.:	8a	**Date of Sample:**	16.08.90.
CDC group:	III	**CD4+:**	100
VI:	positive	**plasma p24 Ag:**	positive
Treatment:	none		
Amp aliquot:	1.5×10^5		

DNA PCR results	***pol***	***env***	**1LTR**	**2LTR**	**LUVD**
	+	+	+	+	nd

Sample No.:	8b	**Date of Sample:**	14.09.90.
CDC group:	III	**CD4+:**	nd
VI:	positive	**plasma p24 Ag:**	positive
Treatment:	on AZT 30.08.90.		
Amp aliquot:	2×10^4		

DNA PCR results	***pol***	***env***	**1LTR**	**2LTR**	**LUVD**
	+	+	+	+	-

Sample No.:	8c	**Date of Sample:**	26.03.91.
CDC group:	III	**CD4+:**	nd
VI:	positive	**plasma p24 Ag:**	negative
Treatment:	on AZT 30.08.90.		
Amp aliquot:	7×10^4		

DNA PCR results	***pol***	***env***	**1LTR**	**2LTR**	**LUVD**
	+	+	+	+	-

Sample No.:	9a	Date of Sample:	07.08.90.
CDC group:	not infected	**CD4+:**	nd
VI:	nd	**plasma p24 Ag:**	negative
Treatment:	none		
Amp aliquot:	1×10^5		

DNA PCR results	***pol***	***env***	**1LTR**	**2LTR**	**LUVD**
	-	-	-	-	-

Sample No.:	9b	**Date of Sample:**	14.08.91.
CDC group:	not infected	**CD4+:**	nd
VI:	nd	**plasma p24 Ag:**	nd
Treatment:	none		
Amp aliquot:	2.5×10^5		

DNA PCR results	***pol***	***env***	**1LTR**	**2LTR**	**LUVD**
	-	-	-	-	nd

Sample No.:	10	**Date of Sample:**	07.06.91.
CDC group:	III	**CD4+:**	468
VI:	negative	**plasma p24 Ag:**	negative
Treatment:	none		
Amp aliquot:	4×10^4		

DNA PCR results	***pol***	***env***	**1LTR**	**2LTR**	**LUVD**
	+	+	-	-	nd

Sample No.:	11a	**Date of Sample:**	18.01.91.
CDC group:	III	**CD4+:**	221
VI:	nd	**plasma p24 Ag:**	negative
Treatment:	none		
Amp aliquot:	nq		

DNA PCR results	***pol***	***env***	**1LTR**	**2LTR**	**LUVD**
	+	+	-	-	-

Sample No.:	11b	**Date of Sample:**	05.04.91.
CDC group:	III	**CD4+:**	176
VI:	positive	**plasma p24 Ag:**	negative
Treatment:	on AZT 21.01.91.		
Amp aliquot:	*pol* & *env* 2×10^4; 1 & 2 LTR 4×10^4		

DNA PCR results	***pol***	***env***	**1LTR**	**2LTR**	**LUVD**
	+	+	-	-	-

Sample No.:	12a	**Date of Sample:**	24.07.89.
CDC group:	IV	**CD4+:**	nd
Sample type	cultured cells	**plasma p24 Ag:**	negative
Treatment:	none		
Amp aliquot:	4×10^4		

DNA PCR results	***pol***	***env***	**1LTR**	**2LTR**	**LUVD**
	+	+	+	+	nd

Sample No.:	12b	**Date of Sample:**	12.11.90.
CDC group:	IV	**CD4+:**	260
VI:	nd	**plasma p24 Ag:**	negative
Treatment:	on AZT 08.08.89.		
Amp aliquot:	4.5×10^5		

DNA PCR results	***pol***	***env***	**1LTR**	**2LTR**	**LUVD**
	+	+	+	+	-

Sample No.: 13 **Date of Sample:** 21.11.91.
CDC group: III **CD4+:** nd
VI: negative **plasma p24 Ag:** negative
Treatment: on AZT 28.10.91.
Amp aliquot: 2×10^4

DNA PCR results	***pol***	***env***	**1LTR**	**2LTR**	**LUVD**
	+	+	+	+h	nd

Sample No.: 14a **Date of Sample:** 30.07.90.
CDC group: IV **CD4+:** 240
VI: positive **plasma p24 Ag:** positive
Treatment: on AZT 03.05.90.; on Pentamidine 14.05.90.
Amp aliquot: 4×10^4

DNA PCR results	***pol***	***env***	**1LTR**	**2LTR**	**LUVD**
	+	+	-	-	nd

Sample No.: 14b **Date of Sample:** 09.10.90.
CDC group: IV **CD4+:** 260
VI: positive **plasma p24 Ag:** positive
Treatment: on AZT 03.05.90.; on Pentamidine 14.05.90.
Amp aliquot: 8×10^4

DNA PCR results	***pol***	***env***	**1LTR**	**2LTR**	**LUVD**
	+	+	+	+	nd

Sample No.: 15 **Date of Sample:** 17.01.91.
CDC group: IV **CD4+:** 40
VI: positive **plasma p24 Ag:** negative
Treatment: none
Amp aliquot: nq

DNA PCR results	***pol***	***env***	**1LTR**	**2LTR**	**LUVD**
	+	+	+	+	nd

Sample No.: 16 **Date of Sample:** 04.03.91.
CDC group: III **CD4+:** 360
VI: negative **plasma p24 Ag:** negative
Treatment: none
Amp aliquot: 2×10^4

DNA PCR results	***pol***	***env***	**1LTR**	**2LTR**	**LUVD**
	+	+	+	+	-

Sample No.:	17a	**Date of Sample:**	04.09.90.
CDC group:	II	**CD4$^+$:**	240
VI:	positive	**plasma p24 Ag:**	negative
Treatment:	none		
Amp aliquot:	1.5×10^5		

DNA PCR results	***pol***	***env***	**1LTR**	**2LTR**	**LUVD**
	+	+	+	+	nd

Sample No.:	17b	**Date of Sample:**	21.11.90.
CDC group:	II	**CD4$^+$:**	90
VI:	nd	**plasma p24 Ag:**	negative
Treatment:	none		
Amp aliquot:	2×10^4		

DNA PCR results	***pol***	***env***	**1LTR**	**2LTR**	**LUVD**
	+	+	+	+	nd

Sample No.:	17c	**Date of Sample:**	10.01.91.
CDC group:	II	**CD4$^+$:**	100
VI:	nd	**plasma p24 Ag:**	negative
Treatment:	none		
Amp aliquot:	2×10^4		

DNA PCR results	***pol***	***env***	**1LTR**	**2LTR**	**LUVD**
	+	+	-	-	-

Sample No.:	18a	**Date of Sample:**	30.07.90.
CDC group:	IV	**CD4$^+$:**	nd
VI:	positive	**plasma p24 Ag:**	negative
Treatment:	on AZT 03.90.		
Amp aliquot:	8×10^4		

DNA PCR results	***pol***	***env***	**1LTR**	**2LTR**	**LUVD**
	+	+	+	+	nd

Sample No.:	18b	**Date of Sample:**	01.11.90.
CDC group:	IV	**CD4$^+$:**	nd
VI:	nd	**plasma p24 Ag:**	negative
Treatment:	on AZT 03.90.		
Amp aliquot:	6×10^4		

DNA PCR results	***pol***	***env***	**1LTR**	**2LTR**	**LUVD**
	+	+	+	+	nd

Sample No.:	19a	**Date of Sample:**	07.03.91.
CDC group:	III	**CD4$^+$:**	560
VI:	positive	**plasma p24 Ag:**	negative
Treatment:	none		
Amp aliquot:	2 x 10^4		

DNA PCR results	***pol***	***env***	**1LTR**	**2LTR**	**LUVD**
	+	+	-	-	-

Sample No.:	19b	**Date of Sample:**	20.11.91.
CDC group:	III	**CD4$^+$:**	440
VI:	nd	**plasma p24 Ag:**	negative
Treatment:	none		
Amp aliquot:	2 x 10^5		

DNA PCR results	***pol***	***env***	**1LTR**	**2LTR**	**LUVD**
	+	+	+	+	-

Sample No.:	20a	**Date of Sample:**	16.10.90.
CDC group:	III	**CD4$^+$:**	340
VI:	negative	**plasma p24 Ag:**	negative
Treatment:	none		
Amp aliquot:	nq		

DNA PCR results	***pol***	***env***	**1LTR**	**2LTR**	**LUVD**
	+	+	+	+	-

Sample No.:	20b	**Date of Sample:**	25.02.91.
CDC group:	III	**CD4$^+$:**	350
VI:	positive	**plasma p24 Ag:**	negative
Treatment:	none		
Amp aliquot:	2 x 10^4		

DNA PCR results	***pol***	***env***	**1LTR**	**2LTR**	**LUVD**
	+	+	nd	nd	nd

Sample No.:	20c	**Date of Sample:**	21.11.91.
CDC group:	III	**CD4$^+$:**	nd
VI:	negative	**plasma p24 Ag:**	negative
Treatment:	none		
Amp aliquot:	1 x 10^6		

DNA PCR results	***pol***	***env***	**1LTR**	**2LTR**	**LUVD**
	+	+	+	+	nd

Sample No.: 21a **Date of Sample:** 06.08.90.
CDC group: III **CD4$^+$:** 100
VI: positive **plasma p24 Ag:** negative
Treatment: none
Amp aliquot: 2 x 10^4

DNA PCR results	***pol***	***env***	**1LTR**	**2LTR**	**LUVD**
	+	+	-	-	nd

Sample No.: 21b **Date of Sample:** 08.01.91.
CDC group: III **CD4$^+$:** 99
VI: positive **plasma p24 Ag:** negative
Treatment: on AZT 07.01.91.; on Pentamidine 15.12.90.
Amp aliquot: *pol* & *env* 7 x 10^3; 1 & 2 LTR 2 x 10^4

DNA PCR results	***pol***	***env***	**1LTR**	**2LTR**	**LUVD**
	+	+	-	-	nd

Sample No.: 22a **Date of Sample:** 08.08.90.
CDC group: III **CD4$^+$:** 130
VI: positive **plasma p24 Ag:** positive
Treatment: on AZT & Pentamidine 23.04.90.
Amp aliquot: 8 x 10^4

DNA PCR results	***pol***	***env***	**1LTR**	**2LTR**	**LUVD**
	+	+	+	+	nd

Sample No.: 22b **Date of Sample:** 06.12.90.
CDC group: III **CD4$^+$:** 110
VI: nd **plasma p24 Ag:** nd
Treatment: on AZT & Pentamidine 23.04.90.
Amp aliquot: 1 x 10^5

DNA PCR results	***pol***	***env***	**1LTR**	**2LTR**	**LUVD**
	+	+	+	+	-

Sample No.: 23a **Date of Sample:** 02.08.90.
CDC group: IV **CD4$^+$:** nd
VI: positive **plasma p24 Ag:** negative
Treatment: none
Amp aliquot: 4.5 x 10^5

DNA PCR results	***pol***	***env***	**1LTR**	**2LTR**	**LUVD**
	+	+	+	+	-

Sample No.:	23b	**Date of Sample:**	16.10.90.
CDC group:	IV	**CD4$^+$:**	200
VI:	negative	**plasma p24 Ag:**	negative
Treatment:	on AZT 02.08.90.		
Amp aliquot:	5×10^4		

DNA PCR results	***pol***	***env***	**1LTR**	**2LTR**	**LUVD**
	+	-	-	-	-

Sample No.:	24a	**Date of Sample:**	09.05.90.
CDC group:	II	**CD4$^+$:**	280
VI:	nd	**plasma p24 Ag:**	negative
Treatment:	none		
Amp aliquot:	1.2×10^5		

DNA PCR results	***pol***	***env***	**1LTR**	**2LTR**	**LUVD**
	-	-	-	-	nd

Sample No.:	24b	**Date of Sample:**	08.08.90.
CDC group:	II	**CD4$^+$:**	250
VI:	negative	**plasma p24 Ag:**	negative
Treatment:	none		
Amp aliquot:	4×10^4		

DNA PCR results	***pol***	***env***	**1LTR**	**2LTR**	**LUVD**
	+	-	-	-	nd

Sample No.:	25a	**Date of Sample:**	14.02.91.
CDC group:	III	**CD4$^+$:**	470
VI:	negative	**plasma p24 Ag:**	negative
Treatment:	none		
Amp aliquot:	4×10^4		

DNA PCR results	***pol***	***env***	**1LTR**	**2LTR**	**LUVD**
	-	-	-	-	-

Sample No.:	25b	**Date of Sample:**	21.11.91.
CDC group:	III	**CD4$^+$:**	276
VI:	negative	**plasma p24 Ag:**	negative
Treatment:	on AZT 04.04.91.		
Amp aliquot:	2×10^5		

DNA PCR results	***pol***	***env***	**1LTR**	**2LTR**	**LUVD**
	+	+	-	-	-

Sample No.: 26a **Date of Sample:** 07.05.90.
CDC group: IV **CD4+:** nd
VI: nd **plasma p24 Ag:** positive
Treatment: on AZT 24.07.89.
Amp aliquot: 3 x 10^5

DNA PCR results	*pol*	*env*	1LTR	2LTR	LUVD
	+	+	+	+	nd

Sample No.: 26b **Date of Sample:** 15.04.91.
CDC group: IV **CD4+:** nd
VI: nd **plasma p24 Ag:** positive
Treatment: on AZT 24.07.89.
Amp aliquot: *pol* & *env* 4 x 10^4; 1 & 2 LTR 1 x 10^5

DNA PCR results	*pol*	*env*	1LTR	2LTR	LUVD
	+	+	+	-	-

Sample No.: 27a **Date of Sample:** 07.05.90.
CDC group: II **CD4+:** 390
VI: negative **plasma p24 Ag:** negative
Treatment: none
Amp aliquot: 1.6 x 10^5

DNA PCR results	*pol*	*env*	1LTR	2LTR	LUVD
	+	+	+	+	-

Sample No.: 27b **Date of Sample:** 18.03.91.
CDC group: II **CD4+:** 402
VI: negative **plasma p24 Ag:** negative
Treatment: none
Amp aliquot: 7.5 x 10^3

DNA PCR results	*pol*	*env*	1LTR	2LTR	LUVD
	+	+	+	+	-

Sample No.: 28a **Date of Sample:** 02.08.90.
CDC group: IV **CD4+:** nd
VI: positive **plasma p24 Ag:** negative
Treatment: on AZT 12.88 to 04.07.90.
Amp aliquot: *pol* & *env* 1 x 10^4; 1 & 2 LTR 3 x 10^4

DNA PCR results	*pol*	*env*	1LTR	2LTR	LUVD
	+	+	-	-	-

Sample No.:	28b	**Date of Sample:**	21.11.90.
CDC group:	IV	**CD4+:**	46
VI:	nd	**plasma p24 Ag:**	negative
Treatment:	none		
Amp aliquot:	6×10^4		

DNA PCR results	***pol***	***env***	**1LTR**	**2LTR**	**LUVD**
	+	+	+	+	nd

Sample No.:	28c	**Date of Sample:**	25.03.91.
CDC group:	IV	**CD4+:**	nd
Sample type	macrophage cells, isolated by adherence		
Treatment:	none		
Amp aliquot:	3.5×10^4		

DNA PCR results	***pol***	***env***	**1LTR**	**2LTR**	**LUVD**
	-	-	-	-	-

Sample No.:	28d	**Date of Sample:**	10.04.91.
CDC group:	IV	**CD4+:**	25
VI:	positive	**plasma p24 Ag:**	negative
Treatment:	none		
Amp aliquot:	*pol* & *env* 2×10^4; 1 & 2 LTR 5×10^4		

DNA PCR results	***pol***	***env***	**1LTR**	**2LTR**	**LUVD**
	+	+	+	+	-

Sample No.:	29a	**Date of Sample:**	21.01.90.
CDC group:	III	**CD4+:**	170
VI:	nd	**plasma p24 Ag:**	negative
Treatment:	none		
Amp aliquot:	2×10^5		

DNA PCR results	***pol***	***env***	**1LTR**	**2LTR**	**LUVD**
	+	+	-	-	nd

Sample No.:	29b	**Date of Sample:**	09.10.90.
CDC group:	III	**CD4+:**	150
VI:	negative	**plasma p24 Ag:**	negative
Treatment:	none		
Amp aliquot:	*pol* & *env* 4×10^4; 1 & 2 LTR 1.2×10^5		

DNA PCR results	***pol***	***env***	**1LTR**	**2LTR**	**LUVD**
	+	+	-	-	-

Sample No.:	30a	**Date of Sample:**	24.10.90.
CDC group:	IV	**CD4$^+$:**	30
VI:	nd	**plasma p24 Ag:**	positive
Treatment:	on AZT 24.07.89.		
Amp aliquot:	1.5×10^5		

DNA PCR results	***pol***	***env***	**1LTR**	**2LTR**	**LUVD**
	+	+	+	+	-

Sample No.:	30b	**Date of Sample:**	07.06.91.
CDC group:	IV	**CD4$^+$:**	15
VI:	positive	**plasma p24 Ag:**	positive
Treatment:	on AZT 24.07.89.		
Amp aliquot:	5×10^4		

DNA PCR results	***pol***	***env***	**1LTR**	**2LTR**	**LUVD**
	+	+	+	+	nd

Sample No.:	31	**Date of Sample:**	04.03.91.
CDC group:	II	**CD4$^+$:**	280
VI:	negative	**plasma p24 Ag:**	negative
Treatment:	none		
Amp aliquot:	4×10^4		

DNA PCR results	***pol***	***env***	**1LTR**	**2LTR**	**LUVD**
	+	+	-	-	-

Sample No.:	32a	**Date of Sample:**	26.07.90.
CDC group:	II	**CD4$^+$:**	470
VI:	negative	**plasma p24 Ag:**	negative
Treatment:	none		
Amp aliquot:	2×10^5		

DNA PCR results	***pol***	***env***	**1LTR**	**2LTR**	**LUVD**
	+	+	-	-	-

Sample No.:	32b	**Date of Sample:**	18.01.91.
CDC group:	II	**CD4$^+$:**	620
VI:	nd	**plasma p24 Ag:**	negative
Treatment:	none		
Amp aliquot:	*pol* & *env* 3.75×10^5; 1 & 2 LTR 4.5×10^5		

DNA PCR results	***pol***	***env***	**1LTR**	**2LTR**	**LUVD**
	+	-	-	-	nd

Sample No.:	33a	**Date of Sample:**	07.08.90.
CDC group:	II	**CD4$^+$:**	212
VI:	nd	**plasma p24 Ag:**	negative
Treatment:	none		
Amp aliquot:	2 x 10^5		

DNA PCR results	***pol***	***env***	**1LTR**	**2LTR**	**LUVD**
	+	+	-	-	-

Sample No.:	33b	**Date of Sample:**	14.08.91.
CDC group:	II	**CD4$^+$:**	205
VI:	nd	**plasma p24 Ag:**	negative
Treatment:	none		
Amp aliquot:	2 x 10^5		

DNA PCR results	***pol***	***env***	**1LTR**	**2LTR**	**LUVD**
	-	+	-	-	-

Sample No.:	34a	**Date of Sample:**	14.05.91.
CDC group:	IV	**CD4$^+$:**	nd
VI:	nd	**plasma p24 Ag:**	positive
Treatment:	on AZT 09.90.		
Amp aliquot:	1 x 10^5		

DNA PCR results	***pol***	***env***	**1LTR**	**2LTR**	**LUVD**
	+	+	+	+	-

Sample No.:	34b	**Date of Sample:**	27.06.91.
CDC group:	IV	**CD4$^+$:**	nd
VI:	positive	**plasma p24 Ag:**	positive
Treatment:	on AZT 09.90.		
Amp aliquot:	1 x 10^5		

DNA PCR results	***pol***	***env***	**1LTR**	**2LTR**	**LUVD**
	+	+	+	+	-

Sample No.:	34c	**Date of Sample:**	01.08.91.
CDC group:	IV		
Sample type	cultured cells		
Treatment:	none		
Amp aliquot:	3.5 x 10^5		

DNA PCR results	***pol***	***env***	**1LTR**	**2LTR**	**LUVD**
	+	+	+	+	+

nq, not quantified; nd, not done; -, negative; +, positive; +h, band present but heavier than expected; Pentamidine, pentamidine isethionate for treatment of *Pneumocystis carinii*; AZT, Azidothymidine or Zidovudine, inhibitor of HIV-1 replication; $CD4^+$ counts, CD4 surface antigen positive cells per millilitre of blood; LUVD, linear unintegrated viral DNA; 1LTR and 2LTR, covalently closed circular unintegrated viral DNA with 1 and 2 LTR sequences respectively. Patients 2 & 9 were included as high risk seronegative controls.

Appendix V. Sequences and coordinates of oligonucleotides.

Oligonucleotides were synthesized by Oswel DNA Service (Department of Chemistry, The University of Edinburgh), and purified by high-performance liquid chromatography.

HIV-1 specific oligonucleotides. The coordinates (5' to 3') of HIV-1 specific sequences are based on the published sequence of HTLV-III by Ratner *et al.*, (1985). The sources of sequences are given in parenthesis.

5' CATGGGTACCAGCACACAAAGG 3'	Sense primer 160H from *pol* gene, position 3730 to 3751. **(1)**
5' TCTACTTGTCCATGCATGGCTTC 3'	Anti-sense primer 161H from *pol* gene, position 3973 to 3951. **(1)**
5' GGAGGAAATGAACAAGTAGATAAATT 3'	Sense primer 002C from *pol* gene, position 3756 to 3781. **(1)**
5' TCACTAGCCATTGCTCTCCAATT 3'	Anti-sense primer 003C from *pol* gene, position 3883 to 3861. **(1)**
5' GAGGATATAATCAGTTTATGG 3'	Sense primer 401C from *env* gene, position 6117 to 6137. **(1)**
5' AATTCCATGTGTACATTGTACTG 3'	Anti-sense primer 404C from *env* gene, position 6554 to 6532. **(1)**
5' GATCAAAGCCTAAAGCCATG 3'	Sense primer 402C from *env* gene, position 6138 to 6157. **(1)**
5' CAATAATGTATGGGAATTGG 3'	Anti-sense primer 403C from *env* gene, position 6454 to 6435. **(1)**
5' GGTCCTTGTCTTATGTCCAGAATGCTG 3'	Anti-sense primer 077F from *gag* gene, position 1199 to 1173. **(1)**

5' ATCTGATCCCTGGCCCTGGTGTGT 3'	Anti-sense primer 779K from U3 sequence, position -340 to -363, and 8776 to 8753. **(2)**
5' GTCACACAACAGACGGGCACACACT 3'	Anti-sense primer 780K from U5 sequence, position 126 to 102, and 9241 to 9217. **(3)**
5' CTGCATCCGGAGTACTTCAAGAACT 3'	Sense primer 781K from U3 sequence, position -151 to -127, and 8965 to 8989. **(3)**
5' CAAGTGGTCAAAAAGTAGTCTGGTT 3'	Sense primer 782K from *env* gene, position 8382 to 8406. **(4)**
5' GCGTTCAGCAAGCCGAGTCCTGCG 3'	Anti-sense primer 783K from *gag* gene, position 257 to 234. **(4)**
5' TAG 3'	Sense primer 556L from *pol* gene, position 3771 to 3773. **(1)**
5' AGTAG 3'	Sense primer 557L from *pol* gene, position 3769 to 3773. **(1)**
5' CAAGTAG 3'	Sense primer 558L from *pol* gene, position 3767 to 3773. **(1)**
5' AACAAGTAG 3'	Sense primer 559L from *pol* gene, position 3765 to 3773. **(1)**
5' GGTACTAGCTTGTAGCACCATCCAA 3'	Anti-sense primer 585N from U3 sequence, position -303 to -327. **(3)**
5' GGAGGTTTGACAGCCGCCTAGCATT 3'	Sense primer 586N from U3 sequence, position -195 to -171. **(3)**
5' CTTGCCGCTAGACTGCGTCG 3'	Ligation sequence 021N. **(3)**
5' CAGTCTAGCGGCAAGACTGC 3'	Anti-sense primer 022N for ligation sequence and U5 termini of LTR. **(3)**

5' CAGTCTAGCGGCAAGACTGG 3'	Sense primer 915R for ligation sequence and U3 termini (full length). **(3)**
5' CAGTCTAGCGGCAAGTGGAA 3'	Sense primer 916R for ligation sequence and U3 termini (integration precursor). **(3)**

Sources of primer sequences.

(1) Based on those of Simmonds et al., (1990a).

(2) Adapted from the primer M844 used by Pang et al., (1990).

(3) Selected from the LTR sequences of Starcich et al., (1985) and Hammarskjöld & Rekosh, (1989).

(4) Modified from the *nef* and *gag* sequences used by Pauza, (1990).

HBV specific oligonucleotides. The coordinates (5' to 3') of HBV specific sequences are based on the published sequence of an *adr* HBsAg subtype genome by Kobayashi & Koike, (1984).

5' GAGGGATAGCAGGAGGATGAAGAGG 3'	Anti-sense primer 057C, position 301 to 277.
5' CATGAGGATTCCTAGGACCCCT 3'	Sense primer 058C, position 42 to 63.
5' GGAAAGCCCTACGAACCAATGAAC 3'	Anti-sense primer 869C, position 589 to 566.
5' CGTGTTACAGGCGGGGTTTTTCTTG 3'	Sense primer 870C, position 67-91.
5' CTTGAGCAGTAGTCATGCAGGTCCG 3'	Anti-sense primer 907E, subtype *y* specific, position 416 to 392.
5' CTTGAGCAGGAGTCGTGCAGGTTTT 3'	Anti-sense primer 029H, subtype *d* specific, position 416 to 392.

5' CTTGAGCAGTAGTCATGCAGGTCC 3'	Anti-sense primer 394J, subtype *y* specific, position 416 to 393.

HBV specific oligonucleotides were derived from the analyses of 5 human HBV DNA sequences of subtypes *adrcg*, *adw*, *adyw*, *ayw*, and adw_2 (GenBank accession numbers: X01587; V00866 J02201; J02202; J02203 V01460; and X02763 respectively). Primers 057C, 058C, 869C, and 870C, were selected from conserved regions of the S-gene. Primers 907E and 394J were selected from the *y* specific sequence, and 029H from the *d* specific sequence, hybridizing to codon 122 at their 3' ends (Figure 9).

Appendix VI. Amino acid sequence of recombinant gp120 protein.

The amino acid sequence of the mature recombinant protein gp120 (residues 31 to 510 inclusively). Amino acids are supplied as 3 letter code, the key to this code is supplied in Table 42. Variable amino acids are given in italics, and tyrosine and histidine residues are in bold. The protein was produced using a Baculovirus expression system in Chinese hamster ovary cells (Appendix II). The protein sequence was kindly supplied by Dr Gary Clements from Celltech Research (Slough).

```
 31                                                  40
Thr Glu Lys Leu Trp Val Thr Val Tyr Tyr Gly Val Pro Val
                        50
Trp Lys Glu Ala Thr Thr Thr Leu Phe Cys Ala Ser Asp Ala
     60                                                 70
Lys Ala Tyr Asp Thr Glu Val His Asn Val Trp Ala Thr His
                                 80
Ala Cys Val Pro Thr Asp Pro Asn Pro Gln Glu Val Val Leu
               90                                          100
Val Asn Val Thr Glu Asn Phe Asn Met Trp Lys Asn Asp Met
                                    110
Val Glu Gln Met His Glu Asp Ile Ile Ser Leu Trp Asp Gln
                        120
Ser Leu Lys Pro Cys Val Lys Leu Thr Pro Leu Cys Val Ser
    130                                                 140
Leu Lys Cys Thr Asp Leu Lys Asn Asp Thr Asn Thr Asn Ser
                                 150
Ser Ser Gly Arg Met Ile Met Glu Lys Gly Glu Ile Lys Asn
               160                 Lys                  170
Cys Ser Phe Asn Ile Ser Thr Ser Ile Arg Gly Lys Val Gln
                                    180
Lys Glu Tyr Ala Phe Phe Tyr Lys Leu Asp Ile Ile Pro Ile
                        190
Asp Asn Asp Thr Thr Ser Tyr Thr Leu Thr Ser Cys Asn Thr
    200                                                 210
Ser Val Ile Thr Gln Ala Cys Pro Lys Val Ser Phe Glu Pro
                                 220
Ile Pro Ile His Tyr Cys Ala Pro Ala Gly Phe Ala Ile Leu
               230                                          240
Lys Cys Asn Asn Lys Thr Phe Asn Gly Thr Gly Pro Cys Thr
                                    250
Asn Val Ser Thr Val Gln Cys Thr His Gly Ile Arg Pro Val
                        260
Val Ser Thr Gln Leu Leu Leu Asn Gly Ser Leu Ala Glu Glu
    270                 Val                             280
Glu Val Val Ile Arg Ser Ala Asn Phe Thr Asp Asn Ala Lys
```

290/*Thr*
Thr Ile Ile Val Gln Leu Asn Gln Ser Val Glu Ile Asn Cys
300 *Lys* 310
Thr Arg Pro Asn Asn Asn Thr Arg Lys Ser Ile Arg Ile Gln
320
Arg Gly Pro Gly Arg Ala Phe Val Thr Ile Gly Lys Ile Gly
330
Asn Met Arg Gln Ala **His** Cys Asn Ile Ser Arg Ala Lys Trp
340/*Ala* *Ala* 350
Asn Asn Thr Leu Lys Gln Ile Asp Ser Lys Leu Arg Glu Gln
360
Phe Gly Asn Asn Lys Thr Ile Ile Phe Lys Gln Ser Ser Gly
370 380
Gly Asp Pro Glu Ile Val Thr **His** Ser Phe Asn Cys Gly Gly
390
Glu Phe Phe **Tyr** Cys Asn Ser Thr Gln Leu Phe Asn Ser Thr
400
Trp Phe Asn Ser Thr Trp Ser Thr Glu Gly Ser Asn Asn Thr
410 420
Glu Gly Ser Asp Thr Ile Thr Leu Pro Cys Arg Ile Lys Gln
Ile 430
Phe Ile Asn Met Trp Gln Glu Val Gly Lys Ala Met **Tyr** Ala
440
Pro Pro Ile Ser Gly Gln Ile Arg Cys Ser Ser Asn Ile Thr
460 *Ser*
Gly Leu Leu Leu Thr Arg Asp Gly Gly Asn Asn Asn Asn Glu
470
Ser Glu Ile Phe Arg Pro Gly Gly Gly Asp Met Arg Asp Asn
480 490
Trp Arg Ser Glu Leu **Tyr** Lys **Tyr** Lys Val Val Lys Ile Glu
500
Pro Leu Gly Val Ala Pro Thr Lys Ala Lys Arg Arg Val Val
510
Gln Arg Glu Lys.

Table 42. Three letter abreviations for amino acid residues.

Code	Amino acid	Code	Amino acid
Ala	Alanine	Ile	Isoleucine
Arg	Arginine	Leu	Leucine
Asn	Asparagine	Lys	Lysine
Asp	Aspartic acid	Met	Methionine
Cys	Cysteine	Phe	Phenylalanine
Gln	Glutamine	Ser	Serine
Glu	Glutamic acid or glutamine	Thr	Threonine
		Trp	Tryptophan
Gly	Glycine	Tyr	Tyrosine
His	Histidine	Val	Valine

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Comparison of Hepatitis B Virus Subtyping of *d/y* Determinants by Radioimmunoprecipitation Assay and the Polymerase Chain Reaction

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Using a double polymerase chain reaction a method was devised for detecting and subtyping hepatitis B virus DNA in serum samples. Primers from the S-gene were selected from the sequence analyses of five HBV HBsAg subtypes, to amplify HBV DNA and subtype for *y* specific DNA. Thirty-eight samples were subtyped for *d* and *y* determinants by radioimmunoprecipitation assay (RIPA) and the polymerase chain reaction (PCR). Subtyping by PCR and RIPA was in agreement in 100% of subtype *y* samples and 83.3% of subtype *d*, giving an overall correlation of 92.1%. As a third comparison, 12 amplified samples were digested by the restriction enzyme *Sau* 3A, which differentiates between subtypes *y* and *d*. The digest results agreed with PCR in 83.3% of the samples. In addition, we compared our standard phenol/chloroform extraction against a rapid one step method. The phenol/chloroform stage was found to be essential for the removal of nucleases and polymerase inhibitors present in sera.

KEY WORDS: conformational determinant, linear determinant, oligonucleotide primers

INTRODUCTION

It has been shown that HBeAg is a good indicator of ıral replication and is, therefore, related to the presıce of HBV DNA in serum samples. Utilising the ɔlymerase chain reaction [Saiki et al., 1985], HBV NA has been detected in 100% of HBeAg positive ɛra, and in up to 75% of HBeAg/anti-HBe negative and ıti-HBe positive sera [Bonino et al., 1986; Chen et al., 986; Okamoto et al., 1989]. Hybridisation studies ɛtected HBV DNA in 4–21% of similar HBeAg negave samples [Weller et al., 1982; Karayiannis et al., 985; Carloni et al., 1987; Cheng et al., 1989], demonrating the improved sensitivity offered by PCR.

The surface antigen of hepatitis B virus (HBsAg) has an immunodominant determinant *a*, and two further sets of determinants *d/y* [Le Bouvier, 1971] and *w/r* [Bancroft et al., 1972] that are generally believed to be mutually exclusive. This gives four major subtypes of HBsAg, *adw, adr, ayw,* and *ayr,* which are determined by conventional serology. At the molecular level, the *d/y* and *w/r* determinants were shown to be coded for by amino acid residues 110 to 160 of the HBsAg gene [Gerin et al., 1983; Takeshima et al., 1985]. From site-directed mutagenesis, Okamoto et al. [1987b] demonstrated that a base change of G to A resulted in amino acid 122 changing from arginine to lysine, and the subtype from *ay* to *ad*. Similarly, a G to A point mutation at amino acid 160 resulted in a change from *aw* to *ar* [Okamoto et al., 1987a].

We compared the detection of HBV DNA by PCR with HBsAg, anti-HBs, anti-HBc, and HBe status, and developed a method of subtyping for the *d/y* determinants of the HBsAg, using a subtype specific primer from the S-gene.

MATERIALS AND METHODS

Source of HBsAg and Anti-HBs Positive Sera

Sera submitted to the Hepatitis Reference Laboratory (University of Edinburgh, Medical School) were tested by standard radioimmunoassays for HBsAg (Blood Products Laboratory, Elstree), anti-HBs and anti-HBc (Hepatitis Reference Laboratory, Edinburgh), and HBeAg/anti-HBe (Middlesex Hospital). The 109 samples studied were selected to include a range of HBV serological markers: 54 HBsAg/HBeAg positive; 6 HBsAg positive, HBeAg/anti-HBe negative; 23 HBsAg/anti-HBe positive; 11 HBsAg negative, anti-HBs positive, HBeAg/anti-HBe negative; 9 HBsAg negative, anti-HBs/anti-HBe positive; 3 HBsAg negative, anti-HBc positive, HBeAg/anti-HBe negative; and 3 HBsAg negative, anti-HBc/anti-HBe positive. These

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were tested for HBV DNA by PCR. Thirty-eight of the HBsAg positive/HBV DNA positive samples (32 HBeAg positive, 7 anti-HBe positive, and 1 HBeAg/anti-HBe negative) were subtyped by RIPA and PCR. Samples from 10 low-risk seronegative individuals were used as negative controls. Reference HBsAg preparations and antisera were supplied by the Research Resources Branch, National Institutes of Health (Bethesda, MD). The panel of HBsAg subtypes [Couroucé et al., 1976], as described at the first HBsAg Subtype Workshop, Paris 1975, was kindly supplied by Dr. A.M. Couroucé, Centre National de Transfusion Sanguine (6 Rue Alexandre-Cabanel, Paris, France).

HBsAg for purification and the subtyping RIPA was prepared from HBsAg-positive blood donations supplied by the Glasgow and West of Scotland Blood Transfusion Service, Law Hospital (Carluke, Lanarkshire).

Purification of HBsAg

HBsAg was purified from separate samples of antigen-positive plasma as described by Burrell [1975].

Preparation of Rabbit Anti-HBs

Antisera were prepared in rabbits by immunisation with purified HBsAg emulsified in Freund's incomplete adjuvant.

HBsAg Absorption of Rabbit Anti-HBs Sera

To produce monospecific antisera, aliquots of rabbit sera were cross-absorbed with appropriate subtype antigens. After absorption the samples were centrifuged at 37,000*g* for 2 hr and the supernate stored at 4°C.

Radiolabelling Purified HBsAg

Samples of purified HBsAg were radiolabelled with iodine (^{125}I) by the modified chloramine-T method as described by Burrell et al. [1973].

Analyses of Rabbit Anti-HBs After Absorption With HBsAg

Standard titration curves of the absorbed antisera with radio-labelled antigen were used to determine the working dilution of the antiserum in RIP subtype assays. Fourfold dilutions from 1:100 (in 1:160 carrier rabbit serum diluted in RIP buffer) of absorbed rabbit anti-HBs serum were incubated with ^{125}I-HBsAg and neat HBsAg-negative human serum for 16 hr at 4°C. The rabbit antibody was precipitated by adding anti-rabbit IgG serum (donated by Scottish Antibody Production Unit) in RIP buffer, and incubated for a further 16 hr at 4°C. The percentage of ^{125}I-HBsAg bound to rabbit anti-HBs was determined as described by Burrell et al. [1973]. The dilution of absorbed anti-HBs serum, giving 40–70% binding of labelled antigen, was used as the working dilution of antiserum in the assays of antigen subtypes. The anti-HBs activity of each absorbed rabbit antiserum was characterised by RIP assay with eight known subtypes (Paris Panel of Antigen Subtypes). After precipitation with anti-rabbit IgG serum the percentages of ^{125}I-HBsAg bound in the precipitates of the control and the test samples were calculated as before.

RIP Assay for Subtyping

HBsAg samples from positive patients were tested for subtype determinants by RIP assays using the absorbed rabbit anti-HBs sera of "defined-subtype" activity. One in ten and 1:100 dilutions of the samples were assayed in parallel with samples of the neat diluent. Also included in each subtype assay were 100 μl samples of the "determinant-negative" control at 1:10 dilution, i.e., the subtype antigen used in the absorption of the rabbit antiserum. The technique was described by Burrell et al. [1978], for HBsAg detection.

DNA Extractions

Sera (200 μl) were incubated for 45 min on ice with proteinase K (Sigma) at 100 μg/ml in an equal volume of Lysis buffer (200 mM NaCl, 100 mM EDTA, 100 mM Tris, pH 8.0). N-lauroylsarcosine was added to a final concentration of 1% (w/v), and samples were incubated at 65°C for 3 hr. After phenol/chloroform extraction, the aqueous phase was precipitated with ethanol (2 × volume) and 4M potassium acetate (0.1 volume). DNA pellets were resuspended in 100 μl of 0.1 × STE (10 × STE: 10 mM Tris, 100 mM NaCl, 1 mM EDTA, pH8.0).

Rapid DNA Extractions

Patient sera (100 μl) were incubated with proteinase K (100 μg/ml) and N-lauroylsarcosine (1% w/v) at 60°C for 60 min, then boiled for 10 min. The samples were centrifuged at 9,800*g* for 10 min, and the supernates used directly for PCR.

DNA Amplification

Resuspended DNA was amplified essentially as described by Simmonds et al. [1990], excluding dimethylsulphoxide and EDTA from the reaction. To reduce the risk of contamination and monitor performance, samples were amplified in batches of ten, each batch consisting of five samples and five controls numbered randomly. The three negative controls were obtained from low risk, seronegative individuals. The two positive controls, a *d* and *y* subtype, were patient samples found to subtype consistently by RIPA and PCR. To avoid the possibility of gross contamination, no cloned HBV genomes were used. Patient samples, although not standardised, offered a more appropriate control than cloned material as they included possible serum contaminants, known to inhibit *Taq* polymerase [Zeldis et al., 1989].

To ensure that the initial amplification of viral DNA was specific, the secondary and subtype-specific amplifications used nested inner primers to amplify an aliquot of primary amplified material [Mullis et al., 1986].

Each batch was amplified and analysed by gel electrophoresis. False positive or negative results obtained in any control resulted in the batch being discarded and

plification repeated. Each sample was subtyped and samples found to differ were then subtyped ier five times.

Oligonucleotide PCR Primers

onucleotide primers were synthesised by Oswel Service, Department of Chemistry, University of urgh, and purified by high-performance liquid atography. Their sequences were derived from nalyses of 5 human HBV DNA sequences of es *adrcg, adw, adyw, ayw,* and *adw*[2]. Primers 058C, 869C, and 870C, were selected from con- regions of the S-gene. Primers 907E and 394J elected from the *y*-specific sequence, and 029H ne *d*-specific sequence, hybridising to codon 122 at end. Primer sequences included 057C 5′GAGG-GCAGGAGGATGAAGAGG-3′, 058C 5′-CAT-ATTCCTAGGACCCCT-3′, 869C 5′-GGAAAGC-CGAACCAATGAAC-3′, 870C 5′-CGTGTTACA-GGGTTTTTCTTG-3′, 907E 5′-CTTGAGCAGT-ATGCAGGTCCG-3′, 029H 5′-CTTGAGCAGG-GTGCAGGTTTT-3′, 394J 5′-CTTGAGCAGTA-TGCAGGTCC-3′.

uots (10 μl) of the amplification products were ed by gel electrophoresis, against ladders of digested by *Hae* III (Gibco BRL) and DNA ular-weight marker VI (Boehringer Mannheim). rs 058C and 869C were used for primary ampli-n, yielding a band of 548 bp. The nested primers and 870C) gave a band of 235 bp, and the e-specific primers used in conjunction with resulted in bands of 350 bp.

Sau 3A Digest of PCR Products for Confirmatory Subtyping

generate sufficient HBV DNA to visualise the tion digest products on an agarose gel, an aliquot of primary amplified product was re-amplified ne outside primer pair 058C/869C. The amplifi-product was restricted with 2.5 units of *Sau* 3A inger Mannheim) at 37°C for 1.5 hr. The restric-igest (15 μl) was electrophoresed on a 1.5% e gel, against DNA molecular-weight marker VI inger Mannheim), at 150 V.

RESULTS

Comparison of Rapid and Full DNA Extraction Methods

teen samples were extracted by both methods, nplified as described. When analysed by agarose ectrophoresis, 13 (100%) of the samples were e for HBV DNA after the full extraction method, 44%) after the rapid extraction. The difference in vity observed was probably due to the presence of erase inhibitors in the rapid extracts. In addition , rapid extracts stored at −20°C for a period of onths, often gave negative results when re-ied. This may be accounted for by the presence of ses not removed during extraction. All subse-DNA for HBV detection and subtyping, was ted by the full phenol/chloroform method.

TABLE I. Comparison of HBV DNA Detection in Sera Against Markers of Infection (HBsAg, Anti-HBs, and Anti-HBc), Correlated to HBe Status*

	HBeAg positive	HBe negative	Anti-HBe positive	Total No.
HBsAg positive	54	6	23	83
(DNA +)	(54)	(2)	(8)	(64)
Anti-HBs Anti-HBc positive	0	11	9	20
(DNA +)	(0)	(0)	(2)	(2)
HBsAg & anti-HBs negative	0	3	3	6
Anti-HBc positive				
(DNA +)	(0)	(0)	(0)	(0)

*Nos. in parentheses are total No. of HBV DNA positive samples by PCR.

Detection of HBV DNA by PCR

The HBsAg, anti-HBs, anti-HBc, and HBe status of 109 samples were determined by radioimmunoassays. Eighty-three were HBsAg positive; 20, anti-HBs/anti-HBc positive; and 6, HBsAg/anti-HBs negative, anti-HBc positive. DNA was extracted and an aliquot used for the specific amplification of HBV DNA by a double PCR, using primer pairs 058C/869C and 057C/870C (Fig. 2). HBV DNA was detected in 64 (77%) of HBsAg positive samples (54 HBeAg positive, 2 HBeAg/anti-HBe negative, and 8 anti-HBe positive), and 2 (10%) of the anti-HBs positive samples (both anti-HBe positive). The results are summarised in Table I.

Subtyping of HBV

Thirty-eight HBV DNA positive samples were subtyped by RIP assay. Eighteen samples were subtyped *d*, 20 subtyped *y*. These samples were also subtyped by PCR, using the *y*-specific primer (907E), and 870C as a nested primer pair.

Subtyping by PCR is based on the theory that a 3′ mismatch of a primer with the template DNA results in a decrease in amplification efficiency [Newton et al., 1989]. Utilising this theory, two subtype-specific primers (029H and 907E) were selected to subtype samples *d* and *y*, respectively. These primers were selected from our sequence analysis which showed the three bases of codon 122 to be involved in *d/y* subtyping (Fig. 1). In contrast, only the central base of this codon is published as being essential for *d/y* subtyping [Okamoto et al., 1987b]. To correlate with published data a third primer 394J was selected, identical to 907E except for the loss of the 3′ terminal guanine residue. When tested, it was found that primers 209H and 394J acted like subtype common primers, amplifying both *d* and *y* subtype DNA. The primers 029H and 394J had a T:C and C:A 3′ mismatch, respectively. These mismatches were shown to allow significant amplification by Kwok et al. [1990], which explains their inability to distinguish

adr			g	t
adw	t			
adyw		t c	g	
adw2	t			
ayw			cgg	t a
Consensus	AGCACGGGAC	CATGCAAAAC	CTGCACGACT	CCTGCTCAAG
Primer 907E			GCCTG	GACGTACTGA TGACGAGTTC
029H			TTTTG	GACGTGCTGA GGACGAGTTC
394J			CCTG	GACGTACTGA TGACGAGTTC

Fig. 1. Sequence analysis of S-gene from codon 117 to 130. The consensus sequence of the above 5 HBV subtypes is given in uppercase, and their subtypic variation in lowercase. The primers selected to differentially amplify the *d* and *y* subtypes are given in bold, demonstrating their 3′ mismatches with codon 122.

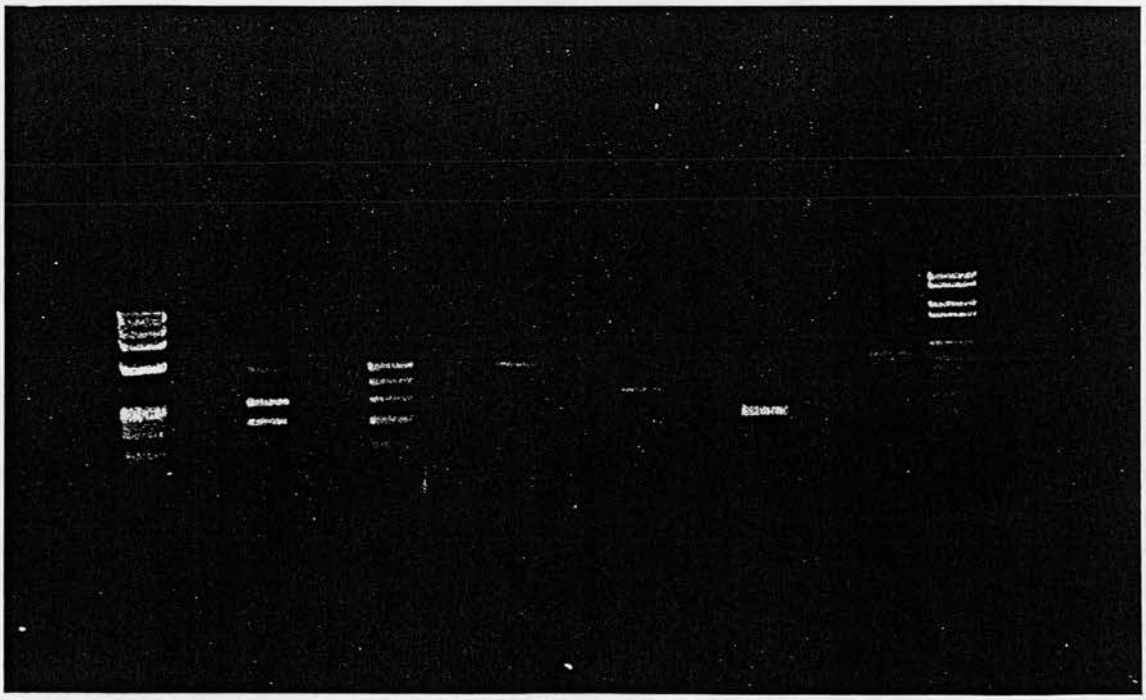

Fig. 2. Ethidium bromide stained agarose gel of HBV DNA detection and subtyping by PCR, and *Sau* 3A digest results. **Lane 1:** øX174/*Hae* III digest ladder. **Lane 3:** *Sau* 3A digest of sample 11 (subtype y). **Lane 5:** *Sau* 3A digest of sample 2 (subtype *d*). **Lane 7:** Subtype-specific amplification of sample 2. **Lane 9:** Subtype-specific amplification (870C/907E) of sample 11. **Lane 11:** Secondary amplification (057C/870C). **Lane 13:** Primary amplification (058C/869C). **Lane 14:** DNA molecular-weight marker VI. **Lanes 2,4,6,8,10,12** were negative controls.

etween subtypes. The *y*-specific primer (907E) was elected with a 3′ G:A mismatch, shown to reduce the CR product yield 100-fold. In addition to this, the econd and third base mismatches enhanced the effect Kwok et al., 1990]. Primer 907E, therefore, distinuished between subtypes *d* and *y* by not amplifying *d* ubtype samples sufficiently for ethidium bromide viualisation on agarose gels. Due to the lack of a d pecific primer, *d* subtyping was based on a negative esult with the *y*-specific primer pair. To account for this, negative samples with the *y*-specific primers were only accepted as true subtype *d*, if the primary amplification band was visible, indicating the presence of amplified viral DNA (Fig. 2). Due to these preliminary studies all PCR subtyping used the nested primer pair 870C/907E.

PCR and RIPA subtyping agreed in 20 (100%) of subtype *y* samples, and 15 (83.3%) of subtype *d* samples, giving an overall correlation of 92.1% for the 38 samples tested (Table II).

TABLE II. Comparison of RIPA and PCR Subtyping of HBsAg

subtype	*d*	*y*
No.	18	20
subtype *d*	15	0
subtype *y*	3	20

e to the three samples with conflicting RIPA and results, a third method of subtyping was used. restriction maps of five HBV subtypes (*adw, ayw, adw2,* and *adrcg*), it was observed that a 3A digest of the S-gene would discriminate be- n *d* and *y* subtype genomes. These maps predicted *d* subtype genomes would result in three bands 204, 227bp) and *y* subtype two bands (227 and 321 When analysed by agarose gel electrophoresis, it found that *d* subtype genomes resulted in five s, and *y* subtype three bands (Fig. 2). Due to the s of template DNA generated by PCR, neither t was complete. The predicted bands for a partial t were six for *d* subtypes (117, 204, 227, 321, 431, 48 bp), and three for *y* subtypes (227, 321, and 548 Due to the limitation of agarose gels, 2 of the *d* ic bands (204 and 227 bp) migrated together, ting in five bands for *d* subtype samples. Twelve les were tested, nine of which confirmed PCR and results. Of the three remaining samples, one ple 32) confirmed PCR, and two (samples 35 and IPA (Table III).

DISCUSSION

e polymerase chain reaction is the most sensitive od for the detection of specific DNA sequences, and onsequently improved the rate of detection of HBV in patient samples with serological markers of ion [Larzul et al., 1988, 1989, 1990; Kaneko et al., Liang et al., 1989; Zeldis et al., 1989; Lin et al., . The main disadvantage of this increased sensi- is the possibility of false-positive results from mination of samples by template DNA [Lo et al., . To avoid contamination, we developed a rapid od of extraction which minimised handling of les. This proved to be less sensitive when com- with the full extraction, and so it was concluded the phenol/chloroform step of the extraction od was essential to remove inhibitors which re- the efficiency of PCR, and nucleases which can de DNA in stored samples.

V has previously been subtyped by serology on the of three-dimensional epitopes. The region of the e involved in subtyping (amino acids 110–160) ertiary structure due to the formation of disul- bridges by cross linking of cysteine residues, g a conformational determinant for antibody rec- on [Gerin et al., 1983; Takeshima et al., 1985]. In arison, our method of subtyping by PCR is re- ed to a linear epitope, or sequential determinant, consisting of three bases coding for amino acid 122. Like RIPA, subtyping by *Sau* 3A digest is based on multiple sites of the S-gene. Due to this similarity in subtyping, RIPA and *Sau* 3A restriction digest results were expected to correlate more closely than with PCR. However, codon 122, although involved in antibody:antigen recognition, is not part of a restriction site for *Sau* 3A. It is, therefore, possible for codon 122 to code for one determinant and for the other sequences involved in restriction sites and antibody binding to be the other determinant. This phenomenon could explain the results obtained with samples 35 and 38.

Alternatively, PCR subtyping relies upon a 3′ mismatch to differentially amplify viral DNA. By reducing the overall efficiency of PCR for amplification with the subtype specific primer pair, it may be possible to further optimise the reaction for HBV DNA subtyping, and obtain a better correlation with standard methods of subtyping. To support this theory, Norder et al. [1990] were able to use a primer similar to 029H (which we found to be indiscriminant) to detect *d* subtype samples. Their detection was based on a 3′ T:C mismatch of both nested inner primers, used in a single PCR, and detected by hybridisation with a ^{32}P-labelled probe. Their use of two subtype-specific primers for a single PCR followed by hybridisation, has overcome the reported amplification efficiency of T:C mismatches, and allowed discrimination between subtypes *d* and *y*. The third sample (32) with aberrant PCR and RIPA results when digested by *Sau* 3A confirmed the PCR result. Although the digest and RIPA subtyping methods involve different sites of the S-gene, their conflicting results highlight problems associated with RIP assays. RIPA subtyping involves a number of antibody purifications from polyclonal sera, whose subtypic determinants are characterised by blocking by HBsAg of known subtype. As with PCR, subtyping by RIPA is not absolute and, therefore, requires similar careful optimisation.

Two HBsAg negative samples gave HBV DNA positive results on repeated extractions. Although no clinical information was available, both had antibodies to core and surface antigen indicating exposure to infection. HBV DNA detected in HBsAg negative samples could be due to the presence of immune complexes of hepatitis B virions in the blood [Liang et al., 1989], or to the release of HBV DNA from necrotic hepatocytes [Carman et al., 1989]. These samples demonstrate the sensitivity of PCR in detecting HBV DNA in patient sera with serological markers indicative of recovery. Since PCR can detect one molecule of template DNA from patient samples [Simmonds et al., 1990], it is therefore advantageous over RIP assays, which are dependent on high HBsAg concentrations which decline with anti-HBs production.

In conclusion, the polymerase chain reaction offers a rapid method of subtyping hepatitis B virus from sera of patients at various stages of infection. In contrast to RIPA, increased sensitivity is achieved by PCR with the ability to detect a single molecule of HBV DNA.

TABLE III. Comparison of RIPA, PCR, and *Sau* 3A Restriction Digest Subtyping of HBsAg

Sample No.	HBsAg result	HBeAg/Ab positive	PCR result	RIPA subtype	PCR subtype	DIGEST subtype
11	+	Ag	+	*y*	*y*	*y*
2	+	Ag	+	*d*	*d*	*d*
5	+	Ag	+	*y*	*y*	*y*
13	+	Ag	+	*d*	*d*	*d*
26	+	Ag	+	*y*	*y*	*y*
27	+	Ag	+	*d*	*d*	*d*
32	+	Ag	+	*d*	*y*	*y*
25	+	Ag	+	*y*	*y*	*y*
10	+	Ag	+	*d*	*d*	*d*
35	+	Ab	+	*d*	*y*	*d*
38	+	Ab	+	*d*	*y*	*d*
4	+	Ab	+	*y*	*y*	*y*

Since PCR detects HBV directly, it can be used in the detection and subtyping of HBV in blood, liver biopsies, frozen or fixed tissue, and for studies on hepatoma tissues. The sensitivity and flexibility of PCR will hopefully help advance the study of the molecular epidemiology of the hepatitis B virus.

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