THE INVOLVEMENT OF VIRUSES IN THE AETIOLOGY OF HUMAN LEUKAEMIAS AND LYMPHOMAS.

by

Sarah Gledhill, B.Sc.(Hons), B.V.Sc., M.R.C.V.S..

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in the Faculty of Veterinary Medicine, University of Glasgow.

Department of Veterinary Pathology,
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ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

For Richard.

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DECLARATION.

I declare that this thesis is entirely my own composition and that the studies described herein are the results of my own work, except where otherwise acknowledged.

SUMMARY

The experiments described in this thesis illustrate three approaches to the investigation of leukaemias and lymphomas for evidence of a viral aetiology. These approaches are (1) to target specific disease entities for investigation on the basis of epidemiological and clinical features suggestive of a viral aetiology, (2) to investigate the molecular association between a known virus and leukaemia and lymphoma tissue and (3) to search for novel viruses related to a known leukaemogenic virus.

Some of the clinical and epidemiological features of Hodgkin's disease (HD) are suggestive of an infectious or transmissable aetiology. As part of an investigation into the pathogenesis of HD 35 samples from HD patients were characterized by the molecular analysis of the T-cell receptor (TCR) and immunoglobulin (Ig) genes. Ig gene rearrangements were detected in 9/35 HD samples. No TCR gene rearrangements were detected. Seven of the cases in which IgH gene rearrangements were detected contained high numbers of Reed-Sterberg cells and their mononuclear variants. The data are consistent with the hypothesis that HRS cells are derived from lymphocytes at varying stages of differentiation.

Forty seven HD samples were analysed for the presence of human herpesvirus 6- (HHV-6-) specific DNA sequences. HHV-6-specific DNA sequences were not detected in any of the 35 HD samples. The results do not support a direct role for HHV-6 in the aetiology of HD. HHV-6 DNA sequences were detected in 2/53 samples obtained from patients with non-Hodgkin's lymphoma (NHL). The positive cases were a patient with angioimmunoblastic lymphadenopathy (AIL), which had progressed to T-zone lymphoma, and a patient with a B-cell NHL occurring in the context of Sjögren's syndrome. Viral DNA sequences were detected in tumour and in non-tumour tissue from both patients. HHV-6 has been previously isolated from a patient with AIL (Salahuddin *et al.* 1986) and HHV-6-specific DNA sequences have been detected in a B-cell lymphoma occurring in a patient with Sjögren's syndrome (Josephs *et al.* 1988a). The data obtained in this study do not support a direct role for HHV-6 in the aetiology of either condition, however the possibility that the virus could be indirectly involved in disease pathogenesis remains. In particular the association between HHV-6 and lymphomas occurring in the context of Sjögren's syndrome appears worthy of further investigation.

The presence of antibodies that cross-react with proteins encoded by the *gag*-gene of human T-lymphotropic virus type I (HTLV-I) in sera from persons who do not appear to be HTLV-I-infected may indicate infection with an HTLV-I-related retrovirus. A novel approach to investigating this possibility was devised. Overlapping octapeptides spanning the length of the p19 gag protein of HTLV-I were synthesized and used in an enzyme-linked immunoassay to identify the regions of p19 that bound antibodies in cross-reactive sera. Cross-reactive serum samples obtained from persons who appeared to be HTLV-I negative were shown to react with epitopes near the C-terminus of p19. HTLV-I positive serum samples also reacted with octapeptides near the C-terminus of p19. The data obtained from the analysis of 9 cross-reactive sera obtained from residents of Papua New Guinea provide some support for the hypothesis that these persons may be infected with a retrovirus related to HTLV-I. The data also provide information that could be used for further testing of this hypothesis.

ABBREVIATIONS.

AIDS: acquired immune deficiency syndrome
AIL: angioimmunoblastic lymphadenopathy
ATL: adult T-cell leukaemia/lymphoma
BL: Burkitt's lymphoma
BLV: bovine leukaemia virus
CHEF: contour clampled hexagonal electric field
CTCL: cutaneous T-cell lymphoma
C-terminal: carboxy-terminal
DMF: dimethyformamide
eBL: endemic Burkitt's lymphoma
EBV: Epstein-Barr virus
EDTA: disodium ethylene diamine tetraacetate
ELISA: enzyme linked immunoassay
FIV: feline immunodeficiency virus
Fmoc: fluorenylmethoxycarbonyl
GM-CSF: granulocyte-macrophage-colony stimulating factor
GPA: gel particle agglutination
GST: glutathione-S-transferase
HCL: hairy cell leukaemia
HCMV: human cytomegalovirus
HD: Hodgkin's disease
HERV: human endogenous retrovirus-related sequences
HHV-6: human herpesvirus type 6
HIV: human immunodeficiency viruses
HIV-2: human immunodeficiency virus type 2
HLA: human leukocyte antigen
HOBt: 1-hydroxybenzotriazole
HRPO: horse radish peroxidase
HRS cells: Reed-Sternberg cells and mononuclear Hodgkin's cells
HSV: herpes simplex virus
HTLV: human T-cell leukaemia viruses
HTLV-I: human T-cell leukaemia virus type I
HTLV-II: human T-cell leukaemia virus type II
IF: immunofluorescence
Ig: immunoglobulin
IgH: immunoglobulin heavy chain
IgL: immunoglobulin light chain
IgLκ: immunoglobulin kappa light chain
IgLλ: immunoglobulin lambda light chain
IL-2: interleukin-2

ABBREVIATIONS (continued)

IL-2Rα: interleukin-2 receptor alpha chain
IM: infectious mononucleosis
IPTG: isopropylthio-β-D-galactoside
IR: indeterminate reactivities
IVDA: intravenous drug abusers
kb: kilobases
LB: Luria-Bertani
LCL: lymphoblastoid cell lines
LD: lymphocyte depleted
LGLL: large granular lymphocyte leukaemia
LMP: latent membrane protein
LP: lymphocyte predominant
LTR: long terminal repeat
MBR: major breakpoint cluster region
MC: mixed cellularity
MDV: Marek's disease virus
MF: mycosis fungoides
MoAb: monoclonal antibody
mRNA: messenger RNA
MS: multiple sclerosis
MTPBS: mouse tonicity phosphate-buffered saline
NHL: non-Hodgkin's lymphoma
NPC: nasopharyngeal carcinoma
NS: nodular sclerosing
O.D.: optical density
OND: other neurological diseases
OPC: oligonucleotide purification cartridge
PAGE: polyacrylamide gel electrophoresis
PBMCs: peripheral blood mononuclear cells
PBS: phosphate-buffered saline
PCR: polymerase chain reaction
PFGE: pulse field gel electrophoresis
phage: bacteriophage
PNG: Papua New Guinea
RIPA: radioimmunoprecipitation assay
rp19: recombinant p19
rpm: revolutions per minute
RR: repeatedly reactive
RS: Reed-Sternberg
sBL: sporadic Burkitt's lymphoma
SDS: sodium dodecyl sulphate

ABBREVIATIONS (continued)

SS: Sezary syndrome
TBS: tris-buffered saline
TCR: T-cell receptor
TCRα: T-cell receptor α chain
TCRβ: T-cell receptor β chain
TCRγ: T-cell receptor γ chain
TCRδ: T-cell receptor δ chain
TEMED: N,N,N',N'-tetramethylethyldiamine
TSP: tropical spastic paraparesis
Vβ: variable region of the T-cell receptor β chain
WB: western blot

CHAPTER ONE

GENERAL INTRODUCTION:

THE INVOLVEMENT OF VIRUSES IN THE AETIOLOGY OF HUMAN LEUKAEMIAS AND LYMPHOMAS.

1. INTRODUCTION.

Members of two families of viruses, the Retroviridae and the Herpesviridae, are known to be involved in the aetiology of leukaemias and lymphomas in animals. Leukaemias known to have a viral aetiology provide useful models for developing approaches to the investigation of viral involvement in other haematopoietic neoplasms. Sections 1.1-1.2 provide a brief review of the associations between retroviruses and herpesviruses and lymphoid malignancies. The leukaemias and lymphomas of humans that are considered to have a viral aetiology are discussed in more detail.

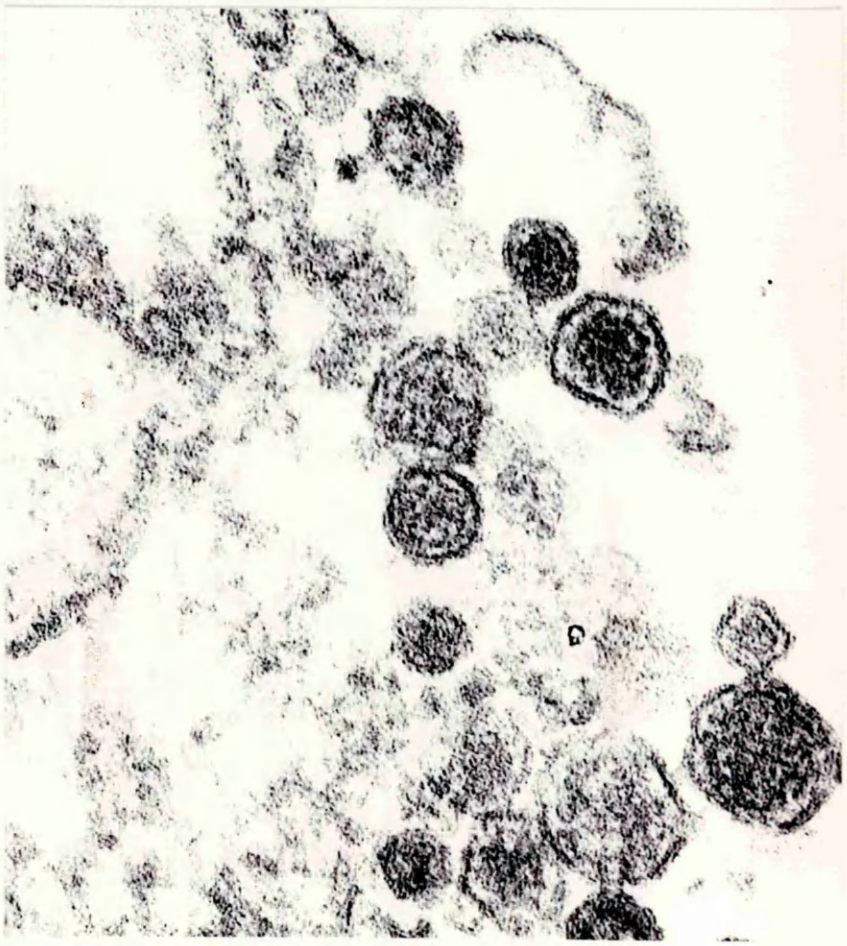
In sections 1.3-1.5 three general approaches to the investigation of leukaemias and lymphomas for evidence of a viral aetiology are described. These may be summarized as follows: 1.3, the targeting of specific disease entities on the basis of clinical and/or epidemiological evidence suggestive of an infectious or viral aetiology; 1.4, the analysis of leukaemias and lymphomas for evidence of a molecular or serological association with a specific virus; 1.5, the use of molecular or serological techniques to search for unidentified viruses that are related to known leukaemogenic viruses. These approaches have provided the justification for the experiments described in this thesis.

1.1. RETROVIRUSES.

The family Retroviradae includes a group of related viruses which possess an RNA genome and an RNA dependant DNA polymerase. Members of the family have a number of common morphological and biochemical characteristics (Teich 1984). Morphologically the viruses are spherical enveloped particles of 80-120nm in diameter, which may have visible surface projections on electron microscopy (Figure 1.1).

The replication of retroviruses has been reviewed by Varmus and Swanstrom (1984). Infection of an host cell occurs following interaction between the viral envelope glycoprotein and a specific receptor on the cell surface. Following entry of the core particle, the viral RNA genome is released into the cytoplasm and transcribed into DNA by the viral reverse transcriptase. A number of molecules of this enzyme are contained within the virion. The DNA copy of the viral genome is integrated into the host cell genome, the integrated viral DNA is known as a provirus. The mechanism of integration has been recently reviewed by Brown (1990); no specific target sequences

Figure 1.1. Electron micrograph of retrovirus virions (HTLV-I. x 150 000)



are required, though some regions of the host cell genome appear to be less accessible than others. Once integrated, the provirus is stably maintained in the cell and replicates when cell division occurs. Infection is thus transmitted to the daughter cells with cellular DNA. During viral replication proviral DNA is transcribed into messenger RNA (mRNA) encoding viral proteins and into genomic RNA, the latter being incorporated into new virions. Virions are assembled as they are released from the host cell by budding through the plasma membrane. In the majority of retroviral infections, virion release is not associated with a cytopathic effect.

The family Retroviridae has been subdivided into three subfamilies on the basis of differences in biological behaviour and pathological effects (Teich 1984): (1) The Oncovirinae, which includes the oncogenic viruses and closely related non-oncogenic viruses, (2) Lentivirinae, which are viruses inducing non-oncogenic diseases with a long latent period, (3) Spumavirinae, viruses which produce a characteristic "foamy" cytopathic effect in vitro, and induce persistent infections without known clinical consequences. More recently, the availability of nucleotide sequence data for many retroviruses has led to attempts to classify this family on the basis of sequence similarity and genomic organization. Doolittle *et al.* (1990) have identified four major subgroups of retroviruses using this approach: (a) the slow viruses, or lentiviruses, (b) a group including the human T-cell leukaemia viruses (HTLV) and bovine leukaemia virus (BLV), (c) a group of exogenous and endogenous viruses and intracisternal particles showing diverse morphology and (d) a mixed group of various leukaemia viruses, several endogenous viruses and the spumaviruses.

1.1.1. Leukaemogenic retroviruses.

Exogenous retroviruses from the subfamily Oncovirinae have been identified as aetiological agents of haematopoietic malignancies in a number of animal species, including chickens, mice, cats, cattle, apes and humans. Some of these viruses have also been shown to cause other neoplastic and non-neoplastic diseases. The development of disease usually occurs after a prolonged latent period and is associated with persistent virus infection of the host. The disease associations of a number of leukaemogenic retroviruses are shown in Table I.I. Retroviruses that cause immunosuppression in their hosts, such as the human immunodeficiency viruses (HIV) and the feline immunodeficiency virus (FIV) may be indirectly involved in leukaemogenesis, as discussed further in section 1.4.2.

Table I.I. Retroviruses associated with lymphoproliferative disorders.

Virus	Lymphoproliferative disease	Other disease associations
Avian leukaemia viruses	B-cell lymphomas	Osteopetrosis, erythro-blastosis, myeloblastosis
Murine leukaemia viruses	T- or B-cell lymphomas	Posterior paralysis
Feline leukaemia viruses	Thymic T-cell lymphomas B-cell lymphomas	Immunosuppression, myelo-proliferative diseases, anaemia, enteritis, reproductive failure
Bovine leukaemia virus	Multicentric B-cell lymphomas, persistent lymphocytosis	
Gibbon ape leukaemia virus	Lymphosarcoma?	Myeloid leukaemia
Simian T-lymphotropic virus type I	Malignant lymphoma?	
Human T-cell leukaemia virus type I	T-cell leukaemia/lymphoma	Spastic paraparesis

Derived from Homma et al. 1984; Teich et al. 1984; Tsujimoto et al. (1987) and Blattner (1989).

1.1.2. Human T-cell leukaemia viruses (HTLV).

Human T-cell leukaemia virus type I (HTLV-I).

HTLV-I was the first oncogenic human retrovirus to be isolated (Poiesz *et al.* 1980) following the development of techniques allowing the long term culture of T-lymphocytes. HTLV-I was subsequently shown to be aetiologically associated with an aggressive T-cell maligancy, adult T-cell leukaemia/lymphoma (ATL) first described by Uchiyama *et al.* (1977) in southern Japan. The virus has also been aetiologically linked with a progressive demyelinating neurological disorder known as tropical spastic paraparesis (TSP) in the Carribean, and as HTLV-1 associated myelopathy (HAM) in Japan (Gessain *et al.* 1985; Osame *et al.* 1986).

Associations between HTLV-I and other diseases are less well established. An increased incidence of HTLV-I antibodies in B-CLL relative to control sera was reported by Blattner *et al.* (1983a). More recently, a serological association between HTLV-I infection and polymyositis in Jamaica has been identified (Morgan *et al.* 1989).

Human T-cell leukaemia virus type II (HTLV-II).

A second human retrovirus, designated HTLV-II, was first isolated from a patient with a T-cell variant of hairy cell leukaemia (HCL) (Kalyanaraman *et al.* 1982). An additional isolate has been reported from a patient with the more common B-cell form of HCL (Rosenblatt *et al.* 1986) though in this patient the virus was shown to be present in T-cells and not in the leukaemic B-cells (Rosenblatt *et al.* 1988). Serological and molecular studies have failed to show a consistent association between HTLV-II and HCL (Rosenblatt *et al.*1987; Lion *et al.* 1988). Further HTLV-II isolates have been made from persons with immunodeficiency (Hahn *et al.* 1984; Chorba *et al.* 1985). However an association between HTLV-II infection and disease has not yet been demonstrated.

A number of studies have identified intravenous drug abusers as a population at increased risk of HTLV-II infection (Tedder *et al.* 1984; Ehrlich *et al.* 1989; Lee *et al.* 1989; Kwok *et al.* 1990a; Zella *et al.* 1990). Lairmore *et al.* (1990) have reported the isolation of HTLV-II from a member of the Guaymi Indian population of Panama. HTLV-II infection was detected in additional, seropositive, members of this population by using the polymerase chain reaction (PCR) to analyse peripheral blood mononuclear cells (PBMCs) (Heneine *et al.* 1990). These authors suggest that HTLV-II may be endemic in this population.

Cell tropism.

Both HTLV-I and HTLV-II are tropic for, and transform, CD4 positive T-lymphocytes in vitro (Yamamoto *et al.* 1982a; Chen *et al.* 1983). HTLV-I has been detected and shown to replicate in B-lymphocytes and in a variety of non-lymphoid cells and cell lines in vitro (Yamamoto *et al.* 1982b; Clapham *et al.* 1983; Longo *et al.* 1984; Hiramatsu *et al.* 1986; Akagi *et al.* 1988). However it is not known whether these cell types are susceptible to infection in vivo.

The mechanism by which HTLV-I and HTLV-II are thought to transform T-cells in vitro is discussed further in section 1.1.5.

1.1.3. Adult T-cell leukaemia/lymphoma (ATL).

The clinical features of ATL have been reviewed by Uchiyama (1988) and Yamaguchi *et al.* (1990). The median age of onset of disease has been estimated at 55-56 years, with an age range of 16-63 years. Typically the disease has an acute onset, more than 50% of cases show symptoms of lymphadenopathy, hypercalcaemia and hepatomegaly. Up to 50% of affected patients also have splenomegaly and skin lesions. Opportunistic infections frequently develop, reflecting an underlying immunodeficiency. Patients with acute forms of ATL show a poor response to treatment and have mean survival times of 4.4-7.5 months (Uchiyama 1988). Less acute "preleukaemic" and "smouldering" forms of the disease have been described (Kinoshita *et al.* 1985; Yamaguchi *et al.* 1985).

Molecular analysis of leukaemic cells from ATL patients has shown monoclonal or, rarely, oligoclonal integration of the complete provirus (Wong-Staal *et al.* 1983; Yoshida *et al.* 1984; Clark *et al.* 1988). Occasionally partially deleted proviruses have been detected (Wong-Staal *et al.* 1983; Clark *et al.* 1988).

The cell surface phenotype of the neoplastic cells in ATL is in most cases CD1-, CD2+, CD3+, CD4+, CD8-, HLA-DR+/-, CD25+, identifying the neoplastic cells as mature peripheral T-cells of the T-helper subtype (Uchiyama *et al.* 1988). In vitro studies have, however, failed to detect helper activity by the neoplastic cells (Uchiyama *et al.* 1983). Clonal chromosomal abnormalities have been detected in the leukaemic cells of ATL patients (Ueshima *et al.* 1981; Miyamoto *et al.* 1984). No consistent abnormalities were reported in these studies, though a high incidence of trisomy 7 was detected by Ueshima *et al.* (1981), while Miyamoto *et al.* (1984) reported frequent rearrangements of chromosomes 6, 1 and 3.

1.1.4. Epidemiology of HTLV-I infection and of ATL.

The epidemiology of HTLV-I infection has been recently reviewed by Blattner (1989). The seroprevalence of HTLV-I in endemic areas of Japan and the Caribbean varies from 5-10%. HTLV-I infection also appears to be endemic in parts of Africa (Saxinger *et al.* 1984; Biggar *et al.* 1984; Delaporte *et al.* 1989; Goubau *et al.* 1990). Familial clustering of seropositive persons is observed, reflecting the requirement for intimate contact for virus transmission. Transmission of HTLV-I occurs through the transfer of virus-infected cells by three major routes: the transfer of cellular blood components from infected donors by blood transfusion (Okochi *et al.* 1984) or intravenous drug abuse (Tedder *et al.* 1984; Lee *et al.* 1989; Kwok *et al.* 1990a), transmission of virus-infected cells from HTLV-I positive mothers to children in breast milk (Nakano *et al.* 1986) and transmission during sexual intercourse (Nakano *et al.* 1984).

ATL shows pronounced geographical clustering in south Japan and the Carribean (Hinuma *et al.* 1981; 1982; Yoshida *et al.* 1982; 1984; Blattner *et al.* 1983b), areas where HTLV-I infection is endemic. The epidemiology of HTLV-I associated disease in Africa is poorly described, though TSP and ATL-like disorders have been reported in HTLV-I-infected Africans (Williams *et al.* 1984; Delaporte *et al.* 1989; de-The *et al.* 1989a)

HTLV-I infected persons have a low risk of developing ATL; the annual incidence rate for Japanese virus carriers older than 40 years has been estimated at 0.6-1.7% (Tajima *et al.* 1990). Studies of populations that have migrated from endemic areas indicate that a long latent period may elapse between infection by the virus and the development of disease (Greaves *et al.* 1984). Murphy *et al.* (1989) have suggested that HTLV-I infection in childhood is an important risk factor for the development of ATL. In this study the cumulative risk of developing ATL for seropositive children under 20 years old was estimated at 4.0% for males and 4.2% for females.

The low incidence of ATL in HTLV-I infected persons indicates that HTLV-I is a relatively inefficient leukaemogenic virus and that additional factors are likely to be involved in disease development. The monoclonal origin of the virus infected tumour cells also suggests that multiple events are required for oncogenesis. However the high level of correlation between the epidemiology of ATL and the seroepidemiology of HTLV-I indicates that the major risk factor for ATL development is HTLV-I infection. Other risk factors have not yet been convincingly identified, though the age at which infection is acquired may be important.

1.1.5. HTLV-I in T-cell transformation and leukaemogenesis.

HTLV-I and the related retroviruses, HTLV-II and BLV have a genome organization which differs from that of other non-defective, leukaemogenic retroviruses. The viral genomes possess an additional protein coding region at the 3' end of the *env* gene which extends into the viral long terminal repeat (LTR) (Seiki *et al.* 1983; Shimotohno *et al.* 1984; Sagata *et al.* 1985). The tax protein of HTLV-I and HTLV-II, which is encoded by this region, has been shown to activate transcription from the viral LTR in *trans* (Yoshida and Seiki 1987; Green and Chen 1990). Expression of the tax protein is also associated with *trans*-activation of a number of cellular genes, including the genes for the interleukin 2 receptor α-chain, (IL-2Rα), interleukin 2 (IL-2), granulocyte/macrophage colony stimulating factor (GM-CSF), c-fos and c-sis (Greene *et al.* 1986; Inoue *et al.* 1986; Fujii *et al.* 1988; Green and Chen 1990).

The involvement of tax in leukaemogenesis by HTLV-I in vivo via the *trans*-activation of cellular genes has been proposed by many groups (e.g. Wong-Staal and Gallo 1985; Arima *et al.* 1987; Yoshida and Seiki 1987; Tanaka *et al.* 1990). A similar mechanism has been proposed for the immortalization of T-lymphocytes by HTLV-I and HTLV-II

in vitro. T-cells transformed in vitro by HTLV-I express large amounts of mRNA for both structural and non-structural proteins (de Rossi *et al.* 1985). Events in addition to virus infection appear to be required for transformation in vitro, as an initial poly- or oligoclonal proliferation of infected cells is followed by outgrowth of a dominant clone which may eventually become independent of IL2 (de Rossi *et al.* 1985).

The infrequent development of ATL in HTLV-I infected persons and the long latent period between infection and the onset of leukaemia indicate that the pathogenesis of ATL is also likely to require multiple events. Expression of the viral genome in fresh leukaemic cells from ATL patients is limited, suggesting that HTLV-I infection may be important in the early stages of cell transformation. In early studies, the expression of mRNA coding for viral proteins was not detected by Northern blotting, though rare exceptions were reported (Franchini *et al.* 1984; Umadome *et al.* 1988). More recent studies using reverse transcription followed by amplification of DNA sequences with the PCR have detected low levels of tax RNA in PBMCs and lymph node tissue from ATL patients (Kinoshita *et al.* 1989; Greenberg *et al.* 1990). However neither of the latter reports demonstrated that viral mRNA expression was occurring in the leukaemic, rather than the normal, virus-infected lymphocytes.

Experimental evidence identifies tax as a candidate transforming gene (Hinrichs *et al.* 1987; Grassmann *et al.* 1989; Tanaka *et al.* 1990). It is possible that low level expression of tax is sufficient to maintain the transformed phenotype, though it should be noted that tax expression was not detectable in all ATL cases in the studies of Kinoshita *et al.* (1989) and Greenberg *et al.* (1990). Alternatively tax, or some other viral function, may be involved in an early step in leukaemogenesis, and virus gene expression may not be required for maintenance of the neoplastic state. A model in which HTLV-I is involved in the early stages of leukaemogenesis in vivo has been described by Yoshida and Seiki (1987). In this model HTLV-I infected T-cells are induced to proliferate due to *trans*-activation of cellular genes by tax. The virus induced polyclonal proliferation creates a large pool of rapidly dividing cells which are then susceptible to additional transforming events.

1.2 HERPESVIRUSES.

The characteristics of Herpesviruses have been reviewed by Roizman (1982). The Herpesviridae are a family of DNA viruses with a large, linear, double stranded genome. Morphologically the virions are enveloped particles of 120-150nm in diameter with surface projections that are visible with an electron microscope (Figure 1.2). The viral nucleic acid is contained in the nucleocapsid, which is an icosahedron made up of 162 capsomeres.

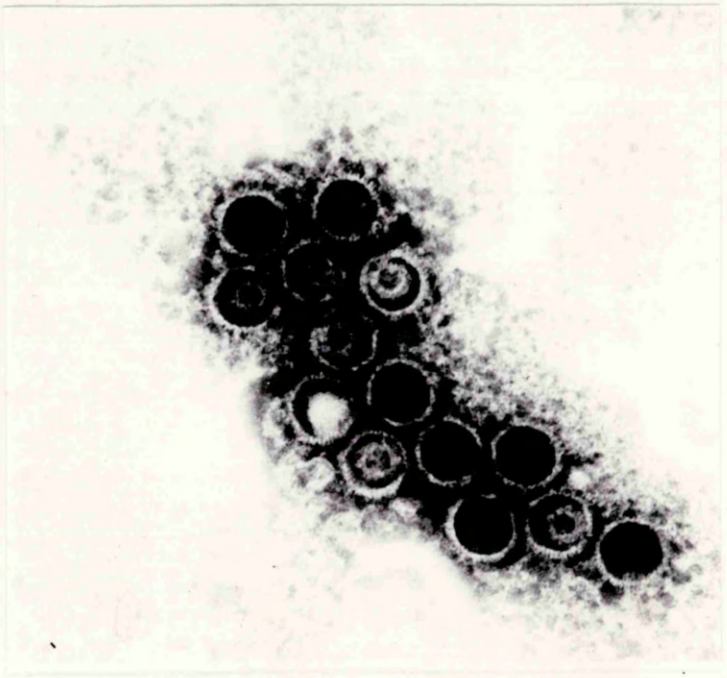
The replication of herpesviruses has been reviewed (Kieff and Liebowitz 1990; Roizman and Sears 1990; Stinski 1990). The virus gains access to the cell following interaction with a specific cell surface receptor. After entering the cell nucleus, the herpesvirus DNA molecules are thought to circularize or to form concatemers. Transcription and replication of viral DNA takes place in the nucleus, followed by the assembly of capsids. Immature nucleocapsids appear to bud through the inner lamella of the nuclear membrane. Enveloped viral particles are released from the cell surface. Productive herpesvirus replication results in lysis of the host cell.

Herpesviruses are also able to establish latent infections in some infected cell types (Roizman 1982). During latent infection limited regions of the virus genome are expressed. Viral DNA is maintained in various forms in latently infected cells. EBV genomes are maintained as covalently closed circular episomes, integration into the cellular genome has also been detected (Lindahl *et al.* 1976; Matsuo *et al.* 1984). The genomes of other herpesviruses may be maintained as circular, concatemeric or linear forms in latently infected cells (Rziha *et al.* 1986; Mellerick and Fraser 1987).

Herpesviruses have been divided into three subfamilies, the Alphaherpesvirinae, Betaherpesvirinae and Gammaherpesvirinae. Viruses were originally assigned to these subfamilies on the basis of their biological properties (Roizman 1982).

The alphaherpesviruses have a variable host range in vivo and in vitro and have a short reproductive cycle. These viruses are highly cytopathic in vitro and frequently establish latent infections in ganglia. The betaherpesviruses show a restricted host range in vivo and in vitro and have a relatively long reproductive cycle. The cytopathic effect in vitro is associated with the enlargement of infected cells. The site(s) of latency for most betaherpesviruses are unknown. The gammaherpesviruses show a narrow host range in vivo and replicate and establish non-lytic persistent infections in lymphoblastoid cells

Figure 1.2. Negatively stained electron micrograph of herpesvirus virions (HHV-6, x 75 000).



in vitro. The duration of the reproductive cycle is variable. Latent infections are frequently established in lymphoid tissue in vivo.

Grouping of herpesviruses according to the structure of their genomes, in cases for which information is available, does not give consistent agreement with the above classification system (Roizman 1982). Molecular analysis of herpesvirus genomes has resulted in the reclassification of some viruses on the basis of their nucleotide sequence homology with other herpesviruses and similar genome structure. Thus Marek's disease virus (MDV), originally considered a gammaherpesvirus, has been shown to be more closely related to the alphaherpesviruses following the analysis of nucleotide composition, genome organization and gene sequences (Cebrian *et al.* 1982; Buckmaster *et al.* 1988; Honess *et al.* 1989).

1.2.1 Herpesvirus associated lymphoid malignancies.

The associations between herpesviruses and lymphoproliferative diseases are summarized in Table I.II. Two herpesviruses have been associated with the induction of lymphoid malignancies in their natural hosts: Marek's Disease virus (MDV) in chickens and Epstein-Barr virus (EBV) in man. Both these viruses infect and immortalize lymphoid cells in vitro (Powell *et al.* 1974; Henle *et al.* 1967; Pope *et al.* 1968). A number of herpesviruses cause lymphoproliferative diseases when experimentally innoculated into animals other than their natural hosts (Table I.II).

Other herpesviruses that have not been directly associated with lymphoproliferative malignancies, have in some cases been shown to have oncogenic properties in vitro. The oncogenic properties of human herpesviruses have been reviewed by Sugden (1986). Genomic fragments of the human alphaherpesviruses, herpes simplex virus type 1 and type 2, have been shown to have transforming potential in vitro. A fragment of human cytomegalovirus (HCMV), a betaherpesvirus, has also been shown to induce transformation of cultured rodent cells. Galloway *et al.* (1986) report that the transforming region of HCMV may act as a transcriptional enhancer and suggest that the transforming region of herpes simplex virus type 2 may be a weak mutagen. The relevance of these findings to the pathogenic effects of these viruses in vivo is not certain.

Table I.II. Herpesviruses associated with lymphoproliferative disorders.

Virus	*Lymphoproliferative disease*	*Other disease associations*
Leporid herpesvirus 1 (herpesvirus sylvilagus)	Lymphoproliferation and lymphomas in experimentally infected animals	
Cercopithecine herpesvirus 12 (herpesvirus papio)	B-cell lymphoproliferation	
Gallid herpesvirus 2 (Marek's disease virus)	Lymphoproliferation and infiltration of nervous tissue, acute T-cell lymphomas	
Saimirine herpesvirus 2 (herpesvirus saimiri)	Does not cause disease in the natural host T-cell lymphomas in other New World primates	
Ateline herpesvirus 2 (herpesvirus ateles)	Does not cause disease in the natural host T-cell lymphomas in other New World primates	
Human herpesvirus 4 (human cytomegalovirus)	Infectious mononucleosis	Pneumonia in immuno-suppressed persons
Human herpesvirus 5 (Epstein-Barr virus)	Infectious mononucleosis, Burkitt's lymphoma B-cell lymphomas in immunosuppressed persons, X-linked lymphoproliferative syndrome, polyclonal lymphomas in Cotton Top marmosets	Nasopharyngeal carcinoma

Derived from zur Hausen 1981; Griffiths and Grundy (1987) and Evans and Niederman (1989).

1.2.2 Epstein-Barr virus (EBV).

EBV was first isolated by Epstein *et al.* (1964) and has since been linked to the aetiology of a number of lymphoproliferative disorders. The association between EBV and a high grade B-cell lymphoma, Burkitt's lymphoma (BL) in Africa is discussed further in sections 1.2.3-1.2.4.

The virus has been identified as the aetiological agent of infectious mononucleosis (IM), which occurs predominantly in young adults of between 15 and 25 years of age (Henle *et al.* 1968; Evans and Neiderman 1989). IM is characterized clinically by fever, pharyngitis and lymphadenopathy, these symptoms are associated with a rapid lymphoproliferative response, which is usually self-limiting (Purtillo *et al.* 1985; Evans and Niederman 1989). Persons with inherited immunodeficiency syndromes are susceptible to chronic and fatal forms of IM (Purtillo *et al.* 1985).

EBV has also been associated with the development of B-cell lymphoproliferative disorders and lymphomas in persons with primary or secondary immunodeficiency (Hanto *et al.* 1985). EBV-associated benign polyclonal B-cell hyperplasias, oligoclonal B-cell lymphomas and monoclonal lymphomas have been described in immunosuppressed tissue-allograft recipients (Hanto *et al.* 1985). These disorders show a spectrum of histological appearances, large non-cleaved cell and immunoblastic lymphomas are frequently reported. EBV DNA has been detected in 28-50% of lymphomas occurring in HIV infected individuals (Suber *et al.* 1988; Ernberg 1989; Hamilton-Dutoit *et al.* 1989). The virus-positive lymphomas are histologically diverse; immunoblastic lymphomas are seen in patients with more severe immune deficiency, while BL-like tumours appear to be associated with a less compromised immune system (Evans and de The 1989).

EBV has been been linked with the aetiology of an epithelial tumour, nasopharyngeal carcinoma (NPC). NPC is an aggressive squamous cell carcinoma that is common in south-east Asia (de-The *et al.* 1989b; Evans and de-The 1989). Patients with NPC have high levels of antibodies to a number of EBV antigens and EBV DNA has been detected in the majority of NPC biopsies (zur Hausen *et al.* 1970; de Schryver *et al.* 1972; Desgranges *et al.* 1975; de The *et al.* 1978a). The epidemiology of NPC indicates that genetic and environmental factors are important in disease pathogenesis (de-The *et al.* 1989b). The interactions between these factors and EBV and the role played by the virus in the development of this tumour are not understood.

Molecular associations between EBV and other B- and T-cell non Hodgkin's lymphomas (NHLs) have been reported (Bornkamm *et al.* 1976; Jones *et al.* 1988; Chappuis and Muller-Hermelink 1989; Murphy *et al.* 1990; Richel *et al.* 1990; Rouah *et al.* 1990). The role of EBV in the pathogenesis of these lymphomas is unknown. The serological and molecular evidence linking EBV to the aetiology of Hodgkin's disease (HD) is discussed in Chapter 4 of this thesis.

In vitro cell tropism.

EBV is tropic for resting human B-cells in vitro and transforms infected cells into permanent lymphoblastoid cell lines (LCL) (Henle *et al.* 1967; Pope *et al.* 1968). The viral genome is maintained in multiple episomal copies in immortalized cells and shows a restricted range of gene expression (Speck and Strominger 1989). Productive viral replication occurs in a small proportion of transformed cells and is associated with loss of the transformed phenotype and B-cell differentiation (Wendel-Hansen *et al.* 1987). The relationship between EBV-induced immortalization of B-cells in vitro and the pathogenesis of EBV-associated B-cell lymphomas in vivo is discussed further in section 1.2.5.

EBV is also tropic for, and replicates in, oropharyngeal epithelial cells in vivo (Sixbey 1989). Infection of epithelial cells by EBV has not been succesfully reproduced in vitro.

1.2.3 Burkitt's lymphoma (BL).

The epidemiology of BL has been reviewed by Evans and de-The (1989). BL occurs in two epidemiologically distinct forms, an endemic, or high incidence form and a sporadic, or low incidence form. The geographical distribution of endemic BL (eBL) shows a close correlation with the incidence of malaria caused by *Plasmodium falciparum* (*P. falciparum*). Sporadic BL (sBL) occurs at a low frequency in many geographical areas; the distribution of disease is unrelated to the prevalence of malaria.

The histopathological features of both forms of BL are similar (Berard 1985; Wright 1985). The tumour is classified as a high grade lymphoma of small noncleaved cell type according to the "working formulation" (Rosenberg 1982). The malignant cells are

monomorphic and express surface immunoglobulin (Ig). Some differences in the pattern of cell surface antigen expression by the tumour cells of sBL and eBL have been described; the malignant cells in eBL appear to have a less mature B-cell phenotype than those in sBL (Preud'homme *et al.* 1985).

Differences in the clinical features of sBL and eBL have also been reported. African cases of BL usually present with lymphomas occurring in the jaw and/or abdomen (Ziegler 1977). Jaw tumours are rarely seen in sBL, which frequently presents with abdominal masses and lymph node tumours (Levine *et al.* 1982). The peak age incidence of eBL is 6-8 years old, while the peak incidence of sBL is in older children of 10-12 years (Levine *et al.* 1982). However BL occurring in non-endemic areas is an heterogeneous disease and a significant proportion of cases cannot be distinguished clinically or morphologically from eBL (Levine *et al.* 1982).

The association between BL and EBV has been recently reviewed by Evans and de-The (1989) and Rowe and Gregory (1989). The results of seroepidemiological and molecular studies support a role for EBV in the aetiology of eBL. African patients with BL have significantly higher antibody titres to EBV than control populations (Henle *et al.* 1969) and elevated antibody titres to the EBV viral capsid antigen are associated with an increased risk of developing BL (de-The *et al.* 1978b). EBV DNA is detected in 98-99% of biopsies obtained from African patients with BL (Zur Hausen *et al.* 1970; Nonoyama *et al.* 1973). Viral DNA is present in the tumour cells as multiple episomal copies, occasionally integrated virus has been detected (Kaschka-Dierich *et al.* 1976; Lawrence *et al.* 1988). EBV genomes have also been detected in the tumour cells of 15-20% of sBL cases (Andersson *et al* 1976; Philip 1985). Raab-Traub and Flynn (1986) have shown that the EBV-infected tumour cells are clonal in origin, indicating that virus-infection has occurred prior to malignant transformation.

Chromosome translocations occurring in BL.

Both sBL and eBL are consistently associated with the presence of one of three chromosomal translocations (Croce 1986). Croce (1986) has reviewed the characteristics of the three types of chromosome translocation, each of which involves the *c-myc* locus on chromosome 8. In 75% of cases the translocation also involves the immunoglobulin heavy chain (IgH) locus on chromosome 14. The remaining 25% of tumours have either a t(8;22)(q24;q11) or a t(2;8)(p11;q24) chromosomal translocation,

involving the immunoglobulin light chain (IgL) lambda or kappa loci respectively. The results obtained in a number of studies indicate that juxtaposition of the *c-myc* gene and the immunoglobulin loci results in constitutive and elevated expression of *c-myc* (e.g. Dalla Favera *et al.* 1983; Erikson *et al.* 1983).

The translocations occurring in BL have been shown to involve different sites on chromosomes 8 and 14 in the endemic and sporadic forms of the tumour (Erikson *et al.* 1982; Dalla Favera *et al.* 1983; Neri *et al.* 1988). In most African cases of BL the translocation breakpoint on chromosome 14 occurs within, or 5' to the joining region of the IgH locus. The breakpoint on chromosome 8 usually occurs several kilobases (kb) upstream of the *c-myc* gene. In the sporadic form of BL the translocation appears to involve the heavy chain switch region on chromosome 14 and the first intron of the *c-myc* gene.

1.2.4 Epidemiology of EBV infection and BL.

The epidemiology of EBV infection has been reviewed by Evans and Niederman (1989). EBV infection is acquired at an earlier age in developing countries than in developed countries. Seroprevalence increases with age and 90-100% of adults over the age of 50 years are EBV-antibody-positive (Schmader *et al.* 1989). Epidemiological data suggest that transmission of EBV in adults occurs mainly through intimate oral contact with virus-excreting healthy carriers. Consistent with this, excretion of EBV in saliva has been detected in the majority of healthy virus-infected individuals (Yao *et al.* 1985).

Despite the ubiquitous distribution of EBV infection, the incidence of EBV associated tumours shows significant geographical variation. The risk of developing BL is strongly associated with *P. falciparum* infection. This may be due to reduced EBV-specific T-cell immunity (Moss *et al.* 1983), or related to *P. falciparum*-induced proliferation of B-cells (Morrow 1985). In addition de-The (1977) has suggested that the acquisition of EBV infection at an early age may be important in the development of BL.

The risk of developing other EBV-associated diseases depends on a number of factors. The association between various types of immune deficiency and EBV-associated lymphoproliferative disease indicates the importance of immune status in determining the outcome of EBV infection. The age at which EBV infection is acquired is considered to be a significant factor in the development of IM (Evans and Niederman 1989).

1.2.5. EBV, B-cell transformation and BL.

The mechanism by which EBV immortalizes B-cells in vitro is not fully understood. Knutson and Sugden (1989) have recently reviewed the evidence for the involvement of viral gene products in cell transformation. A number of viral proteins are expressed in LCL, the EBV nuclear antigens- (EBNA-) 1-6, the latent membrane protein (LMP) and the terminal protein. EBNA-2 has been shown to be required for the immortalization of B-lymphocytes, while LMP has been shown to have a transforming function when expressed in rodent cells. EBNA-1 appears to *trans*-activate a viral promoter and is required for maintenance of the viral episomes in dividing cells. Whether this protein is able to *trans*-activate any cellular genes or plays an additional role in B-cell immortalization is not known.

Expression of EBV gene products in BL tumour cells is more restricted than in LCL. EBNA-1 expression is readily detectable in fresh biopsy tissue and BL cell lines, but EBNA-2 and LMP do not appear to be expressed at detectable levels (Rowe *et al.* 1987). In addition deletion of EBNA-2 has been reported in BL cell lines, indicating that EBNA-2 expression is not required to maintain the transformed state (Knutson and Sugden 1989).

Two alternative models for the role of EBV in BL have been proposed. Klein (1979; 1987) has suggested that EBV-induced immortalization of B-cells creates an expanded target cell population, which is able to proliferate at an increased rate in persons with malaria-related immune dysfunction. This proliferating population of cells is then more susceptible to a second transforming event, i.e. a chromosomal translocation involving

c-myc. This model has some features in common with the model for HTLV-I involvement in ATL proposed by Yoshida and Seiki (1987) and discussed in section 1.1.5. In both models the virus plays a role in the early stages of leukaemogenesis by stimulating cell proliferation and therefore increasing the probability that additional transforming events will occur.

An alternative model has been proposed by Lenoir and Bornkamm (1987). These authors suggest that *P. falciparum* infection induces chronic immunological stimulation of B-cells, thus increasing the probability that the *c-myc* translocation will occur. At least one additional event is needed for acquisition of the full neoplastic phenotype and this event may, in eBL, be EBV infection. In EBV-negative sBL, some other event is substituting for EBV infection.

1.2.6. HTLV-I and EBV as models for human leukaemia viruses.

The evidence described above for the involvement of HTLV-I and EBV in the aetiology of ATL and BL respectively may be summarized under four headings.

1. Virus-determined antigens and/or viral genomes are present in the tumour cells.

2. Antibodies to virus-determined antigens occur at higher frequency and/or titres in patients with these diseases than in appropriate controls.

3. Both viruses are capable of transforming normal human cells in vitro.

4. Both viruses, or closely related viruses, are capable of tumour induction in animals other than humans.

These four lines of evidence are similar to the criteria suggested by Henle (1971) for the assessment of viruses as aetiological agents of cancer in humans. However it is debatable whether or not these criteria should still be considered appropriate. In particular, failure to fulfil the third criterion could reflect lack of an appropriate system for in vitro culture of the target cells for viral infection. Failure to fulfil the fourth criterion could reflect inability of the virus or related viruses to establish infection in animals other than humans.

An additional criterion that has been used by some authors to assess the potential role of viruses in human neoplasia is that the seroprevalence of the virus infection should parallel the geographic distribution of the neoplasm (e.g. Henderson 1989). HTLV-I clearly fulfills this criterion with regard to ATL, however, as discussed previously the geographic distribution of BL bears no relationship to the seroprevalence of EBV. A possible explanation for the latter finding, i.e. the importance of *P. falciparum* infection as a cofactor in disease pathogenesis, has been described above. Thus while a correlation between the distribution of virus infection and of disease may provide powerful support for an aetiological association, the absence of a correlation does not necessarily provide evidence against it.

The mechanisms by which EBV and HTLV-I are thought to contribute to leukaemogenesis involve direct effects on virus infected cells (section 1.4.2). However other possible mechanisms by which viruses could take part in the pathogenesis of leukaemias and lymphomas have been proposed, not all of which require virus infection of the tumour cells. The use of the above criteria may be inappropriate for the assessment of the pathogenic role of a virus that induces lymphoproliferative diseases by an indirect mechanism. A number of mechanisms of viral leukaemogenesis and some of the difficulties that may be encountered in determining the association between a virus and disease are discussed in section 1.3.

1.3. APPROACHES TO THE INVESTIGATION OF VIRAL INVOLVEMENT IN THE AETIOLOGY OF LEUKAEMIAS AND LYMPHOMAS: (A) THE TARGETING OF SPECIFIC DISEASES FOR INVESTIGATION.

The investigation of specific disease entities for evidence of a viral aetiology may be prompted by the identification of clinical or epidemiological features that are characteristic of an infectious disease. Similarities with a type of leukaemia or lymphoma known to have a viral cause may also identify a condition with an unknown aetiology as a potential target for investigation. The accurate classification of leukaemias and lymphomas into distinct entities is critical if this is to be a useful approach.

1.3.1. The classification of haematopoietic malignancies.

Leukaemia and lymphoma classification has become increasingly complex with the evolution of techniques permitting more detailed characterization of the malignant cells. Early classification systems were based mainly on the morphology of malignant tissues visualized under a light microscope (e.g. Bennet *et al.* 1976). The development of immunological techniques for the characterization of cell surface phenotypes has permitted further subclassification of many leukaemias and lymphomas. Immunophenotyping has been particularly useful for the identification of distinct subtypes of lymphoid leukaemia (Foon *et al.* 1982; Schroff *et al.* 1982; Nadler *et al.* 1984) and NHL (Picker *et al.* 1987; Salter *et al.* 1988). More recently molecular techniques for the determination of the B- or T-cell lineage of lymphoid neoplasms have been developed (Arnold *et al.* 1983; Toyanaga and Mak 1987). The application of the latter techniques for the classification and diagnosis of malignant lymphomas is discussed further in Chapter 3.

The identification of distinct disease entities may reveal epidemiological features suggestive of a viral aetiology that were previously obscured by inappropriate classification. For example, the identification of ATL followed the development of techniques for the immunophenotyping of leukaemias (Uchiyama *et al.* 1977). This study describes the first identification of ATL as an aggressive form of T-cell leukaemia with characteristic clinical and pathological features. The identification of ATL as an entity distinct from other leukaemias permitted the epidemiological observations described by Uchiyama *et al.* (1977). The geographical clustering of patients with ATL was strongly suggestive of a viral aetiology. Following the isolation of HTLV-I by Poiesz *et al.* (1980) a serological association between HTLV-I infection and ATL was rapidly established (Hinuma *et al.* 1982; Robert-Guroff *et al.* 1982).

Two lymphoproliferative disorders which have epidemiological and/or clinical features suggestive of an infectious or viral aetiology are HD and angioimmunoblastic lymphadenopathy (AIL). These disorders are discussed further in the following sections and are the subjects of some of the investigations described in Chapters 3 and 4. The clinical similarities between ATL and other cutaneous T-cell lymphomas (CTCLs) has stimulated interest in the possibility that a retrovirus related to HTLV-I may be involved in the aetiology of CTCL. The relevant clinical features of these conditions are described in section 1.3.4. The evidence supporting a role for an HTLV-I-related retrovirus in the aetiology of CTCL is discussed further in Chapter 5.

1.3.2 Hodgkin's Disease (HD).

Controversy over the nature of HD has persisted since the first description of the disease. This debate has been reviewed by Kaplan (1972). Some early investigators considered HD to be neoplastic in nature, though others suggested that the disease was a form of tuberculosis. Alternative proposals included an auto-immune origin for HD and a secondary inflammatory reaction to virus infection. Until relatively recently the neoplastic nature of HD was still being debated (Lukes and Butler 1966).

Clinical features.

The clinical features of HD have been reviewed by Selby and McElwain (1987). The majority of patients present with lymphadenopathy, while approximately one third of patients have systemic signs ("B" symptoms) at presentation. Interest in a possible infectious origin for HD was stimulated by some of the systemic signs observed in patients, as the occurrence of persistent pyrexia, night sweats, pruritis and weight loss are features more commonly associated with infectious diseases.

Histopathological features.

The histological appearance of tissues affected by HD, in particular the reactive nature of the cellular infiltrate, led to early suggestions that the disease was caused by a virus (e.g. Order and Hellman 1972; DeVita 1973).

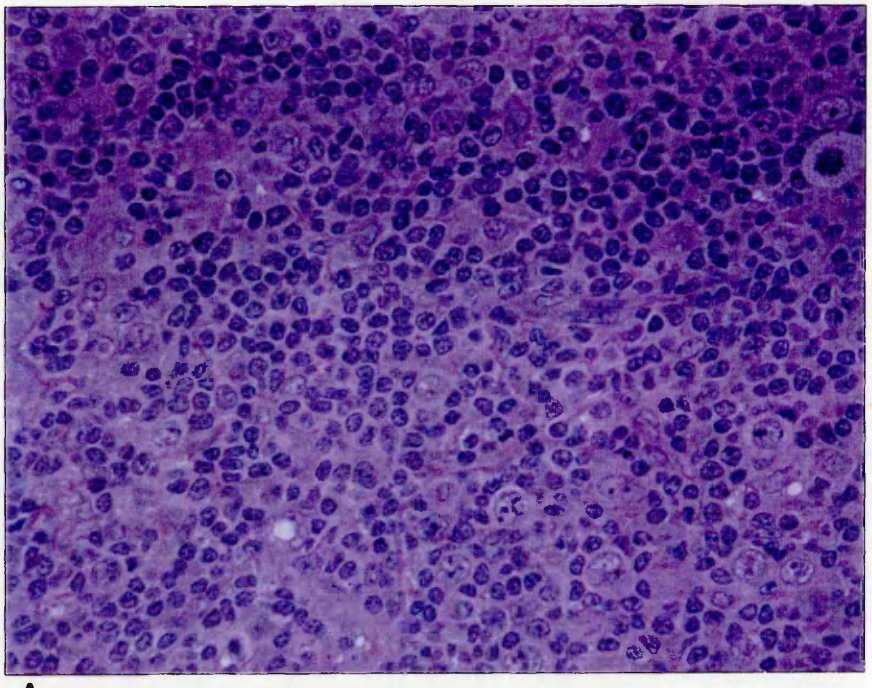
The histological features of HD have been reviewed by Lukes and Butler (1966), Williams (1985) and Lee (1989). Affected tissues show diverse morphological features, but are characterized by the presence of variable numbers of Reed-Sternberg (RS) cells. RS cells show some variation in appearance, but classically are large with either a bilobed nucleus or two nucleii, each of which has a large nucleolus (Figure 1.3). The detection of these cells is a prerequisite for a diagnosis of HD. RS cells and their mononuclear variants (collectively abbreviated as HRS cells) are usually relatively scarce and are intermixed with large numbers of inflammatory cells.

The numbers of HRS cells and the nature of the inflammatory infiltrate have been used to subclassify HD (Lukes *et al.* 1966; Lukes and Butler 1966). The Rye classification (Lukes *et al.* 1966) divides HD into four subtypes, lymphocytic predominance (LP), nodular sclerosis (NS), mixed cellularity (MC) and lymphocytic depleted (LD). LPHD is characterized by the presence of "L & H" type RS cells, or "popcorn" cells, large numbers of mature lymphoctyes and very few classical RS cells (Figure 1.3, panel A). LPHD is divided by some authors into nodular and diffuse forms, histiocytes may be the predominant cell type in the latter form (Lukes and Butler 1966; Williams 1985). The NS subtype of HD shows varying degrees of fibrosis, with connective tissue subdividing the cellular infiltrate into nodules (Figure 1.3, panel B). A variant form of RS cell, the lacunar cell, is frequently seen in NSHD. Diagnostic RS cells are usually scarce but may be numerous in some cases. NSHD has been divided into two types

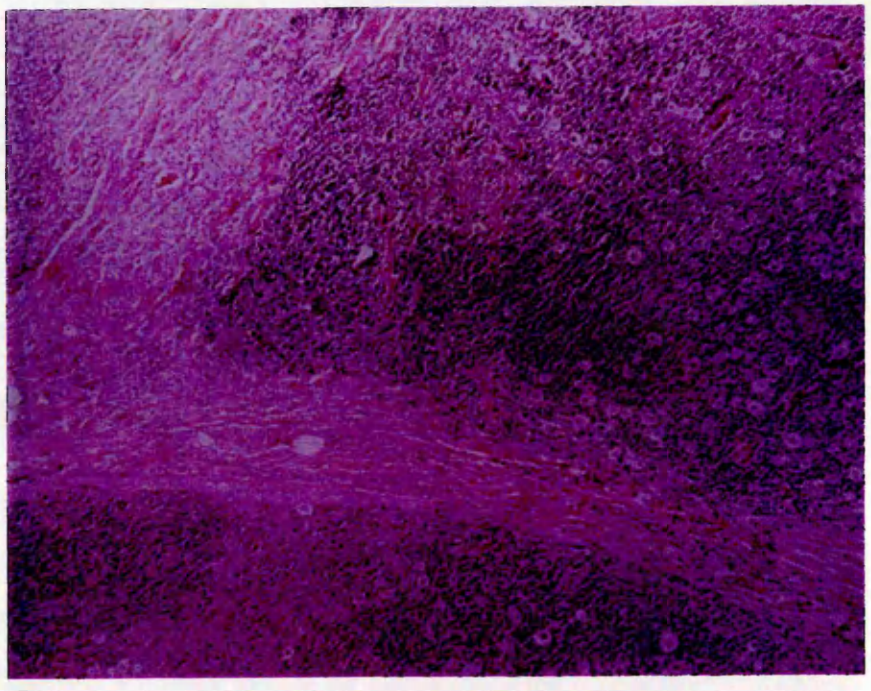
Figure 1.3. A, lymphocyte predominant HD. The lymph node contains a polymorphic cellular infiltrate with many small lymphcytes and small numbers of large binucleate and mononuclear cells (RS cells and their variants. The two large cells with multilobated nucleii in the upper left quadrant are similar to the "popcorn" cell variants of RS cells.

B, nodular sclerosing HD. Bands of fibrous connective tissue divide the lymph node into nodules. Many "lacunar" cells are visible.

Figure 1.3. Lymphocyte predominant (A) and nodular sclerosing (B) Hodgkin's disease. (Haemotoxylin and eosin-stained sections).



A



B

based on the predominant cell types in the nodules (Lee 1989). MCHD has a polymorphic cellular infiltrate which includes many cell types, HRS cells may be numerous (Figure 1.4, panel A). LDHD is characterized by small numbers of lymphocytes and a variable extent of disordered fibrosis (Figure 1.4, panel B). The number of classical RS cells may be large.

The four histopathological subtypes show differences in the sites of disease involvement, clinical stage at presentation, prognosis and age and sex distribution (Keller *et al.* 1968; Symmers 1978). NSHD is the commonest subtype in developed countries, making up 50-75% of cases; this subtype occurs mainly in adults less than 40 years old (Lee 1989). Patients with NSHD who are between 15 and 24 years of age represent the only group of HD cases to show an excess in females (McKinney *et al.* 1989). MCHD accounts for 17-31% of HD cases and occurs more frequently in older adults (Symmers 1978; Lee 1989). LPHD and LDHD are less common, forming 10-17% and 4-14% of cases respectively (Symmers 1978; Lee 1989). LPHD and NSHD-type I have a better prognosis than MCHD and NSHD-type II, while the prognosis for patients with LDHD is poor (Linch and Vaughan-Hudson 1988). Grufferman and Delzell (1984) have suggested that different aetiological factors may be associated with the different subtypes of HD.

Epidemiology.

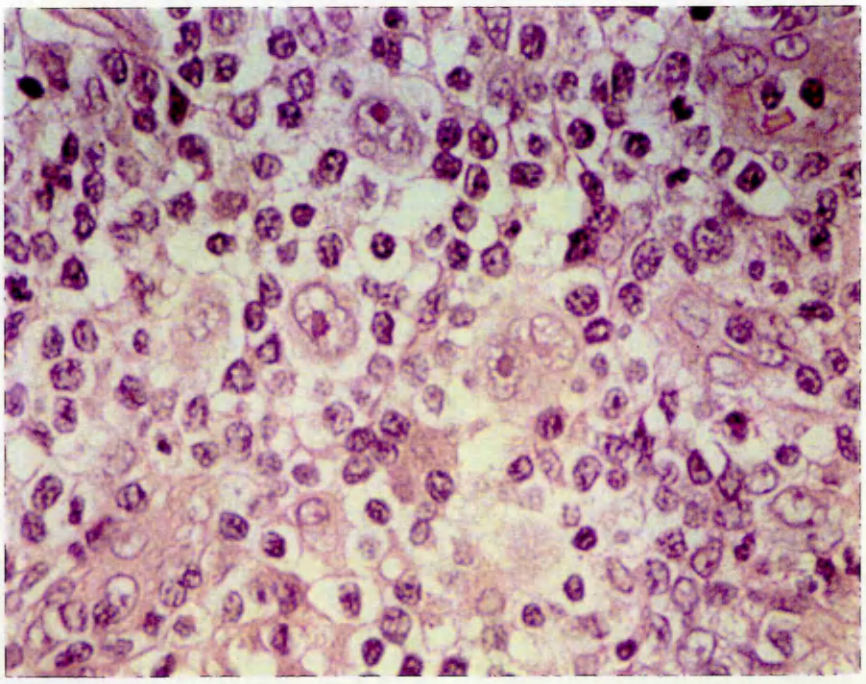
Subdivision of HD cases according to age of onset has also suggested the existence of distinct forms of the disease (MacMahon 1966; Grufferman and Delzell 1984). MacMahon (1966) identified three subgroups of HD associated with a clinical onset of disease at ages of 0-14 years, 15-34 years and over 50 years. The descriptive epidemiology of HD occurring in persons in these age-groups differed. MacMahon advanced the hypothesis that the aetiology of HD occurring in young and old adults is different and that the disease occurring in young adults has an infectious aetiology.

The age-related incidence of HD has been reviewed by MacMahon (1966), Gutensohn and Cole (1980) and Grufferman and Delzell (1984). Most studies performed in developed countries report a bimodal age-incidence curve with a peak in incidence between the ages of 15 and 40 years and a second peak at over 50 years. A recent study

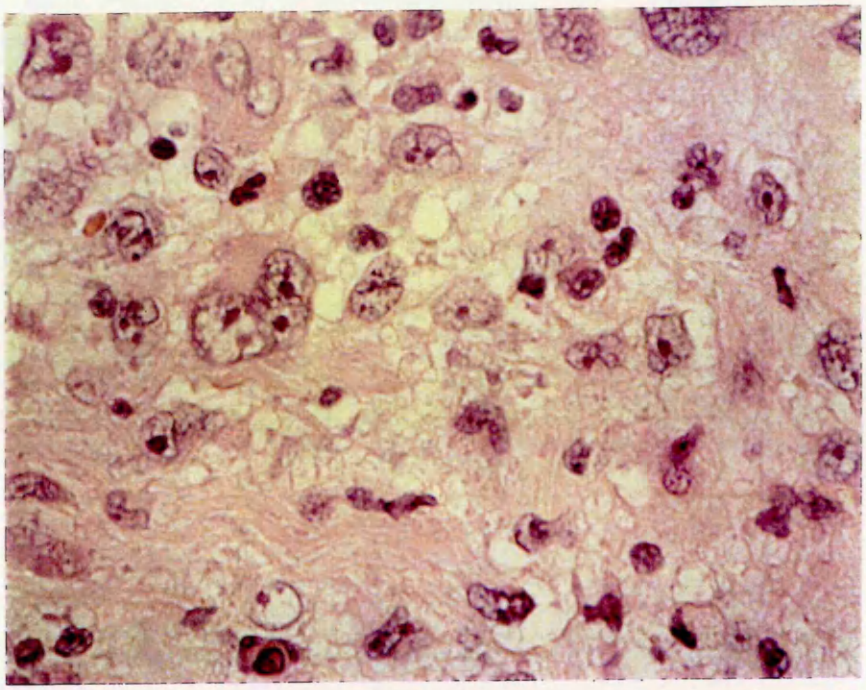
Figure 1.4. A, mixed cellularity HD. There is a polymorphic cellular infiltrate with many small lymphocytes and occasional large mononuclear and multinuclear cells.

B, lymphocyte depleted HD. There is extensive fibrosis with a scanty polymorphic cellular infiltrate.

Figure 1.4. Mixed cellularity (A) and lymphocyte depleted (B) Hodgkin's disease. (Haemotoxylin and eosin-stained sections)



A



B

in the U.K. did not detect the second, age-related incidence peak, but found that separate consideration of HD subtypes revealed different incidence peaks (McKinney *et al.* 1989). In this study, NSHD showed a peak incidence in young persons aged 14-35 years, while the other subtypes showed an increase in incidence with increasing age.

High socioeconomic status and small family size have been associated with an increased risk of HD (Gutensohn and Cole 1981; Gutensohn 1982; Bernard *et al.* 1987). Gutensohn (1982) found that risk of HD was associated with higher social class in young adults, but not in older adults.

These epidemiological data are consistent with MacMahon's proposals (1966) that HD in young adults is distinct from HD occurring in older persons and that the former may have an infectious aetiology. Increasing economic development has been associated with a shift in the peak age incidence of HD from childhood to young adults. This has been compared to changes in the epidemiology of poliomyelitis in developing and developed countries (Grufferman and Delzell 1984). The parallel with poliomyelitis led a number of groups to suggest that HD in young adults represents an unusual host response to a common infection, the risk of which is related to the age at infection (Gutensohn and Cole 1980; Grufferman and Delzell 1984).

Supportive evidence for the involvement of a transmissable agent in the aetiology of HD is provided by numerous reports in the literature documenting temporal or geographical clusters of HD (e.g. Grufferman *et al.* 1977; Grufferman and Delzell 1984; Mangoud *et al.* 1985; Davis 1986). Most studies have focused on HD occurring in young adults, though Mangoud *et al.* (1985) reported clustering of older HD cases. Alexander *et al.* (1989) found evidence of spatial clustering of HD in young adults (0-34 years). Analysis of their data according to histopathological subtype indicated that clustering might, rather, be associated with NSHD, the predominant subtype in this age range.

HD classification: The nature of the HRS cell.

The above discussion indicates that HD is an heterogeneous disorder. Classification of HD cases according to histopathological subtype or age of onset reveals variation in the clinical behaviour and epidemiology of the disease. Different aetiological factors may be associated with the different histological subtypes, or with HD occurring in different age groups. The evidence that HD may have a transmissable aetiology is strongest for cases occurring in young adults. The studies by McKinney *et al.* (1989) and Alexander *et al.* (1989) emphasize that it may be difficult to separate an association with age from an association with histological subtype. It is possible that both age and histological subtype are inadequate parameters for the classification of HD and do not accurately reflect the heterogeneity of the disease.

Detailed characterization of HD is therefore important, in order to establish criteria for improved classification systems for this disease. The present histopathological classification is based mainly on the variations seen in the non-neoplastic components of HD. Characterization of the HRS cells, the putative malignant cells, has been difficult due to the small numbers of these cells that are usually present in affected tissues. The use of immunological techniques to study the surface phenotype of HRS cells has been reviewed by Jones (1987), Lee (1987) and Drexler and Leber (1988). Despite extensive study, the cellular origin and lineage of HRS cells have not been conclusively identified. Many studies suggest that HRS cells are lymphocytic in origin (Falini *et al.* 1987; Casey *et al.* 1989; Drexler *et al.* 1989; Herbst *et al.* 1989), though this remains a controversial issue (Hsu and Hsu 1989; Hsu 1990; Naumovski *et al.* 1990). The results obtained in these studies are discussed in more detail in Chapter 3.

More recently, the availability of molecular techniques for the detection of clonal populations of B- or T-lymphocytes has permitted further evaluation of the possible lymphoid origin of HRS cells. Chapter 3 describes the application of these techniques for the characterization of HD material.

The experiments reported in Chapter 3 were performed in parallel with investigations into the involvement of viruses in the aetiology of HD. The evidence supporting a transmissable aetiology for HD in young persons, suggests that the aetiological agent(s) is likely to be ubiquitous and transmitted during close person-to-person contact. Members of the human herpesviruses show these features and have been proposed as candidate transmissable agents. Many studies have demonstrated serological

associations between human herpesviruses and HD (Henle and Henle 1973; Hesse *et al.* 1977; Evans and Gutensohn 1984; Ablashi *et al.* 1988; Biberfeld *et al.* 1988; Clark *et al.* 1990). This evidence in discussed in more detail in Chapter 4, which describes the results of experiments to determine the involvement of human herpesvirus type 6 (HHV-6) in HD.

1.3.3. Angioimmunoblastic lymphadenopathy (AIL).

AIL shares a number of clinical and morphological features with HD, and these two conditions may be difficult to differentiate (Lukes and Tindle 1975; Krajewski *et al.* 1988). Interest in the possibility that AIL has a viral aetiology has been stimulated by the clinical and pathological characteristics of the disease. AIL was originally described as a distinct clinicopathological entity by Frizzera *et al.* in 1974. A disorder showing similar features was described as immunoblastic lymphadenopathy by Lukes and Tindle (1975).

Clinical features.

The clinical features of AIL have been reviewed (Frizzera *et al.* 1974; 1975; Lukes and Tindle 1975; Azevedo and Yunis 1985; Steinberg *et al.* 1988). The clinical manifestations of AIL include systemic signs resembling those of HD: night sweats, pyrexia, weight loss, skin rashes, pruritis, generalized lymphadenopathy and hepatosplenomegaly. Patients with AIL usually also show immunological abnormalities not typical of HD patients, such as hypergammaglobulinaemia and Coombs' test-positive haemolytic anaemia.

The clinical course of AIL is variable (Lukes and Tindle 1975; Pangalis *et al.* 1983). In the majority of patients the disease is rapidly fatal, though up to a third of patients may show complete and prolonged remission of symptoms (Frizzera *et al.* 1975; Pangalis *et al.* 1983). Infectious complications are the most frequent cause of death (Frizzera *et al.* 1975; Nathwani *et al.* 1978; Pangalis *et al.* 1983), reflecting the extent of the immunological abnormalities associated with AIL.

Histopathological features.

Frizzera *et al.* (1974; 1975), Lukes and Tindle (1975) and Nathwani *et al.* (1978) have described the histopathology of AIL. The normal lymph node architecture is replaced to a variable extent by a pleomorphic cellular infiltrate, similar to that seen in HD (Figure 1.5). The distinctive histological features of AIL include the presence of a prominent proliferation of immunoblasts, an arborizing network of "high endothelial venules" and the interstitial deposition of an amorphous acidophilic material. Immunoblasts resembling RS cells may be detected, but classical RS cells are not usually seen in AIL (Nathwani *et al.* 1978).

Pathogenesis.

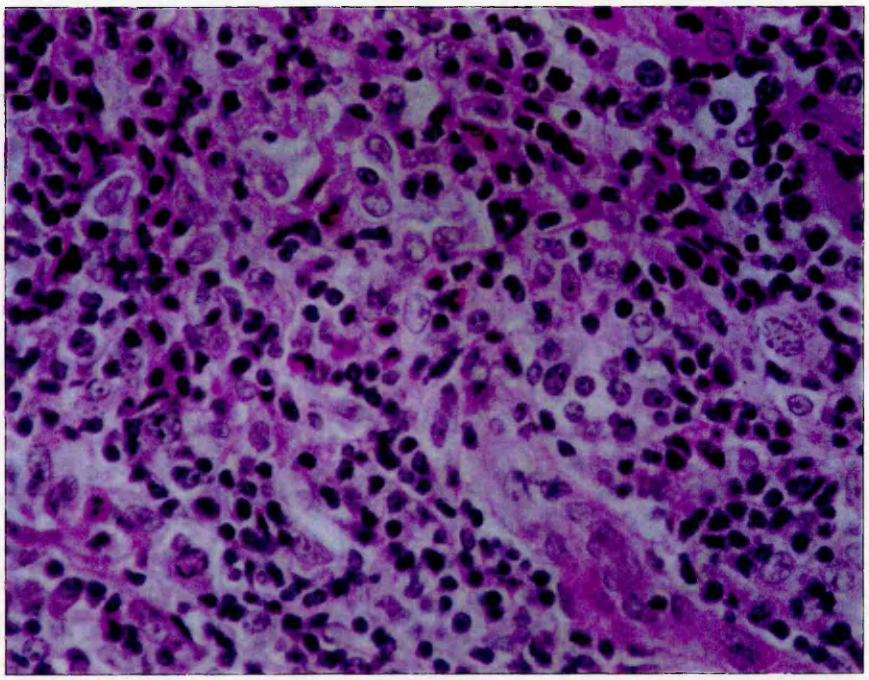
Many alternative mechanisms for the pathogenesis of AIL have been proposed. When first described, AIL was considered to be non-neoplastic (Frizzera *et al.* 1974; Lukes and Tindle 1975). Frizzera *et al.* (1974) suggested that the clinical and pathological features of AIL were consistent with those of a graft-versus-host reaction. Lukes and Tindle (1975) proposed that AIL represented a benign hyperimmune disorder, in which there was an increased tendency to develop malignant lymphoma. Drug exposure resulting in hypersensitivity or autoimmunity has been suggested as a pathogenetic mechanism by some authors (Frizzera *et al.* 1975; Lukes and Tindle 1975).

The clinical features of AIL are consistent with those of an infectious disease and the histological features of AIL are predominantly those of a reactive process. Prominent proliferations of postcapillary venules and immunoblasts may also be seen in conditions such as IM and postvaccinial lymphadenitis (Frizzera *et al.* 1975). The involvement of both herpesviruses and retroviruses in AIL has been proposed (Azevedo and Yunis 1985; Honda *et al.* 1985). Evidence relevant to the possible viral aetiology of AIL is discussed further in Chapter 4.

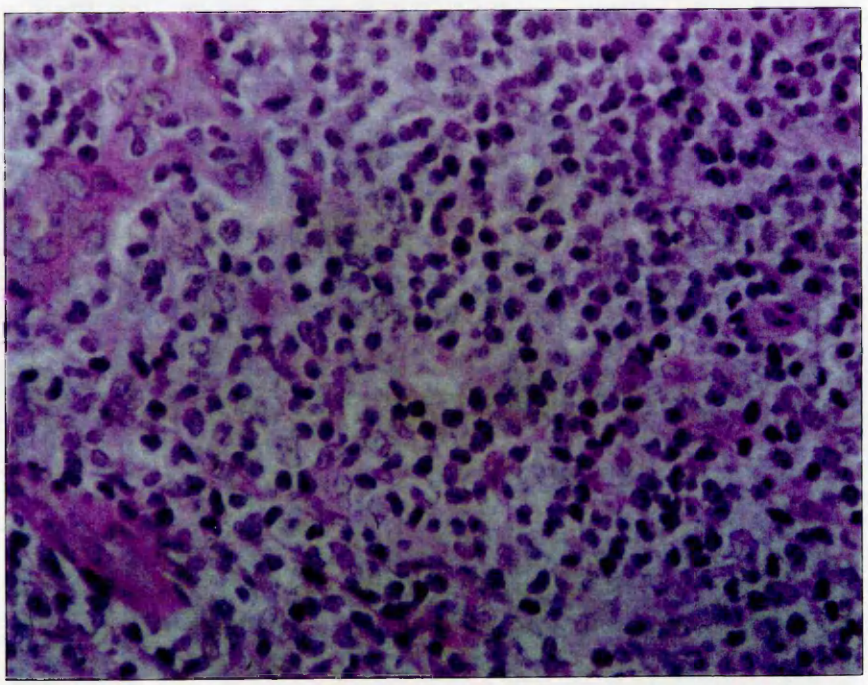
Figure 1.5 A, AIL-like T-cell lymphoma. There is a polymorphic infiltrate of lymphocytes with irregular nucleii, eosinophils, plasma cells and a number of blast cells. The lower right quadrant contains a longitudinal section through a high endothelial venule.

B, AIL-like T-cell lymphoma. This section is from case 107, described in Chapter 3. The infiltrate is composed mainly of lymphocytes, many with irregular nucleii. There are a few blast cells with clear cytoplasm. HIgh endothelial venules can be seen on the left.

Figure 1.5. Angioimmunoblastic lymphadenopathy-like T-cell lymphoma.
(Haemotoxylin and eosin-stained sections)



A



B

Classification.

In early studies, the development of malignant lymphoma was reported in occasional cases of AIL (Lukes and Tindle 1975; Fisher *et al.* 1976; Yatanagas *et al.* 1977). Later reports suggested that a higher proportion of cases showed features of malignancy (Nathwani *et al.* 1978; Shimoyama *et al.* 1979; Watanabe *et al.* 1980). These findings led to suggestions that lymphomas with histological features of AIL represented a distinct disease entity (Shimoyama *et al.* 1979) or alternatively, that AIL was a malignant disease from the outset (Watanabe *et al.* 1980; 1986).

The results of immunophenotypic, karyotypic and genotypic investigations have suggested that neither of these interpretations is correct, but that AIL is an heterogeneous disorder. These studies are discussed further in Chapter 3.

The accurate characterization of AIL cases will therefore be important in any investigation into the possible role of viruses in the aetiology of AIL. Chapter 3 describes the use of molecular analysis for the investigation of the clonality and lineage of 5 cases of AIL. These cases were also included in a study investigating the role of herpesviruses in lymphoproliferative diseases. The results of the latter investigations are discussed in Chapter 4.

1.3.4. Cutaneous T-cell lymphoma (CTCL).

The CTCLs include a range of lymphoproliferative disorders characterized by skin infiltration by mature T-lymphocytes. The clinical and morphological features of CTCL have been reviewed by Lutzner *et al.* (1975), Stansfeld (1985) and MacKie (1989). Mycosis fungoides (MF) presents clinically with skin eruptions appearing as plaques, ulcers or tumours. A proportion of cases progress to lymph node and visceral involvement. Sezary syndrome (SS) also frequently presents with skin lesions, but in addition involves lymph nodes, spleen and peripheral blood. SS may represent a leukaemic variant of MF.

A number of additional disorders have been described, in which the skin lesions are difficult to differentiate from those seen in MF. Some of these conditions, such as lymphomatoid papulosis and large plaque parapsoriasis, are considered to be premycotic lesions as a proportion evolve into MF (Slater 1987; MacKie 1989).

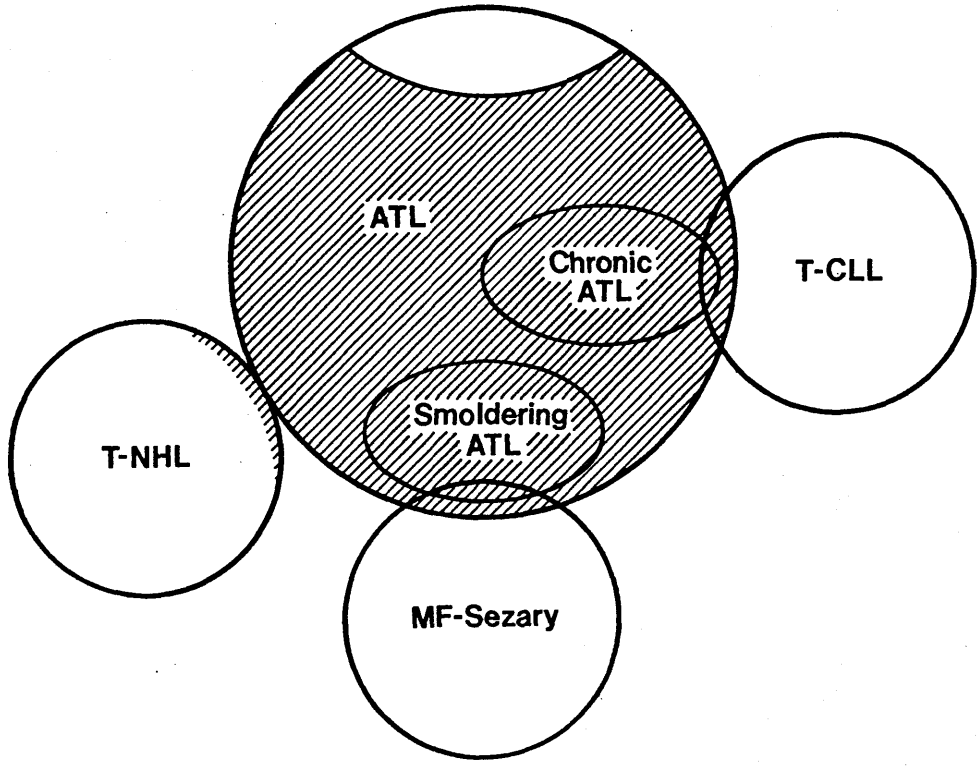
The first two isolates of HTLV-I were obtained from patients reported to have MF (Poiesz *et al.* 1980) and SS (Poiesz *et al.* 1981). These findings stimulated considerable interest in the possibility that HTLV-I might be the aetiological agent of CTCL, however antibodies to HTLV-I were rarely detected in the sera of CTCL patients (Gallo *et al.* 1983). A later review of the clinicopathological features of the two original cases indicated that both were consistent with ATL (Blattner *et al.* 1983b).

Most cases of ATL can be readily distinguished clinically and morphologically from MF and SS (Stansfeld 1985; Suchi *et al.* 1987). However there is some clinical and pathological overlap between ATL and CTCL. The clinical features of ATL have been described briefly in section 1.1.3. Clinically CTCL is associated with a better prognosis and more indolent course than ATL. The median survival for patients with MF was estimated at 10-12 years by Epstein *et al.* (1972) and 5 years by Cohen *et al.* (1980). Neither MF nor SS are associated with hypercalcaemia.

It may however, be more difficult to distinguish between the less aggressive forms of HTLV-I associated malignancy, as described by Yamaguchi *et al.* (1985) and Kinoshita *et al.* (1985), and other forms of CTCL. The cases of "smouldering" and "preleukemic" ATL described by Yamaguchi *et al.* (1985) and Kinoshita *et al.* (1985) did not follow an aggressive course and many presented with skin lesions. In addition, an aggressive form of Sezary cell-like leukaemia has recently been described (Matutes *et al.* 1990). HTLV-I antibodies were detected in one of the cases described in this study and the authors suggest that some cases of SS may be variant forms of ATL. Blattner (1989) has reviewed the evidence suggesting that the clinical features of the malignancies associated with HTLV-I show overlap with T-cell chronic lymphocytic leukaemias and T-cell NHLs in addition to MF and SS (Figure 1.6). In agreement with this, HTLV-I infection has been reported in a small number of cases of typical MF and SS (Kaplanski *et al.* 1986; Detmar *et al.* 1991).

The histopathology of the skin lesions seen in ATL, MF and SS is similar. MF and SS are characterized by the presence of small T-lymphocytes with many nuclear indentations ("cerebriform" cells), larger abnormal cells are also usually seen and both cell types may accumulate in the epidermis (Stansfeld 1985; Suchi *et al.* 1987). Clusters

Figure 1.6. T-cell malignancies associated with HTLV-I infection.



ATL, adult T-cell leukaemia; MF-Sezary, mycosis fungoides or Sezary syndrome; T-CLL, T-cell chronic lymphocytic leukaemia; T-NHL, T-cell non-Hodgkin's lymphoma. The shaded area indicates the proportion of cases which are either positive for HTLV-I antibodies, or in which integration of HTLV-I genomes may be detected. From Blattner (1989).

of these cells (Pautrier microabscesses) are usually present in MF and may be seen in the skin lesions of patients with SS. In ATL the malignant cells seen in skin lesions are also small or medium sized T-lymphocytes with convoluted nucleii; Pautrier microabscesses and epidermal infiltration may be observed (Suchi *et al.* 1987).

The cell surface phenotype of the malignant cells of SS and MF is similar to that of the malignant cells in ATL (Picker *et al.* 1987; Slater 1987). In contrast to ATL, the malignant cells in CTCL have been reported to be CD25- by some authors (e.g. Matutes *et al.* 1990). However other studies have reported CD25 expression in CTCL (Nasu *et al.* 1985; Ralfkiaer *et al.* 1985; Slater 1987) and rare cases of ATL are CD25+ (Pandolfi *et al.* 1985; Yoshida *et al.* 1989).

The majority of patients with CTCL are not seropositive for HTLV-I (Gallo *et al.* 1983). However, the clinical and pathological overlap between ATL and other CTCLs has stimulated interest in the possibility that retroviruses related to HTLV-I might be involved in the aetiology of the latter conditions. A number of ultrastuctural and serological studies have provided evidence supporting this notion (van der Loo *et al.* 1979; Fullbrandt *et al.* 1983; Slater *et al.* 1985; Lange-Wantzin *et al.* 1986; Ranki and Krohn 1987; Saal *et al.* 1989; Srivastava *et al.* 1990). These studies are discussed further in Chapter 5 and an experimental approach to the detection of HTLV-I related retroviruses is described.

1.4. (B) INVESTIGATION OF THE DISEASE ASSOCIATIONS OF A KNOWN VIRUS.

A complementary approach to the analysis of specific lymphoproliferative diseases for evidence of a viral aetiology is to investigate the disease-associations of a previously identified virus. Such investigations are likely to focus on viruses that are related to known leukaemogenic agents, viruses that show transforming potential in vitro, or viruses that have been isolated from patients with lymphoproliferative disease. The role of a known virus in the pathogenesis of lymphoid malignancy may be investigated by determining the molecular association between the viral genome and tumour tissues (section 1.4.1), and the serological association between virus infection and disease (section 1.4.2).

Some features of the recently isolated virus, HHV-6, have stimulated interest in the possible involvement of this virus in haemopoietic malignancies. HHV-6 was first isolated from patients with a variety of lymphoproliferative disorders (Salahuddin *et al.* 1986) and is lymphotropic in vitro (Lusso *et al.* 1988). More recently this virus has been shown to have tumorigenic potential in nude mice (Razzaque 1990). Chapter 4 of this thesis describes the results of experiments to investigate the role of HHV-6 in the pathogenesis of human lymphomas.

1.4.1 Molecular analysis of the association between a virus and lymphoproliferative disease.

The association between a candidate virus and lymphoproliferative disorders may be analysed using a number of approaches. The presence of viral nucleic acid or viral protein in tumour cells may be determined using molecular or immunological techniques. The mechanism of viral leukaemogenesis will determine whether a disease association is amenable to molecular analysis. Mechanisms by which viruses may contribute to lymphoproliferative disease are described in the following section.

1.4.2. Mechanisms of viral leukaemogenesis.

Viruses that are known to induce leukaemia and/or lymphoma appear to do so via a variety of different pathogenic mechanisms. The mechanism of involvement of a virus in the induction of disease will determine the molecular association between the virus and the tumour cells. The following sections describe the direct and indirect mechanisms that may be involved in viral leukaemogenesis.

Direct mechanisms of viral leukaemogenesis.

Direct mechanisms of viral leukaemogenesis may be defined as those in which the presence of all or a part of the virus in the neoplastic cell is required at some stage in the transformation process. A number of different types of direct mechanism have been shown, or proposed, to occur in virus-induced haematopoeitic neoplasms.

a) *Cis*-acting

Cis-acting mechanisms have been shown to be important in retroviral leukaemogenesis (e.g. Hayward *et al.* 1981; Neil *et al.* 1984). This type of mechanism involves the activation of cellular oncogenes by viral transduction or by insertional mutagenesis (Figure 1.7).

Transduction of *c-myc* has been shown to be a frequent event in naturally occurring FeLV-induced thymic lymphosarcoma (Mullins *et al.* 1984; Neil *et al.* 1984). However oncogene transduction appears to be relatively rare in lymphoid tumours associated with experimental infections of FeLV and murine and avian retroviruses (Hayward *et al.* 1981; Neil and Onions 1985).

Activation of oncogenes by insertional mutagenesis may occur via a number of mechanisms (Figure 1.7) (Neil and Forrest 1987). Activation of *c-myc* expression by promoter insertion has been reported in a high percentage of ALV-induced lymphomas (Hayward *et al.* 1981). In T-cell lymphomas caused by MuLV, insertion of enhancer elements within the viral LTR into the proximity of *c-myc* are thought to result in increased gene expression (Corcoran *et al.* 1984; Li *et al.* 1984; Selton *et al.* 1984).

Additional *cis*-acting mechanisms are theoretically possible. Insertion of viral DNA into the regulatory sequences of a cellular oncogene could result in abnormal levels of expression (Neil and Onions 1985). Disruption of the regulatory sequences of "tumour suppressor" genes, or of the genes themselves, by viral insertion could result in reduced levels of the gene product.

b) *Trans*-acting.

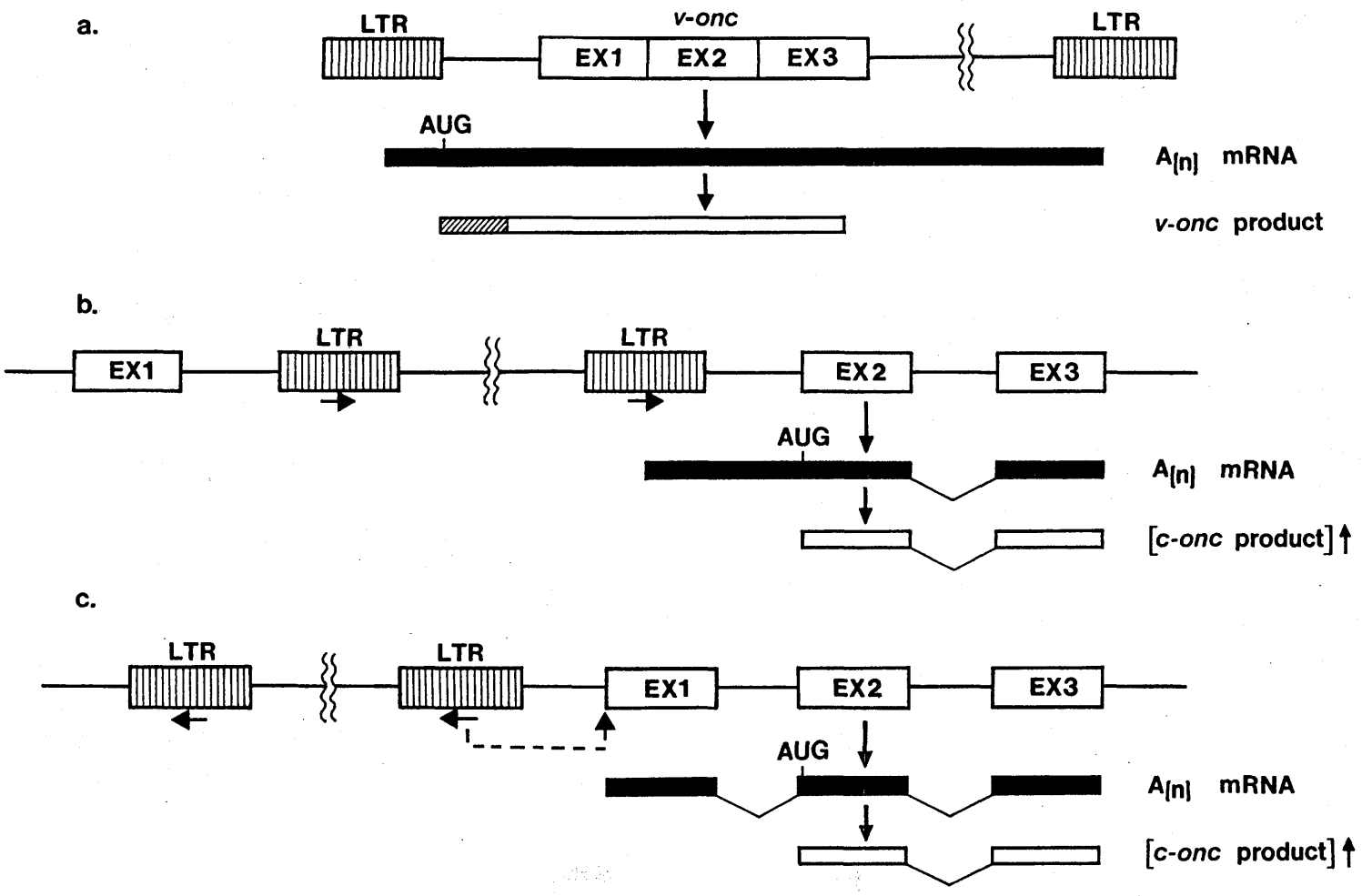
Cell transformation by some retroviruses is not associated with *cis*-activation of cellular oncogenes. For HTLV-I the failure to identify common integration sites in tumour cells has led to the conclusion that cell transformation is induced by a *trans*-acting mechanism (Seiki *et al.* 1984; Wong-Staal and Gallo 1985). Similar findings have been reported for BLV (Gelmann *et al.* 1984; Gregoire *et al.* 1984). EBV also appears to transform cells by a *trans*-acting mechanism.

Figure 1.7. (a) Retroviral transduction of cellular genes. The viral genome has been partially replaced by a transduced oncogene (v-onc) of cellular origin. Expression of the gene is controlled by the viral long terminal repeat (LTR). This may result in expression of elevated levels of the v-onc product, or in the formation of a structurally modified product. The example shown illustrates the initiation of protein expression from a viral initiation codon (AUG), giving rise to an altered v-onc product which is fused with a viral structural protein.

(b) Activation of a cellular oncogene (c-onc) by promoter insertion. The insertion of a retroviral LTR in an appropriate position and orientation relative to a cellular gene may alter expression of the gene. Promoter sequences present in the LTR may cause increased expression of a normal c-onc product , as shown here. Alternatively if viral integration occurs within coding sequences a truncated protein with altered activity may be expressed.

(c) Activation of a c-onc by enhancer insertion. Insertion of a retroviral LTR close to, or within, a cellular gene may result in increased expression of the gene due to the effects of enhancer sequences within the LTR (dashed arrow). In the example shown, the viral LTR is in a 5' position and the opposite orientation relative to the affected gene.

Figure 1.7. Cis-acting mechanisms of retroviral leukaemogenesis.



Trans-acting mechanisms of leukaemogenesis are likely to involve interactions between viral gene products and cellular genes that play a role in the control of cell proliferation and/or differentiation. The mechanisms by which HTLV-I and EBV are considered to transform their target cells in vitro and contribute to leukaemogenesis in vivo have been discussed in sections 1.1.5 and 1.2.5.

c) Receptor-mediated leukaemogenesis.

The possibility that leukaemias could be induced in retrovirus-infected cells via a receptor-mediated effect was proposed originally by McGrath *et al.* (1978). This group reported evidence that the interaction between a radiation leukaemia virus-induced lymphoma and its cognate retrovirus was mediated by the T-cell receptor (TCR) (O'Neill *et al.* 1987). Evidence that this mechanism is important in naturally occurring virus-induced leukaemias has not been reported.

Molecular analysis of tumours induced by direct mechanisms.

Molecular analysis of tumours induced by *cis*-acting effects on cellular genes will show the presence of viral DNA. However the viral genome may be highly defective, as activation of oncogene expression may require only limited regions of the virus, i.e. those containing promoter or enhancer sequences. Viral gene products may not be detectable, or may be expressed as fusion proteins with host gene products.

Molecular analysis of tumours induced by a virus operating through a *trans*-acting mechanism should reveal the presence of viral nucleic acid. Defective viral genomes may be detected if expression of the *trans*-acting function requires only limited viral sequences. Analysis of viral expression should permit the detection of the viral gene product(s) responsible for *trans*-activation.

However, virus involvement may be important only in an early stage of leukaemogenesis, and may not be required for later stages, or for the maintenance of the tumour phenotype. The hosts immune system may exert considerable selection pressure on virus-infected malignant cells, permitting preferential survival of cells in which virus gene expression has been reduced to a minimum. The limited expression of viral gene products in both BL and ATL may be examples of this effect. An alternative scenario may be envisaged, in which selection pressure due to the hosts immune response

permits preferential survival of cells in which viral nucleic acid has been lost from tumour cells. This would represent a "hit and run" effect by the virus. Analysis of the malignant cells would not reveal the presence of viral nucleic acid in tumours induced by this type of mechanism. However the analysis of premalignant lesions may enable an association between virus infection and disease pathogenesis to be established.

Molecular analysis of the tumour cells in a lymphoproliferative disorder induced by a receptor-mediated mechanism should reveal the presence of both viral nucleic acid and viral proteins. Loss of viral gene expression would be associated with loss of the proliferative signal and therefore with the regression of disease.

Indirect mechanisms of viral leukaemogenesis.

Failure to detect evidence of virus infection of tumour cells does not preclude the possibility that the virus is involved in the pathogenesis of disease. Viruses may play a role in the pathogenesis of leukaemias or lymphomas in the absence of infection of the tumour cells. A variety of mechanisms by which viruses may contribute indirectly to leukaemogenesis have been proposed.

a) Virus induced immunosuppression.

Immunosuppression is associated with an increased incidence of lymphomas and leukaemias (Filipovich *et al.* 1990). Neil and Forrest (1987) have reviewed some of the models that could explain this phenomenom. A compromised immune system may be less able to eliminate transformed cells. Alternatively immunosuppression may permit infection by some other oncogenic virus or reactivation of a latent oncogenic virus. An immunosuppressive virus could contribute to leukaemogenesis by increasing the rate of recruitment of undifferentiated haematopoietic cells due to the destruction of mature lymphoid cells. The increased pool of proliferating cells would increase the probability that additional, tumorigenic, genetic events will occur.

A number of viruses induce immunosuppression in infected hosts. HIV infection is associated with an increased risk of developing lymphomas; between 7% and 12% of AIDS patients have been estimated to develop NHL during the course of disease (Cremer *et al.* 1990; Ziegler and McGrath 1990). The majority of these are high grade B-cell lymphomas which frequently occur in extra-nodal sites (Ziegler and McGrath

1990). The pathogenesis of HIV-associated B-cell NHL appears to involve multiple mechansisms. Between 38% and 50% of lymphomas occurring in HIV-infected persons are associated with EBV (Suber *et al.* 1988; Boiocchi *et al.* 1990; Chappuis *et al.* 1990; Cremer *et al.* 1990).

b) Immune stimulation.

Many models of leukaemogenesis have, as a central tenet, the assumption that increased proliferation of immature lymphocytes increases the risk of transforming events occurring in the dividing (and differentiating) cells. Examples are the models proposed by Klein (1979) and Yoshida and Seiki (1987) for the pathogenesis of BL and ATL respectively. These authors suggest that lymphocyte proliferation is induced by virus infection of the target cells.

Chronic activation of the immune system due to other causes would also be expected to result in an increased risk of developing lymphoid malignancies. Virus infections could result in chronic stimulation of the immune system due to continued antigen presentation in persistently infected hosts, or as a result of virus induced release of growth factors. Virus infection may also result in changes in antigen expression by infected cells (Miyagawa *et al.* 1988; Baboonian *et al.* 1989). Such changes could create novel targets for the immune system and thus result in chronic immune stimulation.

Immune stimulation in response to acute encounters with infectious agents may also be important in predisposing proliferating lymphoid cells to tumorigenic events. Stimulation of the immune response in infancy and early childhood, as a result of initial encounters with exogenous antigens, has been proposed as an explanation for the age distribution of the common (B-cell precursor) form of acute lymphoblastic leukaemia (Greaves 1988).

(c) Interactions between viruses.

Infection by a virus may indirectly contribute to leukaemogenesis by activating other oncogenic viruses. This may involve direct effects on viral replication following infection of the same cell, or an indirect effect, through stimulating cell proliferation and activating replication of a latent virus present in the cell. Interactions between herpesviruses and retroviruses are discussed further in Chapter 4.

Molecular analysis of tumours induced by indirect mechanisms.

Molecular analysis of tumour samples from patients with lymphoid malignancies that are induced by the indirect mechanisms described above will not, in most cases, reveal the presence of virus.

However for some of the possible indirect mechanisms described above, molecular analysis may provide circumstantial evidence for viral involvement in disease development. If chronic antigenic stimulation and/or lymphokine release by virus infected cells is important in pathogenesis, evidence of viral antigen expression in the tissues in which lymphomas develop may be detectable.

The detection and interpretation of evidence for the involvement of viral interactions in leukaemogenesis may be difficult. The results obtained in molecular analysis will depend on the mode of interaction between the viruses and on the mechanisms of leukaemogenesis.

1.4.3 Determining the significance of a molecular association.

a) Determination of the clonality of the tumour cells and virus infected cells.

Techniques for determining the clonal nature of lymphoproliferative disorders have been mentioned in section 1.3.2 and are discussed further in Chapter 3. Viruses capable of transforming cells by a single step mechanism would produce polyclonal tumours. Viruses containing transduced oncogenes may induce polyclonal malignancies when experimentally transmitted to susceptible hosts (Frykberg *et al.* 1983; Teich *et al.* 1984). However many oncogene-containing viruses induce monoclonal tumours (e.g. Baumbach *et al.* 1986) suggesting that additional events may be required for transformation in vivo.

The majority of clinically overt lymphomas and leukaemias are monoclonal in nature. Determination of the clonality of virus-infected cells permits an assessment of the possible role of the virus in disease pathogenesis. If the virus is present in the tumour cells, evidence that the clonality with respect to virus infection differs from that of the

tumour suggests that the virus is not playing a direct role in the development of the malignancy. In particular, failure to detect evidence that clonal proliferation of virus-infected cells has occurred indicates that virus infection has taken place after tumour development.

The clonal origin of retrovirus infected cells may be assessed by the molecular analysis of integration sites. Clonal expansion of a single retrovirus-infected cell produces a cell population with a common site of retroviral integration, which can be detected by molecular analysis. A technique for determining the clonal origin of EBV-infected cells has been described by Raab-Traub and Flynn (1986). Size variations in the fused terminal fragments of the virus are generated when replicating linear virus forms circular episomes following cell infection. The detection of a unique fused terminal fragment of EBV in a cell population indicates amplification of a single episome of EBV and by inference, clonal proliferation of a single EBV-infected cell (Figure 1.8).

However the demonstration that the clonality of the virus infected cells coincides with the clonality of the malignancy does not necessarily prove that the virus is involved in disease pathogenesis. The same result could be obtained if malignant transformation occurs in a cell which is already infected by virus. Henderson (1989) has suggested that EBV is purely a passenger virus in NPC and that the association between NPC and EBV is due to malignant transformation of epithelial cells that normally harbour EBV. This is, however, a contentious suggestion (see for example de-The *et al.* 1989b).

b) Determination of the consistency of an association between the presence of a virus and disease.

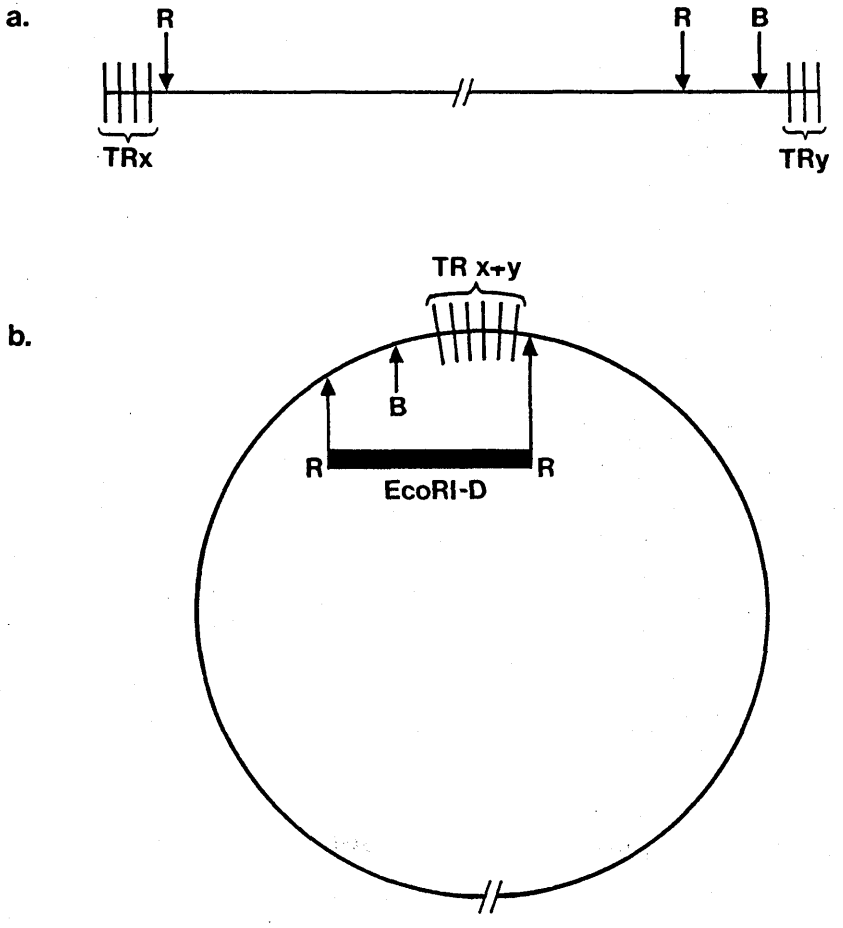
The detection of a consistent association between a particular lymphoid malignancy and the presence of a virus in the tumour cells would be significant evidence supporting a role for the virus in the pathogenesis of that malignancy. However such a finding may not be considered proof that the virus is playing a role in disease development, even if the virus infected cells are shown to be clonal.

As mentioned above, Henderson (1989) has suggested that EBV is a passenger virus in NPC, despite the detection of EBV DNA in nearly 90% of NPC biopsies (Zur Hausen 1970; Nonoyama *et al.* 1973; Desgranges *et al.* 1975). If EBV is not involved in the pathogenesis of NPC, it is necessary to postulate virus infection of approximately 90%

Figure 1.8. (a) Infectious EBV virions contain linear double stranded DNA with direct terminal repeats (TR) of approximately 500bp. The number of TR at each end of the viral DNA is variable (x and y). Cleavage sites for the restriction enzymes EcoRI (R) and BamHI (B) are shown.

(b) After infection of a cell the termini are joined to form a circular episome. If infection has occurred at a multiplicity of one all the episomes within a cell and within the progeny of that cell will have the same number of TR (x+y). If such an infected cell undergoes clonal expansion, the presence of a population of EBV genomes with identical numbers of TR can be detected by molecular analysis. Digestion of DNA with appropriate restriction enzymes (e.g. BamHI and EcoRI) will generate a unique fragment containing the TR which will vary in size depending on the numbers of TR present. If polyclonal expansion of EBV-infected cells has occurred, or if infection has occurred at high multiplicity, many TR-containing fragments of varying sizes will be generated. The presence of a discrete fragment can be detected by hybridization with a probe for the TR, such as the EcoRI-D probe shown here.

Figure 1.8. The detection of clonal populations of EBV genomes.



or greater of the epithelial cells that are the tumour cell precursors. Alternatively virus infection of this cell type must occur in approximately 90% or more of individuals that are susceptible to the development of NPC.

Conclusive proof that EBV is a passenger virus in NPC is likely to be difficult to establish. Seroepidemiological studies demonstrate a characteristic immune response to EBV antigens in NPC patients, suggesting that the host-virus interaction is disrupted in NPC (de-The *et al.* 1989b). However this could conceivably occur as a consequence of tumour development.

Failure to detect a consistent association between the presence of a virus in tumour cells and a specific malignancy may be taken to indicate lack of viral involvement in the aetiology of that disease. However alternative interpretations should also be considered, as discussed below.

(i) Inappropriate disease classification.

The importance of disease classification in investigating the viral aetiology of lymphoproliferative disorders has been discussed in section 1.3. The detection of viral involvement in a consistent proportion of cases of a particular malignancy may indicate the existence of distinct entities within the disease classification.

(ii) Genetic events producing the same phenotypic changes.

For many of the direct and indirect mechanisms of viral leukaemogenesis described above, it is possible to envisage genetic or other non-virus related events that could produce the same effect. The resulting tumour could be phenotypically identical to virus-induced tumours, but molecular analysis would not reveal the presence of virus. A similar explanation was proposed by Shimoyama *et al.* (1986) to explain the occurrence of ATL in five persons, in the absence of molecular or serological evidence of HTLV-I infection. A second example is provided by the occurrence of EBV-positive and EBV-negative sBL.

1.4.4. Seroepidemiological analysis of the association between a virus and lymphoproliferative disease.

Evidence for the involvement of a virus in lympoproliferative disease may also be obtained as the result of seroepidemiological studies. Such analyses may provide the only way to establish the indirect involvement of a virus in disease pathogenesis. The relationship between the occurrence of disease and virus seroprevalence will depend on the efficiency with which the virus induces disease and on the involvement of non-virus associated factors in disease pathogenesis.

Non-virus associated factors that have been identified as influencing the development of virus-induced lymphoid malignancies include the dosage of infecting virus, the age at which virus infection is acquired and the immune status of the host. The importance of the latter two parameters in the development of HTLV-I and EBV-associated lymphomas has already been discussed (sections 1.1.4 and 1.2.2-1.2.4).

The establishment of a correlation between disease development and viral seroprevalence will not be possible if virus infection is ubiquitous, as is the case for many of the human herpesviruses. Virus involvement in disease may, however, be reflected by abnormal or distinctive responses to viral antigens, as has been reported for patients with NPC or BL who show elevated antibody titres to specific EBV antigens (de-The *et al.* 1989b; Evans and de-The 1989).

The use of seroepidemiological studies to evaluate the role of viruses in the aetiology of leukaemias and lymphomas is not discussed further here, as this approach was not used in the experiments described in this thesis. Chapter 4 describes the results of experiments to determine the role of HHV-6 in lymphoproliferative diseases. Molecular analysis of biopsy material from patients with a variety of disorders was used to determine the presence of HHV-6 DNA sequences in tumour samples. These investigations were performed in parallel with a case-controlled study to determine the seroprevalence and titres of HHV-6 antibody in patients with various haematological malignancies (Clark *et al.* 1990). The results obtained in the molecular studies described in Chapter 4 are discussed in the context of findings of Clark *et al.* (1990).

1.5 (C) THE DETECTION OF VIRUSES RELATED TO KNOWN LEUKAEMOGENIC VIRUSES.

A third approach to the identification and isolation of novel leukaemogenic viruses of humans is to search for viruses related to known leukaemia viruses. Members of the Herpesviridae and Retroviridae are known to be leukaemogenic in man and other animals. Both molecular and serological approaches have been used in attempts to isolate novel, related viruses.

1.5.1 Molecular approaches.

A number of groups have used low-stringency hybridization of retroviral probes to search for related DNA sequences in the human genome (e.g. Bonner *et al.* 1982; Noda *et al.* 1982; Callahan *et al.* 1985). The purpose of most of these studies was to search for endogenous retroviral sequences, rather than to identify novel exogenous retroviruses. A similar approach has been employed to analyse lymphoma material for the presence of HTLV-related sequences (Manzari *et al.* 1987; R.F. Jarrett and S. Gledhill, unpublished data). The results obtained by Manzari *et al.* (1987) are discussed further in Chapter 5. Low-stringency hybridization suffers from the disadvantage that related sequences in cellular DNA will give background hybridization and will limit the sensitivity of the technique.

The development of the PCR has permitted a more specific approach based on the use of conserved regions of viral genomes for PCR primers to obtain the amplification of intervening divergent sequences. The use of the PCR allows the analysis of small amounts of sample DNA and permits the precise selection of sequences predicted to be conserved. Selection of appropriate primer sequences may utilize regions that have been identified as being highly conserved between viruses.

Bangham *et al.* (1988) used primers derived from *pol* gene sequences, which are highly conserved between diverse retroviruses, to amplify HTLV-I sequences from DNA samples from patients with TSP. Amplification of a number of retrovirus related sequences was also obtained, these included two endogenous Moloney MuLV-related retroviruses. The results described in this report illustrate one of the limitations of this approach, as selection of highly conserved retroviral sequences is likely to increase the probability of amplifying endogenous retroviral DNA.

1.5.2. Serological approaches.

Serological approaches to the detection of novel leukaemogenic viruses have utilized a variety of assays to detect the presence of antibodies in patient sera that cross-react with viral antigens. A novel retrovirus has been detected using this approach; the patient from which HTLV-II was first isolated was initially identified using assays for HTLV-I antibody (Kalyanaraman *et al.* 1982). Many studies have analysed sera from patients with a variety of malignancies for evidence of antibodies that cross-react with retroviral antigens (e.g. Lange Wantzin *et al.* 1986; Maeda *et al.* 1986; Ranki and Krohn 1987; Kovarik *et al.* 1989; Srivastava *et al.* 1990). Enzyme-linked immunoassays (ELISAs) and Western blots (WB) using HTLV-I proteins have been used by a number of groups to search for evidence that either HTLV-I or a related retrovirus is present in patients with CTCL (Lange Wantzin *et al.* 1986; Ranki and Krohn 1987; Srivastava *et al.* 1990). The results obtained in these studies are discussed further in Chapter 5.

The results of such studies may be difficult to interpret as a variety of alternative explanations for the detection of antibodies to retroviral antigens in sera of non-infected persons have been proposed (Barbacid *et al.* 1980; Snyder and Fleissner 1980; Talal *et al.* 1990a). In addition, confirmation that the cross-reacting antibodies do indicate infection with a related virus requires the isolation or identification of the virus by other means, such as electron microscopy.

1.5.3 A combined approach.

Some of the problems associated with the use of the PCR for the amplification of conserved viral sequences, and with the use of serological assays for the detection of cross-reacting antibodies could be circumvented by a combined approach. The identification of the precise regions of viral proteins that are reacting with antibodies in sera that show cross-reactivity in immunological assays will indicate the location of conserved regions in a related virus. The amino acid sequence of the putative conserved regions could then be used to derive redundant nucleotide sequences for use as PCR primers. Chapter 5 describes the results of experiments designed to identify the regions of HTLV-I core proteins that cross-react with HTLV-I-negative sera. The purpose of these experiments is to generate information that could be used to design PCR primers for the amplification of HTLV-I related retroviruses.

CHAPTER TWO

GENERAL MATERIALS AND METHODS.

Table II.I. Sources of routinely used materials.

Materials	Source
Apparatus for agarose gel electrophoresis	BRL
Apparatus for polyacrylamide gel electrophoresis	Biorad
Corex centrifuge tubes (30ml)	Sarstedt
Falcon tubes (15ml, 50ml)	A & J Beveridge Ltd.
Filter paper (Whatman, grade)	Scotlab
Filter paper (Whatman, 3MM)	Scotlab
Filtration units (Nalgene, 0.2um)	A & J Beveridge Ltd.
Fliptop microcentrifuge tubes (1.5ml)	Scotlab
Nylon membrane (Hybond-N)	Amersham Int. plc.
Oakridge centrifuge tubes (30ml)	Sarstedt
Petri dishes	Scotlab
Pipette tips (Rainin)	Scotlab
Pipette tips (Treff)	Scotlab
Polaroid film (Type 57)	Genetic Research Instrumentation Ltd.

Table II.II. Stock solutions and buffers.

Acrylamide stock solution (30%)

acrylamide	29%
N,N'-methylenebisacrylamide	1%

6% acrylamide solution for denaturing polyacrylamide gels

acrylamide stock solution (30%)	200mls/L
TBE	1 x
urea	7.6M

Alkaki buffer

sodium hydroxide	0.5M
sodium chloride	1.5M
adjusted to >pH12.0	

Denhardt's solution (100 x)

Ficoll	2%
Bovine serum albumin (Fraction V)	2%
polyvinylpyrollidone	2%

Elution buffer for removal of DNA fragments from DEAE membrane

sodium chloride	1M
Tris pH9.0	50mM
EDTA	1mM

Extraction buffer for the purification of high molecular weight DNA

sodium chloride	144mM
Tris	10mM
EDTA	1mM
adjusted to pH8.0	

10 x Loading buffer for nondenaturing gel electrophoresis

bromophenol blue	0.42%
xylene cyanol	0.42%
glycerol	50%

Neutralizing buffer

Tris base	0.5M
sodium chloride	3M
concentrated hydrochloric acid	3.3%
adjusted to pH8.0	

Table II.II. Stock solutions and buffers (continued).

Potassium acetate (3M/5M)

potassium acetate (5M)	60mls
glacial acetic acid	11mls
deionized water	28.5mls

SSC

sodium chloride	0.15M
sodium citrate	0.015M

Southern hybridization buffer

Formamide	50%
sodium dodecyl sulphate	0.1%
Denhardts solution	5 x
Tris pH7.4	50mM
EDTA	10mM
SSC	3 x

TAE

Tris	40mM
sodium acetate	20mM
sodium chloride	20mM
EDTA	2mM
adjusted to pH8.0	

TBE

Tris	90mM
boric acid	90mM
EDTA	2.25mM
adjusted to pH8.0	

TE

Tris pH8.0	10mM
EDTA pH8.0	1mM

TNE

sodium chloride	0.1M
Tris	10mM
EDTA	1mM
adjusted to pH8.0	

Table II.III. Composition of bacterial media.

Luria-Bertani (LB) medium

sodium chloride	170mM
bactotryptone	1%
bacto-yeast extract	0.5%
adjusted to pH7.0	

Minimal medium

potassium hydrogen phosphate (monobasic)	50mM
potassium hydrogen phosphate (dibasic)	10mM
ammonium sulphate	30mM
sodium citrate	8.5mM
magnesium sulphate	0.4mM
glucose	11mM
thiamine	60um

SOB

bactotryptone	2%
yeast extract	0.5%
sodium chloride	10mM
potassium chloride	2.5mM
magnesium chloride	10mM
magnesium sulphate	10mM
adjusted to pH7.0	

2. GENERAL MATERIALS AND METHODS.

The following sections describe the materials and methods that have been used frequently in the experiments reported in this thesis. In addition, the majority of the experimental methods involving DNA manipulations are described in this chapter. The details of the materials and methods employed in additional experiments are given in the relevant chapters.

2.1. MATERIALS.

The sources of routinely used materials are detailed in Table II.I. The sources of additional materials are given elsewhere in the text.

2.1.1. Chemicals.

All chemicals were of Analar or molecular biology grade and were obtained from The Sigma Chemical Company or BDH except where stated. Deionized, filtered water (MilliQ water filtration system, Millipore) was used in all the buffers utilized for enzyme-catalysed reactions and also for the dissolution of DNA and proteins. Deionized water obtained from a MilliQ reverse osmosis system (Millipore) was used to make up all other buffers and for all other procedures.

2.1.2. Bacterial strains.

Eshchericia coli (*E. coli*) strains HB101 (genotype supE44 hsdS20[r_B-m_B-] recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1) and DH5α (genotype supE44 lacU169[Φ80lacZ M15] hsdR17 recA1 endA1 gyrA96 thi-1 relA1) were obtained from Gibco BRL. *E. coli* strains JM105 (genotype supE endA sbcB15 hsdR4 rpsL thi [lac-proAB] F'[traD36 proAB$^+$ lacI^q lacZ M15]) and JM109 (genotype recA1 supE44 endA1 hsdR17 gyrA96 relA1 thi [lac-proAB] F'[traD36 proAB$^+$ lacI^q lacZ M15]) were obtained from Pharmacia.

2.2. PURIFICATION AND SEPARATION OF NUCLEIC ACIDS.

2.2.1. Preparation of high molecular weight DNA from eukaryotic cells.

High molecular weight DNA was prepared from PBMCs or biopsy material as follows. Tissue biopsies were disrupted in 5mls of extraction buffer (Table II.II) in a sterile polythene bag using a Stomacher 80 (Colworth). The resulting cell suspension was pelleted by centrifugation at 2500 revolutions per minute (rpm) for 5 minutes at room temperature and then washed twice in extraction buffer.

PBMCs were isolated from whole blood by centrifugation in Ficoll Paque. Peripheral blood samples were collected into an equal volume of RPMI 1640 containing 20u/ml preservative free heparin. Five to twenty millilitres of the diluted sample was layered onto an equal volume of Ficoll Paque and centrifuged at 2500 rpm for 15 minutes at room temperature. Mononuclear cells were removed from the interphase and washed twice in extraction buffer.

The cell pellets from either source were resuspended in 5-25mls of extraction buffer. Sodium dodecyl sulphate (SDS) and proteinase K were added to final concentrations of 0.5% and 50µg/ml respectively and the mixture incubated at 50°C for 60 minutes or at 40°C for 90 minutes. An equal volume of phenol was added to the lysate and the phases mixed slowly for 10 minutes. Phenol was equilibrated with 0.1M Tris pH8.0 prior to use. Following centrifugation at 2500 rpm for 10 minutes at room temperature the aqueous phase was removed to a sterile tube using a wide-tipped pasteur pipette. An equal volume of chloroform containing 4% isoamylalcohol was added, the phases mixed thoroughly and then separated as before. A wide-tipped pipette was used to transfer the aqueous phase into at least two times the volume of 100% ethanol. High molecular weight DNA was spooled onto a sealed pasteur pipette and transferred to a microcentrifuge tube. The DNA pellet was washed first in 70% ethanol then in 100% ethanol and the pellet dried for 2-3 hours. The DNA pellet was resuspended in TE (Table II.II) and incubated at 37°C for 5-16 hours until the DNA was completely dissolved.

The concentration and purity of the DNA solution were determined by measuring the optical density of the solution at wavelengths of 260nm ($O.D._{260}$) and 280nm ($O.D._{280}$). The concentration of the DNA solution was estimated on the basis of the assumption that an $O.D._{260}$ of 1.0 corresponds to 50ug/ml of double stranded DNA (Maniatis *et al.* 1982). The purity of the solution was determined by calculating the $O.D._{260}/O.D._{280}$ ratio. If this value deviated significantly from the value expected for pure DNA solutions, i.e. 1.8 (Maniatis *et al.* 1982), the estimated DNA concentration was not considered to be reliable.

2.2.2. Preparation of plasmid DNA.

a) Small-scale procedure.

Small quantities of plasmid DNA were prepared for analysis using the alkaline lysis method described by Birnboim and Doly (1979).

A single bacterial colony was inoculated into 5mls of culture medium containing the appropriate antibiotic and incubated overnight at 37°C with vigorous shaking. *E. Coli* strains HB101 and DH5α were cultured in Luria-Bertani (LB) medium (Table II.III), *E. Coli* strains JM105 and JM109 were cultured in minimal medium (Table II.III). A 1.5ml aliquot of the culture was transferred into a microcentrifuge tube and centrifuged briefly. The supernatant was discarded and the pellet resuspended by vortexing in 100µl of ice cold 25mM Tris, 10mM disodium ethylene diamine tetraacetate (EDTA), 50mM glucose, pH8.0 (solution 1). The tubes were left at room temperature for 5 minutes and then 200µl of a freshly prepared solution of 0.2M sodium hydroxide, 1% SDS (solution 2) added. The tube contents were mixed gently and placed on ice for 5 minutes. One hundred and fifty microlitres of an ice cold solution of potassium acetate 3M/5M, pH 4.8 (solution 3, Table II.II) were added, the mixture vortexed gently and then stored on ice for 5 minutes. Cell debris and bacterial DNA were pelleted by centrifuging briefly in a microcentrifuge and the supernatant was removed to a fresh tube. The solution of DNA was further purified by adding an equal volume of 50% phenol, 48% chloroform, 2% isoamyl alcohol (PCI9), vortexing, centrifuging for 2 minutes and transferring the aqueous phase to a clean tube.

The DNA was precipitated by the addition of 2 volumes of 100% ethanol. The mixture was incubated at room temperature for 2 minutes and the DNA pelleted by centrifugation in a microcentrifuge for 15 minutes at room temperature. The supernatant was decanted and the pellet washed by vortexing in 100µl of 70% ethanol. After aspiration of the supernatant the pellet was allowed to dry then resuspended in 50µl of TE.

The identity of purified plasmid DNA was routinely checked by digestion of 10µl of DNA in TE with appropriate restriction endonucleases as described in section 2.3.2 and analysis of the fragments by agarose or polyacrylamide gel electrophoresis (sections 2.2.3 and 2.2.5).

b) Large-scale procedure.

Large quantities of plasmid DNA were prepared using a similar procedure, modified as described in Maniatis *et al.* (1982). Bacteria containing the required plasmid were grown in 500mls of LB or minimal medium, as detailed above, with the appropriate antibiotic at 37°C for 16 to 20 hours.

The cells were harvested by centrifugation at 7000rpm for 10 minutes at 4°C and resuspended in TNE (Table II.II). Cells were washed once in TNE and resuspended in 8mls of solution 1. Lysozyme was added to a final concentration of 5mg/ml and the mixture incubated at room temperature for 5 minutes. The DNA was denatured by the addition of 16mls of freshly made solution 2. The mixture was vortexed and incubated on ice for 10 minutes. Plasmid DNA was reannealed by neutralizing the solution with 12mls of solution 3. After gentle agitation the mixture was replaced on ice for a further 10 minutes.

Cell debris was pelleted by centrifugation at 19000rpm for 30 minutes at 4°C in a JA20 rotor (Beckman). The supernatant was transferred into 30ml glass corex tubes, mixed with 0.6 volumes of isopropyl alcohol and the DNA precipitated by incubation at room temperature for 15 minutes. The precipitate was collected by centrifugation at 10000rpm for 30 minutes at 4°C in a JA20 rotor, washed with 70% ethanol and dried for 10 minutes.

The plasmid DNA was further purified by centrifugation to equilibrium in a caesium chloride/ethidium bromide gradient. The DNA pellet was suspended in 4-5mls of TE and 1g of caesium chloride added per millilitre of DNA solution. Ethidium bromide was added to a final concentration of 740μg/ml and the solution transferred to Beckman 'quick seal' centrifuge tubes. Any air space remaining in the tubes was filled with light paraffin oil and the tubes were then heat sealed. DNA was separated on a density gradient by centrifugation in a Vti 65.2 rotor (Beckman) at 49000rpm for 16 hours at 20°C. Supercoiled plasmid DNA bands at higher density on the gradient than fragmented bacterial DNA due to a lower level of incorporation of ethidium bromide (Radloff *et al.* 1967), and thus may be visualized as the lower band on the gradient. The lower band was removed in a volume of 2mls using a 23 gauge hypodermic needle and 5ml syringe.

The solution was diluted three to four-fold with TE and the ethidium bromide removed by repeated extraction with water saturated 1-butanol. When no visible colour was detectable in the aqueous phase, 2 volumes of 100% ethanol were added and the plasmid DNA was precipitated at -20°C for 2 hours. The DNA was then pelleted by centrifugation at 10000rpm for 30 minutes at 4°C in a JA20 rotor. The pellet was washed with 70% ethanol, allowed to dry for 1 hour and resuspended in TE. Plasmids were routinely stored at 4°C.

Before use in nucleotide sequencing reactions, plasmid DNA was further purified by dialysis. Approximately 1mg of plasmid DNA was diluted in 2.5mls of TE, clamped in prepared dialysis tubing of three quarters of an inch diameter (Gibco BRL) and dialysed against 2L of TE (3 x 8 hours) at 4°C. The DNA was precipitated from solution by the addition of sodium chloride to a final concentration of 0.4M followed by the addition of 2 volumes of ethanol. The DNA was pelleted by centrifugation at 10000rpm for 30 minutes at 4°C, washed in 70% ethanol and resuspended in TE.

2.2.3. Agarose gel electrophoresis.

The following protocol was used for the separation of restriction enzyme digested high molecular weight DNA. Following restriction enzyme digestion, DNA fragments were size separated in agarose on horizontal slab gels. Agarose was made up at 0.6-0.8% weight/volume (w/v) in TBE buffer (Table II.II) and dissolved by heating. The solution was cooled to 55°C before pouring onto a horizontal perspex bed and was left to solidify with the well-forming comb in position. The gel was submerged in an electrophoresis tank, covered with TBE buffer and the well-forming comb removed.

Prior to electrophoresis, restriction endonuclease-digested DNA samples were heated to 65°C for 5 minutes and a 1/10 volume of loading buffer (Table II.II) added. The samples were loaded into the wells and a constant potential difference of 1.3 V/cm applied across the gel for 20 hours. The gel was then immersed in a staining solution of TBE containing 0.5μg/ml of ethidium bromide for 30 minutes and destained in TBE for a further 30 minutes. The DNA was visualized on a transilluminator (Foto/Prep I, Fotodyne Inc.) at 300nm, in analytical mode and photographed on a Polaroid MP4 Land camera with Polaroid Type 57 high speed film.

The sizes of restriction enzyme-digested DNA fragments were estimated by comparison with the migration distances of DNA fragments of known sizes. For this purpose, 0.5-1.0μg of HindIII digested bacteriophage (phage) lambda DNA (Gibco BRL) were electrophoresed alongside each batch of DNA samples.

2.2.4. Purification of DNA inserts from agarose gels.

DNA fragments larger than 0.5kb were purified on agarose gels following restriction endonuclease digestion. Agarose gels were prepared with a 0.8% solution of agarose in TAE buffer (Table II.II) essentially as described above but with the addition of 0.5μg/ml ethidium bromide to the agarose solution immediately prior to pouring the gel. DNA samples were loaded into wells at 2-5μg per 0.5cm well and electrophoresed at 4V/cm for 3-5 hours. The separation of DNA fragments was checked by visualization under ultraviolet light (366nm) using a hand-held transilluminator (Model UVGL-58, UVP Inc., Gallenkamp).

DNA fragments were purified from agarose gels using one of two methods.

a)DEAE membrane.

Following separation of DNA fragments as described above, a scalpel incision was made in the gel immediately in front of the DNA fragment to be purified. A strip of NA45 DEAE membrane (Schleicher and Schuell, Anderman & Co.) was inserted into the incision such that further migration of the DNA fragment would be completely impeded by the membrane. The gel was replaced in the electrophoresis tank and a potential difference reapplied. When the DNA fragment had migrated onto the DEAE membrane this was removed from the gel and excess membrane trimmed away.

The DEAE membrane was then placed in sufficient elution buffer (Table II.II) to just cover the membrane and incubated at 65°C for 1 hour. Complete elution of DNA from the membrane was confirmed by examination under ultraviolet light. An equal volume of PCI9 was added and the mixture vortexed then centrifuged briefly in a microcentrifuge. DNA was precipitated from the aqueous phase by the addition of two volumes of 100% ethanol followed by incubation at -20°C for 2 hours. The precipitate was collected by centrifugation at 13000rpm for 30 minutes at 4°C, the pellet washed with 70% ethanol and then dried for 15 minutes at room temperature. The pellet was resuspended in TE and the DNA concentration estimated following agarose gel electrophoresis by comparison with DNA samples of known concentration. DNA fragments for use as radioactively-labelled probes were stored at 4°C while fragments for subcloning were stored at -20°C until required.

b) Glass beads.

Some DNA fragments were purified from agarose using glass beads supplied with a chaotropic buffer and wash buffer as "Geneclean" (Stratech Scientific). Following electrophoresis the DNA fragment was visualized under ultraviolet light and the agarose block containing the fragment excised using a scalpel. The gel slice was cut into approximately 2mm cubes and transferred to a microcentrifuge tube. The weight of the gel slice was determined and the agarose dissolved by incubation in 2-3 volumes of sodium iodide solution at 55°C. An appropriate volume of "Glassmilk" was added to the solution (5µl for solutions containing less than 5µg of DNA with an additional 2µl

for every additional 1μg) which was then mixed gently and incubated on ice for 5 minutes. The glass beads were pelleted by spinning briefly in a microcentrifuge and washed three times with the wash buffer supplied with the "Geneclean" kit. DNA was then eluted from the glass beads by incubation in an equal volume of TE at 55°C for 2-3 minutes.

2.2.5. Polyacrylamide gel electrophoresis.

Polyacrylamide gel electrophoresis (PAGE) was used for the separation and purification of DNA fragments less than 0.5kb in length. Acrylamide solutions were made up from a stock solution (Table II.II) to a final concentration of 3.5-12% in TBE and polymerized by the addition of 0.06% (w/v) ammonium persulphate and 0.03% TEMED. The gel (20cm x 20cm x 3mm) was poured between glass plates and allowed to polymerize for 1 hour with the well-forming comb in place. After polymerization was completed, the well-forming comb was removed and the wells thoroughly flushed with water and TBE. DNA samples in loading buffer were loaded onto the gel at up to 1μg per 0.5cm well. Electrophoresis was performed in vertical gels, using TBE as the running buffer, at 9V/cm for 4-5 hours or 1.3V/cm overnight. DNA fragments were visualized using ultraviolet light following staining of the gel in ethidium bromide and destaining as previously described.

DNA fragment sizes were estimated by comparison with the migration distances of DNA fragments of known size. For this purpose, 0.5-1.0μg of PhiX174 RF DNA digested with the restriction enzyme HaeIII (Gibco BRL) were electrophoresed alongside each batch of DNA samples.

2.2.6. Purification of DNA from acrylamide gels.

Following PAGE, gel slices containing DNA fragments to be purified were excised from the gel, cut into fine pieces and transferred to a microcentrifuge tube. DNA was eluted from the gel fragments by incubation in two volumes of 0.5M ammonium acetate, 1mM EDTA, pH8 at 37°C for 2-16 hours (the longer times being required for the elution of larger fragments). The gel pieces were pelleted in a microcentrifuge and the supernatant removed. Any remaining acrylamide was removed by extracting the solution with an equal volume of phenol followed by an equal volume of chloroform

containing 4% isoamyl alcohol. The DNA was then precipitated at -20°C after the addition of 2 volumes of 100% ethanol. The precipitate was recovered by centrifugation, rinsed in 70% ethanol, dried and resuspended in TE.

2.2.7. Denaturing polyacrylamide gel electrophoresis.

Denaturing polyacrylamide gels were used for the electrophoretic separation of radioactively-labelled DNA fragments generated by nucleotide sequencing reactions (section 2.3.6).

Preparation of denaturing polyacrylamide gels.

Gel plates for the nucleotide sequencing apparatus (Biorad) were prepared by thorough washing in phosphate-free detergent, water and finally 100% ethanol. One of the gel plates was silicone treated to prevent the polyacrylamide adhering to both plates following electrophoresis. Silicone treatment was accomplished by pouring 1ml of dimethyldichlorosilane solution onto the plate, wiping the fluid gently over the plate and allowing the plate to dry in an extractor hood. This process was repeated twice and then the gel mould assembled, using wedge-shaped spacers (0.25-0.53mm thick) to form a mould of dimensions 40cm x 20cm.

Electrophoresis was performed in 6% denaturing polyacrylamide gels (Table II.II) using TBE as the running buffer. Ten millilitres of the 6% acrylamide solution was polymerized by the addition of 125µl of 10% ammonium persulphate and 27µl of N,N,N',N'-tetramethylethyldiamine (TEMED) and used to seal the base of the gel mould. After polymerization of the seal was complete, an additional 40mls of 6% acrylamide solution was mixed with 270µl of 10% ammonium persulphate and 27µl of TEMED and poured into the gel mould using a syringe and 19 gauge needle. The well-forming comb was inserted and the gel placed at a angle of approximately 20° to the horizontal and allowed to polymerize for 1 hour.

Electrophoresis of nucleotide sequencing reaction products.

Prior to electrophoresis the well-forming comb was removed and the wells flushed with TBE to remove unpolymerized acrylamide. The gel was then placed in a vertical electrophoresis apparatus (Biorad) and pre-heated by applying a potential difference of 1500V across the electrodes for 1 hour prior to loading the DNA samples. The wells were flushed again with TBE before loading the gel. The products of the nucleotide sequencing reactions (section 2.3.3) were heated to 80°C for 2 minutes then immediately placed on ice and 2µl of each reaction mixture loaded onto the gel.

Electrophoresis was performed at a constant voltage of 1500V and continued for 1-5 hours, depending on the length of sequence information required. On some occasions, in order to obtain the maximum amount of information from each sequencing reaction, additional 2µl aliquots of each reaction were loaded onto the gel 2-3 hours after commencing electrophoresis of the initial samples.

Autoradiography of the gels.

Following electrophoresis the apparatus was dismantled and the silicone-treated gel-plate gently lifted away from the gel and the other glass plate. The gel was then fixed and the urea removed from the polyacrylamide by transferring the gel, still attached to the glass plate, into a bath containing 10% methanol, 10% acetic acid. After 15 minutes the gel and glass plate were removed and the gel transferred onto Whatman 3MM paper as follows. Two sheets of Whatman 3MM paper were gently placed on top of the gel, smoothed down and then the paper, gel and glass plate inverted. The glass plate was lifted away, and the gel covered in Saran Wrap (Dow Chemical Company, Genetic Research Instrumentation Ltd.). The 3MM paper and Saran Wrap were trimmed close to the edges of the gel and the gel was then dried under vacuum at 80°C for 3 hours in an automatic gel drier (Biorad, Model 583) Autoradiography was performed by exposing the dried gel to Hyperfilm MP autoradiography film in a metal cassette (Dupont) for 48 hours at room temperature.

Following exposure films were developed in a Kodak X-OMAT automatic processor (Model ME-3) and the nucleotide sequence determined by comparing the fragment sizes obtained in the reaction mixtures containing each dideoxynucleotide triphosphate.

2.2.8. Purification of radioactively labelled DNA fragments by gel filtration.

Radioactively-labelled DNA probes were purified from unincorporated radioactively-labelled nucleotides by filtration down a Sephadex-G50 column.

Sephadex-G50 columns were prepared using 20 x 0.5cm Econo-columns (Biorad). Sephadex-G50 was made up at 120g/L in TE, autoclaved before use and stored at 4°C. The Sephadex-G50 solution was pipetted slowly into the columns, avoiding the introduction of air bubbles and the columns were then equilibrated with TE for 1 hour to allow settling of the Sephadex-G50.

The mixture of incorporated and unincorporated radioactively-labelled nucleotides obtained from the multiprime labelling reaction described in section 2.3.1. was added to 10µl of 0.25% Orange-G and the entire volume pipetted onto the top of the column. A hand held Geiger counter was used to monitor the filtration process. The most rapidly migrating peak of radioactivity was assumed to represent the radioactively-labelled DNA fragment, while the unincorporated radioactively-labelled nucleotides migrated more slowly down the column, forming a second peak of radioactivity.

The radioactively-labelled DNA fragment was collected in a 500µl volume of buffer. The buffer containing the unincorporated labelled nucleotides was discarded. The specific activity of the labelled nucleic acid in counts per minute per microlitre was estimated by adding 1µl of the collected fraction to 5mls of "Ecoscinct" (National Diagnostics) and measuring the emitted radiation on a Beckman LS 1801 scintillation counter.

2.2.9. Purification of oligonucleotides.

Oligonucleotides for use in the PCR or for nucleotide sequence analysis were obtained from an "in house" synthesis facility, employing an automated oligonucleotide synthesizer (Model 381A, Applied Biosystems).

Following synthesis, oligonucleotides were cleaved from their resin supports by flushing the synthesis cartridges with 2mls of 35% ammonia solution over a period of 2.5 hours. The oligonucleotides were then deprotected by incubation at 55°C in 35% ammonia solution for 5-16 hours. The solution volume was made up to 4mls with water before further purification steps.

The trityl groups were removed and the oligonucleotides purified using Oligonucleotide Purification Cartridges (OPC, Applied Biosystems Ltd.). Each OPC was flushed through with 5mls of acetonitrile (Applied Biosystems Ltd.) followed by 5mls 2.0M triethylamine acetate (Applied Biosystems Ltd). The solutions were flushed through the OPC using 5ml syringes, at a rate of 1-2 drops per second.

The deprotected oligonucleotide was then pushed through the OPC at the same rate, repeating the process to ensure efficient binding of the oligonucleotide to the support. The OPC was flushed with 3 x 5mls of 3.5% ammonia solution, followed by 2 x 5mls of water. The oligonucleotide was then detritylated using 2 x 5mls of 2% trifluoroacetic acid (Applied Biosystems Ltd.) and washed with 2 x 5mls of water. The detritylated oligonucleotide was eluted with 3 x 1ml of 20% acetonitrile. The eluate was divided into aliquots, evaporated to dryness by centrifugation under vacuum in a bench-top centrifuge (Univap, Uniscience) and redissolved in water as required. Oligonucleotides were routinely stored as solids at -20°C.

The concentration of redissolved oligonucleotides was determined by measurement of the $O.D._{260}$, assuming that a solution of 20μg/ml has an $O.D._{260}$ of 1.0 (Maniatis *et al.* 1982).

2.3. ENZYMATIC MANIPULATION OF NUCLEIC ACIDS.

2.3.1. Radioactive labelling of nucleic acids.

DNA fragments were radioactively labelled to a high specific activity using a procedure based on that described by Feinberg and Vogelstein (1983). All reagents for the labelling reactions were obtained from Amersham International plc ("Multiprime labelling system").

Twenty five nanograms of a linear double stranded DNA fragment for use as probe were denatured by boiling for 2 minutes and then cooled rapidly on ice. Multiprime buffer solution, containing dATP, dGTP and dTTP, and a primer solution, containing random hexanucleotides, were added according to the manufacturers recommendations. Three Megabecquerels of {α^{32}P}dCTP (30TBq/mmol) were added followed by 2U of DNA polymerase I "Klenow" fragment in a total reaction volume of 50µl, and the reaction allowed to proceed for 3-5 hours at room temperature. Unincorporated radioactively-labelled nucleotides were removed by gel filtration as described in section 2.2.8.

2.3.2. Restriction endonuclease cleavage of DNA.

The required amount of DNA in TE buffer was transferred to a microcentrifuge tube. Water was added to make the final volume up to 50µl, for digestion of high molecular weight DNA, and to 10µl for plasmid DNA. One tenth of the final volume of the appropriate restriction enzyme reaction buffer (supplied by the manufacturers) was added. Spermidine was added to a final concentration of 3mM to reduce the possibility of inhibition by contaminants present in the DNA (Bouche 1981). Two to five units of restriction enzyme were added per microgram of DNA, the contents of the tube mixed gently and then centrifuged briefly in a microcentrifuge. The reaction mixture was incubated at 37°C for 16 hours for the restriction endonuclease digestion of high molecular weight DNA and for 1-2 hours for digestion of plasmid DNA.

2.3.4. DNA amplification using the polymerase chain reaction.

Specific amplification of a target DNA sequence was achieved using oligonucleotide primers and a thermostable DNA polymerase essentially as described by Saiki *et al.* (1988).

One microgram of high molecular weight DNA was included in a reaction mixture containing 1µM primers, 1.5mM magnesium chloride, 50mM potassium chloride, 10mM Tris pH 8.2, 0.05% NP40, 200µM of each deoxynucleotide triphosphate and 3U heat-stable DNA polymerase (Cambio). Amplification was performed in a programmable heat block (Perkin Elmer-Cetus Instruments). Target DNA samples were initially denatured by heating at 95°C for 6 minutes then subjected to 30 cycles in

which they were cooled to 55°C over 2 minutes to anneal the primers, heated to 70°C over 1 minute and incubated at 70°C for 0.5 minutes to allow primer extension and then denatured by heating to 95°C over 1 minute.

Reaction products were visualized on, and purified from, ethidium bromide-stained polyacrylamide gels as described in section 2.2.5.

S2.3.5. Cloning of DNA fragments

The plasmid vector and DNA fragment to be ligated were digested with appropriate restriction enzymes and size separated on agarose or polyacrylamide gels, as appropriate. DNA fragments were purified from agarose gels using DEAE membrane (section 2.2.4) and from polyacrylamide gels as described in section 2.2.6. Molar ratios of insert to vector of between 10:1 and 2.5:1 were combined in the ligation reactions. The insert and vector fragments were mixed to make up a total of 150-200ng of DNA in a final volume of 20µl. DNA ligase reaction buffer (Boehringer Mannheim), 1U of T4 DNA ligase (Boehringer Mannheim) and 0.5mM ATP (Boehringer Mannheim) were included in the reaction mixture.

Control reactions were set up as described above but omitting either insert DNA or vector DNA. The former control reaction indicated the frequency at which religation or incomplete digestion of vector was occurring. The reaction omitting vector DNA permitted the detection of incomplete purification of insert, where the insert was being subcloned into a new vector. Ligation reactions were incubated at room temperature for 4 hours then 5µl of each reaction and 10µl of a 1:10 dilution of each reaction were used to transform an appropriate *E.Coli* host as described in section 2.5.2.

2.3.6. Nucleotide sequence analysis.

Nucleotide sequencing was carried out using the dideoxy chain termination method (Sanger *et al.* 1977). Double stranded DNA was used as the template and reactions were performed using the "T^7 Sequencing Kit" manufactured by Pharmacia LKB.

Template DNA was denatured by adding 2µl of 2M sodium hydroxide to 2µg of DNA in 8µl of TE and incubating at room temperature for 10 minutes. The DNA was precipitated by the addition of 3µl of 3M sodium acetate (pH4.8), 7µl of water and 60µl of 100% ethanol and incubating the mixture on dry ice for 15 minutes. The DNA was pelleted by centrifugation at 13000rpm for 10 minutes at 4°C, washed with 70% ethanol, centrifuged and dried briefly under vacuum. The denatured template DNA was dissolved in 10µl of water and incubated with appropriate primers in the annealing buffer supplied with the kit, at 37°C for 20 minutes. The quantity of each primer added was calculated to give a molar ratio of the primer to the template of 10:1. The primer/template mix was placed at room temperature for 10 minutes prior to commencing the sequencing reactions.

An enzyme premix containing 1µl of water, 3µl of labelling mix (dATP, dGTP and dTTP), 2µl of T^7 DNA polymerase (1.5U/µl) and 1µl of [α-^{35}S]dCTP (370MBq/ml) was prepared. Primer extension with the mix of deoxynucleotide triphosphates was initiated by the addition of 6µl of enzyme premix to the annealed template/primer and the labelling reaction continued for 5 minutes at room temperature. Aliquots (2.5µl) of the four chain termination mixes containing one dideoxynucleotide triphosphate and the remaining three deoxynucleotide triphosphates were prewarmed to 37°C for 1 minute and 4.5µl of the labelling reaction added to each of the four mixes. Chain termination was allowed to proceed for 5 minutes at 37°C before stopping the reactions with 5µl of the stop solution supplied with the kit. Sequencing reactions were analysed by denaturing PAGE (section 2.2.7).

2.4. HYBRIDIZATION ANALYSIS OF DNA.

2.4.1. Southern transfer of nucleic acids.

Nucleic acids were transferred to 'Hybond-N' nylon membranes essentially as described by Southern (1975). After photography gels were agitated gently in 0.25M HCl for 2 x 15 minutes in order to depurinate the DNA molecules as this improves the efficiency of transfer of high molecular weight DNA. Gels were rinsed in deionized water and the DNA denatured by submerging the gels in alkali buffer (Table II.II) for 1 hour at room temperature. After a 1 hour submersion in neutralizing buffer (Table II.II) gels were placed in 10 x SSC (Table II.II) prior to transfer.

Transfer of nucleic acids onto the nylon membrane was assisted by gravity. An absorbent pad of tissues was covered first with a layer of filter paper (Whatmann No. 1, Scotlab) presoaked in transfer buffer (10 x SSC) and then with "Hybond-N" nylon membrane presoaked in deionized water and transfer buffer. Gels were placed onto the membrane ensuring that the lower surface of the gel was apposed to the membrane and were then covered with a sheet of filter paper presoaked in transfer buffer. A sponge soaked in transfer buffer and placed onto the top layer of filter paper was used to provide a buffer reservoir and the transfer apparatus was sealed in a plastic bag to reduce evaporative loss.

After 16-20 hours the nylon membrane was removed, cut to size and rinsed for several minutes in 3 x SSC. The efficiency with which DNA transfer had occurred was checked by restaining the gel with ethidium bromide. DNA was irreversibly bound to the membrane by baking in an oven at 80°C for 2 hours.

2.4.2. Hybridization of radioactive probes to filter bound DNA.

Detection of specific DNA sequences in filter bound DNA samples was achieved by hybridization to radioactive DNA probes labelled by random priming (section 2.3.1).

Filters were presoaked in 0.1% SDS, 3 x SSC and wrapped around the interior of a 50ml Falcon tube with the side of the membrane onto which the DNA was bound facing inside. Care was taken to exclude air bubbles. Filters were prehybridized in 3 mls of hybridization buffer (Table II.II) for a minimum of 4 hours at 37°C, with continuous rotation. The concentration of formamide in the hybridization buffer was adjusted according to the algorithm described by Meinkoth and Wahl (1984) so that the stringency of hybridization was appropriate for the mismatch permitted between the probe and target sequence. Hybridization was routinely performed at approximately 25°C below the calculated T_m of the DNA duplex. DNA probes were added to a final activity of 10^6 counts per minute per ml of buffer and hybridized for 16 hours at 37°C with continuous rotation.

Filters were then rinsed twice for 10 minutes in 2 x SSC, 0.1% SDS at room temperature and washed under conditions appropriate for the permitted degree of mismatching between probe and target sequences and the length of the probe being

used. Washing was routinely performed in 0.1% SDS, 0.5 X SSC at 65°C except when stated otherwise in the description of specific experiments. After a brief rinse in 2 x SSC, filters were air dried to dampness, wrapped in Saran wrap and autoradiographed by exposing the filters to autoradiography film (Hyperfilm MP) in DuPont metal cassettes containing Cronex lightning plus intensifying screens (DuPont). Autoradiographs were developed after exposures of 1-21 days as required.

2.5. GROWTH AND MANIPULATION OF BACTERIA.

2.5.1. Preparation of competent cells.

Bacterial cells were made competent for the uptake of DNA molecules using calcium ions and low temperature, as described by Lederberg and Cohen (1974).

An overnight culture of *E. Coli* was diluted 1:100 in 50mls of LB or minimal medium, as appropriate, and shaken at 225rpm at 37°C until the cell density reached 4-7 x 10^7 cells/ml. The bacterial cell density was calculated from the $O.D._{550}$ of the solution assuming that the approximate $O.D._{550}$ of 5 x 10^7 cells is 0.2 with rec- strains, and is 0.5 with rec+ strains of E.Coli (Maniatis *et al.* 1982). The bacteria were chilled on ice for 10 minutes then pelleted by centrifugation at 4000rpm for 5 minutes at 4°C and the supernatant discarded.

Bacteria were resuspended in 25mls of an ice cold sterile solution of 50mM calcium chloride, 10mM Tris (pH 8.0) and incubated in an ice bath for 15 minutes. The cells were centrifuged at 4000rpm for 5 minutes at 4°C and resuspended in 3.3mls of calcium chloride solution as above. The bacterial suspension was divided into 0.2ml aliquots in prechilled tubes and stored at 4°C for 12-24 hours. The aliquots were then used immediately for transformation of plasmids into *E. Coli* (section 2.5.2) or stored at -70°C for future use. Competent bacteria with transformation efficiencies of 10^6-10^7 transformants per microgram of DNA were routinely made using this procedure.

2.5.2. Transformation of bacterial cells with plasmid DNA.

Competent bacteria were transformed with plasmids essentially according to the method described by Hanahan (1983). Frozen competent bacterial cells were made as described in section 2.5.1 or purchased from Gibco BRL.

Frozen competent cells were thawed on ice and then aliquoted into 100µl volumes in 17 x 100mm polypropylene tubes (Falcon 2059, A. & J. Beveridge). Plasmid DNA was added at approximately 4-40ng DNA per 100µl of cells, mixed briefly and the tubes placed on ice for 30 minutes. Bacteria were then heat shocked at 42°C for 45 seconds and cold shocked on ice for 1-2 minutes.

Nine hundred microlitres of SOB (Table II.III) containing 20mM glucose (SOC) were added and the bacteria allowed to recover by shaking in an orbital incubator at 225rpm for 60 minutes at 37°C. The bacteria were centrifuged at 2500rpm for 5 minutes at 20°C and washed before resuspending in 150µl of SOC and plating onto LB-agar (15g/L of bacto-agar in LB-medium) plates containing the appropriate antibiotic. Plates were incubated overnight at 37°C and then stored at 4°C for up to 1 week.

2.5.3. Identification of recombinants.

Bacteria containing recombinant plasmids were identified in one of two ways.

a) Selection by α-complementation.

Plasmid vectors containing the regulatory sequences and coding sequences of the amino-terminal region of the β-galactosidase gene (*lacZ*) were used in some cloning experiments. The products of ligation reactions and control reactions were transformed into competent bacteria of an *E. coli* strain capable of expressing the carboxy-terminal region of β-galactosidase (*E. coli* strain DH5α). Bacteria containing the non-recombinant vector may be identified, as they produce a functional β-galactosidase and thus will metabolise 5-bromo-4-chloro-3-indoyl-β-D-galactosidase (X-gal) producing blue colonies.

Transformed bacteria were plated onto LB-agar containing 0.05mg/ml isopropylthio-β-D-galactoside (IPTG) and 0.05mg/ml 5-bromo-4-chloro-3-indoyl-β-D-galactoside (X-gal). The plates were incubated at 37°C overnight and then placed at 4°C for 3-4 hours before examination. Bacteria containing recombinant plasmids were identified as white colonies, as insertion of foreign DNA into the cloning site of the vector results in disruption of the *lacZ* gene. The identity of recombinant plasmids was confirmed as described below.

b) Restriction endonuclease digestion of small scale preparations of plasmid DNA.

When the choice of plasmid vector and/or the host *E.coli* strain did not permit use of the above protocol, recombinant plasmids were identified by restriction endonuclease analysis of plasmid DNA.

Bacteria transformed with the products of ligation reactions were plated onto LB-agar and grown overnight at 37°C. The numbers of colonies obtained from bacteria transformed with the control ligations and the vector plus insert ligation reactions were compared. If a significantly greater number of colonies was obtained from the transformation performed using the vector plus insert ligation reaction, compared to the control reactions, between 12 and 24 colonies from the former transformation were selected for analysis. Plasmid DNA was prepared using the small scale procedure described in section 2.2.2, digested with appropriate restriction endonucleases and analysed by agarose gel electrophoresis (section 2.2.3).

CHAPTER THREE.

THE USE OF GENE REARRANGEMENT ANALYSIS FOR THE CHARACTERIZATION OF HODGKIN'S AND NON-HODGKIN'S LYMPHOMAS.

3.1 INTRODUCTION.

The accurate classification of malignant lymphomas and leukaemias is important both clinically, for the selection of appropriate therapies and scientifically, as the identification of specific disease entities is a prerequisite for the effective investigation of their aetiologies.

The classification of lymphomas and leukemias has been greatly assisted by immunophenotyping. For many types of lymphoma determination of the surface phenotype of the malignant cells has allowed the identification of their lineage. The clonality of proliferative disorders of B-lymphocytes may also be evaluated immunophenotypically if expression of IgL is detected. The restricted expression of a single IgL isotype by a B-cell population may be used as an indication of clonality (Isaacson *et al.* 1980; Norton and Isaacson 1987). There are no equivalent immunophenotypic markers for clonal T-cell populations, though monoclonal antibodies (MoAbs) reactive with variable regions of the TCRβ (Vβ) may be useful in a proportion of cases (Clark *et al.* 1986).

Some lymphoproliferative disorders that are morphologically and clinically heterogeneous have proved difficult to characterize immunophenotypically. The morphological and clinical heterogeneity of two such disorders, HD and AIL, have been described in Chapter 1. The immunophenotypic analysis of diseased tissues from persons with these disorders has produced conflicting results. These data are reviewed in sections 3.1.3-3.1.4.

Molecular analysis of the configuration of the Ig and TCR genes provides an alternative approach to the characterization of lymphoproliferative disorders. This chapter describes the results of experiments that use this approach to investigate the lineage and clonality of the malignant cells of HD. Samples from patients with AIL were also analysed. The following sections describe the organization and rearrangement of the IgH and IgL genes and the TCRβ chain (TCRβ) and TCRγ chain (TCRγ) genes. The use of molecular analysis of these genes for the determination of the lineage and clonality of lymphoproliferative disorders is described in section 3.1.2.

3.1.1 The organization and rearrangement of the TCR and Ig genes.

The organization of the genes encoding the Ig and TCR genes is similar. The organization of the genomic loci of the IgH and IgL genes have been recently reviewed by Lai *et al.* (1989). The IgH gene locus consists of clusters of variable (V_H), diversity (D_H) and joining (J_H) regions and a group of constant region (C_H) genes (Figure 3.1, panel a). The Ig κ-light chain (Igκ) gene locus is made up from a number of κ regions, 5 Jκ regions and a single CΛ region gene (Figure 3.1 panel b). The Igλ-light chain (Igλ) gene locus is less well characterized, but contains Vλ and Jλ genes in addition to a cluster of Cλ genes.

The generation of antibody diversity by rearrangement of the immunoglobulin genes during B-cell differentiation has been reviewed by Tonegawa (1983). Rearrangement results in the apposition of V, D (where present) and J regions in an arrangement that is unique to a given B-cell and its progeny (Figure 3.2). The IgH genes rearrange first, followed by Igκ gene rearrangement. If Igκ gene rearrangement is unsuccesful, i.e. the apposition of Vκ and Jκ regions does not produce a functional coding sequence, then Igλ gene rearrangement follows.

The antigen receptors of T-cells are formed from heterodimers of TCRα and TCRβ chains, or of TCRγ and TCRδ chains. The organization of the genes for these molecules and the generation of diversity through rearrangement of the TCR genes has been reviewed by Lai *et al.* (1989) and Davis and Bjorkman (1988). Molecular analysis of the TCRα gene is difficult due to the large size and complexity of the TCRα locus (Yoshikai *et al.* 1985). Molecular analysis of the TCRδ gene is also of limited value due to the frequent occurrence of TCRδ gene deletion in mature and malignant T-cells (Chein *et al.* 1987; Foroni *et al.* 1989). The configurations of the TCRα and TCRδ genes were not analysed in the studies described in this chapter, thus the organization and rearrangement of these genes will not be considered further.

The TCRβ and TCRγ genes consist of clusters of V regions, D regions (in the TCRβ gene only) and J regions, as shown in Figure 3.3 (Toyanaga *et al.* 1985; Lefranc and Rabbitts 1985; Quatermous *et al.* 1986). Rearrangement of the TCR genes occurs at discrete stages of T-cell differentiation, creating combinations of V, D (for TCRβ genes) and J regions that are unique for a given T-cell and its progeny (Toyanaga and Mak 1987).

Figure 3.1. (a) The organization of the immunoglobulin (Ig) heavy-chain gene locus. Multiple variable (VH) and diversity (DH) regions are sited 5' to 6 joining (JH) regions. The shaded areas indicate the positions of exons. The cross-hatched area (uS) indicates the sequences involved in heavy-chain class switching. The constant region expressed in IgM heavy chains is shown (Cu). The constant regions expressed in other Ig classes are located 3' to Cu (Flanagan and Rabbitts, 1982). The location of the cleavage sites for the restrction enzymes EcoRI (R), HindIII (H) and BamHI (B) are indicated and the JH probe used for the molecular analysis of Ig heavy-chain gene rearrangement is shown. The restriction enzyme map is derived from Ford et al. (1983).

(b) The organization of the Ig kappa-light-chain gene locus. Multiple variable (VK) regions are sited 5' to 5 joining (JK) regions and a single constant region (CK). The location of cleavage sites for restriction enzymes are indicated (S, SalI) and the CK probe used for the detection of Ig kappa-light-chain gene rearrangements is shown. The restriction enzyme map is derived from Siminovitch et al. (1985).

(c) A representation of the Ig kappa-light-chain gene locus following an unsuccessful rearrangement. The CK region has been deleted (for explanation see section 3.2.2). The Kde probe used to detect CK deletion is shown. The restriction enzyme map of this region is derived from Siminovitch et al. (1985).

Figure 3.1. Organization and restriction maps of the loci of the immunoglobulin heavy-chain and kappa-light-chain genes.

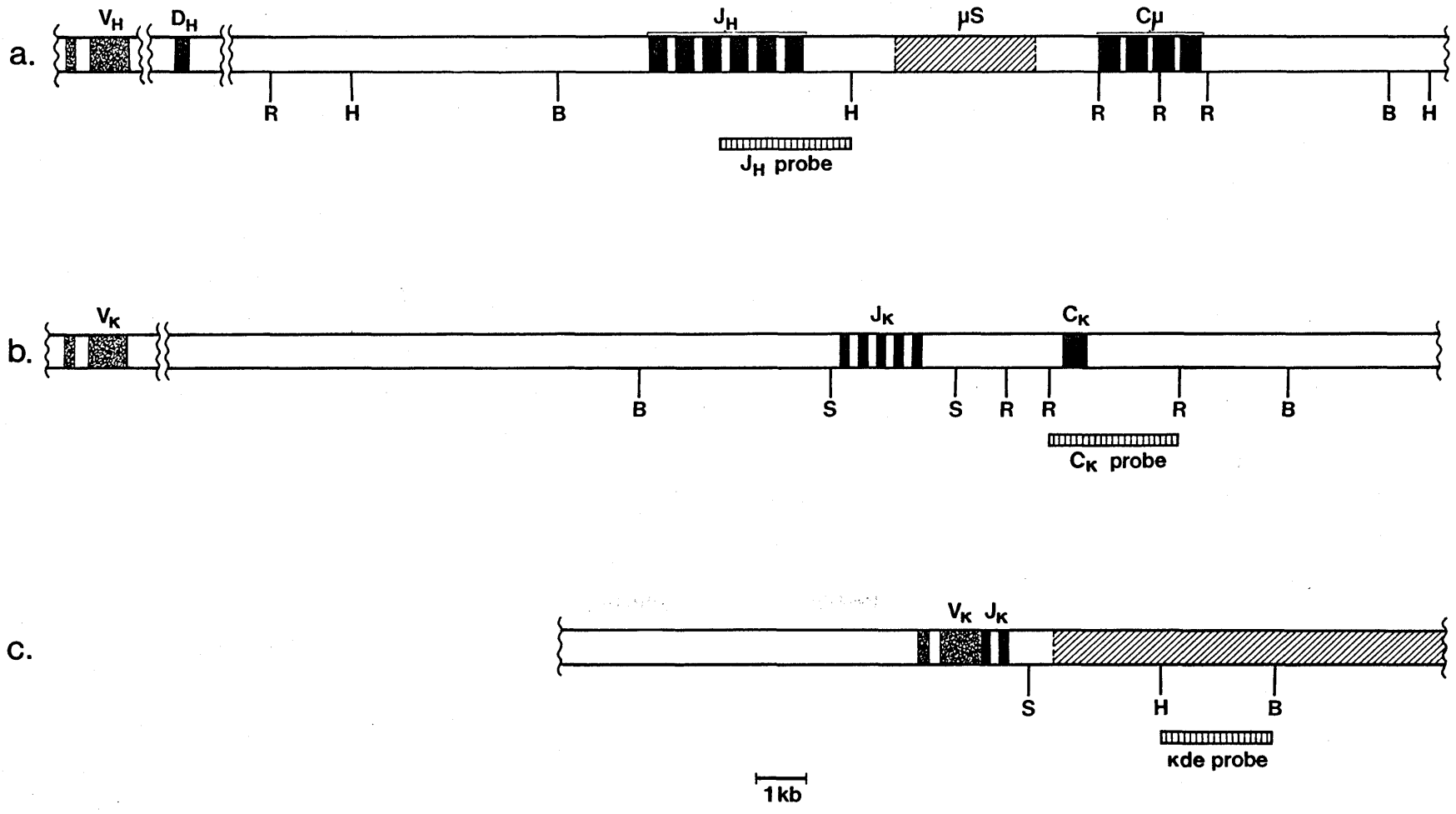


Figure 3.2. (a) The organization of the immunoglobulin (Ig) heavy-chain gene in germline DNA. (b) Rearrangement of the gene involves recombination between specific signal sequences located adjacent to the variable (VH), diversity (DH) and joining (JH) coding regions (shaded boxes) (Ravetch et al. 1981). If rearrangement is productive, i.e. the coding sequences recombine in-frame, the rearranged gene is transcribed (c), then spliced to produce a mature messenger RNA (d) before being translated into protein.

Figure 3.2. Rearrangement and expression of the immunoglobulin heavy-chain gene.

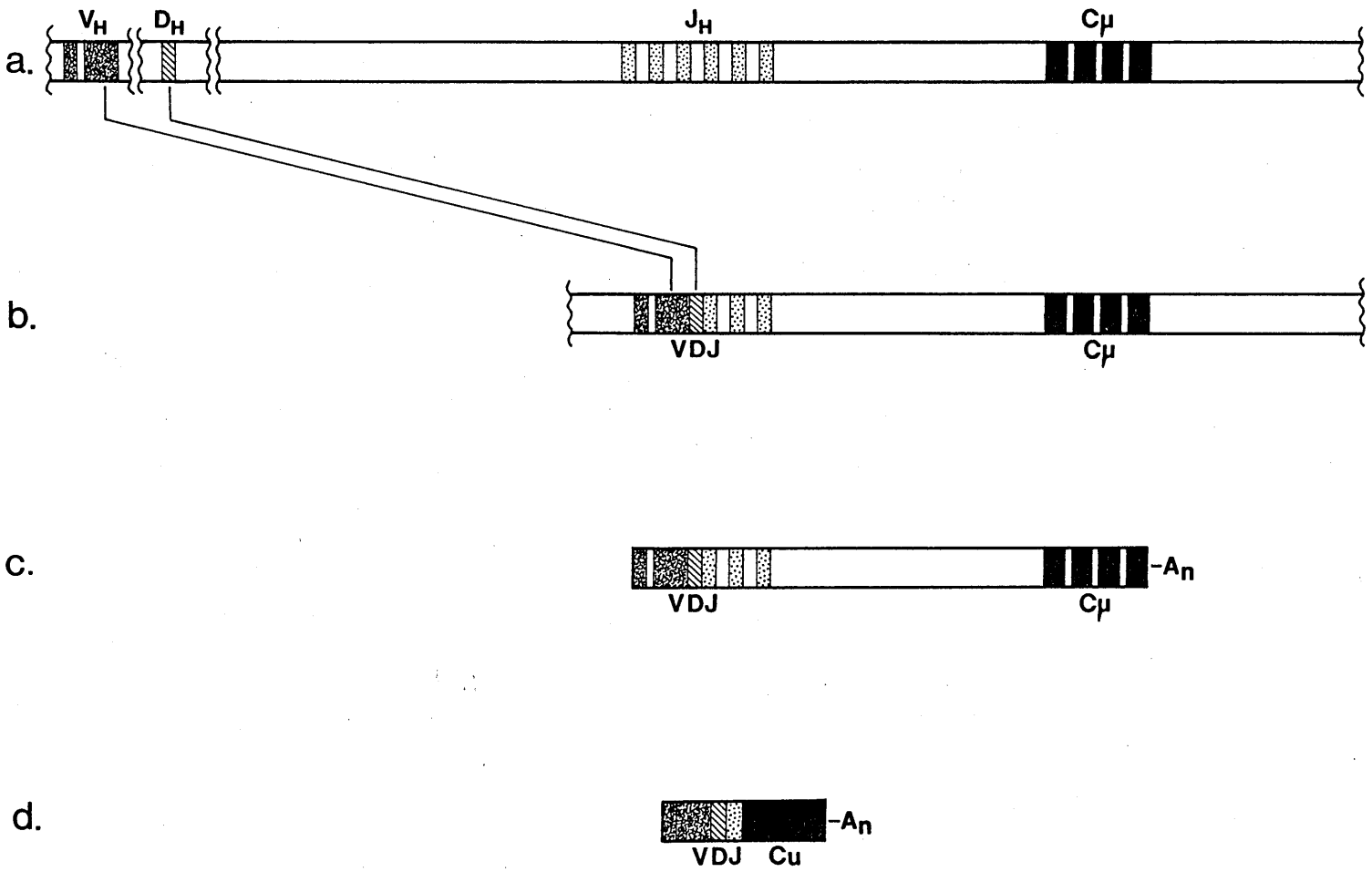
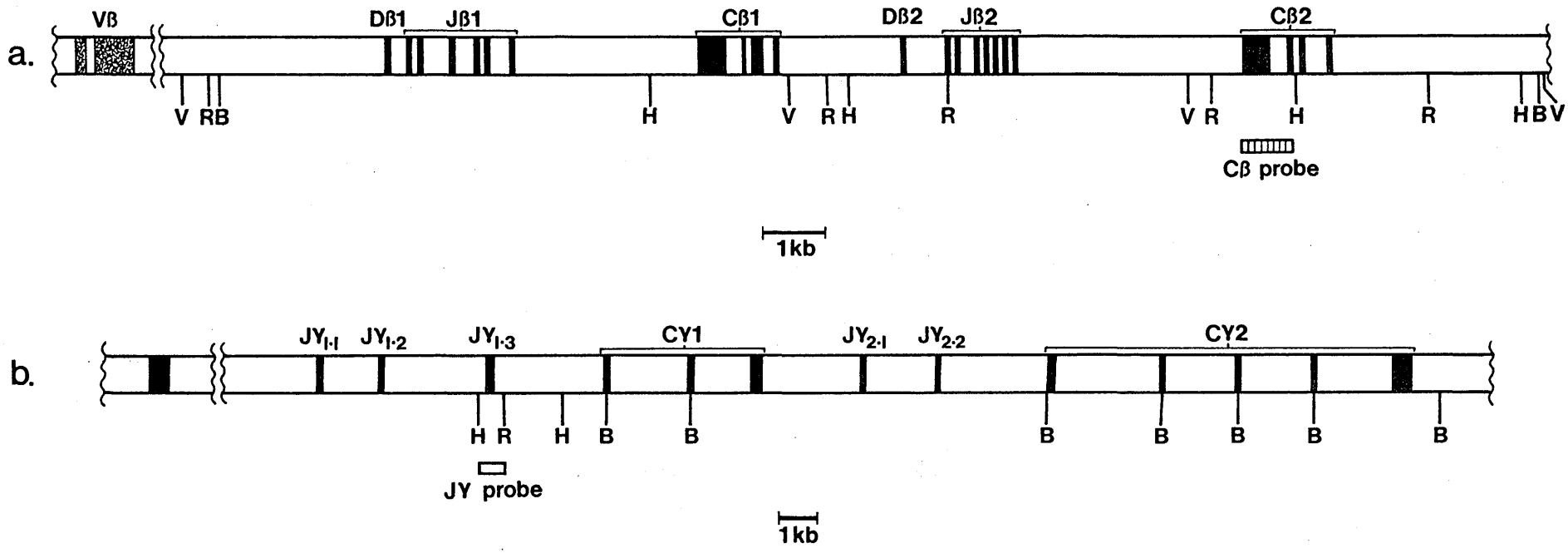


Figure 3.3. (a) The organization of the T-cell receptor beta-chain (TCRß) gene. A single diversity region (Dß) and a cluster of joining regions (Jß) are located upstream of each of the two constant regions (Cß). The shaded areas indicate the positions of exons. The locations of cleavage sites for the restriction enzymes EcoRV (V), EcoRI (R), BamHI (B) and HindIII (H) are shown. The Cß probe used for analysis of TCRß gene rearrangements is derived from the second constant region. The restriction enzyme map is derived from Minden and Mak (1986).

(b) The organization of the T-cell receptor (TCR) gamma-chain gene locus. There are five joining regions (J) and two constant regions (C). The locations of exons and restriction enzyme cleavage sites are indicated as detailed above. The J probe used for detection of TCR gamma-chain gene rearrangement is shown. The restriction enzyme map is derived from Pelicci et al. (1987) and Tawa et al. (1987).

Figure 3.3 Organization and restriction map of the loci of the T-cell receptor beta-chain and gamma-chain genes.



3.1.2 TCR and Ig gene rearrangement analysis.

Clonal populations of T-cells or B-cells will have identical TCR or Ig gene rearrangements, which can be detected by molecular analysis. Rearrangement of the TCR or Ig genes causes alterations in the sizes of the restriction endonuclease fragments that span the rearranging sequences, as illustrated in Figure 3.4. The altered fragments can be detected by Southern hybridization of molecular probes derived from the TCR or Ig genes to restriction endonuclease digested DNA.

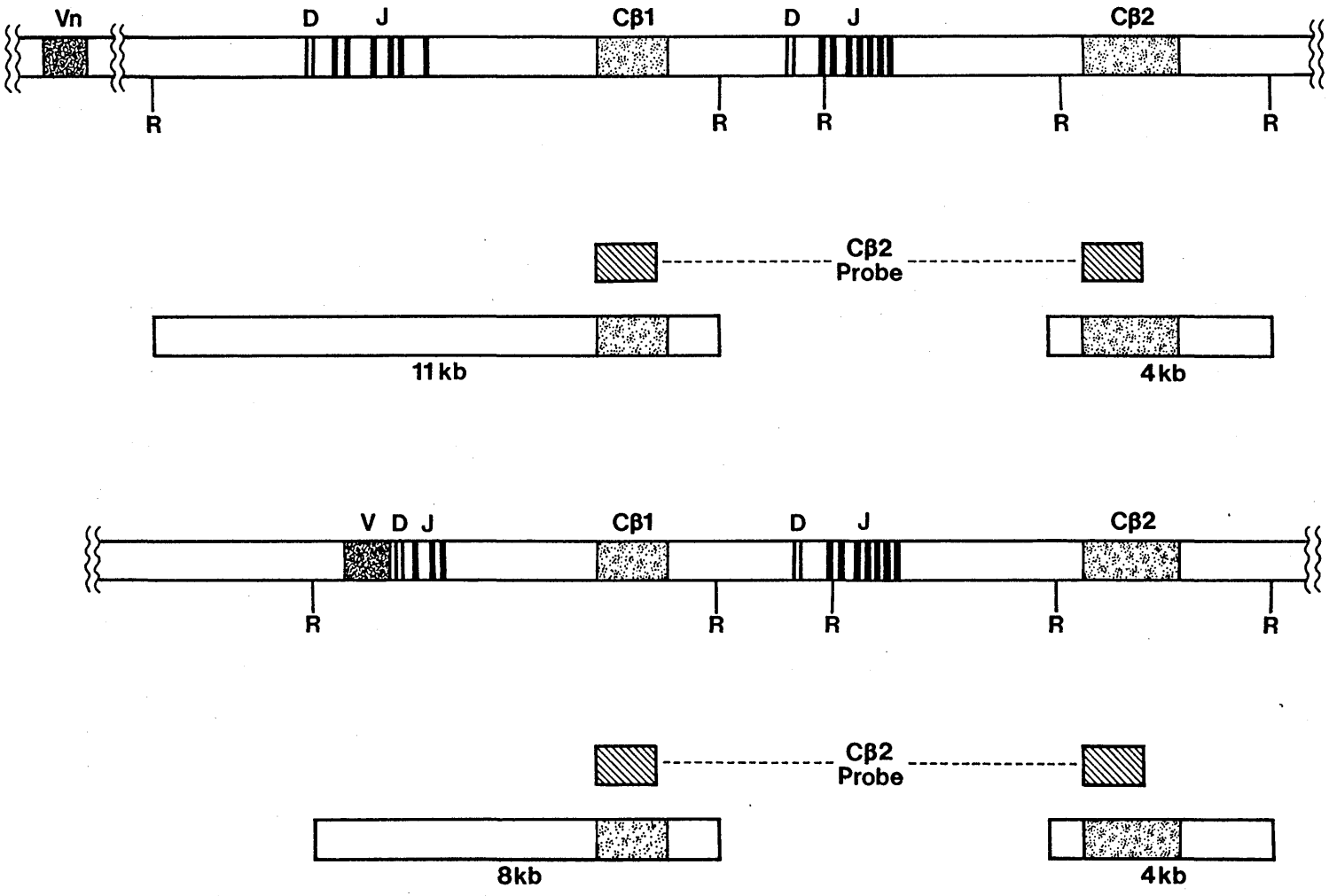
Ig and TCR gene rearrangement analysis has been extensively applied to lymphoma material. In the majority of cases of B-cell NHL clonal rearrangements of the IgH and IgL genes are detectable (Arnold *et al.* 1983; O'Connor 1987). Similarly in the majority of cases of T-cell NHL clonal rearrangements of the TCRβ and TCRγ genes are present (Bertness *et al.* 1985; O'Connor *et al.* 1985; Griesser *et al.* 1986a; Minden and Mak 1986; Toyanaga and Mak 1987). The analysis of lymphoproliferative disorders for the presence of Ig and TCR gene rearrangements thus allows the lineage and clonality of the proliferating cells to be determined.

Rearrangement of these genes is not completely lineage specific as clonal IgH gene rearrangements have been reported in T-cell NHL and TCRβ gene rearrangements have been detected in B-cell NHL (Pelicci *et al.* 1985; Waldmann *et al.* 1985; O'Connor 1987). Rearrangement of the TCRγ genes has been reported in a high proportion of B-cell neoplasms and is thus a poor indicator of cell lineage (Griesser *et al.* 1986a; Pelicci *et al.* 1987a). Rearrangements of the IgH and/or TCRβ genes have also been detected in leukaemic cells from patients with AML (Boehm *et al.* 1987a; Foa *et al.* 1987). The detection of Ig or TCRβ gene rearrangement in a tumour sample cannot, therefore, be taken as definitive proof of its lymphoid origin.

Despite these caveats, the analysis of the configuration of the TCR and Ig genes has permitted the more accurate characterization of a variety of lymphoproliferative disorders including, for example, hairy cell leukaemia (Cleary *et al.* 1984) and lymphomatoid papulosis (Weiss *et al.* 1986a). The technique has also proved valuable for the investigation of lymphoproliferative disorders that appear to be heterogeneous in nature, such as HD and AIL.

Figure 3.4. (a) The organization of the T-cell receptor beta-chain gene locus in germline DNA. The cleavage sites for the restriction enzyme EcoRI (R) are shown. (b) The probe for the second constant region hybridizes to both constant regions, which are contained in two fragments of 4kb and 11kb in EcoRI-digested DNA. (c) Rearrangement of the gene results in apposition of the variable (V), diversity (D) and joining (J) regions. (d) Following gene rearrangment the constant region probe hybridizes to a germline fragment of 4kb and a rearranged fragment of variable size (8kb in the diagram) in EcoRI-digested DNA.

Figure 3.4. Molecular detection of T-cell receptor beta-chain gene rearrangement.



3.1.3 HD: the immunophenotypic analysis of HRS cells.

The use of immunological methods for the characterization of the cell surface phenotype of the HRS cells has failed to identify convincingly normal cellular counterparts and thus a potential origin for these cells. Many different cell types have been proposed as the normal counterparts of HRS cells including B- and T-lymphocytes, macrophages, monocytes, myeloid cells, and interdigitating reticulum cells (Drexler and Leber 1988).

A lymphoid origin for HRS cells.

The majority of recent studies support a lymphocytic origin for HRS cells. This evidence is most convincing for the LP subtype of HD. The HRS cells of LPHD express cytoplasmic J chains (Poppema 1980; Stein *et al.* 1986), the common leukocyte antigen, CD45 (Pinkus and Said 1985; Hall *et al.* 1988) and antigens recognized by the B-cell-specific MoAbs LN1 and L26 (Hall *et al.* 1988; Dallenbach and Stein 1989; Nicholas *et al.* 1990). The HRS cells of cases of other subtypes of HD do not express J chains and rarely react with LN1 or L26 (Hall *et al.* 1988; Dallenbach and Stein 1989; Leoncini *et al.* 1990). Conflicting results have been reported for CD45 expression in NS and MCHD (Hsu *et al.* 1985; Hall *et al.* 1988; Casey *et al.* 1989; Leoncini *et al.* 1990). The HRS cells of the majority of cases of LPHD do not express CD15 and CD30, further distinguishing this subtype from the other HD subtypes (Hall *et al.* 1988; Nicholas *et al.* 1990). The results of these studies suggest that LPHD differs from the other subtypes of HD in its histogenesis and that LPHD is a B-cell malignancy.

The derivation of the HRS cells in NS, MC and LDHD is more controversial. Many studies have reported HRS cell expression of antigens normally associated with activated lymphocytes, such as CD30, HLA-DR and CD25 (e.g. Falini *et al.* 1987; Hall *et al.* 1988; Casey *et al.* 1989; Dallenbach and Stein 1989). The consistent expression of CD15 by HRS cells was considered initially to be evidence against their lymphoid derivation, as CD15 expression is associated with myeloid cells (Stein *et al.* 1982a). However CD15 expression by a variety of malignant T- and B-cells has since been reported (Lee 1989).

The use of antibodies to T-cell and B-cell antigens has given conflicting results. In some studies the expression of T-cell markers has been detected in a high proportion of cases (Falini *et al.* 1987; Casey *et al.* 1989; Dallenbach and Stein 1989; Herbst *et al.* 1989), while others have failed to detect any T-cell antigen expression by HRS cells (Hall *et al.* 1988). The expression of B-cell antigens by HRS cells has been reported less frequently (Falini *et al.* 1987; Casey *et al.* 1989; Herbst *et al.* 1989) though Hall *et al.* (1988) found that one third of HD cases (excluding LPHD) expressed B-cell antigens. Cases showing expression of both B- and T-cell markers have also been reported (Casey *et al.* 1989; Herbst *et al.* 1989). However between one third and two thirds of cases fail to show detectable expression of either B- or T-cell antigens (Falini *et al.* 1987; Hall *et al.* 1988; Herbst *et al.* 1989). Cell lines derived from HD express B- or T-cell surface markers, supporting the derivation of HRS cells from T- or B-lymphocytes (Drexler *et al.* 1989; Herbst *et al.* 1989).

A monocyte/histiocyte origin for HRS cells.

Hsu *et al.* (1990) have reported that the HD-derived cell lines HDLM-1 and KM-H2 can be induced to differentiate into cells that have the morphological, immunophenotypic and functional properties of histiocytes. These authors suggest that detection of T- or B-cell markers on HRS cells reflects aberrant antigen expression by malignant cells derived from moncytes/histiocytes, or alternatively, surface binding of antigens released by adjacent lymphocytes. Limited support for this hypothesis has been provided by studies from the same group (Hsu *et al.* 1985; Hsu and Hsu 1989) and by some of the morphological and functional properties of HRS cells (Jones 1987). Hsu and Hsu (1989) have in addition shown that CD30, T-cell and B-cell antigens may be expressed on some monocyte/histiocyte/myelocyte-related cell lines. However other studies have failed to demonstrate the expression of monocyte/histiocyte-associated antigens on HRS cells (Stein *et al.* 1982b; Falini *et al.* 1987).

Some of the variation in the results obtained in the studies described above may be due to technical difficulties encountered because of the scarcity of HRS cells in sample material (Casey *et al.* 1989). In addition the expression of surface antigens by HRS cells may be difficult to identify accurately as these cells are usually surrounded by T-lymphocytes. However many studies indicate that the immunophenotypes of HRS cells are heterogeneous (e.g. Casey *et al.* 1989; Herbst *et al.* 1989) and these data have been considered as evidence in favour of the notion that HD is an heterogeneous entity.

3.1.4 Analysis of HD biopsies for rearrangement of the Ig and TCR genes.

The analysis of fresh HD material for rearrangements of the Ig and TCR genes has permitted further investigation of the lineage and clonality of HRS cells. At the time at which the study described in this chapter was commenced a limited number of such analyses had been reported in the literature.

Weiss *et al.* (1986b) analysed tissues from seven cases of NSHD, selected for the presence of high numbers of HRS cells. Rearrangements of the IgH and/or IgL genes were detected in six cases while the TCRβ genes were germline, leading these authors to suggest that HRS cells could be derived from B-lymphocytes. However, two other published studies produced conflicting results.

Knowles *et al.* (1986) detected minor clonal Ig and/or TCRβ gene rearrangements in only 3/18 cases of HD. There was no relationship between the numbers of HRS cells and the detection of gene rearrangement. These authors did find a correlation between HRS cell numbers and the detection of a pattern of hybridization to the TCRβ constant region probe that was consistent with the presence of a polyclonal T-cell population. Knowles *et al.* (1986) therefore suggested that HRS cells are a population of polyclonal T-cells. These authors further suggested that the absence of significant Ig or TCR gene rearrangements in HD could be used to differentiate HD from NHL.

In contrast, Griesser *et al.* (1986b) reported the detection of TCRβ gene rearrangements in 4/8 cases of HD.

The results obtained in these studies provide no consensus regarding the following two questions.

(a) whether or not significant Ig and/or TCR gene rearrangements are detectable in biopsy material from patients with HD,

(b) whether the rearrangements detected are present in the HRS cells.

In order to investigate these questions, a study combining the molecular analysis of tissues from HD patients with the histopathological determination of HRS cell numbers was undertaken. This chapter describes the results of the analysis of 35 cases of HD for rearrangements of the IgH, IgL and TCRβ genes. The numbers of HRS cells present in the samples were evaluated by the referring pathologists, to assist in determining whether the rearrangements detected were present in the HRS cells.

3.1.5 The immunophenotypic characterization of AIL.

Both the lineage of origin and the neoplastic nature of lesions classified histologically as AIL have been the subject of debate. Early reports considered AIL to be a B-cell disease (Lukes and Tindle 1975; Nathwani *et al.* 1978) and some workers have identified lesions histologically classified as AIL, that are composed mainly of proliferating B-cells (Jones *et al.* 1978; Knecht *et al.* 1985). However immunophenotypic analysis has indicated that most cases of AIL are lymphoproliferative disorders of T-cells (Weiss *et al.* 1986c; Ganesan *et al.* 1987; Namikawa *et al.* 1987).

Similar results have been obtained from the analysis of lymphomas showing histological features of AIL (Watanabe *et al.* 1980; Weiss *et al.* 1986c), though B-cell derived AIL-like lymphomas have also been identified, following the detection of restricted IgL expression by tumour cells (Boros *et al.* 1981; Bauer *et al.* 1982; Knecht *et al.* 1985; Pirker *et al.* 1986).

The controversy over whether AIL should be considered a malignant disease from the outset was discussed in Chapter 1 (section 1.3.3). Most studies have distinguished between AIL cases lacking features consistent with malignant transformation (AIL) and those which show morphological evidence of overt lymphoma (AIL-like lymphoma). Clonal cell populations have been detected by karyotypic and immunophenotypic analyses of AIL biopsies that lacked morphological evidence of lymphoma (Bauer *et al.* 1982; Kaneko *et al.* 1982; Godde-Salz *et al.* 1987). These data have provided evidence to support the notion that AIL might be neoplastic from the outset.

The availability of molecular techniques for the detection of clonal B- and T-cell populations has enabled more detailed investigation of the lineage and clonality of the lymphoproliferations characteristic of AIL.

3.1.6 Ig and TCR gene rearrangements in AIL.

When the experiments described in this chapter were commenced, a limited number of studies describing the analysis of TCRβ and Ig gene rearrangement in AIL had been published (Bertness *et al.* 1985; O'Connor 1986; Weiss *et al.* 1986c). The results of these studies indicated that many cases of AIL contain clonal proliferations of T-cells and that many, but not all, cases of AIL-like lymphoma are T-cell neoplasms.

This chapter describes the results obtained from the analysis of samples from patients with B- and T-cell NHL for the presence of Ig and TCR gene rearrangement. The tissues analysed included 5 samples from patients with AIL. The results obtained are discussed in the context of the probable pathogenesis of this disorder.

3.1.7 Gene rearrangement analysis in the differential diagnosis of Hodgkin's and non-Hodgkin's lymphoma.

The differentiation of HD from NHL is not always straightforward. Particular difficulty may be encountered in distinguishing HD from the AIL-like lymphomas and from some types of T-cell NHL (Lukes and Tindle 1975; Krajewski *et al.* 1988). Knowles *et al.* (1986) have suggested that gene rearrangement analysis may be used to differentiate HD from NHL, as they were unable to detect significant clonal TCRβ or Ig gene rearrangements in HD samples. The technique could therefore be useful in cases in which routine diagnostic procedures have given equivocal results.

Five of the samples included in this study were referred with differential diagnoses that included HD and NHL. The results obtained from the genotypic analysis of these samples are compared with those obtained from the analysis of samples from patients with HD and NHL. The use of Ig and TCR gene rearrangement analysis for the differentiation of HD from NHL is discussed.

3.1.8 The use of Vβ-specific MoAbs for the immunophenotypic detection of clonal T-cell populations in tumour biopsies.

The diagnosis and classification of the peripheral T-cell lymphomas is difficult, in part, due to the diverse clinical and morphological features of these tumours (Wright 1986; Suchi *et al.* 1987). T-cell lymphomas are also immunophenotypically heterogeneous (Ramsay *et al.* 1987; Suchi *et al.* 1987; Krajewski *et al.* 1988). The difficulty of differentiating some T-cell lymphomas from HD has been mentioned above.

The lack of immunohistochemical techniques for the detection of clonality in T-cell lymphomas limits the usefulness of immunophenotyping for lymphoma diagnosis. The development of immunological reagents for the detection of clonal T-cell populations would, therefore, be of considerable diagnostic value.

Recently clonotypic MoAbs, that react with the TCRβ expressed by the T-cell lines HPB-ALL and Jurkat, have been described (Moretta *et al.* 1985; Carrel *et al.* 1986). These MoAbs also react with 2-4% of thymoctyes and with 0.5-13% of PBMCs in healthy humans (Moretta *et al.* 1985; Borst *et al.* 1986). Cloned T-cells, derived by selection for reactivity with the MoAb specific for the HPB-ALL TCR, have been shown to express TCRβs with identical or closely related Vβ (Borst *et al.* 1987). These variable regions are members of the Vβ5 family (Kimura *et al.* 1986; Borst *et al.* 1987). Jurkat T-cells express a TCRβ containing a member of the Vβ8 family (Siu *et al.* 1984; Kimura *et al.* 1986). MoAbs directed against the TCRβ expressed by HPBALL and Jurkat T-cells have been used by Clark *et al.* (1986) to identify clonal T-cell populations in tissue sections.

There are at least 16 families of Vβ, each of which contains a small number of members (Concannon *et al.* 1986; Kimura *et al.* 1986). Thus the Vβ-specific MoAbs are likely to be useful only in a small number of cases. In addition, the specificity of the anti-HPBALL and anti-Jurkat MoAbs for the Vβ5 and Vβ8 regions has not previously been confirmed in tumour samples.

The T-cell NHL samples that were analysed for Ig and TCR gene rearrangements included four samples that showed reactivity with clonotypic antibodies directed against the Vβ5 and/or Vβ8 region of the TCRβ. The specificity of these MoAbs for the Vβ expressed by the tumour cell populations in these samples was investigated using molecular probes for the Vβ gene segments.

3.1.9 Rearrangement of the bcl-2 locus in NHL and HD.

Non-random chromosomal abnormalities have been reported in a number of histological subtypes of NHL (Yunis *et al.* 1982; 1983). Translocations involving the IgH gene locus on chromosome 14(q32) have been described in a significant proportion of follicular and diffuse large cell B-cell lymphomas, BLs, B-cell chronic lymphocytic leukaemias and have also been identified in T-cell leukaemias (Baer *et al.* 1987; Weiss *et al.* 1987a; Aisenberg *et al.* 1988). Such translocations may be detected as rearrangements of the IgH gene locus if the translocation breakpoint occurs within the restriction fragment containing the DNA sequences used as the IgH gene probe.

The t(14;18)(q32;q21) involving the IgH heavy chain locus and the bcl-2 gene has been observed in 60-69% of follicular lymphomas and 20-40% of diffuse large cell lymphomas (Lee *et al.* 1987; Lipford 1987a; Weiss *et al.* 1987a). These studies have shown that in the majority of cases the breakpoint on chromosome 18 occurs within a 4.3kb HindIII fragment at the 3' end of the bcl-2 gene, the major breakpoint cluster region (MBR). DNA sequence analysis of the cross over point on the der (14) chromosome in seven cases has shown that the breakpoints on chromosome 18 occurred within 460bp of each other, while the breakpoints on chromosome 14 were located at the 5' ends of J_H4-J_H6 (Bakshi *et al.* 1985; Cleary and Sklar 1985; Tsujimoto *et al.* 1985; Cleary *et al.* 1986). The HindIII restriction fragment overlapping the translocation breakpoint on the der (14) chromosome would therefore be predicted to be approximately 4.5-6kb in a majority of cases.

The results of IgH gene rearrangement analysis showed that in a significant proportion of the lymphoma samples, the rearrangements detected were giving rise to restriction fragments of similar sizes. The possibility that this could be due to the t(14:18) translocation involving the IgH gene locus and the bcl-2 gene on chromosome 18q22 was investigated.

3.2. MATERIALS AND METHODS.

3.2.1. Clinical cases.

Samples from 35 non-selected cases of HD were obtained from three referral centres. Cases were classified according to the Rye classification (Lukes *et al.* 1966) following histological review. HRS cells were identified on the basis of morphology in haemotoxylin and eosin-stained sections and their relative frequency estimated.

Samples from patients with NHL were obtained from three referral centres. The classification of NHL cases was based on a combination of clinical features, morphological assessment of haemotoxylin and eosin-stained paraffin sections and the results of immunophenotyping. Samples from 5 cases with differential diagnoses of HD and NHL were obtained from the Department of Pathology at Edinburgh University Medical School.

3.2.2 DNA probes.

Ig gene probes: The locations of the Ig gene probes relative to the coding sequences of the Ig gene loci are shown in Figure 3.1. The IgH probe, pHj, was a 3.3kb EcoRI-HindIII fragment which includes 2.2kb of the 3' joining region (J_H) sequences (Erikson *et al.* 1982). The κ light chain probe, Cκ, was a 2.5kb EcoRI fragment containing the constant region (Rabbitts *et al.* 1984). A probe for the κ deleting element, κde, was used to evaluate κ gene loss (Siminovitch *et al.* 1985). This probe detects the deletion of κ gene sequences. B-cells producing λ-chains usually delete both κ genes, more rarely, κ gene rearrangement is detected (Heiter *et al.* 1981). However it may be difficult to detect evidence of deletion of both κ genes when using the Cκ probe for the analysis of tumour biopsies. The detection of germline IgLκ genes in a sample may reflect the presence of non-neoplastic cells in the tumour tissue. In order to determine whether IgL gene rearrangement has occurred in such cases, either the κde probe or probes for the λ gene locus may be used. Use of the κde probe avoids the problems associated with polymorphic sites within the λ locus when using IgL λ chain probes (Brinker *et al.* 1987).

TCRβ gene probes. The TCRβ constant region probe was derived from a cDNA clone, C91β, of the β chain isolated from the HTLV-I infected cell line C91PL (Gledhill *et al.* 1990). Sequence analysis of C91β revealed an XhoI site located precisely at the junction between the joining and constant regions (R. Jarrett unpublished results). The 1.3kb fragment can be excised from the pGEM4 vector with EcoRI, thus simultaneous digestion with XhoI may be used to generate a constant region probe, Cβ, containing all coding sequences and the untranslated 3' sequences of the second constant region. The derivation of the Cβ probe is shown schematically in relation to the TCRβ locus in Figure 3.3. The TCRγ gene probe was a 700bp fragment of genomic DNA encompassing the TCRγ joining region (Lefranc and Rabbitts 1985). The origin of the TCRγ probe is illustrated in Figure 3.3.

Variable region probes: The Vβ8 probe for the Vβ gene segment expressed by the Jurkat cell line was derived from pYTJ2 (Siu *et al.* 1984). Double digestion of the plasmid with PstI and PvuII produces a 256 bp fragment, which corresponds to bases 100-356 of the published sequence of YT35 (Yanagi *et al.* 1984) and includes Vβ sequences only. The Vβ5 probe for the Vβ gene segment expressed by the HPB-ALL cell line was obtained from Dr. M. Owen and is a 120bp fragment which hybridizes with members of the Vβ5 family (Dr. M. Owen, personal communication).

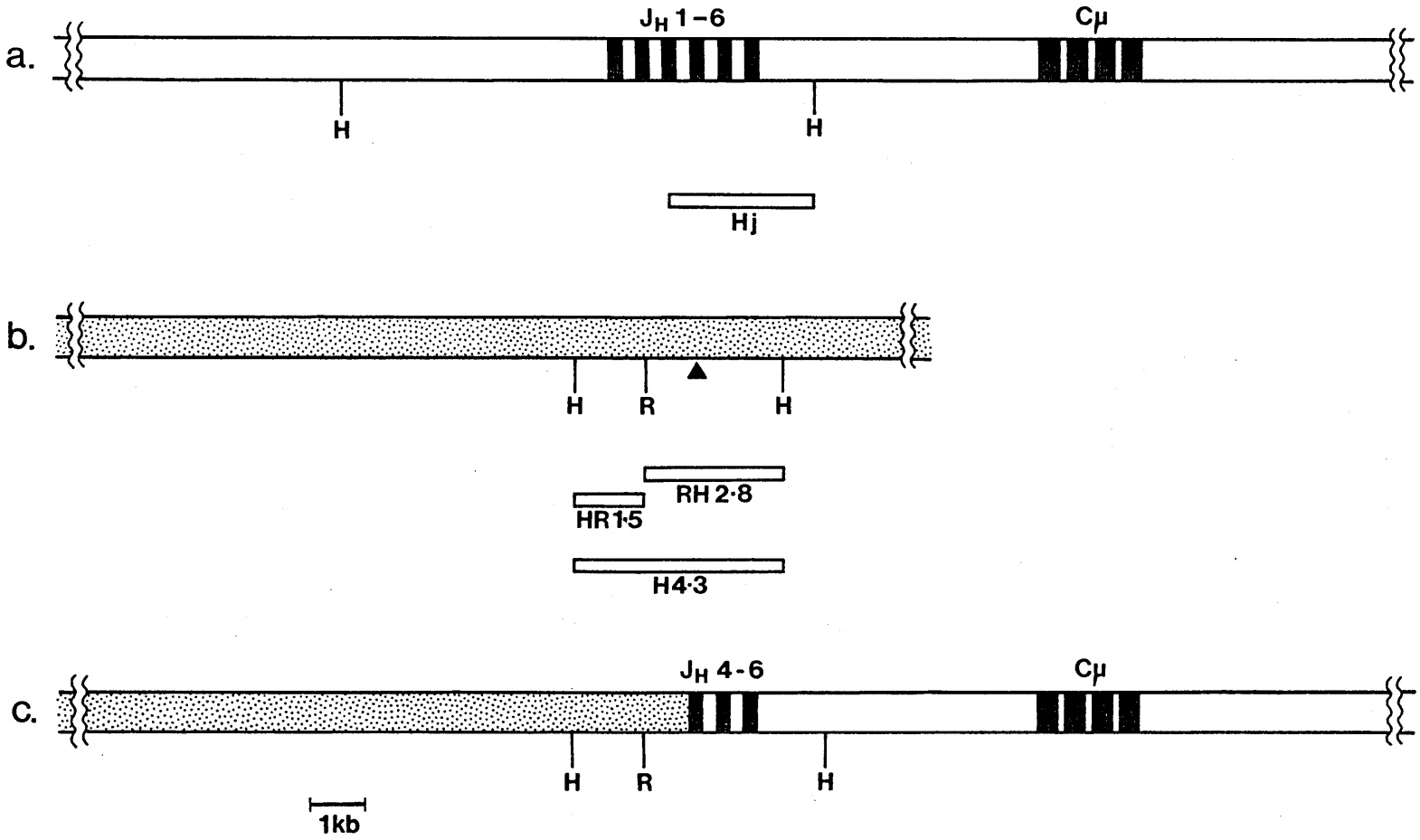
Bcl-2 probes: The probe for the bcl-2 gene on chromosome 18q21 was obtained from Dr. S.J. Korsmeyer and is a 4.3kb HindIII fragment (H4.3) from the bcl-2 exon 3 (Bakshi *et al.* 1987). For some experiments this fragment was further digested with EcoRI and either the 2.8kb EcoRI-HindIII fragment (RH2.8) containing the major breakpoint cluster region (MBR), or the 1.5kb HindIII-EcoRI fragment (HR1.5), 5' to the MBR, was used as a probe. Figure 3.5 illustrates the relationships between these probes, and their use for the detection of the t(14;18) translocation.

3.2.3 Molecular analysis.

For IgH gene analysis DNA samples were digested with the restriction enzymes BamHI, EcoRI and HindIII, and electrophoresed on 0.8% agarose gels. The size separated DNA samples were transferred to nylon membranes and hybridized to the radioactively labelled J_H probe. Washing was performed at high stringency in 0.5 x SSC and 0.1% SDS at 65°C. TCRβ genes were analysed as described above, but in addition samples were digested with EcoRV. In order to detect TCRβ gene

Figure 3.5. (a) The organization of the immunoglobulin heavy chain gene locus. Cleavage sites for the restriction enzyme HindIII (H) are shown. (b) Chromosome segment 18q21 and the probes used to detect rearrangements of this region. Cleavage sites for the restriction enzymes HindIII (H) and EcoRI (R) are shown. The arrowhead indicates the major breakpoint cluster region within the bcl-2 gene. (c) A possible structure for the der(14) chromosome following the t(14;18) translocation. The JH probe and the bcl-2 gene probes would hybridize to the same rearranged DNA fragment in HindIII-digested DNA following the translocation illustrated. The restriction enzyme map of chromosome segment 18q21 is derived from Bakshi et al. (1987).

Figure 3.5. Detection of the t(14;18) translocation using probes for the bcl-2 gene.



rearrangement it was necessary to run some of the EcoRI and EcoRV digested DNA samples on 0.7% agarose gels. Analyses of the IgL and TCRγ genes were performed using BamHI digested DNA samples. HindIII digested DNA samples were used for the analysis of bcl-2 gene rearrangement.

Vβ genes were analysed using BamHI, EcoRI and HindIII digested DNA samples. In order to permit hybridization of the Vβ probes with related Vβ regions washing was performed under lower stringency conditions, at 60°C in 1 x and 3 x SSC. These conditions were selected as described below, to accommodate a 25% mismatch between the probe and the target sequences as Vβ regions are assigned to families on the basis of a greater than 75% homology.

Meinkoth and Wahl (1984) have reviewed the parameters affecting the stability of DNA hybrids. The melting temperature (Tm) of duplex DNA under the conditions employed for hybridization and washing may be calculated from the formula:

$$Tm = 81.5 + 16.6\log M + 0.41(\%G+C) - 500/n - 0.61(\%F)$$

Where M is the ionic strength of the solution (moles/L), (%G+C) is the percentage of G and C residues in the target sequence, n is the average length of the probe in nucleotides and (%F) is the percentage of formamide in the solution.

The Tm of the Vβ probes hybridized to a perfectly matched target sequence may be estimated at 87°C and 84°C for Vβ8 and Vβ5 respectively, when washed in 3 x SSC. The G+C content was assumed to be 40% (similar to the average for mammalian DNA), while the average probe lengths were assumed to be 128 and 60 nucleotides for Vβ8 and Vβ5 respectively. The Tm of a DNA duplex has been estimated to decrease by 1°C for every 1% of base pairs that are mismatched (Meinkoth and Wahl 1984). Hybridization of the Vβ8 and Vβ5 probes to target sequences that diverge by up to 25% would produce duplexes with Tm of 62°C and 59°C respectively. A washing temperature approximately equal to the calculated Tm was selected.

Densitometry of rearranged bands.

In order to evaluate the relationship between the number of HRS cells in HD biopsies with the proportion of cells estimated to contain a clonal gene rearrangement, the intensity of rearranged bands was measured using densitometry. To obtain a linear relationship between the quantity of radioactivity present on membranes following hybridization and the intensity of the film response, autoradiography was performed using preflashed X-ray film (Laskey and Mills 1975; 1977). Film was preflashed using a photographic flash unit at a distance of 3m, in order to increase the absorbance of developed film by 0.1-0.2 units. The intensity of rearranged bands were measured on a Molecular Dynamics densitometer and analysed using ImageQuant software.

The use of this method to estimate the proportion of cells with a clonal gene rearrangement was validated using reconstitution experiments. DNA derived from the leukaemic cells of a patient with B-cell CLL was mixed with DNA extracted from placenta. Analysis of DNA extracted from the leukaemic cells using the IgH probe showed an absence of germline fragments, thus any germline fragments detected in the mixed samples could be assumed to derive from the placental sample.

The DNA from both sources was digested with the restriction enzyme HindIII, precipitated in ethanol and resuspended in TE. The DNA concentration of the solutions was determined using spectrophotometry as described in Chapter 2 (section 2.2.1). The two solutions were combined in various proportions such that between 2 and 100% of the total sample was derived from leukaemic cell DNA.

3.3 RESULTS.

3.3.1 HD cases

Table III.I summarizes the clinical features, diagnoses and results of genotypic analysis for the HD cases. Thirty five HD cases were analysed, including twenty four cases of nodular sclerosing (NS) HD, six cases of mixed cellularity (MC) HD, two cases of lymphocyte predominant (LP) HD and one case of lymphocyte depleted (LD) HD. In two additional cases no consensus was reached as to the correct diagnosis following histological review. Case 72 was referred to us as NSHD but was considered by one of the reviewing histopathologists to be classifiable as MCHD. For case 210 the possibility of sclerosing mediastinal B-cell lymphoma was raised, but NSHD was not excluded as a differential diagnosis. Cases 167 and 168 were referred with differential diagnoses that included HD and T-cell NHL. Following genotypic analysis and histological review final diagnoses of HD were reached for both cases, as described in section 3.3.3. Cases 167 and 168 have therefore been included in the group of HD samples.

Nine cases of HD had rearranged IgH genes (Figure 3.6), seven of these cases also had rearranged IgL genes. Figure 3.7 shows representative results obtained from Southern blot analysis of the IgLκ genes. IgH rearrangements were detected in 6 cases of NSHD (including case 210, for which a differential diagnosis of B-cell NHL was given), 2 cases of MCHD and 1 case of LDHD. The rearranged bands detected in these cases were faint compared to germline bands, but were readily visible on autoradiography. TCRβ gene rearrangement was detected in none of the thirty five cases. No TCRγ gene rearrangements were detected in the ten cases analysed.

Ig gene rearrangements and HRS cell numbers.

Figure 3.8 illustrates the relationship between the detection of IgH gene rearrangements in the HD cases, and the numbers of HRS cells identified in the tissue sections. In seven of the cases in which IgH gene rearrangements were detected the HRS cell numbers were estimated to form more than 8% of the total population. Six HD samples with more than 8% HRS cells showed germline Ig genes. IgH gene rearrangement was detected in one case in which HRS cell numbers were estimated at less than 3% of the

Table III.I. B, B-cell NHL; G, germline; ND, not done; NK, not known; R, rearranged; *, the percentage of cells containing the IgH gene rearrangement as estimated by densitometry; +++, more than 8%; ++, 3-8%; +, less than 3%; -, less than one HRS cell per high power field; *, the differential diagnoses are shown for cases 72 and 210; #, the results shown for case 382 are from the analysis of a lymph node biopsy with the histological appearance of a reactive node (see text). The final column of the table indicates the results obtained from the analysis of the samples by Southern hybridization for the presence of Epstein-Barr virus (EBV) genomes (Gledhill et al. in press). These data are discussed in section 3.4.1.

Table III.I. Clinical features, diagnoses and the results of molecular analyses for the HD cases.

Patient No.	Age/Sex	HD subtype	% HRS cells	Gene rearrangements TCR *β*	TCR*γ*	IgH*	IgL	EBV
70	36/M	LP	-	G		G	G	+
71	22/M	NS	+	G		G	G	-
72	21/M	NS/MC	+	G		G	G	+
74	20/M	MC	ND	G		G	G	-
75	24/M	NS	ND	G		G	G	-
76	19/M	NS	+	G		G	G	-
78	31/F	NS	+ + +	G		R + + +	R *K*	-
79	48/F	MC	+	G		G	G	+
167	35/M	NS	+ +	G	G	G	G	-
168	69/M	NS	-	G		G	G	+
210	50/F	NS/B	+ + +	G		R + + +	R *K*	+
237	25/M	MC	+ + +	G	G	G	G	-
289	43/F	NS	+ + +	G	G	G	G	-
373	66/M	NS	+ + +	G	G	R + + +	R *Kde*	+
382#	23/M	NS	-	G	G	R + + +	R *Kde*	-
384	28/F	NS	+	G	G	G	G	-
385	31/M	NS	+	G	G	G	G	-
386	80/M	MC	+ + +	G	G	R + + +	R *K*	+
387	14/M	MC	+	G	G	G	G	+
390	68/F	NS	+ + +	G	G	G	G	+
420	19/M	LD	+ + +	G		R + + +	R *K*	-
430	67/M	NS	+ + +	G		R + + +	R *K*	-
923	24/M	NS	+ +	G		G	G	-
924	19/M	NS	+ +	G		G	G	-
925	77/M	MC	+	G		R + +	G	+
926	26/M	NS	+ +	G		G	G	-
927	13/M	NS	+ +	G		G	G	+
928	19/M	NS	+ + +	G		G	G	-
929	17/M	LP	-	G		G	G	-
930	32/M	NS	+ +	G		G	G	-
931	42/M	NS	+ +	G		G	G	-
932	25/F	NS	+ + +	G		R + + +	G	-
933	26/M	NS	+ + +	G		G	G	-
934	21/F	NS	+ + +	G		G	G	-
935	15/NK	NS	ND	G		G	G	-

Figure 3.6. Southern blot analyses were performed using the JH probe. The patient numbers are given at the top of each lane. DNA samples were digested with the restriction enzymes shown at the base of each panel. P, control DNA extracted from placenta; 382a, DNA extracted from spleen showing histological evidence of HDMC; 382b, DNA extracted from histologically "reactive" lymph node. The germline bands are indicated with thick lines and the sizes of the germline fragments given (in kb) on the left of each panel. Rearranged bands are indicated with arrows. The probe hybridizes to a 3.5kb fragment in addition to the germline fragment of 10.5kb in HindIII digested DNA, as shown in panels (a) and (b). This fragment is detected in all samples including the placental controls and is of unknown origin.

Figure 3.6. Detection of IgH gene rearrangements by Southern blot analysis of DNA samples from patients with HD.

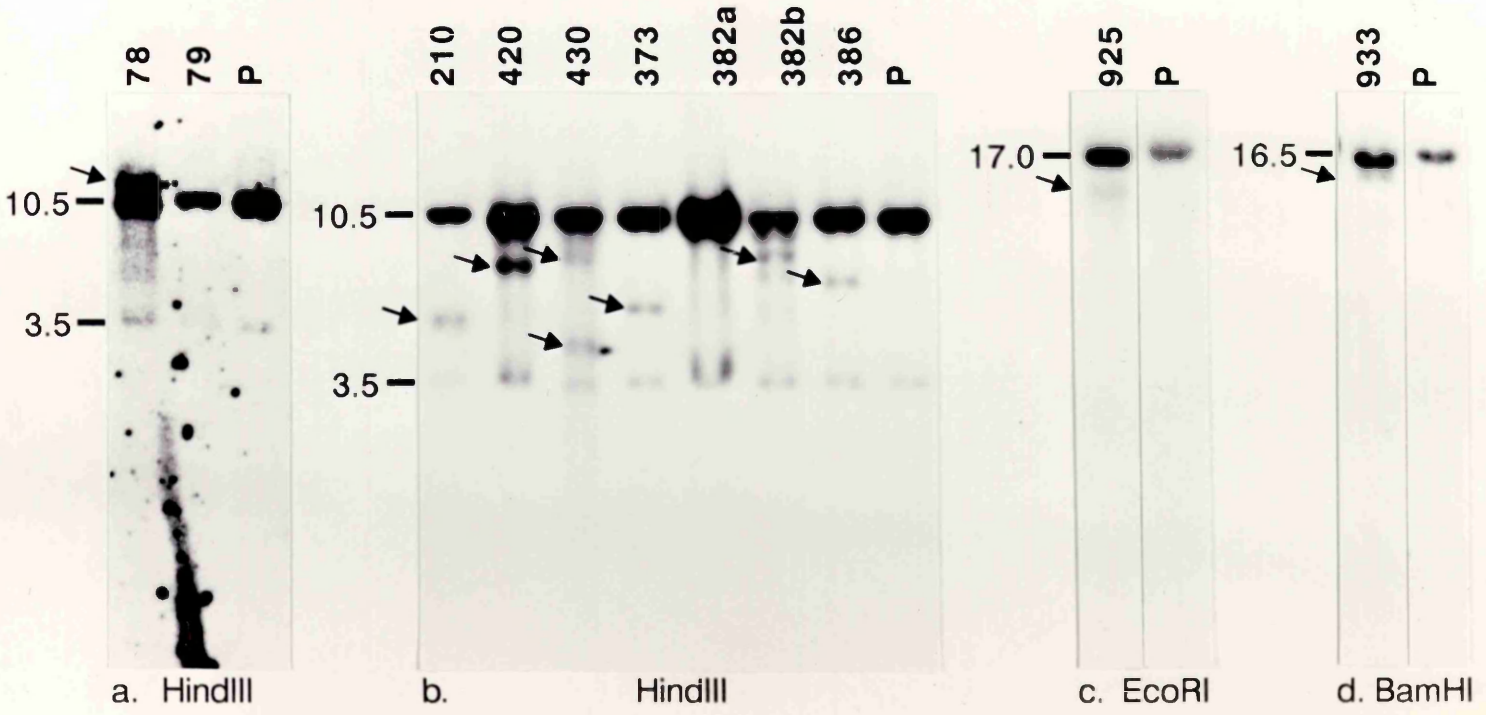


Figure 3.7. Southern blot analyses were performed using the CK probe (panels a, b and c) and the Kde probe (panels d and e). DNA samples were digested with the restriction enzyme BamHI. The germline bands are indicated with thick lines and the sizes of the germline fragments given (in kb) on the left of each panel. The Kde probe hybridizes with a fragment of 3.0kb in addition to the germline fragment of 14.0kb in BamHI digested DNA (panels d and e). This fragment has been previously described as a pseudogene by Williams et al. (1987). The thin lines on the left of panels a, d and e indicate additional bands of unknown origin, these bands are present in control and sample DNAs and therefore are not considered to indicate the presence of rearrangements.

Figure 3.7. Detection of IgL gene rearrangements by Southern blot analysis of DNA samples from patients with HD

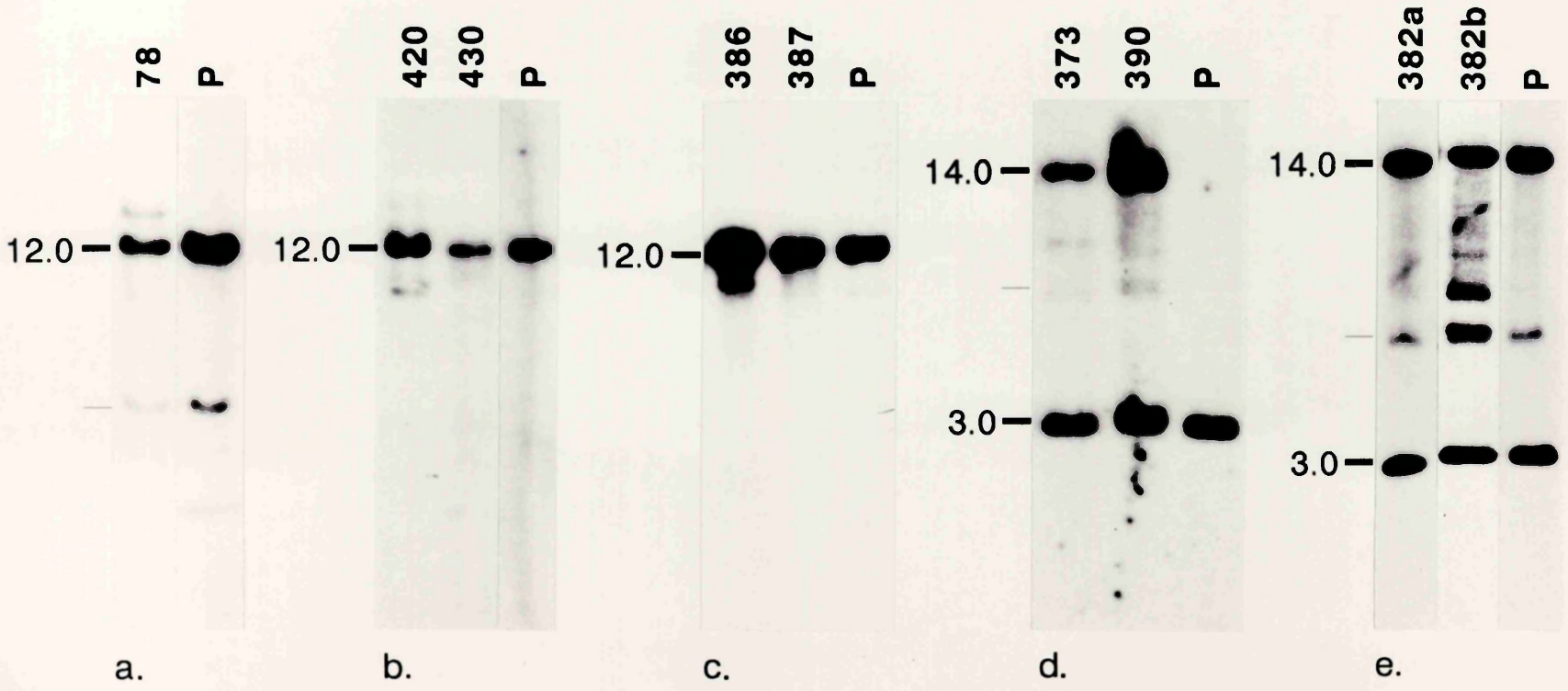


Figure 3.11. Southern blot analyses were performed using the Jy probe. The patient numbers are given at the top of each lane. DNA samples were digested with the restriction enzymes shown at the base of each panel. P, control DNA extracted from placenta. The germline bands are indicated with thick lines and the sizes of the germline fragments given (in kb) on the right of each panel. Rearranged bands are indicated with arrows.

Figure 3.11. Detection of TCRγ gene rearrangements by Southern blot analysis of DNA samples from patients with T-cell NHL.

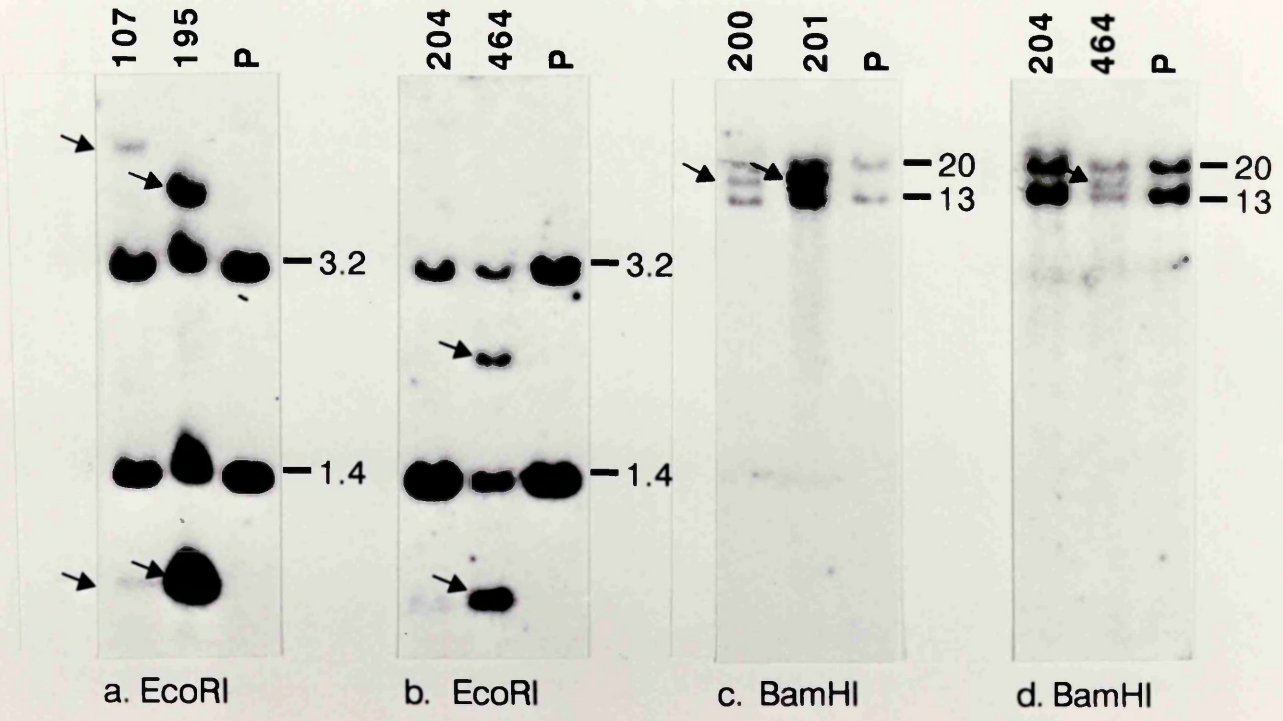
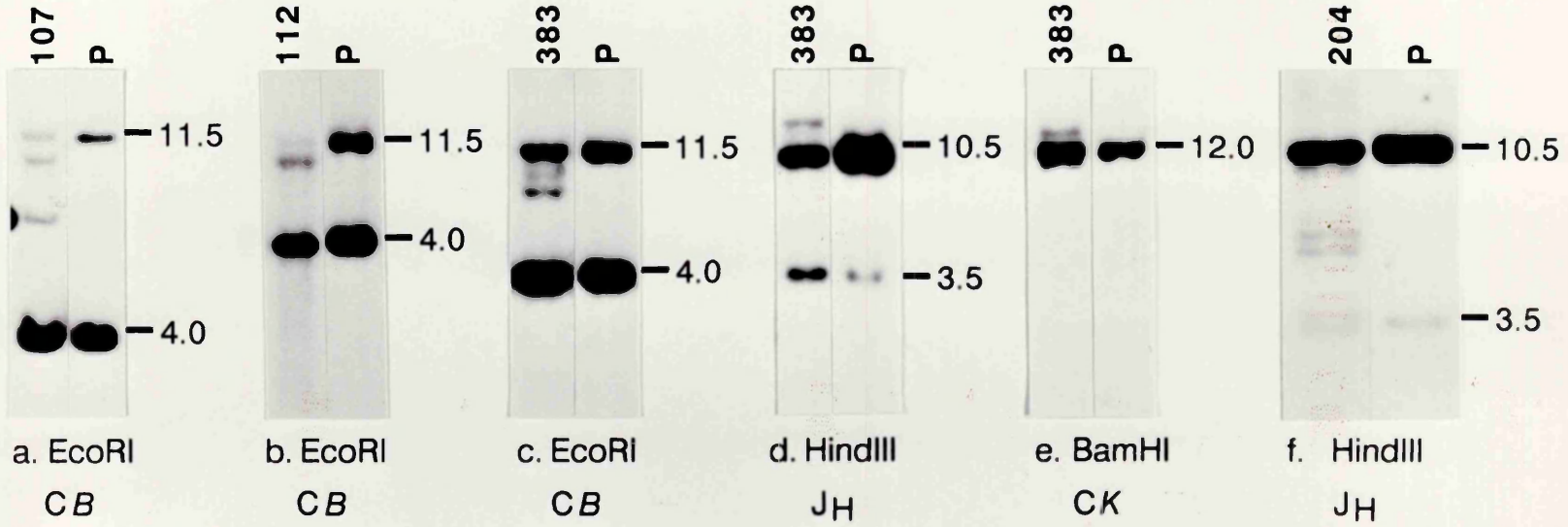


Figure 3.12. Southern blot analyses were performed using the Cß, JH or CK probes, as indicated at the base of each panel. DNA samples were digested with the restriction enzymes shown beneath the panels. P, control DNA extracted from placenta. The germline bands are indicated with thick lines and the sizes of the germline fragments are given (in kb) on the right of each panel. Rearranged bands are indicated with arrows.

Figure 3.12. Detection of TCR and Ig gene rearrangements by Southern blot analysis of DNA samples from patients with AIL-like lymphoma.



total. In sample 382 Ig gene rearrangements were detected in a lymph node biopsy considered to be reactive following histology and immunophenotyping. Some lymphoblasts were identified in the sections, but no classical RS cells were seen. This sample is indicated with an open circle in Figure 3.8.

No gene rearrangements were detected in a spleen sample showing features of NSHD which was also obtained from this patient. Karyotypic analysis also indicated the presence of a clonal cell population in the "reactive" node (Banks *et al.* 1991).

Densitometric analysis of IgH gene rearrangements.

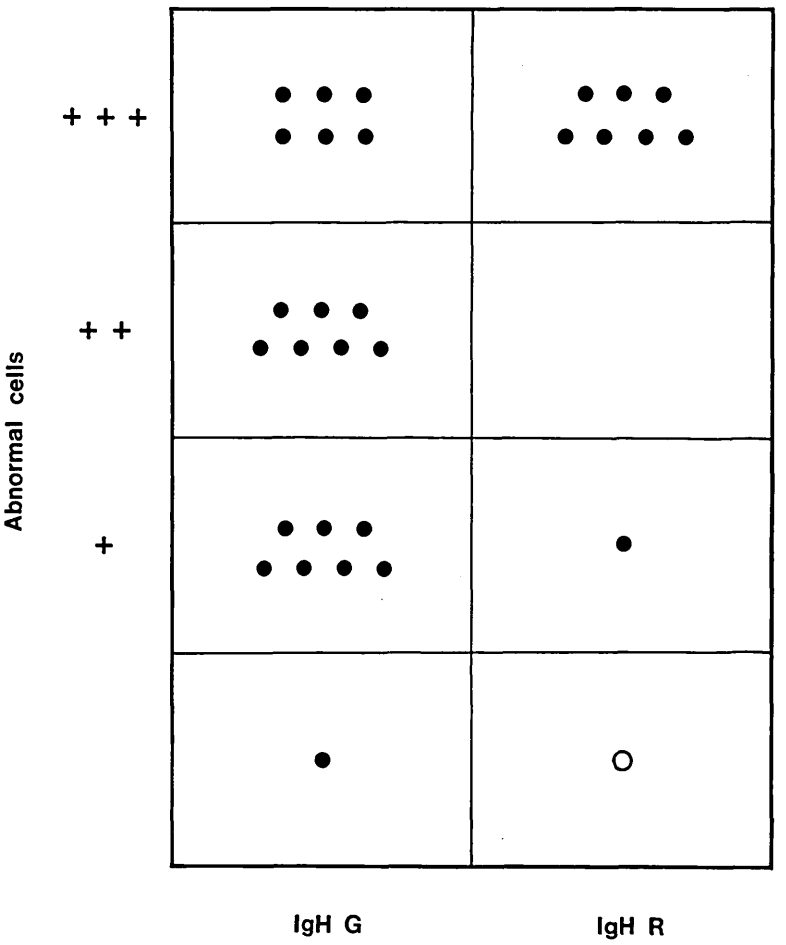
Figure 3.9 shows the detection of IgH gene rearrangements by Southern blot analysis of samples reconstituted from known proportions of leukaemic cell and placental DNA. The densitometric estimations of the proportion of the reconstituted DNA samples containing the clonal IgH gene rearrangements are also shown in Figure 3.9. The results showed good agreement with the estimations based on quantitation of the placental and leukaemic cell DNA samples prior to mixing.

The results obtained from the densitometric analysis of IgH gene rearrangements detected in the HD samples are given in Table III.I. In eight cases the intensity of the rearranged bands indicated that the cells containing the clonal rearrangement formed more than 8% of the total population. The numbers of HRS cells in paraffin sections from seven of these samples were also estimated to represent more than 8% of the total cell numbers. No RS cells were seen in the paraffin sections from the lymph node biopsy obtained from patient 382, as discussed above. Sample 925 was estimated to contain more than 3% HRS cells on the basis of morphology in paraffin sections, while densitometry indicated that the clonal IgH gene rearrangement was present in 3-8% of the cell population.

Figure 3.13. DNA samples were digested with BamHI (panels a,b,e and f) or EcoRI (panels c and d) and analysed using the Cß, Vß5 or Vß8 probes, as indicated below each panel. P, control DNA extracted from placenta. The germline bands are indicated with thick lines and the sizes of the germline fragments are given (in kb) beside the panels. Rearranged bands are indicated with arrows. The 7.0 kb band seen in EcoRI digested DNA hybridized to the Vß8 probe is observed only when filters are washed at low stringency (3 x SSC). The 2.2 kb band seen in BamHI digested DNA from sample 464 when analysed using the Vß8 probe (panel f) is a polymorphic germline band (Flug et al. 1985).

Figure 3.8. The scatter diagram shows the number of samples with germline IgH genes on the left hand side (IgH G) and the number of samples in which IgH gene rearrangements were detected on the right hand side (IgH R). Each sample is indicated by a circle. The number of samples in which HRS cell numbers were estimated at more than 8% of the total is shown in the top panel (+++); the middle two panels indicate the numbers of samples in which the HRS cell numbers were estimated at 3-8% (++), or less than 3% of the total (+); the bottom panel indicates the number of samples in which less than 1 HRS cell was seen per high power field.

Figure 3.8. Comparison between the detection of IgH gene rearrangements and the numbers of abnormal cells in samples from patients with HD.



3.3.2 NHL cases.

The diagnoses and clinical details of the 15 patients with NHL are shown in Table III.II. This table also shows the results of genotypic analysis of the samples obtained from these patients.

T-cell NHLs.

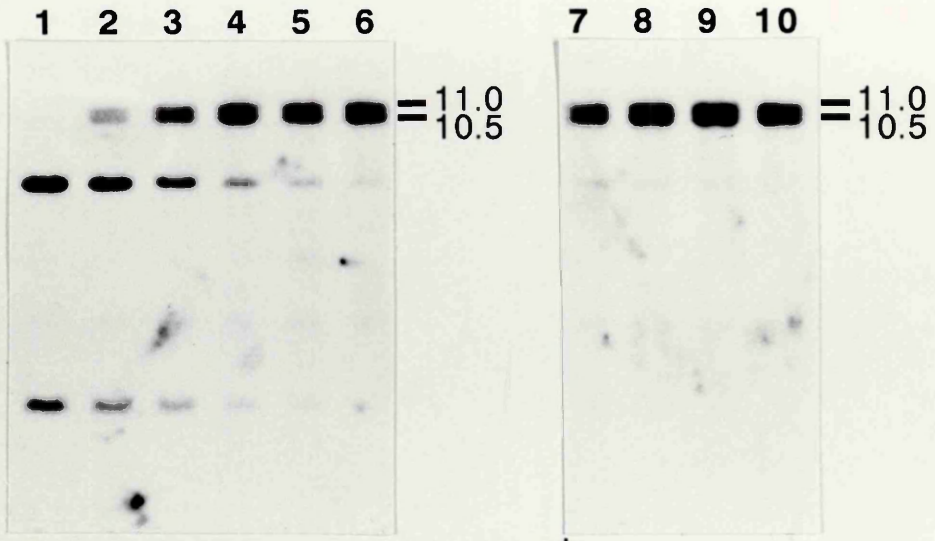
The results obtained from the histological and immunophenotypic analysis of the samples obtained from the majority of this group of patients have been described previously (Krajewski *et al.* 1988).

Three immunoblastic T-cell lymphomas and two cutaneous T-cell lymphomas showed germline Ig genes and rearrangement of the TCRβ gene(s) (Figure 3.10). In cases 200 and 201 TCRβ gene rearrangements were detected only following restriction digestion with EcoRV, or following electrophoresis of EcoRI digested DNA in 0.7% agarose gels (Figure 3.10). Four of the cases also showed rearranged TCRγ genes (Figure 3.11).

Five cases classified as AIL were analysed. In samples 107, 112 and 347 TCRβ gene rearrangement was detected and IgH genes were germline (Figure 3.12). A sample from case 204 showed IgH and IgLκ gene rearrangement and germline TCRβ genes. The IgH gene rearrangement detected in this sample is shown in Figure 3.12. Sample 383 showed TCRβ gene rearrangement in addition to IgH and IgL gene rearrangement (Figure 3.12). Immunophenotyping of cases 204 and 383 indicated that B-cells formed 20-30% of the cellular infiltrate. Restricted expression of IgL was not detected in either case. In the sample obtained from patient 383 there was additional evidence to indicate the presence of a clonal T-cell population in the lymph node biopsy as tumour cells showed reactivity with the clonotypic antibody MX11. However no rearrangements were detected in this sample using the Vβ8 probe. Four of the samples were analysed for TCRγ gene rearrangements. Two rearrangements were detected in sample 107, these are shown in Figure 3.11 (panel a). TCRγ gene rearrangements were not detected in samples 347 and 383. Multiple faint rearranged bands were detected in EcoRI digested DNA from sample 204 (Figure 3.11, panel b). This result indicates the presence of a polyclonal T-cell population in the sample (Boehm *et al.* 1987b).

Figure 3.9. Panels a and b show the results of Southern blot analyses of DNA samples digested with HindIII and hybridized with the JH probe. DNA extracted from placenta was mixed with DNA extracted from leukaemic cells, as described in the text. The proportion of leukaemic cell DNA in each sample is shown in the table (panel c). *, densitometry was used to estimate the percentage of the DNA sample in which clonal IgH gene rearrangements were present. Densitometric analysis was performed on the autoradiograph shown in panel a. Panel b shows that IgH gene rearrangement can be detected in samples in which 2% or more of the cells contain the rearrangement. The germline bands are indicated with thick lines and the sizes of the germline fragments are given in kb. The placental sample used in these experiments has a DNA polymorphism in the JH region of one of the IgH genes, thus two germline bands are seen. The detection of variant HindIII fragments with the JH probe has been reported previously (Fey and Wainscoat 1988). Polymorphisms in this region are caused by the presence of variable numbers of 50 base pair tandem repeats in an hypervariable region 5' of the JH segments (Silva et al. 1987).

Figure 3.9. Densitometric analysis of IgH gene rearrangements in DNA samples containing known proportions of leukaemic cell DNA.



a. b.

Lane No.	1	2	3	4	5	6	7	8	9	10
Leukaemic cell DNA as % of total DNA	100	75	50	25	15	10	10	5	3	2
DNA with IgH gene rearrangements as % of total DNA*.		72.4	50.5	25.6	17	7.8				

c.

Vβ region rearrangements.

Three cases of T-cell NHL in addition to case 383, discussed above, showed reactivity with the T-cell clonotypic antibodies MX11 or 421C1. Figure 3.13 shows the results of Southern blot analysis of these three cases using the Vβ probes. The rearrangements detected using the Vβ probes are shown alongside the results obtained from Southern blot analysis using the Cβ probes, in order to demonstrate comigration of the Vβ and Cβ containing rearranged fragments.

Case 200 showed reactivity with antibody 421C1 in over 90% of tumour cells and TCRβ gene rearrangement was detected using the Vβ5 probe (Figure 3.13, panel b). No rearrangement was detected in BamHI digested DNA from this sample using the Cβ probe (Figure 3.13, panel a). This was due to comigration of the rearranged fragment with the germline fragment, as shown by the location of the rearranged band that was detected with the Vβ5 probe in the adjacent panel of Figure 3.13 (panel b). Sample 347 showed reactivity with MX11 and a rearrangement was detected with the Vβ8 probe which comigrated with one of the rearrangements detected using the Cβ probe (Figure 3.13, panels c and d). In sample 464, which was obtained from a patient with mycosis fungoides, approximately 70% of tumour cells reacted with MX11 while most of the remaining tumour cells stained with 421C1. A rearrangement was detected with the Vβ8 probe and this comigrated with the rearrangement detected in BamHI digested DNA using the Cβ probe. However no rearrangement was detected using the Vβ5 probe to analyse this sample.

B-cell NHLs.

In 5/6 cases of B-cell NHL rearrangements of the IgH and IgL genes were detected (Figures 3.14 and 3.15). In 4/5 cases with rearranged Ig genes monotypic IgL expression was reported. Samples 236 and 238 showed IgLκ gene rearrangement (Figure 3.15, panel a) and were reported to show IgLκ light chain restriction. In sample 238 a rearrangement was also detected using the κde probe (Figure 3.15, panel c), indicating that deletion of one IgLκ gene had occurred prior to successful rearrangement of the second gene. In samples 171 and 239 rearrangement of the κde locus was detected (Figure 3.15, panels b and c) and both samples showed IgLλ light chain restriction. In sample 239 both Cκ alleles were deleted, however in sample 171 only germline fragments were detected using the Cκ probe, most probably reflecting

Table III.II. AIL, angioimmunoblastic lymphadenopathy; B, B-cell NHL; C, cutaneous; CB, centroblastic; CC, centrocytic; CTCL, cutaneous T-cell lymphoma; D, deleted; Foll, follicular; G, germline; Imm, immunoblastic; MF, mycosis fungoides; NK, not known; P, pleomorphic; R, rearranged; R2, rearranged on both alleles; T, T-cell NHL; T-pred, T-cell predominant.

Table III.II. Clinical features, diagnoses and the results of molecular analyses for the NHL cases.

Patient No.	Age/Sex	Diagnosis	Clinical stage	Gene rearrangements						
				IgH	Ck	kde	TCR*B*	TCR*γ*	V*B* 5	V*B* 8
169	46/M	B, T-pred	IA	G	G	G	G			
171	38/F	B, Foll	IVA	R2	G	R	G			
228	45/M	B, Foll	IVA	R2	G	R	G			
236	42/F	B, Foll	IVA	R2	R	G	G			
238	47/M	B, CB	IVB	R2	R	R	G			
239	69/F	B, CC	IVA	R2	D	R	G			
195	15/F	CTCL, P	IVB	G			R	R		
197	65/F	T, Imm	IB	G			R	G		
200	43/M	T, Imm	IIIA	G			R	R	R	
201	74/M	T, Imm	IIIB	G			R	R		
464	22/M	MF	IVB	G			R2	R	G	R
107	62/M	AIL	IIIA	G			R2	R		
112	52/F	AIL	NK	G			R			
204	65/F	AIL	IIIB	R2	R	G	G			
347	52/F	AIL	IIIB	G			R2	G		R
383	66/F	AIL	IIIA	R	R	G	R	G		G

Figure 3.10. Southern blot analyses were performed using the Cß probe. The patient numbers are given at the top of each lane. DNA samples were digested with the restriction enzymes shown at the base of each panel and electrophoresed on 0.8% (panels a and c) or 0.7% (panels b, d and e) agarose gels. P, control DNA extracted from placenta. The germline bands are indicated with thick lines and the sizes of the germline fragments given (in kb) on the right of each panel. Rearranged bands are indicated with arrows.

Figure 3.10. Detection of TCRB gene rearrangements by Southern blot analysis of DNA samples from patients with T-cell NHL.

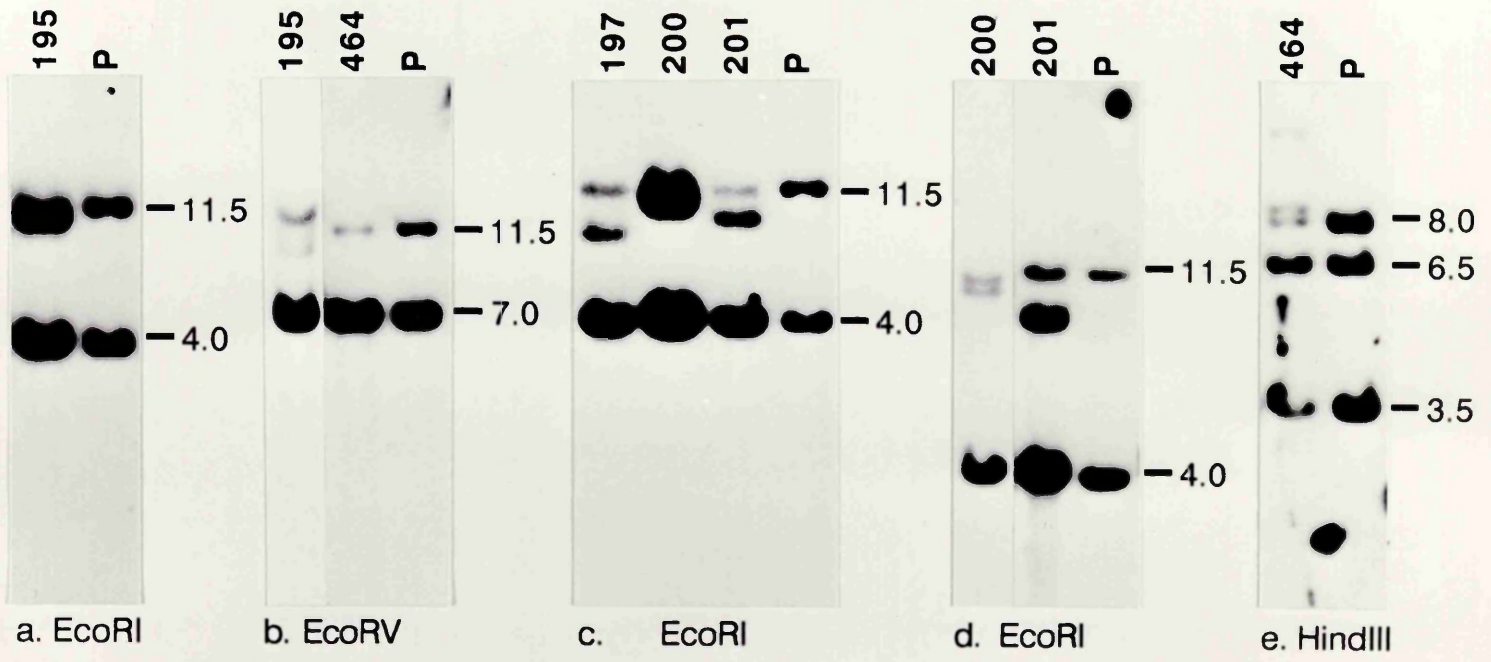


Figure 3.13. Detection of TCRB V-region rearrangements by Southern blot analysis of DNA obtained from tumour samples that showed reactivity with MoAbs MX11 and 421C1.

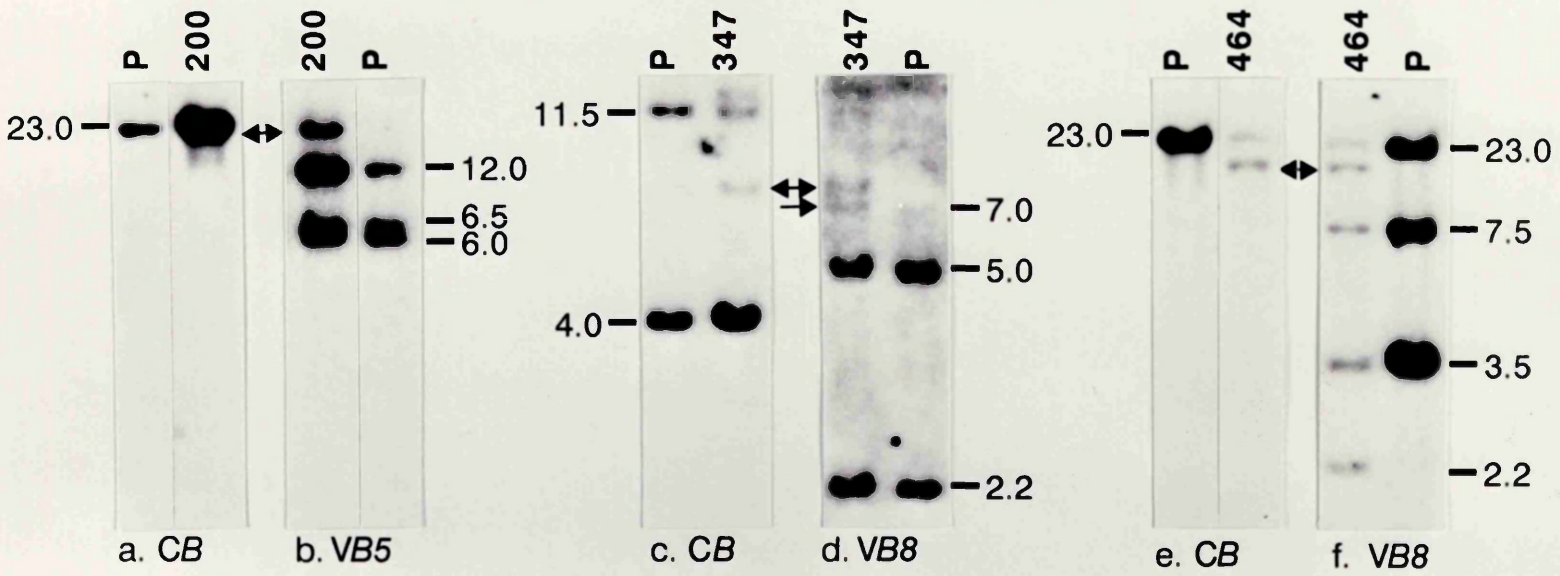


Figure 3.14. DNA samples were digested with HindIII and analysed using the JH probe. P, control DNA extracted from placenta. Germline bands are indicated with thick lines and the sizes of the germline fragments (in kb) given on the right of the panels. The unrearranged fragment that contains the JH region is 10.5 kb. The 3.5 kb band that is seen in sample and control lanes is of unknown origin.

Figure 3.14. Detection of IgH gene rearrangements by Southern blot analysis of DNA samples from patients with B-cell NHL.

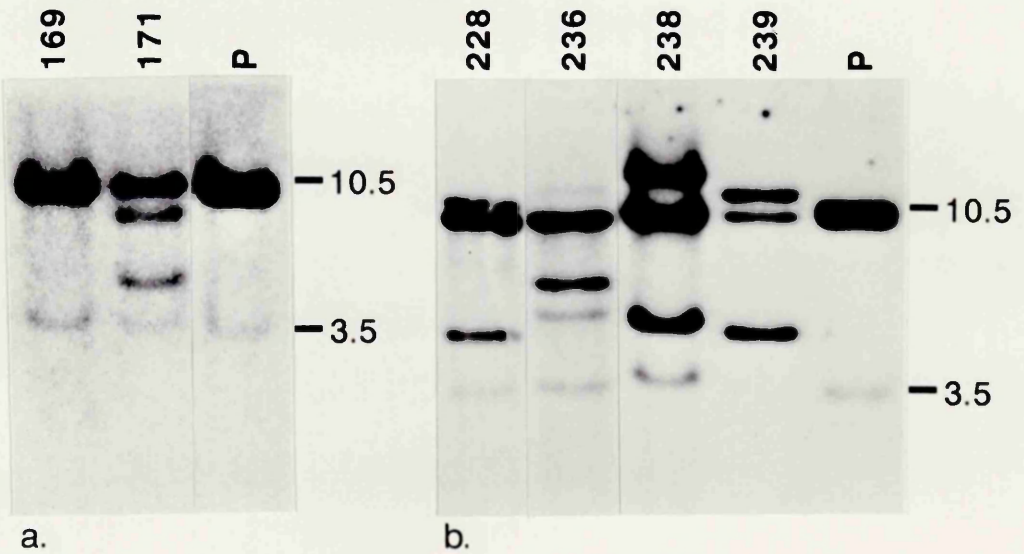
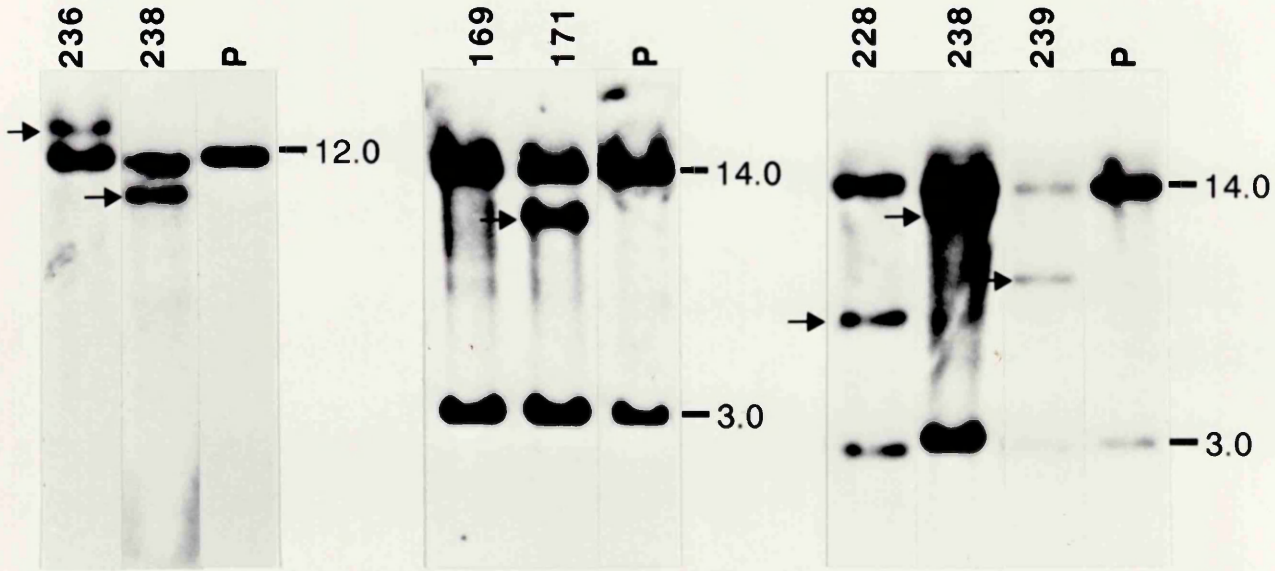


Figure 3.15. DNA samples were digested with BamHI and analysed with the CK or Kde probe as indicated at the base of each panel. P, control DNA extracted from placenta. Germline bands are indicated with thick lines and the sizes of the germline fragments given (in kb) on the right of each panel. The germline Kde-containing fragment is 14.0 kb, the 3.0 kb band detected in BamHI digested DNA using the Kde probe has been previously described as a pseudogene (Williams et al. 1987). Rearrangements are indicated with arrows.

Figure 3.15. Detection of IgL gene rearrangements by Southern blot analysis of DNA samples from patients with B-cell NHL.



the presence of non-tumour cells in the biopsy. In case 228, a follicular lymphoma, no IgH or IgL chain expression had been detected on immunophenotyping, however rearrangements were detected with the J_H and κde probes (Figure 3.14, panel b; Figure 3.15, panel c). No TCRβ gene rearrangements were detected in these samples.

Case 169 was classified as a T-cell predominant B-cell lymphoma. Immunophenotypic analysis indicated that the lymph node was infiltrated by small lymphocytes with T-cell markers (90% CD3+, CD4+ and 10% CD8+) and 1-2% blast cells including a small number of multinucleated cells. The blast cells expressed B-cell markers (CD19 and CD22) and monotypic IgLκ. No Ig or TCRβ gene rearrangements were detected in the sample from this case (Figures 3.14 and 3.15). Ig gene rearrangements were detected only on prolonged autoradiograph exposure in reconstituted DNA samples in which 2% of the total DNA was known to contain a clonal gene rearrangement (see previous section and Figure 3.9). It is therefore likely that the neoplastic cell population in sample 169 was too small for the detection of any gene rearrangements that might be present in this population.

3.3.3 Diagnostically difficult cases.

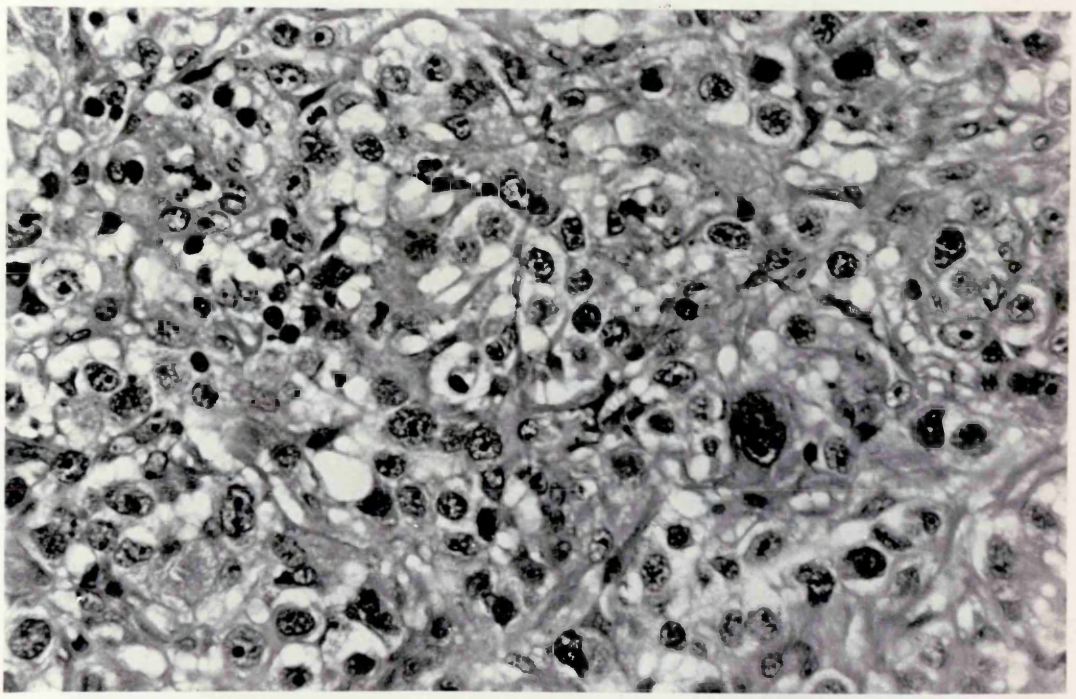
The biopsies from the patients in this group were histologically and immunophenotypically heterogeneous. The clinical features, diagnoses and the results of immunophenotypic and genotypic analysis are shown in Figures 3.16-3.12 together with photomicrographs of tissue sections.

Case 124: NSHD or B-cell NHL?

Case 124 presented with an anterior mediastinal tumour, which when biopsied showed sheets of large mononuclear blast cells, with small numbers of multinucleated cells surrounded by collagenous septae (Figure 3.16). Morphologically the differential diagnoses included NSHD and sclerosing mediastinal B-cell lymphoma. The blast cells were CD45-, CD15+, CD30+, HLA-DR+ and CD25+ and did not express T- or B-cell antigens. On the basis of the tumour phenotype, histology and clinical presentation, a working diagnosis of NSHD was made.

Figure 3.16. Results of histological, immunophenotypic and genotypic analysis for case 124. The photograph is of an haemotoxylin and eosin-stained section and shows an infiltrate of large blast cells with a small number of multinucleated cells. G, germline; ND, not done; NSHD-2, NSHD type II; R1, one allele rearranged ; R2, two alleles rearranged; -, <5% positive cells; ++, >50% positive cells. The histological and immunophenotypic analyses were performed by Dr. A.S. Krajewski.

Figure 3.16. Case 124.



Patient Age: 40
Patient Sex: F
Clinical stage: IB

Immunophenotype:

CD3	4	8	19/22	*K*	λ	CD15	30
-	-	-	-	-	-	++	++

Differential diagnosis: NSHD/B-NHL

Genotype:

IgH	C_K	Kde	TCR*B*	TCR*γ*
R2	R1	G	G	ND

Final diagnosis: NSHD-2

Molecular analysis of the biopsy from Case 124 showed rearrangement of the IgH and IgLκ genes (Figure 3.21). No TCRβ gene rearrangements were detected.

Case 167: MCHD or T-cell NHL?

The initial biopsy obtained from case 167 was an extradural tumour (frontal lobe). The biopsy contained a mixed infiltrate of small lymphocytes with small numbers of larger mononuclear blast cells, but no RS cells (Figure 3.17). A diagnosis of pleomorphic T-cell lymphoma was made. The patient subsequently developed axillary lymphadenopathy and a lymph node biopsy was considered to show features of MCHD, with numerous RS cells expressing CD15 and CD30.

Unfortunately no tissue from the extradural tumour was available for genotyping. Analysis of the lymph node biopsy showed germline IgH (Figure 3.21), IgL and TCRβ genes, though the hybridization pattern observed with the Cβ probe was consistent with the presence of a polyclonal T-cell population.

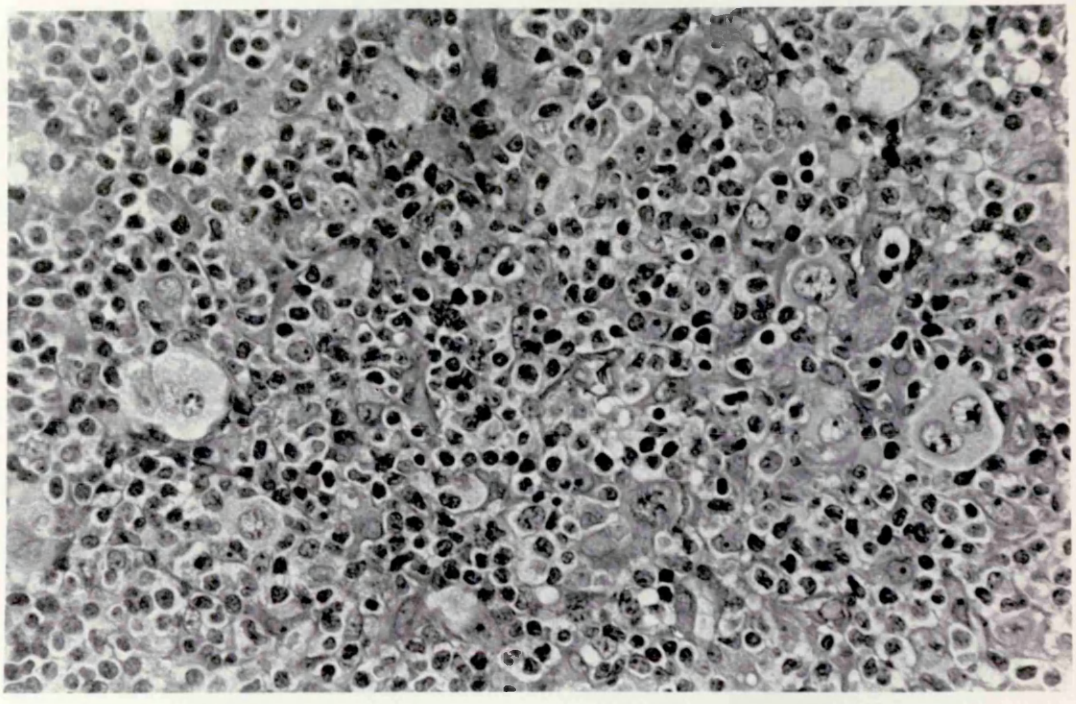
Case 168: NSHD or T-cell NHL?

Case 168 presented with lymphadenopathy and a pulmonary infiltrate. A lymph node biopsy showed infiltration by small and medium-sized lymphocytes, predominantly T-cells, with B-cells expressing either IgLκ or IgLλ present in small residual follicles. No RS cells were seen, though small numbers of large mononuclear cells expressing CD15 and CD30 were present. A second lymph node biopsy showed a similar infiltrate to the first biopsy, with complete effacement of normal node architecture, capsular fibrosis and collagenous septae dividing the lymph node (Figure 3.18). No typical RS cells were seen. The differential diagnosis included NSHD and T-zone lymphoma and despite the lack of RS cells, a diagnosis of HD was considered most likely on the basis of the histological findings.

The second biopsy was analysed for gene rearrangements and showed germline IgH, IgL and TCRβ genes.

Figure 3.17. Results of histological, immunophenotypic and genotypic analysis for case 167. The photograph is of an haemotoxylin and eosin-stained section and shows several typical Reed-Sternberg cells. G, germline; +, 5-50% positive cells; ++, >50% positive cells; #, positive staining of large mononuclear cells and RS-like cells. The histological and immunophenotypic analyses were performed by Dr. A.S. Krajewski.

Figure 3.17. Case 167.



Patient Age: 35
Patient Sex: M
Clinical stage: IVB

Immunophenotype:

CD3	4	8	19/22	*K*	λ	CD15	30
++	++	+	+	+	+	#	#

Differential diagnosis: MCHD/T-cell NHL

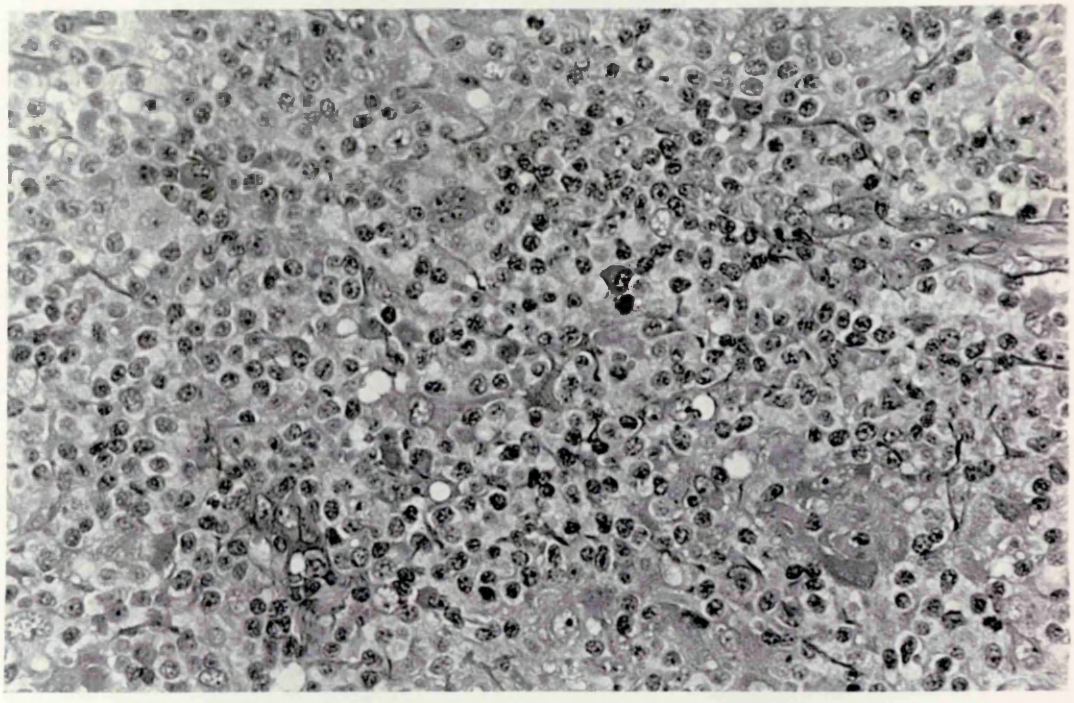
Genotype:

IgH	C*K*	Kde	TCR*B*	TCR*y*
G	G	G	G	G

Final diagnosis: MCHD

Figure 3.18. Results of histological, immunophenotypic and genotypic analysis for case 168. The photograph is of an haemotoxylin and eosin-stained section and shows an infiltrate of lymphocytes and plasma cells with a single binucleated cell. G, germline; ND, not done; +, 5-50% positive cells, ++; >50% positive cells; #, positive staining of large mononuclear cells and RS-like cells. The histological and immunophenotypic analyses were performed by Dr. A.S. Krajewski.

Figure 3.18. Case 168.



Patient Age: 69
Patient Sex: M
Clinical stage: IVB

Immunophenotype:

CD3	4	8	19/22	*K*	λ	CD15	30
++	++	+	+	+	+	#	#

Differential diagnosis: NSHD/T-cell NHL

Genotype:

IgH	*Cκ*	Kde	TCR*B*	TCR*γ*
G	G	G	G	ND

Final diagnosis: NSHD

Case 172: MCHD or AIL?

Case 172 had a prodromal history of fever and malaise for 2 years, and presented as an emergency with right upper quadrant pain, pneumonia and splenomegaly. A lymph node biopsy showed obliteration of the lymph node architecture (Figure 3.19) and replacement with a mixed cellular infiltrate mostly composed of T-cells (80% CD8+, CD3+ and 10% CD4+, CD3+). There were small numbers (less than 1%) of multinucleated cells, which were CD30+ and showed fine granular CD15 staining, but no typical RS cells were seen. Some B-cells showing polytypic IgL expression were present in small residual follicles. Plasma cells, eosinophils and prominent high endothelial vessels were also present. In this case the histological features and high numbers of CD8+ cells were considered to be more consistent with AIL, though MCHD could not be excluded.

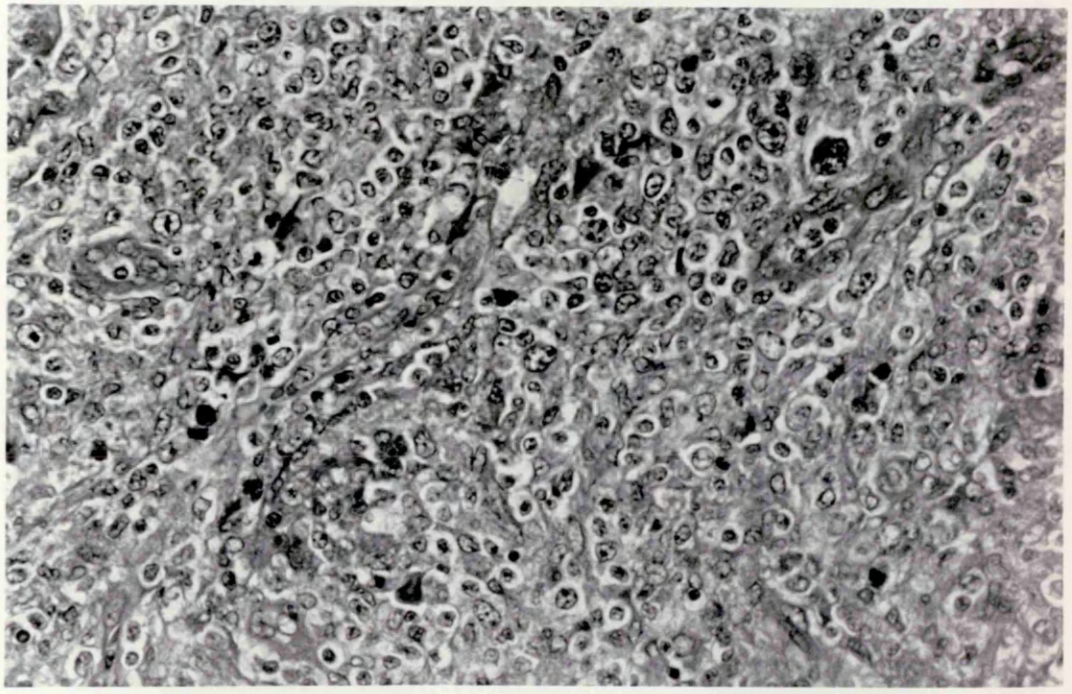
A rearrangement of the IgH gene locus was detected in the sample from patient 172, though the rearranged band was very faint and only detectable following prolonged exposure of the filters to autoradiographic film (Figure 3.21). TCRβ genes and TCRγ genes were germline, though the hybridization pattern detected using the Cβ probe was consistent with that of a polyclonal T-cell population.

Case 196: LPHD or T-cell NHL?

Case 196 had a history of night sweats and fatigue for 4 months prior to presentation with massive hepatomegaly and splenomegaly. Samples obtained from spleen, abdominal lymph nodes and liver showed a diffuse infiltrate of small lymphocytes, many showing nuclear irregularity, occasional macrophages and 1-2% blast cells (Figure 3.20). No multinucleated cells were identified. Over 90% of the cells were CD3+ and CD4+, less than 5% were CD8+ and 1% expressed B-cell markers. Morphologically, many of the features were consistent with nodular lymphocyte and histiocyte predominant HD and this possibility was raised clinically. However, the immunophenotypic findings, the lack of RS cells and the pleomorphic nature of the lymphoid infiltrate was considered to be more consistent with T-lymphocytic, or small cell pleomorphic, T-cell NHL.

Figure 3.19. Results of histological, immunophenotypic and genotypic analysis for case 172. The photograph is of an haemotoxylin and eosin-stained section and shows an infiltrate of small to large blast cells with occasional multinucleated cells (top right). G, germline; R1, one allele rearranged; +, 5-50% positive cells, ++; >50% positive cells; #, positive staining of large mononuclear cells and RS-like cells. The histological and immunophenotypic analyses were performed by Dr. A.S. Krajewski.

Figure 3.19. Case 172.



Patient Age: 56
Patient Sex: M
Clinical stage: IIIB

Immunophenotype:

CD3	4	8	19/22	*K*	λ	CD15	30
++	+	++	+	+	+	#	#

Differential diagnosis: MCHD/AIL

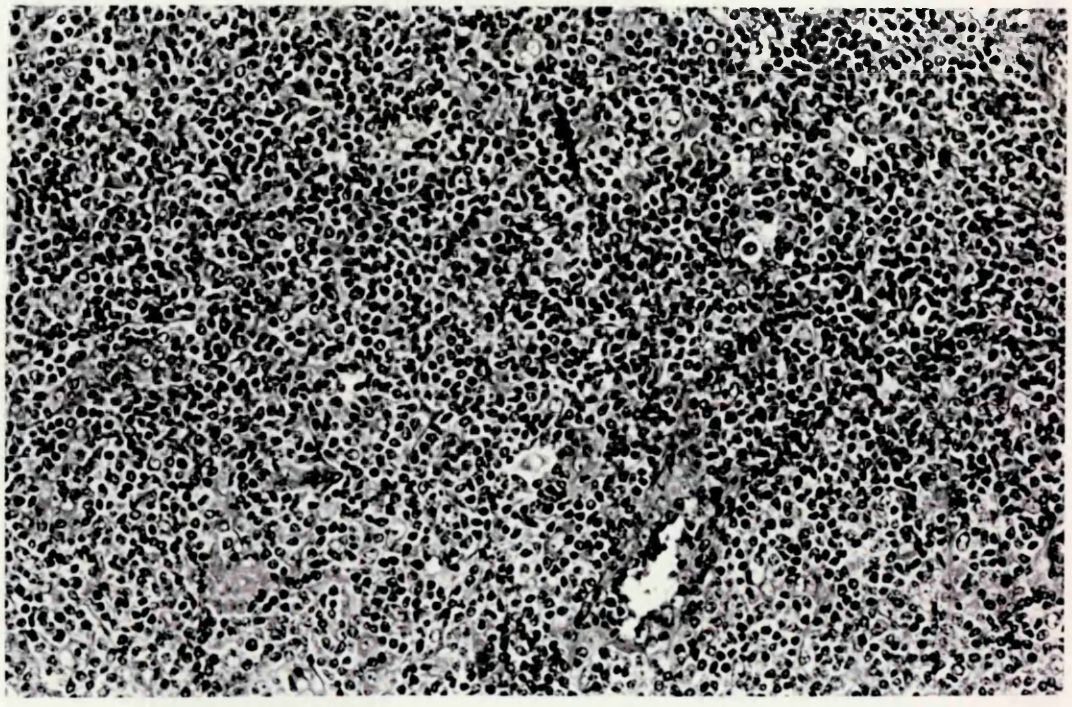
Genotype:

IgH	*Cκ*	Kde	TCR*B*	TCR*γ*
R1	G	G	G	G

Final diagnosis: AIL

Figure 3.20. Results of histological, immunophenotypic and genotypic analysis for case 196. The photograph is of an haemotoxylin and eosin-stained section and shows an infiltrate of small lymphocytes with occasional blast cells. G, germline; R1, one allele rearranged; -, <5% positive cells; ++; >50% positive cells. The histological and immunophenotypic analyses were performed by Dr. A.S. Krajewski.

Figure 3.20. Case 196.



Patient Age: 35
Patient Sex: F
Clinical stage: IVB

Immunophenotype:

CD3	4	8	19/22	*K*	λ	CD15	30
++	++	-	-	-	-	-	-

Differential diagnosis: LPHD/T-cell NHL

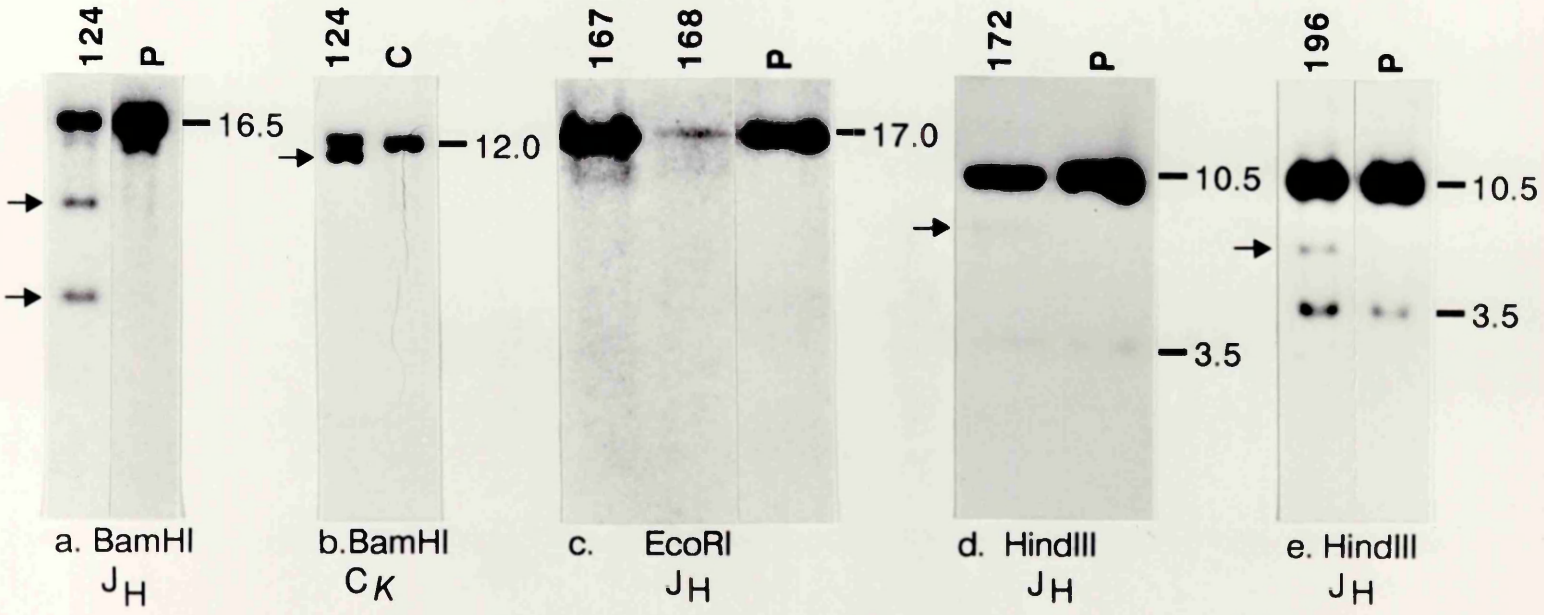
Genotype:

IgH	*Ck*	Kde	TCR*B*	TCR*y*
R1	G	G	G	G

Final diagnosis: T-cell NHL

Figure 3.21. DNA samples were analysed using the restriction enzymes and probes indicated at the bottom of each panel. P, control DNA extracted from placenta; C, control DNA extracted from PBMCs of patient 124. The germline bands are indicated with thick lines and the sizes of the germline fragments are given (in kb) on the right of each panel. Rearranged bands are indicated with arrows. The faint band seen below the germline band in BamHI- and EcoRI-digested DNA hybridized with the JH probe (panels a and c) is seen in samples and controls and is of unknown origin. The faint 3.5 kb band seen in HindIII-digested DNA hybridized with the JH probe (panels d and e) is seen in sample and control lanes and is of unknown origin.

Figure 3.21. Detection of Ig gene rearrangements by Southern blot analysis of DNA samples from the diagnostically difficult cases.



Genotypic analysis of the sample from case 196 showed an IgH gene rearrangement (Figure 3.21) and germline TCRβ and TCRγ genes. The hybridization pattern seen with the constant region probe suggested that there was a significant population of polyclonal T-cells present.

3.3.4 Bcl-2 gene analysis.

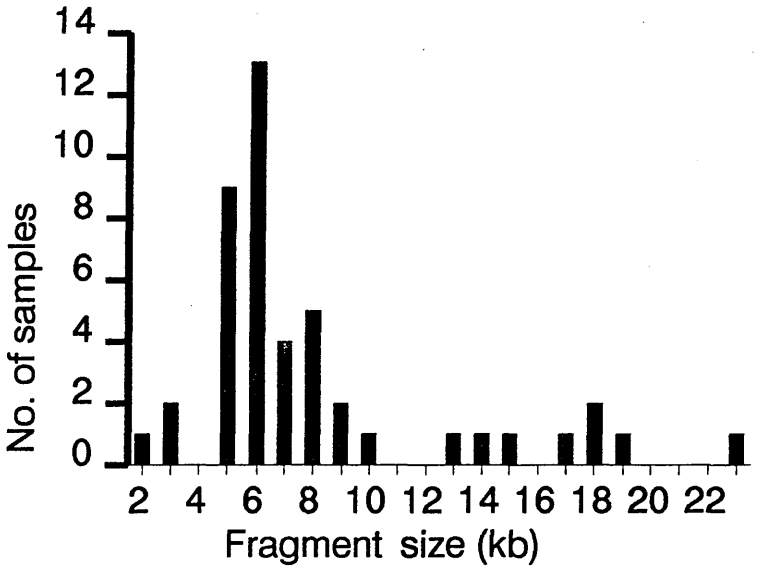
In each of the 5 B-cell lymphomas in which IgH gene rearrangement was detected, one of the rearranged fragments detected in HindIII digested DNA appeared to be between 5kb and 5.6kb in size (Figure 3.14). The size distribution of rearranged IgH J_H fragments detected in HindIII digested DNA in other samples analysed was evaluated.

The rearrangements detected in the HD and AIL samples described in sections 3.3.1 and 3.3.2 and those detected in an additional 12 B-cell lymphomas and 1 unclassified lymphoma (sample number 369) were included in the analysis. A total of 45 rearranged fragments were detected in 29 lymphomas. The size distribution of rearranged bands detected following HindIII digestion is shown in Figure 3.22. Twenty two of a total of forty five rearranged fragments were between 4.0 and 6.0kb in size.

In order to determine whether the consistent size of these rearranged fragments was related to the t(14;18)(q32;q21) chromosomal translocation that is present in a substantial proportion of follicular and diffuse B-cell NHLs, selected samples were analysed using the bcl-2 probes. Fourteen samples with rearranged fragments of between 4.0 and 7.0kb in size were analysed with either the H4.3 probe or both the HR1.5 and RH2.8 probes. Three additional samples were analysed using the RH2.8 probe only (Table III.III).

Rearrangements of the bcl-2 locus were detected in two B-cell NHLs (cases 228 and 352) as shown in Figure 3.23. In both cases one of the rearranged bands detected using the bcl-2 probes comigrated with a rearranged band that was detected with the J_H probe.

Figure 3.22. The size distribution of rearranged JH region fragments detected in HindIII digested DNA samples.



DNA samples were digested with the restriction enzyme HindIII and hybridized with the JH probe. The sizes of rearranged fragments were estimated using the fragments of HindIII-digested phage lambda DNA as standards.

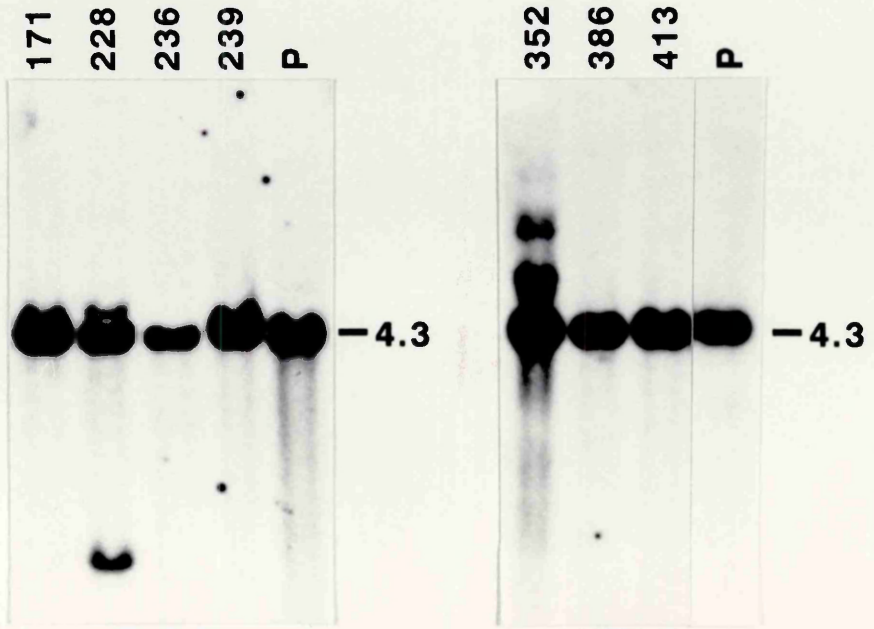
Table III.III. Southern blot analysis of lymphoma samples for rearrangements of the major breakpoint cluster region of the bcl-2 gene.

Patient No.	Diagnosis	Size of rearranged HindIII fragments hybridizing to pHj	Configuration of bcl-2 (*)
51	B (LG)	4.5	G (c)
53	B (FoII)	5.5, 19	G (c)
59	B (FoII)	2.7, 5.4	G (c)
62	B (CC)	2.8, 4.5	G (c)
124	HD/B	5.8, 8.0	G (a,b)
171	B (FoII)	5.1, 8.4	G (a,b,c)
204	AIL	5.4, 6.0	G (a,c)
228	B (FoII)	5.2, 13	R (a,b)
236	B (FoII)	5.6, 6.8	G (a,b,c)
238	B (CB)	5.4, 18	G (a,b)
239	B (CC)	5.0, 15	G (a,b)
352	B (CB)	4.9, 6.6	R (b)
373	HDNS	1.7, 4.7	G (b)
386	HDMC	6.0	G (a,b,c)
413	B (CB)	4.7, 5.8	G (c)
430	HDNS	4.8, 7.4	G (b)
433	B (FoII)	5.0, 5.4	G (a,b,c)

B, B-cell NHL; CC, centrocytic; CB, centroblastic; FoII, follicular; G, germline; L, low grade; R, rearranged (*) the probes used for bcl-2 gene rearrangement analyses are indicated in parenthesis; (a), HR1.5; (b), RH2.8; (c), H3.4.

Figure 3.23. DNA samples were digested with HindIII and hybridized with the bcl-2 probe RH2.8, which contains the MBR, or HR1.5, as indicated below the panels. P, control DNA extracted from placenta. The germline bands are indicated with thick lines and the size of the germline fragment given (in kb) on the right of each panel.

Figure 3.23. Detection of rearrangements of the bcl-2 gene by Southern blot analysis.



3.4 DISCUSSION.

3.4.1 Hodgkin's Disease.

As part of a study into the aetiology and pathogenesis of HD, 35 unselected cases of HD were analysed for the presence of Ig and TCR gene rearrangements.

Ig gene rearrangements in HD.

Ig gene rearrangements were detected in approximately one quarter (9/35) of the cases, while none of the cases showed TCRβ gene rearrangement. These data indicate that clonal cell populations showing genotypic features of B-cells may be found in a significant proportion of HD biopsies. The data are in agreement with a number of other reports, in which Ig gene rearrangements but not TCRβ gene rearrangements were detected in HD samples (O'Connor *et al.* 1986; Weiss *et al.* 1986b; Brinker *et al.* 1987; Raghavachar *et al.* 1988) (Table III.IV). In two of the HD cases with rearranged IgH genes, IgL gene rearrangement was not detected, suggesting that the clonal cell population has been expanded at an early stage in B-cell differentiation. IgL gene rearrangement was not detected in cases without rearranged IgH genes, though this has been reported by others (Weiss *et al.* 1986b; Brinker *et al.* 1987; Herbst *et al.* 1989).

TCRβ gene rearrangements in HD.

No TCRβ gene rearrangements were detected in the 35 cases analysed in this study and no TCRγ gene rearrangements were detected in the 10 cases analysed with the TCRγ gene probe. Griesser *et al.* (1987) found TCRβ and TCRγ gene rearrangements in 4/22 and 15/22 HD samples respectively, while Herbst *et al.* (1989) detected TCRβ gene rearrangement in 6/39 cases of HD. In addition Dallenbach and Stein (1989) have reported that in 30% of HD cases the RS cells express TCRβ. However in the majority of other reports TCRβ gene rearrangements were not detected in HD samples (Table III.IV). The reasons for the variations in the findings of different groups are obscure. Sundeen *et al.* (1987) suggested that technical problems may have been responsible for the high number of TCRγ gene rearrangements reported by Griesser *et al.* (1987).

Table III.IV. a, O'Connor et al. (1986); b, Weiss et al. (1986); c, Knowles et al. (1986); d, Brinker et al. (1987); e, Sundeen et al. (1987); f, Griesser et al. (1987); g, Raghavachar et al. (1988); h, Roth et al. (1988); i, Herbst et al. (1989); j, this study. *, the cases analysed by Weiss et al. (1986) and Brinker et al. (1987) were selected for high numbers of HRS cells. #, the numbers given in parenthesis show the results obtained by Sundeen et al. (1987) after enriching the samples for HRS cells. The results obtained with unfractionated samples are included in the totals.

Table III.IV. Detection of Ig and TCR gene rearrangements in HD samples.

No. of cases	Ig gene rearrangement			TCR gene rearrangement			Reference
	IgH	IgH+ IgL	IgL	TCR*B*	TCR*B* + TCR*y*	TCR*y*	
36	0	2	0	0		ND	a
8*	2	2	3	0		ND	b
18	2	0	0	2		ND	c
11*	0	4	1	0		ND	d
8#	0(2)	0	0(1)	0(0)		ND	e
22	2		ND	0	4	11	f
32	5	0	0	0	0	0	g
18	0	0/11	0/11	1		ND	h
39	4	0	1	5	1	1	i
35	2	7	0	0		0/9	j
227	32		20/198	13		17/102	Total
	14.1		10	5.7		16.6	%

Difficulties may be encountered in differentiating HD from some T-cell lymphomas, in particular the T-zone lymphomas, including those classified as AIL (Lukes and Tindle 1975; Krajewski *et al.* 1988). Thus differences in lymphoma classification in different centres could explain some of the variations in the reported results.

Gene rearrangements in different subtypes of HD.

In this study IgH gene rearrangements were detected in cases of NS, MC and LDHD. These results combined with data from other groups show that IgH gene rearrangements may be detected in the NS, MC and LP subtypes of HD at similar frequencies (using the X^2 test, $p>0.5$) (Table III.V). The number of cases of LDHD are too small to include in this analysis. However comparison of the frequency of gene rearrangements detected in LDHD relative to the frequency detected in all other subtypes combined again shows no significant difference (Fisher's exact test, two tailed, $p=0.16$). The cases analysed by Brinker *et al.* (1987) and Weiss *et al.* (1986b) have been excluded from the analyses, as these cases were selected on the basis of high HRS cell numbers.

These data do not support the suggestion that LPHD is a distinct B-cell disorder. However the criteria used for the diagnosis of LPHD have recently altered (Nicholas *et al.* 1990). The summarized data is therefore likely to include cases that would no longer be classified as LPHD. In addition HRS cells are usually very scarce in LPHD, thus if the Ig gene rearrangements are present in the HRS cells the data do not provide convincing evidence against the hypothesis. The two cases of LPHD analysed in this study were estimated to contain less than 1% HRS cells, thus clonal Ig gene rearrangements present in these cells would not be detectable.

Are the Ig gene rearrangements present in HRS cells?

HD biopsies contain a variety of different cell populations in addition to HRS cells. In order to draw any conclusions about the lineage and clonality of HD from the results of gene rearrangement analysis it is necessary to determine whether the rearrangements are present in the HRS cells. The detection of Ig gene rearrangements in a minority of cases of all subtypes of HD, may reflect the low numbers of HRS cells present in most tumour biopsies. HRS cells frequently make up less than 2% of the total cell population

Table III.V. Detection of TCR and Ig gene rearrangements in samples from patients with different subtypes of HD.

Subtype	No. of cases	Ig gene rearrangements		TCR gene rearrangements	
		IgH	IgL	TCR*B*	TCR*γ*
NS	111	19	15/98	7	7/46
MC	44	5	1/38	3	4/33
LP	21	3	0/16	1	3/14
LD	13	4	1/5	0	3/9

The data presented in this table are summarized from the reports by Knowles et al. (1986); Weiss et al. (1986); Brinker et al. (1987); Griesser et al. (1987); Sundeen et al. (1987); Raghavachar et al. (1988); Roth et al. (1988); Herbst et al. (1989) and this study. The cases of NSHD analysed by Weiss et al. (1986) and the cases analysed by Brinker et al. (1987) were selected on the basis of high HRS cell numbers. The study of Brinker et al. (1987) included two cases of NSHD and one case of MCHD for which differential diagnoses including LDHD were given. These cases have been classified in the table as NS or MCHD, all three cases had IgH gene rearrangements. The results obtained by Sundeen et al. (1987) from the analysis of unfractionated samples have been included in the table.

in HD biopsies and this is close to the limit of sensitivity of gene rearrangement analysis. However a number of authors have suggested that the gene rearrangements detected in HD samples may be present in lymphoid cell populations unrelated to HRS cells (Knowles *et al.* 1986; Griesser *et al.* 1987; Raghavachar *et al.* 1988).

A correlation between the detection of Ig gene rearrangement and HRS cell numbers was observed in this study. Seven of the nine HD cases in which Ig gene rearrangements were detected had more than 8% HRS cells (Figure 3.8). Some other reports have also described a correlation between HRS cell numbers and the detection of Ig and/or TCR gene rearrangement. Weiss *et al.* (1986b) detected Ig gene rearrangements in 7/8 cases of NSHD selected on the basis of high RS cell numbers. Sundeen *et al.* (1987) analysed HD samples enriched for HRS cells, and were able to detect Ig gene rearrangements in the enriched fractions of 3/6 samples, but not in the original material. Herbst *et al.* (1989) also evaluated HRS cell numbers on the basis of CD30 positivity, and found that Ig/TCR gene rearrangements were detected more frequently in HD cases with large numbers of HRS cells.

The relationship between HRS cell numbers and the cells containing clonal Ig gene rearrangements was evaluated for the nine cases in which rearrangements were detected. The proportion of cells containing a clonal gene rearrangement was estimated from densitometric measurement of the intensity of the rearranged band detected following autoradiography. The numbers of cells estimated by densitometry to contain clonal gene rearrangements were similar to the numbers of HRS cells (as assessed morphologically) present in eight of the nine samples. The result obtained from sample 382 is discussed further below.

Additional indirect evidence suggesting that the Ig gene rearrangements detected in case 386 are present in the HRS cells is provided by the results of karyotypic analysis. Detailed karyotyping was performed on 5 of the cases analysed in this study, cases 237, 382, 384, 385, 386 and 387 (Banks *et al.* 1991). Abnormal karyotypes were detected in cases 382 and 386, both of which showed IgH and IgL gene rearrangement. No karyotypic abnormalities and no Ig gene rearrangements were detected in the other three cases. Teerenhovi *et al.* (1988) found that the abnormal clonal karyotypes detected in 2 cases of HD were restricted to the HRS cells. If this finding can be extrapolated to other cases, the results suggest that the Ig gene rearrangements and

karyotypic abnormalities detected in case 386 were present in the same cell population, probably the HRS cells. The results obtained for case 382 are not consistent with this interpretation, as no HRS cells were observed in the biopsy in which Ig gene rearrangements and karyotypic abnormalities were detected.

The above data offer support for the hypothesis that the Ig gene rearrangements detected in HD biopsies are present in the HRS cells.

Are there HRS cells with germline Ig genes?.

Some HD cases with large numbers of HRS cells do not have detectable Ig gene rearrangements. In this study Ig gene rearrangements were not detected in 6 cases in which HRS cell numbers were estimated to be over 8% and 7 cases in which HRS cell numbers were estimated to be 3-8% (Figure 3.8). Others have reported similar findings (Knowles *et al.* 1986; O'Connor *et al.* 1987; Sundeen *et al.* 1987; Herbst *et al.* 1989).

There are two possible interpretations of the data. The results are consistent with the possibility that HRS are derived from lymphocytes at varying stages of differentiation. Alternatively clonal B-cell populations unrelated to HRS cells may be present in a minority of HD biopsies.

Restricted expression of IgL on B-cells in HD biopsies has not been reported, suggesting that the latter interpretation is unlikely to be correct (Brinker *et al.* 1987; Herbst *et al.* 1989). Restricted IgL expresssion was not observed on B-cells in the HD biopsies with IgH and IgL gene rearrangements included in this study.

Case 382.

The results obtained for patient 382 offer some support for the hypothesis that Ig gene rearrangements are present in a cell population distinct from HRS cells. IgH and IgL gene rearrangements were detected in a lymph node biopsy that was classified histologically as "reactive", but were not detected in a spleen sample showing features of NSHD. Alternatively this result could be due to differences in the extent of disease involvement in the parts of the lymph node sent for genotypic and histological analysis.

It should also be noted that some lymphoblasts were present in the "reactive" lymph node. A clonal karyotypic abnormality was also detected in this sample (Banks *et al.* 1991), suggesting that this latter explanation may be correct. Other investigators have shown that karyotypic abnormalities in HD are restricted to the HRS cells (Teerenhovi *et al.* 1988).

The detection of EBV genomes in HD.

The 35 HD cases included in this study were also analysed for the presence of EBV genomes in a parallel investigation (Gledhill *et al.* in press). EBV DNA was detected by Southern hybridization in 11 of the 35 HD cases included in this study. These data have been included in Table III.I. In the 10 EBV positive cases analysed, the viral episomes were shown to be monoclonal in origin implying that the virus-infected cell has undergone clonal expansion. Similar results have been reported by a number of other groups (Weiss *et al.* 1987b; 1989; Anagnostopoulos *et al.* 1989; Boiocchi *et al.* 1989; Staal *et al.* 1989; Uccini *et al.* 1989).

The expression of EBV antigens has been shown on HRS cells in some of these cases (numbers 79, 210, 386, 387 and 390; R.F. Jarrett and A. Armstrong, unpublished results). EBV has also been shown to be present in HRS cells of HD biopsies using in situ hybridization (Anagnostopoulos *et al.* 1989; Uccini *et al.* 1989; Weiss *et al.* 1989). The results of these studies have demonstrated that HRS cells are clonal in origin.

It seems probable that the clonal Ig gene rearrangements detected in case 386 are also present in the EBV infected, clonal HRS cells. However in cases 390 and 927 which were EBV positive, no Ig gene rearrangements were detected although HRS cell numbers were estimated at 3-8% and 8% respectively. Similar results were reported by Weiss *et al.* (1987b) and Herbst *et al.* (1989). In these cases the virus may have infected a cell at an early stage of differentiation prior to the rearrangement of Ig/TCR genes. In support of this possibility, Gregory *et al.* (1987) have shown that EBV will infect and immortalize lymphocytes at varying stages of maturity. An alternative explanation is that polyclonal rearrangements of the Ig and/or TCR genes have occurred in EBV-infected cells following transformation.

The results described in this chapter provide support for the hypothesis that HRS cells are derived from lymphoid cells at various stages of differentiation. The characteristic morphologic and phenotypic features of HRS cells may develop as a result of a common event occurring as part of the transformation process. Evidence from a number of groups suggests that in some cases of HD the mechanism of transformation is related to EBV infection (Anagnostopoulos *et al.* 1989; Uccini *et al.* 1989; Weiss *et al.* 1989; Gledhill *et al.* in press).

3.4.2 AIL.

TCR and Ig gene rearrangement in AIL-like lymphomas and AIL.

Analysis of the configuration of the TCR and Ig genes in 5 cases of AIL-like lymphoma demonstrated that this disease is not associated with a consistent pattern of TCR/Ig gene rearrangement. The results indicate that clonal T-cell populations (cases 106, 112, 347 and 383) or clonal B-cell populations (cases 204 and 383) may be detected in affected tissues. In case 383 the data suggest that more than one clonal population is present, as although IgH gene rearrangement has been described in T-cell lymphomas, IgL gene rearrangement has been reported very infrequently in non-B-cell tumours (Pelicci *et al.* 1985; Ha Kawa *et al.* 1986; O'Connor *et al.* 1987). It is unlikely that the TCRβ gene rearrangement detected in this sample was present in the clonal B-cell population, as the presence of a T-cell clone was also indicated by reactivity of tumour cells with MoAb MXII, which is specific for the Vβ region of the TCR.

AIL-like lymphoma has been included in the updated Kiel classification as a low grade T-cell NHL (Stansfeld *et al.* 1988). Most studies have reported the detection of TCRβ gene rearrangements in the majority of cases of AIL-like lymphoma, supporting this classification (Watanabe *et al.* 1986; Weiss *et al.* 1986c; Suzuki *et al.* 1987; Tobinai *et al.* 1988). However, in agreement with the data reported in this study, some samples have been shown to have rearranged Ig genes, while others have germline Ig and TCR genes (Weiss *et al.* 1986c; Suzuki *et al.* 1987; Tobinai *et al.* 1988). Evidence for the occasional presence of multiple clonal cell populations in AIL-like lymphoma has also been derived from karyotypic and gene rearrangement analysis (Fujita *et al.* 1986; Story *et al.* 1987; Kaneko *et al.* 1988).

The results that have been obtained from the analysis of AIL samples lacking morphological evidence of overt lymphoma do not differ markedly from those described above (O'Connor *et al.* 1986; Weiss *et al.* 1986c; Lipford *et al.* 1987b; Suzuki *et al.* 1987; Kaneko *et al.* 1988). From the above and previous discussions (sections 1.3.3 and 3.1.5) it is apparent that the results of immunophenotypic, molecular and karyotypic analyses do not suggest that a clear distinction may be drawn between AIL and AIL-like lymphoma.

Frizzera *et al.* (1989) have proposed a classification system for AIL and related lesions, in which the histological and immunophenotypic features are considered in the context of the results of molecular and karyotypic investigations. The results described in this chapter suggest that monoclonal T- or B-cell populations were present in samples from cases 112, 204 and 347. These cases would be classified, on these and on morphological grounds, as AIL-like-lymphomas as suggested by Frizzera *et al.* (1989). Clonal TCRβ and TCRγ gene rearrangements were also detected in the sample from case 107, however this case subsequently resolved spontaneously. Frizzera *et al.* (1989) have suggested that such cases, in which evidence of sequential changes in the predominant clonal cell population(s) are found, could be classified as AIL-like dysplasias. The clinical usefulness of this classification may be limited, however, by the requirement for sequential investigations. Case 383, in which evidence for the presence of multiple clonal cell populations was detected, would also be classified as AIL-like dysplasia.

The pathogenesis of AIL.

O'Connor *et al.* (1986) suggested that one possible interpretation of their data is that AIL is initially a polyclonal disease of B- and T-lymphocytes, in which monoclonal cell populations were likely to arise as secondary events. Lipford *et al.* (1987a) propose a similar model of disease pathogenesis, with expansion of clonal B- or T-cell populations occurring in the context of an underlying disorder of immune regulation. In the latter study changes in the predominant clonal lymphocyte population were detected in sequential samples. Similar findings were reported by Kaneko *et al.* (1988). Lipford *et al.* (1987b) make the observation that the presence of clonal lymphocyte populations in AIL biopsies does not necessarily imply overt malignancy. The spontaneous resolution of disease seen in patient 107 is consistent with this notion.

According to this model AIL, AIL-like dysplasia and AIL-like lymphoma represent overlapping stages in disease pathogenesis. Polyclonal or oligoclonal lymphocyte proliferation may be the first stage in a multistep pathogenic process, followed by the expansion of a single predominant clone. The occurrence of additional transforming events in cells of this clone may, in some cases, give rise to the development of an overt lymphoma.

3.4.3. Analysis of Vβ rearrangement in samples reacting with T-cell clonotypic antibodies.

Molecular probes for the Vβ regions used by rearranged TCRβ genes in the Jurkat and HPB-ALL cell lines were used to analyse biopsies showing reactivity with the clonotypic antibodies MX11 and 421C1, which were raised against these cell lines. Rearrangement of the Vβ5 locus was demonstrated in 1/2 cases showing reactivity with MoAb 421C1, while rearrangements of the Vβ8 locus were detected in 2/3 cases showing reactivity with MoAb MX11.

These results support the use of these antibodies for the identification of monoclonal T-cell populations in tumour biopsies, but suggest that the antibodies may have a wider range of specificities that the relevant DNA Vβ probes. It is possible that the antibodies may react with epitopes that are conserved between more than one Vβ family.

Jack *et al.* (1990) have recently reported that the tumour cells of a high proportion of CTCLs show reactivity with a MoAB reactive with TCRβ containing Vβ8 regions. These authors suggest that proliferation and transformation of T-cells expressing a particular Vβ could arise from immune stimulation by a specific antigen. A similar model of leukaemogenesis has been described in Chapter 1 (section 1.4.2). Jack *et al.* (1990) also raise the possibility that Vβ8-containing TCRβ could be functioning as receptors for an oncogenic virus.

In order to assess the significance of results such as those obtained by Jack *et al.* (1990), it is important that the specificity of the Vβ-reactive MoAbs are accurately determined. A PCR strategy similar to those recently described by d'Auriol *et al.* (1989) and McCarthy *et al.* (1990) for the detection of TCR*y* and IgH gene rearrangements respectively could be used to resolve this question. A similar study would also be useful to confirm the specificities of the additional Vβ-specific MoAbs that have recently become available commercially (Laboratory Impex Limited).

3.4.4. The use of gene rearrangement analysis for lymphoma diagnosis.

Gene rearrangement analysis has been extensively used for the investigation of the lineage and clonality of lymphoma material, as in the study of HD described above. This has allowed a number of conditions with defined clinical and pathological features to be classified as B- or T-cell neoplasms. However the results of genetic analysis in individual cases which prove difficult to classify on clinical and pathological criteria may be more difficult to interpret. We analysed the configuration of the Ig and TCR gene loci in 5 cases in which difficulty had been encountered in reaching a diagnosis following routine examination.

The 5 cases analysed had differential diagnoses which included HD and NHL. Knowles *et al.* (1986) have suggested that the detection of significant gene rearrangements in lymphoma material may be used to exclude a potential diagnosis of HD. However the detection of significant gene rearrangements in HD cases in these studies and those of others (Brinker *et al.* 1987; Griesser *et al.* 1987; Herbst *et al.* 1989) indicates that this is not a useful criterion for distinguishing NHL and HD. Failure to detect Ig or TCR gene rearrangements in such cases would be significant supportive evidence for a diagnosis of HD, provided that adequate numbers of the presumptive neoplastic cells were present for detection. The importance of the latter point is illustrated by the lack of detectable Ig gene rearrangement in case 169, a T-cell predominant B-cell lymphoma, in which the numbers of neoplastic cells were estimated at less than 2%.

Thus in cases 26 and 27, for which the differential diagnoses included HD and T-cell NHL, the lack of TCRβ gene rearrangements is more consistent with a diagnosis of HD. Case 29 also had a differential diagnosis which included HD and T-cell NHL. The detection of an IgH gene rearrangement in this case and the lack of TCRβ and TCR*y*

gene rearrangement are more consistent with a diagnosis of HD. However no typical RS cells were observed in biopsies from this patient and the histological and immunophenotypic features were considered to be more consistent with a diagnosis of T-cell NHL. This case is difficult to classify with any degree of certainty as occasional cases of T-cell NHL lacking TCRβ gene rearrangements have been reported (O'Connor *et al.* 1987) and rearranged IgH genes have been described in such cases (Ogawa *et al.* 1986; Weiss *et al.* 1988).

The differential diagnosis for case 28 included HD and AIL. Our results and those of others indicate that lymphomas classified as AIL may show a variety of genotypes (section 3.4.2). Detection of a clonal IgH gene rearrangement in this sample does not resolve the diagnostic difficulty, as Ig gene rearrangements may be detected in both HD and AIL.

A similar problem is encountered with sample 25, in which IgH and IgL gene rearrangements were detected. The diagnosis for this case lay between HD and B-cell NHL, but as discussed earlier, Ig gene rearrangements may be detected in HD samples. The results of genetic analysis of case 25 did not therefore assist in making a definite diagnosis.

This series of cases suggests that the analysis of TCR and Ig genes may be useful for lymphoma samples in which immunophentotyping and histology have been inconclusive. However because variation in genotype may be seen in certain groups of conditions the results of genetic analysis may not be helpful in every case. In order to ensure that gene rearrangements are not missed it is essential that individual samples are subject to extensive analysis. In some of the cases included in this study analysis with 4 restriction enzymes and electrophoresis on low percentage agarose gels was required for the detection of TCRβ gene rearrangement (see Figures 3.10). In addition, care is necessary in the interpretation of results if the putative malignant cells are present in small numbers. In spite of these potential problems, genotypic analysis should form a useful adjunct to routine diagnostic procedures as long as the results are assessed in the context of histological and immunophenotypic findings.

3.4.5 Bcl-2 gene rearrangement analysis.

Analysis of 29 lymphoma samples with the J_H probe showed that nearly half (22/45) the rearranged fragments detected in HindIII digested DNA were between 4.0 and 6.0kb in size. In order to determine whether detection of HindIII restriction fragments of this size indicated the presence of a t(14;18)(q32;q21), HindIII digested DNA from 17 of these samples was analysed with probes for the MBR on chromosome 18. Rearrangements of the MBR were detected in 1/6 follicular lymphomas and 1/3 centroblastic B-cell lymphomas. The results indicate that detection of a restriction fragment of this size with the IgH J-region probe in HindIII digested DNA is not a good indicator of the presence of a translocation involving the bcl-2 MBR on chromosome 18, even in follicular and diffuse B-cell NHL. In addition the incidence of rearrangements of the MBR was low relative to that described in other studies (Lee *et al.* 1987; Lipford *et al.* 1987a; Weiss *et al.* 1987a). In a recent study, translocations involving the MBR of the bcl-2 gene and the J_H region of the IgH gene were detected in 32% of HD samples using the PCR (Stetler-Stevenson *et al.* 1990). However bcl-2 gene rearrangements were not detected in three HD cases analysed in this study, or in case 124, one of the diagnostically difficult cases discussed earlier. The low numbers of MBR rearrangements detected in this study may reflect the small number of samples included in the analysis.

3.4.6. Conclusions.

The results obtained from the analysis of Ig and TCR gene rearrangement in HD are consistent with the derivation of HRS cells from lymphocytes at various stages of B-cell differentiation. Definitive proof that the Ig gene rearrangements are present in the HRS cells will be important for the development of an accurate model for the pathogenesis of HD. This must account for the evidence indicating that EBV may play a role in the aetiology of HD. The epidemiological and serological evidence supporting a viral aetiology for HD is discussed further in the following chapter.

Molecular analysis of the TCR and Ig genes in samples from patients with AIL provides an opportunity to study different stages in a multistep pathogenic process. The emergence of dominant clonal cell populations from a background of abnormally proliferating lymphoctyes may arise as a result of an underlying immunological abnormality. A variety of mechanisms by which viruses could induce immune dysfunction and thus contribute to lymphomagenesis were discussed in Chapter 1 (section 1.4.2). The possibility that virus infection could be involved in the pathogenesis of AIL is explored further in Chapter 4.

CHAPTER FOUR.

THE MOLECULAR ANALYSIS OF TUMOUR BIOPSIES FOR THE PRESENCE OF HUMAN HERPESVIRUS TYPE 6 (HHV-6) DNA SEQUENCES.

4.1. INTRODUCTION.

Human herpesvirus-6 (HHV-6), initially designated human B-lymphotropic virus, was first isolated from 6 patients with a variety of lymphoproliferative and immunosuppressive disorders (Salahuddin *et al.* 1986). This report stimulated interest in the possibility that HHV-6 could be involved in the pathogenesis of lymphoproliferative disease. The study described in this Chapter was designed to investigate the molecular association between HHV-6 and lymphoid malignancy and was commenced immediately following the initial isolation of the virus. At this time little information about the structure or biology of HHV-6 had been reported.

The following sections provide a brief review of subsequently published studies that have investigated the structure, biology and disease associations of HHV-6.

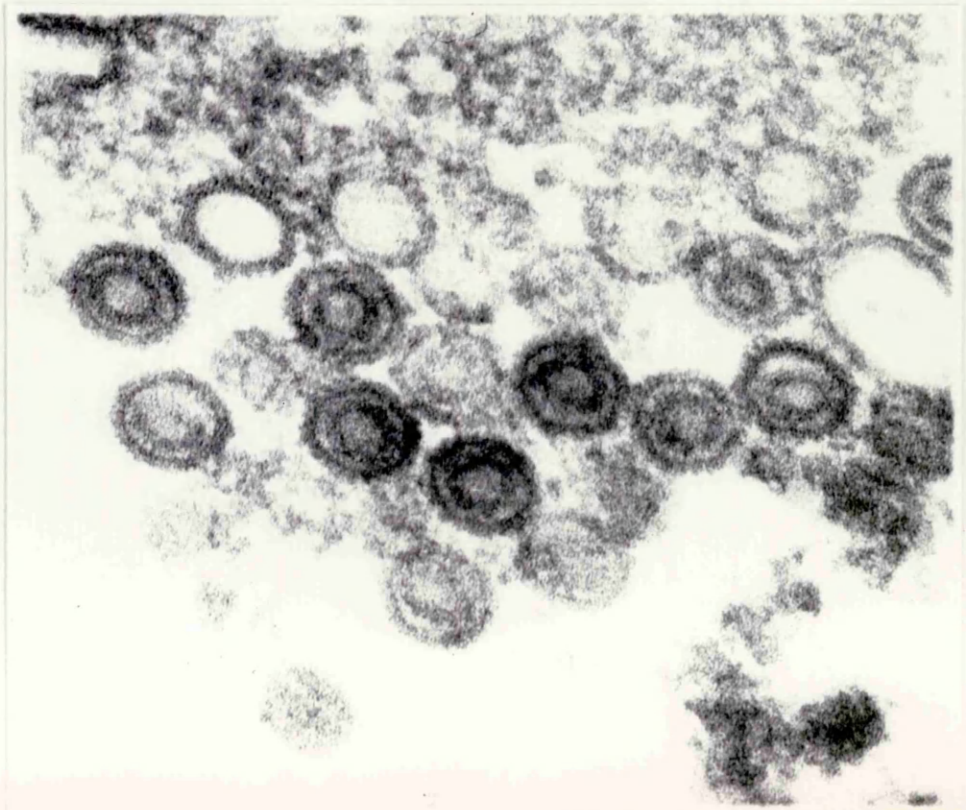
4.1.1. Virus structure and genomic organization.

HHV-6 shows typical herpesvirus morphology when visualized with an electron microscope (Figure 4.1). The virions are enveloped, have a diameter of approximately 200 nm and contain a nucleocapsid with icosahedral symmetry (Salahuddin *et al.* 1986).

Early studies did not detect evidence of serological cross-reactivity or of nucleic acid homology with other animal or human herpesviruses (Josephs *et al.* 1986). Subsequently limited regions of DNA sequence homology with MDV (Kishi *et al.* 1988) and HCMV (Efstathiou *et al.* 1988) were identified.

HHV-6 was initially considered to be a gammaherpesvirus because of its lymphotropism and the lack of evidence indicating a close relationship with any other human herpesviruses (Kishi *et al.* 1988; Lopez *et al.* 1988). Recently nucleotide sequence analysis of a 22kb region of the HHV-6 genome has revealed a closer resemblance to HCMV than to the other human herpesviruses (Lawrence *et al.* 1990). Lawrence *et al.* (1990) have therefore suggested that HHV-6 would be more appropriately classified with HCMV, as a betaherpesvirus.

Figure 4.1. Electron micrograph of intracellular HHV-6 virions. (x 150 000)



4.1.2. Seroepidemiology.

The seroprevalence of HHV-6 in healthy adult populations exceeds 50% and seroconversion appears to occur early in childhood for the majority of individuals (Briggs *et al.* 1988; Knowles *et al.* 1988; Clark *et al.* 1990). The virus therefore appears to be ubiquitous and readily transmitted.

Seroconversion has been reported in association with exanthem subitum, a febrile disease of children (Knowles *et al.* 1988; Takahashi *et al.* 1988; Yamanishi *et al.* 1988). HHV-6 has also been isolated from the peripheral blood of children with exanthem subitum and has been linked to the aetiology of this disease (Yamanishi *et al.* 1988). Seroconversion has also been reported in a number of additional clinical syndromes, including an infectious mononucleosis-like illness (Kirchesch *et al.* 1988; Irving and Cunningham 1990); hepatitis (Irving and Cunningham 1990) and febrile illnesses following renal transplants (Morris *et al.* 1989).

Serological studies have identified an association between high seroprevalence and high antibody titres to HHV-6 and a number of lymphoproliferative disorders, including HD, ALL and Burkitt's lymphoma in Africans (Ablashi *et al.* 1988). An increased seroprevalence to the virus was also detected in patients with sarcoidosis, high grade NHL and Sjögrens syndrome (Ablashi *et al.* 1988; Biberfeld *et al.* 1988). However neither of these studies were case-controlled and thus the significance of the findings is uncertain.

A case-controlled study of the seroepidemiology of HHV-6 in patients with leukaemia and lymphoma was performed in parallel to the experiments that are described in this Chapter (Clark *et al.* 1990). Clark *et al.* (1990) found that HHV-6 seroprevalence and antibody titres were higher in patients with acute myeloid leukaemia, high-grade NHL and HD compared to controls.

4.1.3. In vitro and in vivo tropism.

HHV-6 can infect a number of different cell types in vitro, including B- and T-lymphocyte cell lines, a megakaryocyte cell line and glioblastoma cells (Ablashi *et al.* 1987). Lusso *et al.* (1988) have shown that when cultures of PBMCs are infected with HHV-6, the virus is preferentially tropic for immature CD4+ T-cells. HHV-6 has a cytopathic effect in culture, characterized by the appearance of large refractile cells which express viral antigens (Salahuddin *et al.* 1986; Downing *et al.* 1987; Tedder *et al.* 1987).

The in vivo tropism of HHV-6 may not be the same, though the virus has been detected by PCR in the PBMCs of healthy individuals (Gopal *et al.* 1990; Jarrett *et al.* 1990). Reports that HHV-6 may be isolated from cell-free and unfractionated saliva samples from healthy individuals (Pietroboni *et al.* 1988a; Harnett *et al.* 1990; Levy *et al.* 1990) suggest that the oropharynx is a site of viral replication in vivo. This has been supported by the detection of viral DNA sequences in saliva samples from healthy persons by the PCR (Gopal *et al.* 1990; Jarrett *et al.* 1990). In addition Fox *et al.* (1990) have reported the detection of HHV-6 DNA in salivary gland tissue using in situ hybridization.

4.1.4. Interactions between HHV-6 and HIV.

The possibility that interactions between viruses may be important in the pathogenesis of lymphoproliferative disease was discussed in Chapter 1 (section 1.4.2). A number of lines of evidence suggest that interactions between HHV-6 and HIV are likely to occur *in vivo* and may contribute to the development of HIV-associated disease.

Co-infection of CD4+ T-cells with HIV and HHV-6 results in increased HIV replication and an increased rate of cell death (Lusso *et al.* 1989). HHV-6 infection of T-cells has been shown to result in trans-activation of the HIV LTR, providing a possible mechanism for the effect on viral replication (Horvat *et al.* 1989). HHV-6 infection of CD4- T-cells has been reported to upregulate CD4 expression, rendering the cells susceptible to HIV infection (Lusso *et al.* 1991). HHV-6 may thus increase the number of potential target cells for HIV infection *in vivo*.

It is also possible that HHV-6 replication may be increased in HIV-infected persons, as HHV-6 has been isolated frequently from the peripheral blood of patients with HIV infection (Downing *et al.* 1987; Tedder *et al.* 1987; Lopez *et al.* 1988; Peitroboni *et al.* 1988b). However seroepidemiological studies have failed to detect any differences in HHV-6 antibody titres between HIV antibody-negative, HIV antibody-positive and HIV-p24 antigenaemic persons (Brown *et al.* 1988; Fox *et al.* 1988; Spira *et al.* 1990). The importance of HHV-6 as a cofactor in the pathogenesis of HIV-associated disease is therefore uncertain.

4.1.5. The oncogenic potential of HHV-6.

Cell transformation has not been reported following HHV-6 infection in vitro. However in a recent study transfection of HHV-6 genomic DNA or a subclone, pZVH14, into NIH3T3 cells resulted in morphological transformation of the cells (Razzaque 1990). The transformed cells were tumorigenic in nude mice, however viral DNA sequences were not detected in transformed or tumour cell lines in all cases. Genomic fragments of a number of other human herpesviruses have been shown to induce rodent cell transformation in vitro (Sugden 1986; Galloway *et al.* 1986), as discussed earlier (Chapter 1, section 1.2.1). However the relevance of these findings to virus infections in vivo remains to be established.

In order to investigate the role of HHV-6 in the pathogenesis of lymphoproliferative disease, the molecular association between the virus and a variety of lymphoid malignancies was investigated. This chapter describes the results of molecular analysis of tissues from patients with a variety of lymphoproliferative disorders for the presence of HHV-6 specific sequences. Disorders that had been previously linked to HHV-6 in serological studies were of particular interest, thus a large group of samples from patients with HD were examined. A parallel study into the involvement of EBV in HD was also performed using this material (Gledhill *et al.* in press; Jarrett *et al.* manuscript submitted). Samples from patients with sarcoidosis and Sjögren's syndrome were also selected for investigation.

4.2. MATERIALS AND METHODS.

4.2.1. Clinical samples.

Samples from patients with a variety of diseases were collected from a number of clinical centres. Sample material was categorized into three groups. The first group included samples from patients with lymphomas and leukaemias. This group included 53 samples from patients with NHL and 47 samples from patients with HD. The cases were classified by the referring pathologists according to the Working formulation as detailed by the Non-Hodgkin's lymphoma Pathologic classification project (Rosenberg 1982). A second group, designed as a control group, contained 26 biopsies from patients with non-lymphoid disorders and included reactive nodes, secondary neoplasms and non-lymphoid primary tumours. The third group included material from 9 patients with sarcoidosis or Sjögren's syndrome, conditions which had been associated with HHV-6 in early seroepidemiological studies.

4.2.2. Viral isolates.

The A72 isolate of HHV-6 was obtained from Dr. R. Tedder and was originally isolated from the PBMCs of a Gambian patient with HIV-2-associated AIDS (Tedder *et al.* 1987). The 1102 isolate of HHV-6 was a gift from Dr. R.G. Downing and was obtained from the cultured PBMCs of a Ugandan patient with AIDS (Downing *et al.* 1987).

4.2.3. Molecular analysis.

DNA samples were digested with the restriction enzymes EcoRI or HindIII unless otherwise indicated, size separated on 0.8% agarose gels, transferred to nylon membranes and hybridized to radioactively-labelled DNA probes as described in Chapter 2.

The HHV-6 probe used was the insert from the pZVH14 plasmid, which consists of 9kb of viral DNA and does not cross-react with sequences from any other known herpesvirus (Josephs *et al.* 1986). This probe was obtained from the prototype isolate of

HHV-6, HHV-6_{GS} (Salahuddin *et al.* 1986). Probes for the 5' and 3' regions of the clone, pZVH 5'H/E and pZVH 3'E/H, respectively, were prepared by purification of fragments following double digestion of the plasmid with HindIII and EcoRI (Figure 4.2).

Selected samples were also analysed for rearrangements of the TCRβ and IgH genes as described in Chapter 3.

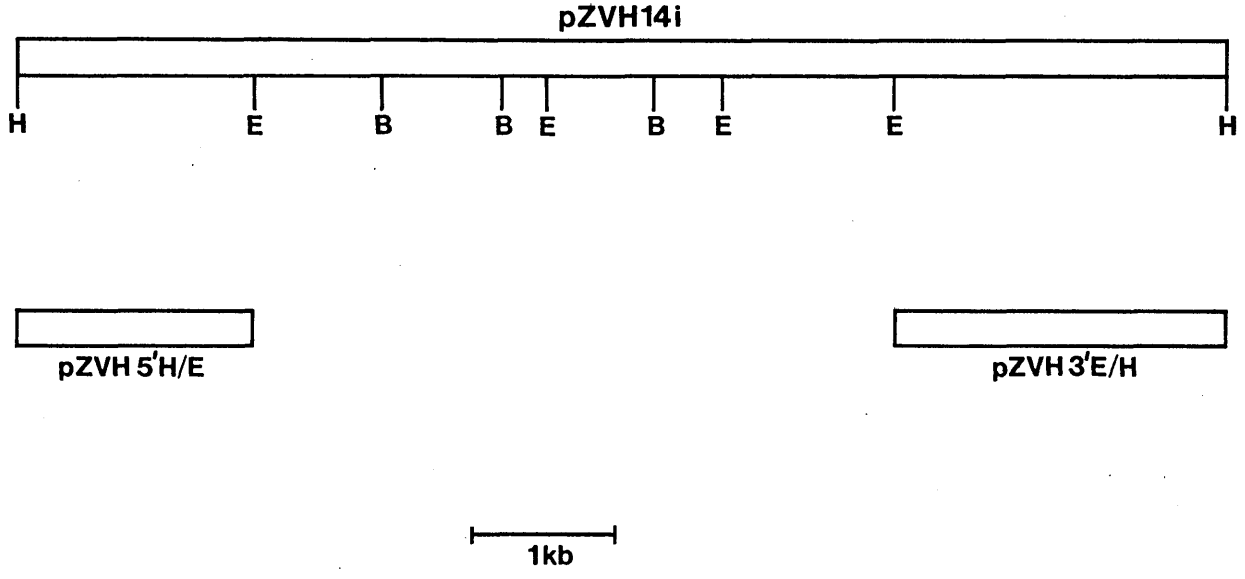
4.2.4. Pulse field gel electrophoresis.

Pulse field gel electrophoresis (PFGE) was used to analyse virus-infected cells and HHV-6 positive samples, in order to investigate the size and state of the viral genome.

Cells were lysed in agarose, as follows, to avoid shearing long DNA molecules. Cultured cells, or cell suspensions prepared from disrupted tissues (as described in section 2.2.1) were washed once in phosphate-buffered saline (PBS; 144mM sodium chloride, 16mM sodium phosphate, dibasic, 4mM sodium phosphate, monobasic, pH 7.4), then resuspended in PBS at between 5 x 10^5 and 10^7 cells per ml. An equal volume of 1% low melting point agarose (BRL) in PBS was added and 100µl aliquots dispensed into prechilled moulds (LKB). The agarose blocks were allowed to set for more than 10 minutes then removed from the moulds and incubated in 10 volumes of lysis buffer (0.5M EDTA, 1% sarcosyl, 2mg/ml proteinase K [Boehringer Mannheim]) at 50°C for 48 hours. The blocks were rinsed twice at 50°C in TE containing 40µg/ml of phenyl methyl sulfonyl fluoride and stored at 4°C in 0.5M EDTA (pH8.0).

DNA molecules were separated by the application of a contour clamped hexagonal electrophoretic field (CHEF), using an apparatus obtained from Dr. D. Green (MRC Human Cytogenetics Unit, Edinburgh). Optimum conditions for the separation of DNA molecules of 50-250kb in length were established in experiments with phage lambda concatemers. Agarose blocks containing the lysed cells were loaded into preformed wells and sealed in place with molten low melting point agarose. Electrophoresis was performed at 4.5 V/cm for 48 hours with a pulse time of 30 seconds. Samples were electrophoresed in 1.0% agarose gels made up in 0.5 x TBE. The running buffer (0.5 x TBE) was constantly recirculated and maintained at 12°C.

Figure 4.2. Restriction map of the pZVH14 insert.



The restriction enzyme sites shown are HindIII (H), EcoRI (E) and BamHI (B). The pZVH 5'H/E and pZVH 3'E/H were derived from the 5' and 3' ends of pZVH14i, as shown.

4.2.5. Preparation of phage lambda concatemers.

Concatemers of phage lambda were prepared using the lysogenic bacterial strain N1323, containing phage lambda (strain C_1857). A single colony was inoculated into 25mls of L-broth and incubated at 30°C overnight. Twelve millilitres of the overnight culture was inoculated into 500mls of L-broth containing 0.01M magnesium sulphate and incubated at 33°C until the optical density at 600nm was 0.35 (approximately 3 hours). Replication of phage lambda in the bacterial culture was induced by incubation at 43°C for 15 minutes and the culture was then incubated for a further 2.5 hours at 39°C. The bacteria were pelleted by centrifugation at 7000rpm for 10 minutes at 4°C and resuspended in 8.8mls of TE. Phage particles were released by lysing the bacterial cells with 0.5mls chloroform and shaking the culture at 37°C for 15 minutes. Ribonuclease A (Boehringer Mannheim) and Deoxyribonuclease I (Boehringer Mannheim) were added to final concentrations of 20µg/ml and the incubation continued for a further 15 minutes. Bacterial debris was removed by centrifugation at 10000rpm for 15 minutes at 4°C. The supernatant was mixed with an equal volume of 1% low melting point agarose and poured into prechilled moulds. The phage particles were lysed in agarose blocks as described above. Concatemerization of the phage lambda genomes was encouraged by incubating the blocks at 50°C in 0.1M EDTA for 72 hours.

4.3. RESULTS.

4.3.1. Analysis of biopsy material for HHV-6 DNA.

A total of 135 cases were analysed for the presence of HHV-6 DNA sequences by Southern hybridization (Table IV.I). Viral sequences were detected in 2/53 patients with NHL. HHV-6 was not detected in 47 biopsies from patients with HD, or in 35 biopsies from patients with other conditions. The two positive cases were patient 112, who had a diagnosis of AIL which had progressed to T-zone lymphoma, and patient 287, who had a gastric lymphoma and a history of Sjögren's syndrome.

4.3.2. Clinical histories and molecular analysis of HHV-6-positive cases.

Case 112.

This patient was a 52 year old white female who first presented in 1979 with generalized lymphadenopathy and hepatosplenomegaly. These symptoms had been preceded by an influenza-like illness which had been associated with a skin rash on the arms and legs. At presentation the patient was profoundly anaemic, she had a normal WBC count with 30% abnormal leukocytes and plasma-like cells and showed Bence-Jones proteinuria. A lymph node biopsy showed partial effacement of the normal node architecture by a polymorphic infiltrate containing numerous large immunoblasts and an arborizing vascular network. The biopsy was not considered to indicate the presence of overt lymphoma. A diagnosis of AIL was made. Therapy was commenced with chlorambucil and prednisolone. The patient also received a blood transfusion.

Over the next few years the patient experienced repeated episodes of lymphadenopathy, involving different lymph node groups. She reported intermittent influenza-like symptoms and periods of extreme tiredness. These episodes were treated with steroids and several courses of more aggressive chemotherapy. The patient developed Coombs-positive haemolytic anaemia on three occasions and was treated with blood transfusions. Protein electrophoresis revealed the presence of an IgGλ band.

Table IV.I. Samples analysed for the presence of HHV-6-specific sequences.

Clinical Condition	Number analysed	HHV-6 +
B cell lymphomas		
Lymphocytic, plasmacytoid	6	0
Follicular		
Small cell and mixed	7	0
Predominantly large cell	1	0
Diffuse		
Small cleaved	4	0
Mixed cell	3	0
Large cell	6	1
Immunoblastic	3	0
Lymphoblastic	1	0
Burkitt's	1	0
Unclassified	3	0
Hodgkin's disease	47	0
T-cell lymphomas		
Lymphocytic	1	0
T-zone and pleomorphic	9	1
Immunoblastic	4	0
Lymphoblastic	1	0
Unclassified	3	0
Nonlymphomatous		
Reactive nodes	10	0
Secondary neoplasms	3	0
Primary neoplasms	4	0
Sarcoidosis	7	0
Sjogren's syndrome	2	0
Miscellaneous	9	0
Total	135	2

Early in 1987 her condition deteriorated. She had systemic symptoms of increased tiredness and sweating. When examined, she had cervical and axillary lymphadenopathy. A cervical lymph node biopsy revealed expansion of the paracortex and infiltration of the cortex by a diffuse proliferation of large clear cells. The original features of AIL could still be discerned. Cells in the paracortical area of the node reacted with T-cell antibodies (CD3, CD4 and CD8), while the cortical areas contained a mixture of CD3+, CD4+, CD8+, CD5+, CD7+ and CD21+ cells. A diagnosis of T-zone lymphoma was made.

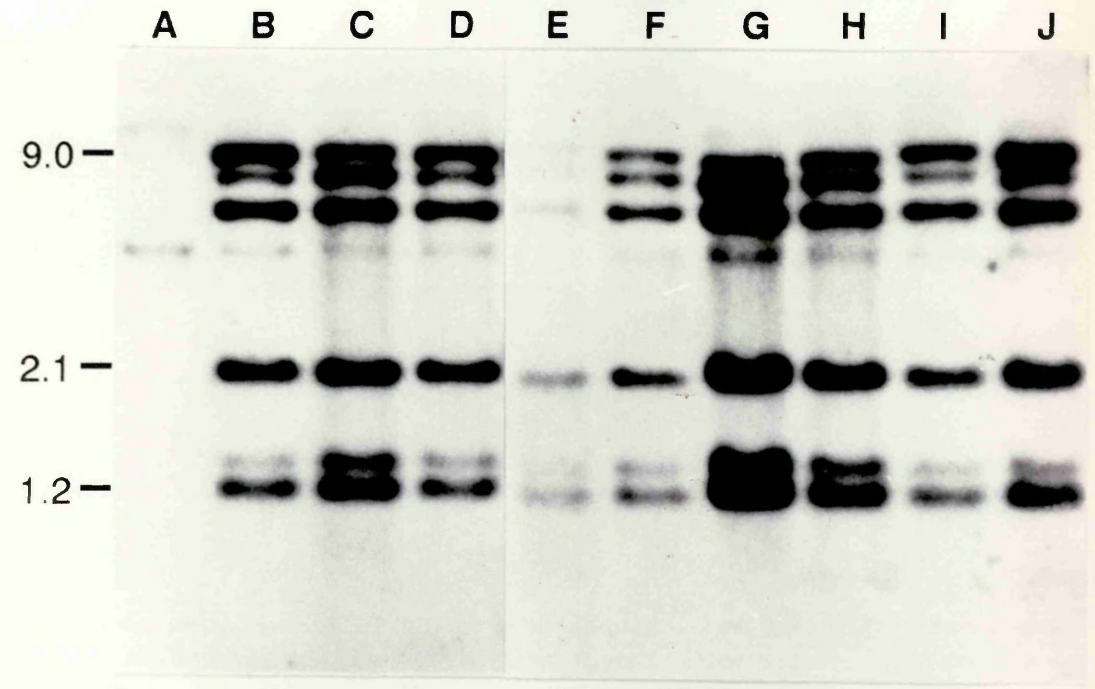
Southern blot analysis of the cervical node biopsy showed the presence of HHV-6 DNA (Figure 4.3, panel a, lane B). The results obtained from the analysis of the lymphoma biopsy from this patient for the presence of TCRβ and Ig gene rearrangements have been described in Chapter 3 (section 3.3.2). A clonal rearrangement of the TCRβ gene was detected (Figure 4.3, panel b, lane B), while the IgH genes were germline.

The patient was treated with ifosfamide, dexamethasone, and vincristine, followed by radiotherapy, but failed to respond and died in July 1987. Disseminated lymphoma was reported on post-mortem examination.

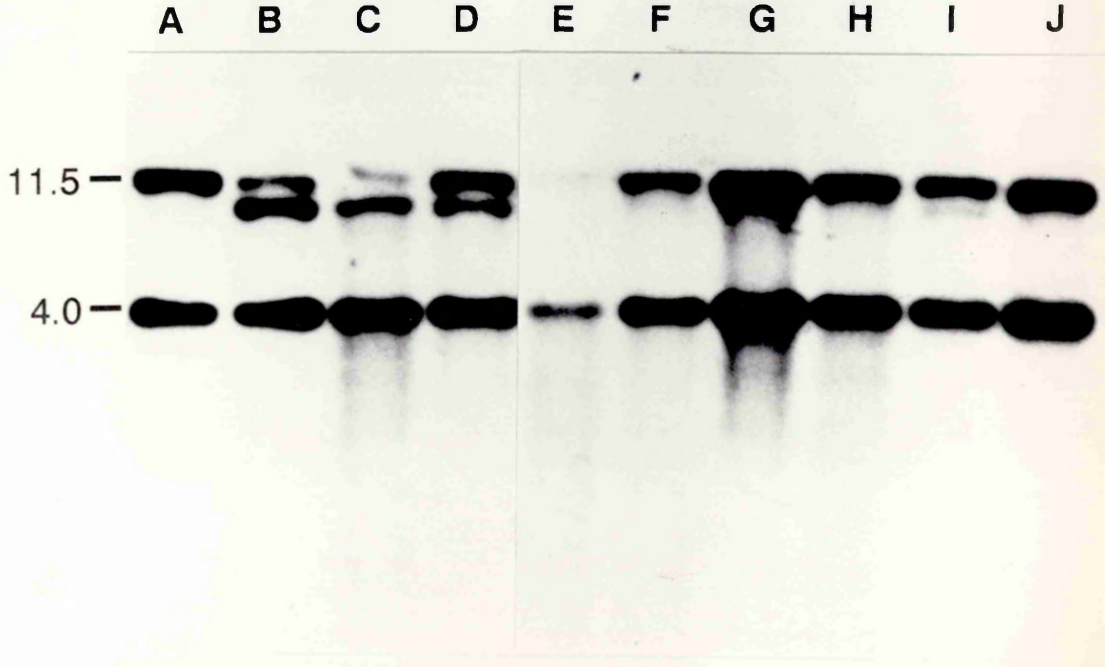
Multiple tissues were obtained at autopsy and were analysed for the presence of HHV-6 DNA by Southern hybridization. Viral DNA was detected in all tissues analysed (Figure 4.3, panel a). Duplicate filters were hybridized with the TCRβ gene probe (Figure 4.3, panel b). The intensity of the rearranged band detected in EcoRI digested DNA using the Cβ probe permits the extent of tumour infiltration of the tissue to be assessed. In the three lymph node samples an intense rearranged band is observed at approximately 8.5kb (Figure 4.3, panel b, lanes B-D) indicating substantial replacement of the lymph nodes by lymphoma. A faint band at the same position can be observed in samples from spleen and parotid salivary gland (Figure 4.3, panel b, lanes F and I), suggesting that small numbers of tumour cells have infiltrated these tissues. The rearranged EcoRI fragment is a similar size to that of a frequently detected partial digestion product. However the 8.5kb fragments detected in these samples were considered to represent TCRβ gene rearrangements, as similar results were obtained from the analysis of BamHI-digested DNA.

Figure 4.3. DNA samples were digested with the restriction enzyme EcoRI. DNA was extracted from placenta, as a negative control (lane A) and from a cervical lymph node biopsy (lane B) obtained from patient 112. The other DNA samples were extracted from tissues obtained at autopsy and included hilar lymph node (lane C), retroperitoneal lymph node (lane D), liver (lane E), spleen (lane F), bone marrow (lane G), kidney (lane H), parotid salivary gland (lane I) and brain (lane J). Duplicate filters were hybridized with the HHV-6 probe, pZVH14 (panel a) or the TCRß gene probe, Cß (panel b).

Figure 4.3. Southern blot analysis of multiple tissues from patient 112.



a.



b.

The amounts of DNA present in different lanes on the filter were assessed using the intensity of the 4kb band observed in EcoRI digested DNA hybridized to the Cβ probe, as this fragment is not altered by TCRβ gene rearrangement. The intensity of this band is similar across all lanes of the filter, thus the amount of viral DNA detected in the samples is not dependent on the extent of tumour infiltration, but appears to be equivalent in all the tissues examined.

Lymph node biopsies from 4 additional patients with AIL-like lymphomas were examined, all were negative for HHV-6 DNA sequences.

Case 287.

This patient, a 77 year-old white female, had a longstanding history of Sjögren's syndrome and Raynaud's phenomenon. She also had seropositive rheumatoid arthritis, fulfilling the American Rheumatoid Association criteria, from the age of 35 years. Biopsies obtained from the left and right parotid glands in 1963 and 1965 respectively, revealed histological features consistent with Sjögren's syndrome. Her recent clinical history included the onset of acute right parotitis in 1980, followed by intermittent swelling of the left parotid gland. Over this period she had progressively worsening pulmonary interstitial fibrosis. She had received numerous courses of steroidal and non-steroidal anti-inflammatory agents.

In August 1987 she presented with progressive weight loss, decreasing appetite and lymphadenopathy. A prepyloric ulcer close to the lesser curvature was detected on endoscopy and biopsied. Histological and immunophenotypic examination resulted in a diagnosis of diffuse large cell NHL with a B-cell phenotype. The patient received therapy with chlorambucil and prednisolone.

Repeat gastric biopsies were obtained in December 1987, but revealed no evidence of tumour infiltration though inflammatory cells were present. DNA extracted from these samples was positive for HHV-6 sequences (Figure 4.4, panel a). IgH gene rearrangement was not detected in EcoRI digested DNA from this sample, though there was insufficient DNA for analysis with additional restriction enzymes.

A lymph node biopsy was obtained in May 1988. Analysis of DNA extracted from this sample showed the presence of HHV-6 sequences (Figure 4.4, panel b). A clonal rearrangement of the IgH gene was also detected in EcoRI digested DNA from this sample (Figure 4.4, panel e). This result shows that the initial biopsy analysed was predominantly non-tumour tissue, as indicated by histology, as insufficient numbers of tumour cells were present for the detection of the IgH gene rearrangement.

Biopsies from two additional patients with Sjögrens syndrome were included in this study, neither of these patients had lymphomas. HHV-6 DNA sequences were not detected in these samples and IgH and TCRβ genes were germline in both cases.

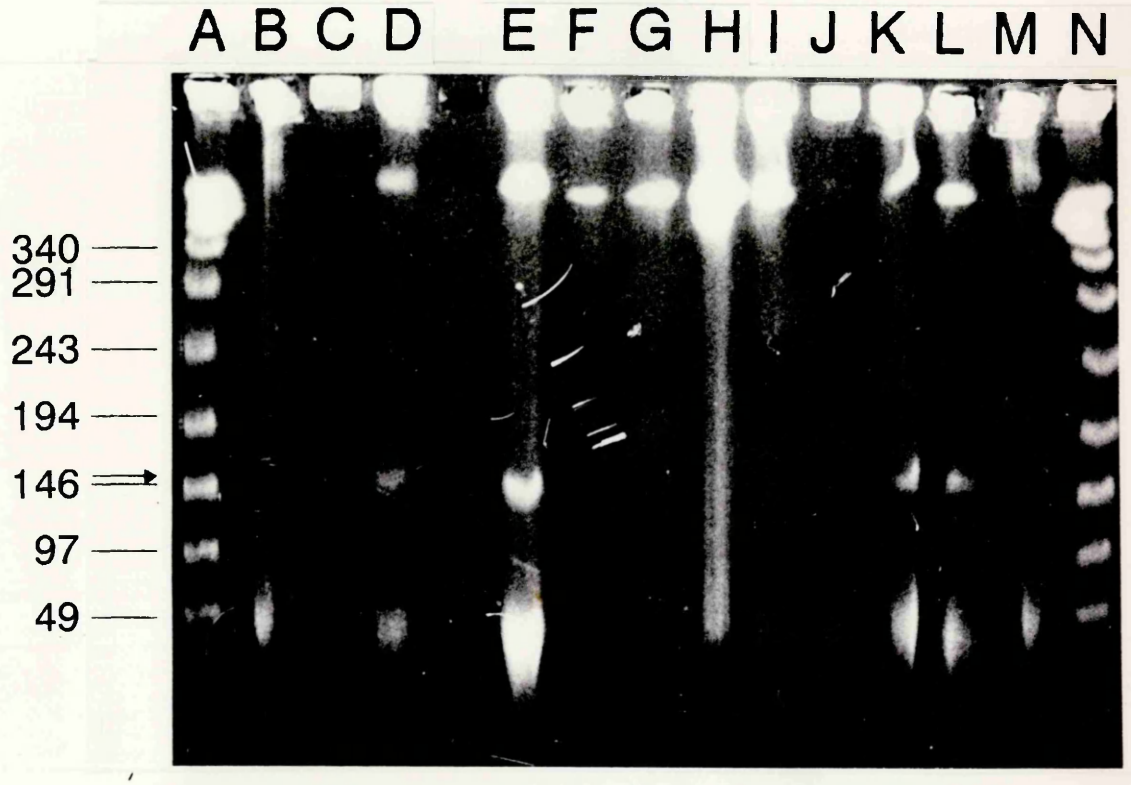
4.3.3. Characterization of HHV-6 genomes.

The pZVH14 homologous sequences in cases 112, 287 and in the A72 and 1102 isolates of HHV-6 were compared with the pZVH14 clone itself, using multiple restriction enzyme digestions.

Hybridization of the pZVH14 probe to sequences present in J JHAN cells infected with the A72 and 1102 isolates of HHV-6 and to viral sequences present in cases 112 and 287 was detected using stringent conditions. The data indicate that there is a high level of homology between the prototype HHV-6_{GS} isolate and the A72 and 1102 isolates over the region spanned by the pZVH14 probe (Downing *et al.* 1987; Tedder *et al.* 1987). The viral sequences present in cases 112 and 287 also show a high degree of homology to the pZVH14 clone.

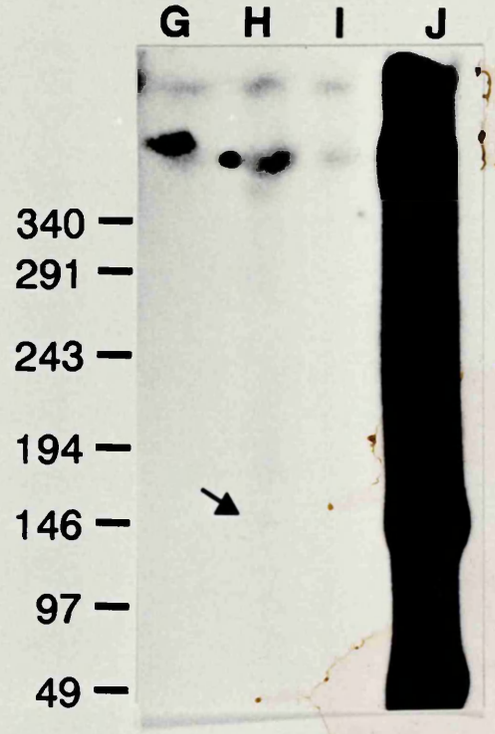
Differences in the restriction enzyme cleavage pattern between the sequences present in case 112 and the three viral isolates were detected when DNA was cleaved with each enzyme (Figure 4.5). Differences between the viral sequences obtained from all four sources were detected with HaeIII, which recognizes a four-base-pair restriction site (Figure 4.5, panel d). The EcoRI restriction pattern demonstrates that the three internal EcoRI sites and the 5' flanking site are conserved in the viral sequences present in case 112 (Figure 4.5, panel c). Hybridization of EcoRI-digested DNA samples with the pZVH 3'E/H probe shows that the 3' flanking fragment is replaced by a doublet (Figure 4.6, panel a). Hybridization of the pZVH14 clone to HindIII-EcoRI double digested DNA samples demonstrates the absence of the 5' 1.8kb HindIII/EcoRI fragment and the 5' internal 2.1kb EcoRI fragment in the 112 virus (Figure 4.6, panel b).

Figure 4.7. Ethidium bromide stained DNA samples from HHV-6-infected cells and tissues following PFGE.



DNA samples were from phage lambda concatemers (lanes A and N), 10^7 J JHAN cells (lanes B and M), 5 x 10^5 (lanes C and J), 10^6 (lanes D and K) and 2 x 10^6 (lanes E and L) J JHAN cells infected with the A72 isolate of HHV-6, 10^7 cells from the brain of patient 112 (lane F), 10^7 (lane G) and 10^8 (lane H) cells from the spleen of patient 112 and 5 x 10^7 cells from the brain of patient 112 (lane I). The approximate sizes (in kb) and migration distances of concatemers of phage lambda are given on the right of the panel. The arrow indicates the location of a band of approximately 160kb which is seen in HHV-6 infected J JHAN cells (lanes D, E, K and L).

Figure 4.8. Southern blot analysis of HHV-6-infected cells and tissues following PFGE.



Lanes G-J of the gel shown in Figure 4.7 were Southern blotted and hybridized with the insert from the pZVH14 plasmid. DNA samples were from 10^7 cells (lane G) and 10^8 cells (lane H) from the spleen of patient 112, 5 x 10^7 cells from the brain of patient 112 (lane I), 5 x 10^5 J JHAN cells infected with the A72 isolate of HHV-6 (lane J). The arrow indicates a faint band in lane H. The migration distances and the sizes (in kb) of concatemers of phage lambda are shown on the left of the figure.

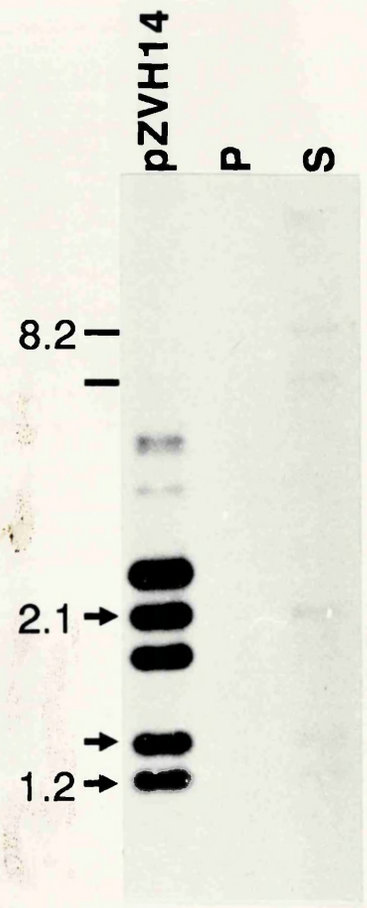
These results indicate that the 112 virus lacks the 5' HindIII site present in the pZVH14 clone and has an extra HindIII site between the 5' internal EcoRI sites. The pZVH3'E/H probe hybridizes to an extra fragment of approximately 5.5kb in DNA digested with HindIII and EcoRI, demonstrating that the extra EcoRI fragment detected with this probe is not a product of an EcoRI restriction site polymorphism in the 3' flanking sequences. The data suggest that there are sequences elsewhere in the viral genome that hybridize to the pZVH14 probe.

Detailed analysis of the viral genome present in case 287 was not possible due to the limited availability of material. Analysis of the HHV-6 DNA sequences present in the lymph node biopsy from patient 287 showed that the restriction fragments detected in BamHI and HindIII digested DNA were identical to those detected in case 112 (Figure 4.4, panels b and c). Thus the virus present in case 287 appears to lack the HindIII site that defines the 5' end of the pZVH14 clone and has an extra HindIII site, similar to that detected in the virus present in case 112. In addition one of the internal EcoRI fragments of the virus present in case 287 appears to be absent (Figure 4.4, panels a and d).

4.3.4. Analysis of the viral genome by PFGE.

Separation of viral DNA from cellular DNA was achieved, using PFGE to analyse HHV-6_{A72} infected JURKAT (J JHAN) cells. In lanes containing lysed virus-infected cells a band of approximately 160kb was visualized on ethidium bromide stained gels (Figure 4.7). No bands were seen in this position in lanes containing uninfected cells, thus this band is likely to represent HHV-6 DNA. Lanes G-J of the gel illustrated in Figure 4.7 were Southern blotted and hybridized to the pZVH14 probe. Intense hybridization was seen along the length of lane J, with the most intense hybridization visible in the 160kb region (Figure 4.8). Hybridization of the pZVH14 probe along the length of this lane is likely to reflect entrapment of HHV-6 DNA in chromosomal DNA, which has undergone partial degradation. Evidence of DNA degradation was visible on the ethidium bromide stained gel, in lanes containing higher numbers of virus-infected cells (Figure 4.7, lanes D, E, K and L). A similar finding was reported by Harris and Bentley (1988), who used PFGE to separate EBV genomes from infected cells.

Figure 4.9. Southern blot analysis of HHV-6 DNA. purified following PFGE.

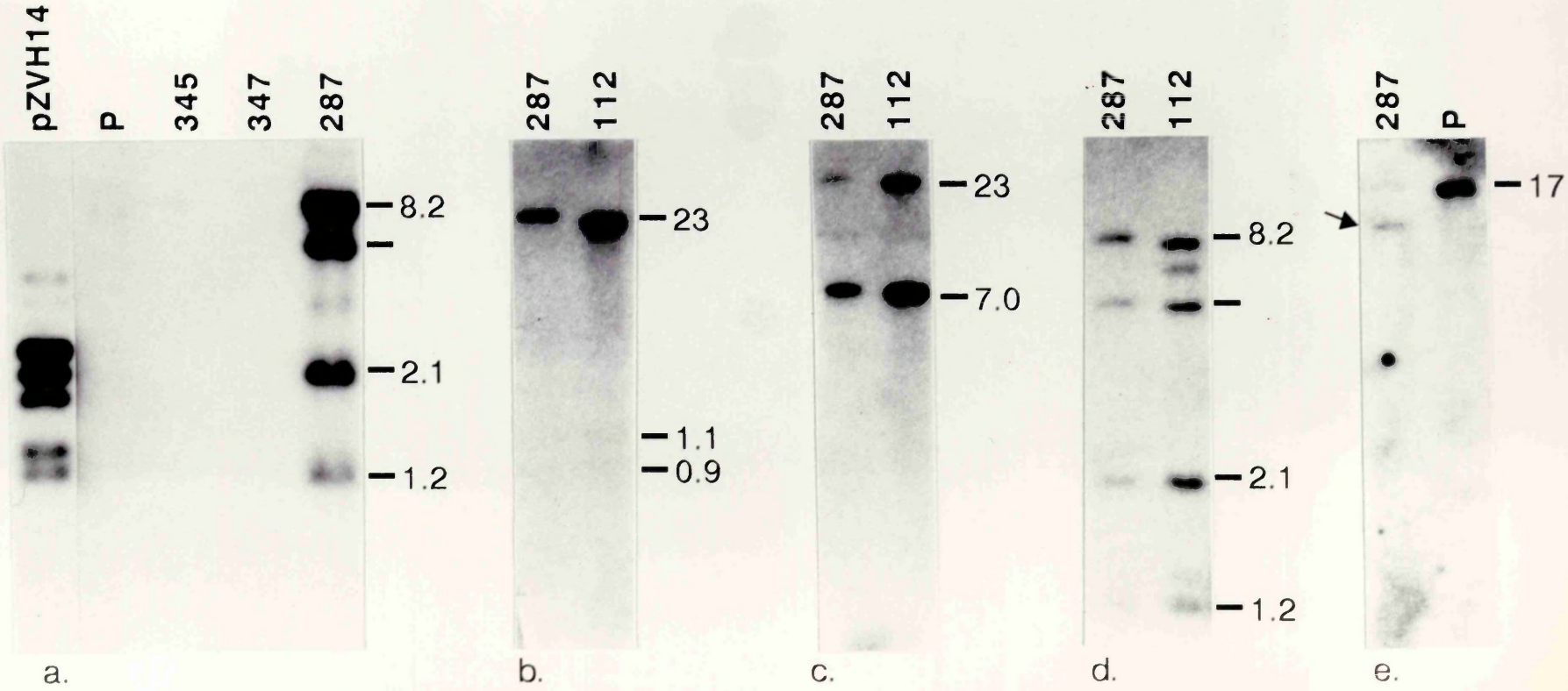


DNA samples were from the pZVH14 plasmid, placenta (P) and J JHAN cells infected with the A72 isolate of HHV-6 (S). The latter sample was extracted from a gel slice containing DNA of approximately 160kb in size, following PFGE of lysed cells (see text for further explanation). DNA was digested with the restriction enzyme EcoRI and the filter was hybridized with the insert from the pZVH14 plasmid. DNA fragments internal to the pZVH14 insert are indicated with arrows, flanking fragments are indicated with lines. The sizes of DNA fragments are shown in kb.

Figure 4.4. Panel a, DNA samples were obtained from the insert of pZVH14, as a positive control, placenta (P), a case of systemic lupus erthymatosus (case 345), a case of AIL-like lymphoma (case 347) and a gastric biopsy obtained from patient 287. DNA was digested with EcoRI and hybridized with the pZVH14 probe. Panels b, c, d, DNA samples were from a lymph node biopsy obtained from patient 287 and the spleen of patient 112. DNA was digested with BamHI (panel b), HindIII (panel c) and EcoRI (panel d) and hybridized with the pZVH14 probe. The sizes of the viral DNA fragments are given in kb on the right of each panel.

Panel e, Detection of an IgH gene rearrangement in the lymph node biopsy from patient 287. DNA samples were from the lymph node biopsy and from placenta (P) and were digested with EcoRI. The filter was hybridized with the JH probe. The size of the germline fragment is shown on the right of the panel. The rearranged fragment is indicated with an arrow.

Figure 4.4. Detection of HHV-6 DNA sequences in non-tumour and tumour tissue of patient 287.



In order to confirm that the 160kb band did represent HHV-6 DNA, the ethidium bromide stained bands in lanes D, E, K and L were excised from the gel and the DNA extracted using glass beads, as described in Chapter 2 (section 2.2.4). The extracted DNA was digested with EcoRI, electrophoresed on a 0.8% agarose gel and Southern blotted as described in Chapter 2. Hybridization with the pZVH14 probe revealed the presence of the three internal EcoRI fragments and two flanking fragments (Figure 4.9).

A number of tissues obtained at autopsy from patient 112 were analysed by PFGE under the same conditions. No discrete bands were observed in ethidium bromide stained gels, but there was evidence of considerable DNA degradation in lane H, which contained 10^8 spleen cells (Figure 4.7). Hybridization with the pZVH14 probe showed a barely detectable signal at approximately 160kb in this lane (Figure 4.8).

Figure 4.5. DNA samples were from the insert of the pZVH14 plasmid (lane A), J JHAN cells infected with the A72 isolate of HHV-6 (lane B), J JHAN cells infected with the 1102 isolate of HHV-6 (lane C) and the spleen of patient 112 (lane D). Samples were digested with the restriction enzymes HindIII (panel a), BamHI (panel b), EcoRI (panel c) and HaeIII (panel d). All filters were hybridized with the pZVH14 insert. Internal fragments of the pZVH14 insert are indicated with arrows and flanking fragments are indicated with lines, the sizes of the fragments are shown in kb.

Figure 4.5. Restriction enzyme analysis of HHV-6 DNA sequences using the pZVH14 probe.

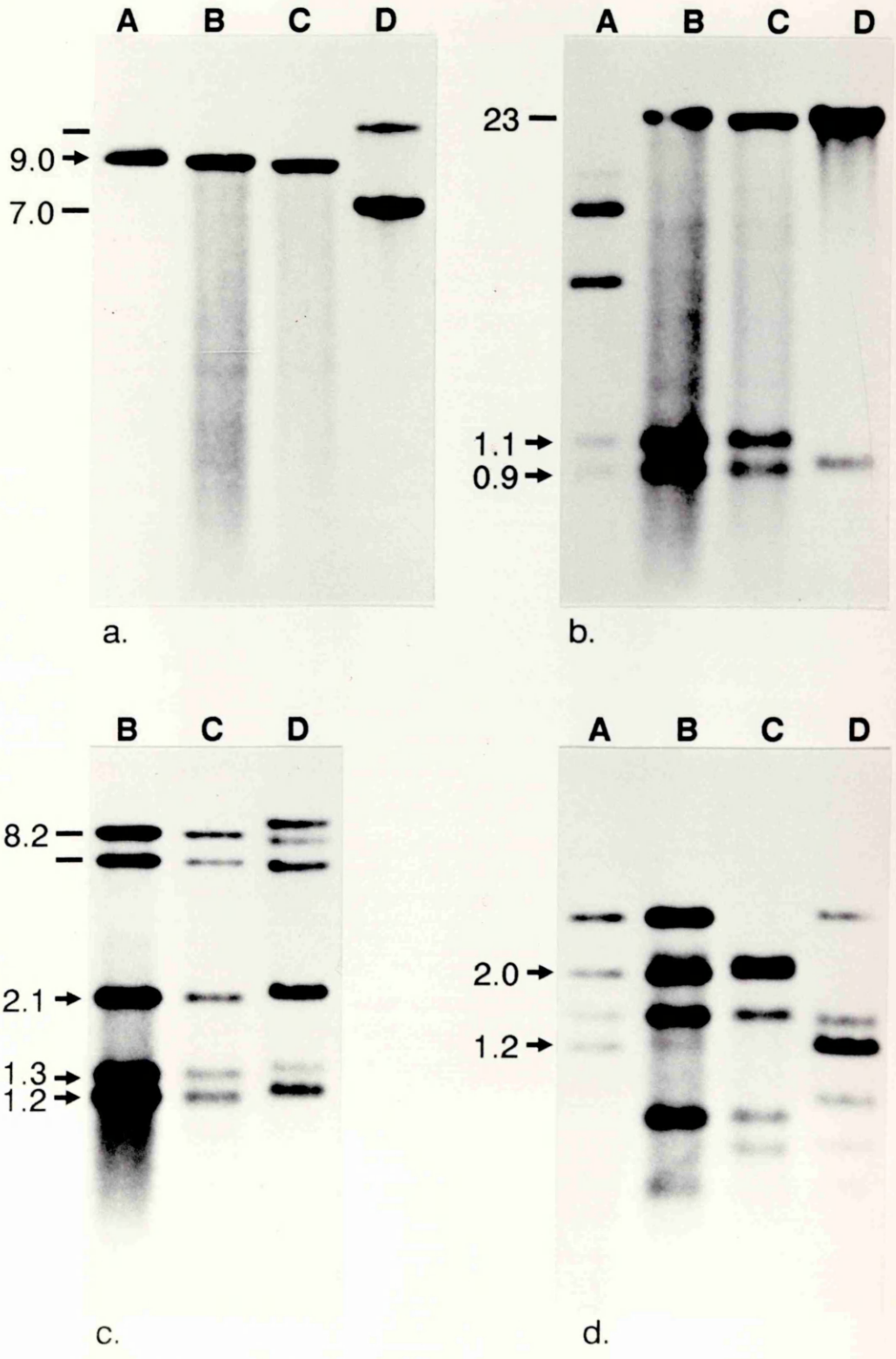
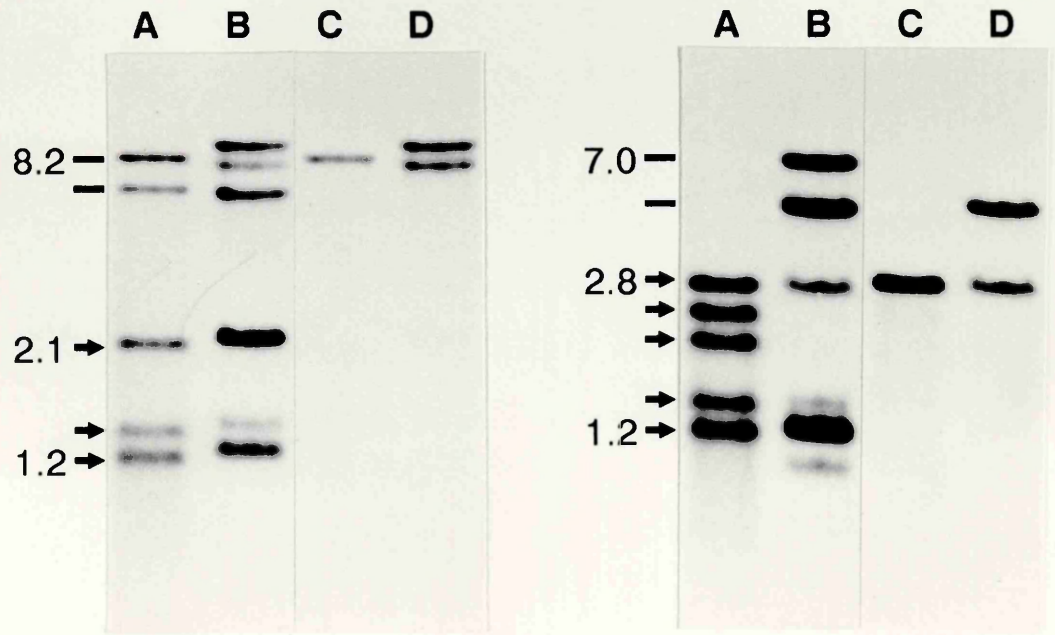


Figure 4.6. DNA samples were from J JHAN cells infected with the 1102 isolate of HHV-6 (lanes A and C) and the spleen of patient 112 (lanes B and D). DNA was digested with the restriction enzymes shown at the base of each panel. The filters were hybridized with pZVH14 (lanes A and B) or pZVH 3'E/H (lanes C and D). Arrows indicate internal fragments of the pZVH14 insert and lines indicate flanking fragments. Fragment sizes are given in kb.

Figure 4.6. Restriction enzyme analysis of HHV-6 DNA sequences present in case 112.



4.4. DISCUSSION.

The involvement of HHV-6 in human lymphoid malignancies was investigated by analysing tumour tissues for the presence of viral DNA. HHV-6-specific sequences were detected in samples from 2 of 135 cases.

4.4.1. Characterization of HHV-6 genomes.

PFGE enables large DNA molecules to be separated according to size and was therefore used in this study to investigate the size and state of the HHV-6 genome in infected cells.

The size of the viral genome.

Analysis of the cells infected with the A72 isolate of HHV-6 by PFGE indicated that the size of the viral genome was approximately 160kb. It is likely that this represents linear viral DNA. Josephs *et al.* (1986) had previously estimated the size of the HHV-6_{GS} genome as greater than 110kb. Subsequently published estimates of the length of HHV-6 viral DNA, derived by summation of the lengths of restriction enzyme fragments (Josephs *et al.* 1988b; Lawrence *et al.* 1990) have shown good agreement with the result obtained by PFGE in the experiments described here.

The state of the viral genome in cells infected in vitro.

Analysis of DNA samples from CMV and HSV-infected cells by PFGE has shown that linear viral genomes are the predominant forms of viral DNA visible on ethidium bromide-stained gels (van den Berg *et al.* 1988). Multimeric forms of CMV genomes were also detected by these workers, who suggest that their presence may reflect a rolling-circle mechanism of viral replication. High molecular weight forms of EBV and of herpesvirus saimiri, thought to represent circular or concatameric forms, have also been detected using field inversion gel electrophoresis to analyse virus-infected cells (M.R. Gopal, personal communication). No multimeric forms of HHV-6 were detected

in virus-infected cells analysed on ethidium bromide-stained gels in this study. This may reflect differences in the replication cycles of CMV and HHV-6 or alternatively, technical differences in the experimental procedures used. The results of Southern hybridization analysis did not permit an assessment of whether higher molecular weight forms of HHV-6 were present. However no evidence for the presence of circular or multimeric forms of HHV-6 in infected cells has been found by others (M.R. Gopal, personal communication).

The state of the viral genome in cells infected in vivo.

PFGE was used to analyse the state of the HHV-6 genome in tissues from patient 112. The results were technically unsatisfactory, but suggested that linear viral genomes were present in the tissue analysed. Evidence for the presence of viral episomes was not detected, however this could not be excluded due to technical difficulties. Evidence of considerable DNA degradation in the samples also suggests that the results of these experiments should be interpreted with caution.

The ability of herpesviruses to establish latent infections in host cells has been described in Chapter 1 (section 1.2). Herpesvirus genomes are maintained in circular, concatameric or linear forms in latently infected cells (Lindahl *et al.* 1976; Gardella *et al.* 1984; Rziha *et al.* 1986; Mellerick and Fraser 1987), however the form in which HHV-6 genomes are maintained has not yet been determined. Further characterization of HHV-6 DNA may permit an assessment of whether the state of the viral genome in the tumour tissues from patients 112 and 287 is indicative of latent infection or active replication.

Restriction enzyme polymorphisms in HHV-6 genomes.

The viruses present in patients 112 and 287 show a high level of DNA sequence homology to the prototype HHV-6_{GS} over the region defined by the pZVH14 probe. DNA sequences that hybridized to the HHV-6_{1102}-derived probe, p9.1 were also detected under stringent conditions in DNA extracted from the same samples (Jarrett *et al.* 1989). However restriction enzyme site variation relative to the HHV-6_{GS} was detected in the region covered by the pZVH14 probe. A variant pattern of HindIII sites was detected in the viruses present in both patients.

Previous isolates had shown restriction maps similar to the prototype isolate when hybridized to the pZVH14 probe (Downing *et al.* 1987; Tedder *et al.* 1987), though Josephs *et al.* (1988a) found evidence of genomic differences in HHV-6 isolates elsewhere in the virus. Becker *et al.* (1989) have subsequently reported the detection of a similar HindIII restriction pattern to that found in the viruses present in cases 112 and 287 following the analysis of additional virus isolates using the pZVH14 probe. The variant pattern was detected in isolates made from 4 patients of diverse ethnic origin (Becker *et al.* 1989). The data indicate that the HHV-6 genome is polymorphic and suggest that the variant HindIII pattern detected with the pZVH14 probe in this study is due to a common polymorphism.

4.4.2. The association of HHV-6 with AIL and AIL-like lymphoma.

HHV-6 DNA sequences were detected in tissues obtained from a patient with AIL which had progressed to T-zone lymphoma. One of the original HHV-6 isolates was made from the cultured PBMCs of a patient with AIL (Salahuddin *et al.* 1986). Thus AIL has been previously associated with HHV-6 infection.

An additional 4 AIL-like lymphomas analysed in this study were negative for HHV-6 DNA sequences, while Josephs *et al.* (1988a) did not detected viral DNA in 7 AIL biopsies included in their study. These data indicate that the presence of detectable HHV-6 DNA sequences is not consistently associated with either AIL or AIL-like lymphomas.

In patient 112 the analysis of multiple tissues demonstrated that there was no correlation between the extent of infiltration by tumour and the amount of HHV-6 DNA present. These results demonstrate that the virus is not present exclusively in the tumour cells in this patient. Unfortunately no tissue was available for in situ hybridization so it was not possible to determine the identity of the virus-infected cells.

The clinical and morphological features of AIL that are suggestive of a viral aetiology have been described in Chapter 1 (section 1.3.2). Viral involvement in the pathogenesis of AIL and AIL-like lymphoma was also discussed briefly in Chapter 3 (section 3.4.6). The data described in this chapter do not support a direct role for HHV-6 in the pathogenesis of AIL or AIL-like lymphomas. The possibility that HHV-6 may be playing an indirect role in disease pathogenesis is discussed further in section 4.4.4.

4.4.3. The association between HHV-6 and lymphomas occurring in the context of Sjögren's syndrome.

HHV-6 DNA sequences were detected in a patient with a B-cell lymphoma occuring in the context of Sjögren's syndrome. Samples from an additional 5 patients with Sjögren's syndrome, 2 of which had lymphomas, were found to be negative for HHV-6 DNA sequences (R.F. Jarrett, unpublished data). Josephs *et al.* (1988a) reported the detection of HHV-6 DNA sequences in 3 of 82 B-cell lymphomas analysed. One of the HHV-6-positive B-cell lymphomas had developed in a patient with a 10 year history of Sjögren's syndrome. Samples from an additional 97 patients with a variety of neoplastic and non-neoplastic disorders were negative for HHV-6 specific sequences. Six of the HHV-6-negative patients had Sjögren's syndrome-associated lymphomas. Thus HHV-6 is not consistently associated with lymphomas occurring in the context of Sjögren's syndrome.

However if the data from the two studies are combined, HHV-6-specific sequences were detected in a total of 5/218 patients with lymphoma, whereas 2/10 patients with Sjögren's syndrome-associated lymphoma were positive. The increased frequency of detection of HHV-6 in lymphomas occurring in patients with Sjögren's syndrome relative to other lymphomas is statistically significant ($p<0.05$ by Fisher's exact test).

In patient 287 viral DNA was detected in a biopsy showing no molecular or histological evidence of tumour infiltration. Virus infection was not, therefore, restricted to tumour cells. Josephs *et al.* (1988a) analysed multiple tissues from one of their HHV-6-positive patients and did not find viral DNA sequences in non-tumour tissues. However in this patient, HHV-6 DNA was not detected in the peripheral blood, which did appear to contain tumour cells. Thus the analysis of multiple tissues in three HHV-6 positive cases has shown that the virus is not exclusively or consistently associated with the presence of tumour cells.

The data do not indicate that HHV-6 is directly involved in the aetiology of lymphomas occurring in the context of Sjögren's syndrome. However the possibility that the virus may be playing an indirect role in the pathogenesis of these lymphomas remains and is discussed further in the following section.

4.4.4. Does HHV-6 play an indirect role in lymphomagenesis?

AIL and Sjögren's syndrome have a number of features in common. The clinical and pathological features of AIL have been described previously. The clinical features of Sjögren's syndrome have been described by Bloch *et al.* (1965) and Morrow and Isenberg (1987). Sjögren's syndrome is an autoimmune disorder characterized by xerostomia and keratoconjuncitivitis sicca and is frequently associated with other autoimmune diseases such as rheumatoid arthritis and systemic lupus erythrematosus. The disease is associated with a 44-fold increase in the incidence of lymphoma, the majority of which are of B-cell type (Kassan *et al.* 1978; Zulman *et al.* 1978)

Both AIL and Sjögren's syndrome are characterized by the presence of immunological abnormalities, including polyclonal B-cell activation and T-cell dysfunction (Whaley and Buchanon 1971; Azevedo and Yunis 1985; Honda *et al.* 1985). In addition the lymphoid proliferation that occurs in both disorders shows a similar pattern of clonal evolution. The notion that AIL and AIL-like lymphoma represent stages in the evolution of a polyclonal lymphoproliferation into a monoclonal disorder has been discussed in Chapter 3. A similar pattern of disease progression has been described in Sjögren's syndrome.

Zulman *et al.* (1978) reported the progression from an unrestricted to a restricted pattern of IgL expression in sequential salivary gland biopsies from Sjögren's syndrome patients. Schmid *et al.* (1982) and Isaacson and Hyjek (1987) detected restricted IgL expression by B-cells in salivary glands that lacked histological evidence of lymphoma; some of these patients subsequently developed overt lymphomas. Oligoclonal and monoclonal Ig gene rearrangements have also been detected in salivary glands lacking histological evidence of overt lymphoma (Fishleder *et al.* 1987; Freimark and Fox

1987). The analysis of sequential biopsies has suggested that different clonal B-cell populations may emerge and predominate over time (Fishleder *et al.* 1987). In the latter study the detection of clonal B-cell populations was not invariably associated with clinical features of malignancy.

Thus both AIL and Sjögren's syndrome appear to be characterized by the progression of polyclonal or oligoclonal lymphoproliferations to monoclonal disorders, which in some cases progress to overt lymphoma. It is therefore likely that the pathogenetic mechanisms involved in these two diseases will share common features. Indeed there are a number of reports describing the development of both AIL and Sjögren's syndrome in the same patient (Moore *et al.* 1976; Bisson *et al.* 1978; Le Charpentier *et al.* 1978; Aizawa *et al.* 1979; Pierce *et al.* 1979; Bauer *et al.* 1982). It is possible that HHV-6 could play a role in the induction of the underlying polyclonal lymphoproliferation observed in AIL and Sjögren's syndrome.

A number of indirect mechanisms by which viruses could be involved in lymphomagenesis were described in Chapter 1 (section 1.4.2). The possibility that chronic stimulation of the immune system by HHV-6 could be be involved in the pathogenesis of lymphomas occurring in the context of Sjögren's syndrome and AIL seems worthy of further investigation.

a)Sjögren's syndrome.

Chronic stimulation of the immune system could form the pathogenetic mechanism underlying both the autoimmune phenomena and the lymphomas occurring in Sjögren's syndrome. The evidence indicating that HHV-6 replicates in salivary glands has been reviewed in section 4.1.3. The detection of HHV-6 antigen expression in the salivary gland epithelium of healthy adults indicates that viral replication at this site is not normally associated with pathological consequences. However it is conceivable that in certain circumstances expression of viral antigens in salivary gland could induce chronic stimulation of the immune system.

Alternatively chronic immune stimulation could result from aberrant antigen expression by virus infected salivary gland epithelial cells. Baboonian *et al.* (1989) have suggested that altered antigen expression following virus infection of salivary gland could be involved in the pathogenesis of Sjögren's syndrome. These authors found that

adenovirus infection of human cell lines induces the relocalization of a nuclear protein, La, to the cell surface membrane. Antibodies to La are detected in 50-70% of cases of Sjögren's syndrome (Morrow and Isenberg 1987). Baboonian *et al.* (1989) also found that EBV and CMV infection of cell lines resulted in altered distribution of the La antigen.

The involvement of other human herpesviruses, including HCMV and EBV in the pathogenesis of Sjögren's syndrome has been previously proposed (Lerner *et al.* 1981; Shillitoe *et al.* 1982; Fox *et al.* 1986). The evidence for the involvement of HCMV is limited. Shillitoe *et al.* (1982) reported elevated antibody titres to HCMV in Sjögren's syndrome patients relative to matched controls, though these results were not confirmed by others (Venables *et al.* 1985).

A more substantial body of evidence implicates EBV as a potential aetiological agent. Lerner *et al.* (1981) found that the La antigen binds to EBV-encoded RNAs and suggest that this interaction could lead to autoimmunity. Salivary glands are known to be a site of replication of EBV (Venables *et al.* 1989). The rapid onset of Sjögren's syndrome following IM has been reported (Pflugfelder *et al.* 1987) and in addition EBV DNA sequences have been detected in a lymphoma occurring in the context of Sjögren's syndrome (Fox *et al.* 1989). It is possible that both EBV and HHV-6 may play a role in the pathogenesis of Sjögren's syndrome via one of the mechanisms described above.

b)AIL

Chronic antigenic stimulation due to herpesvirus infection may be involved in the pathogenesis of AIL. HHV-6 DNA has been detected in lymph nodes of patients with lymphoproliferative disorders by in situ hybridization (Kreuger *et al.* 1989). In this study increased numbers of HHV-6 infected cells were detected in nodes showing histological features of malignant lymphoma and "atypical polyclonal lymphoproliferation" than were present in "reactive" nodes. HHV-6 DNA was not detected in the tumour cells of lymphomatous nodes, but was present in scattered small lymphocytes, suggesting that any role that the virus might be playing in the pathogenesis of these disorders is likely to be indirect.

AIL has also been associated with infection by EBV; the occurrence of AIL in persons with evidence of active EBV infection has been reported (Virelizier *et al.* 1978). EBV DNA has been detected in AIL-like lymphomas (Bornkamm *et al.* 1976; Richel *et al.* 1990). In the study reported by Richel *et al.* (1990) EBV gene expression was localized to the tumour cells, however the clonality of the EBV genomes was not determined so the possibility that EBV infection had occurred subsequent to lymphoma development was not excluded.

4.4.5. Herpesvirus reactivation.

An alternative explanation for the detection of HHV-6 in the two patients described in this Chapter is that reactivation of HHV-6 is occurring due to the immune dysfunction. An additional speculative possibility is that HHV-6 reactivation in these cases may be an indicator of underlying retrovirus infection. These two possibilities are discussed further below.

a) Disseminated herpesvirus infection associated with immunosuppression.

It is possible that the detection of HHV-6 DNA sequences in the tissues of patients 112 and 287 is an indication of disseminated herpesvirus infection. Herpesvirus infection is a frequent complication of AIL and pneumonia caused by HCMV is a common cause of death in AIL patients (Frizzera *et al.* 1975; Nathwani *et al.* 1978; Pangalis *et al.* 1983). Fatal herpesvirus infection has also been reported in Sjögren's syndrome (Asherson *et al.* 1987). Thus the detection of HHV-6 and EBV in lymphomas occurring in the context of Sjögren's syndrome may reflect frequent herpesvirus reactivation in this disease.

The recent data obtained by Lawrence *et al.* (1990) showing that HHV-6 is closely related to HCMV is of interest in this context. It is possible that HHV-6 will share some biological properties with HCMV, which is a common cause of disease in immunosuppressed persons (Griffiths and Grundy 1987). However, as discussed earlier, (section 1.2) herpesviruses that are closely related on the basis of genome organization and DNA sequence homology may show widely divergent biological behaviour.

The detection of HHV-6 DNA sequences in multiple tissues obtained from both the virus positive-patients identified in this study is consistent with dissemination of virus infection due to profound immunosuppression. It should be noted, however, that HHV-6 was not detected in samples from a number of cases in which immune function was likely to be severely compromised.

b) Interaction with a retrovirus.

Most of the reported isolations of HHV-6 from the cultured PBMCs of adults have been made from retrovirus-infected persons (Salahuddin *et al.* 1986; Downing *et al.* 1987; Tedder *et al.* 1987; Lopez *et al.* 1988). This suggests that retroviruses may reactivate latent HHV-6 infection in vivo. Evidence that retroviruses may activate herpesvirus replication or expression has been reported. HIV appears to induce EBV to commence productive replication (Ernberg 1989), while preliminary evidence that the HTLV-I tax protein can trans-activate an EBV promoter has been reported (Pagano *et al.* 1988). Tax expression has also been shown to activate the HCMV enhancer in cotransfection experiments (M. Campbell, personal communication).

The involvement of retroviruses in the aetiology of both AIL and Sjögren's syndrome has been suggested (Ganesan *et al.* 1987; Garry *et al.* 1990). Sera from patients with AIL or Sjögren's syndrome have been shown to cross react with human retroviral core proteins (Nunley *et al.* 1987; Szabo *et al.* 1987; Talal *et al.* 1990b).

AIL and HIV-associated AIDS show some clinical and morphological similarities (Azevedo and Yunis 1985; Steinberg *et al.* 1988). HIV-infection and AIL are both characterized by hypergammagloblinaemia and T-cell dysfunction (Steinberg *et al.* 1988). In addition AIL-like lymphomas have been reported in persons with HIV infection and/or AIDS (Blumenfeld and Beckstead 1983; Lust *et al.* 1989).

Some clinical manifestations of HIV infection also overlap with features characteristic of Sjögren's syndrome, including lymphocytic infiltration of the salivary and lachrymal glands, with associated dry eyes and mouth (Itescu *et al.* 1989). In addition transgenic mice expressing the *tax* gene of HTLV-I have been reported to show salivary gland lesions typical of Sjögren's syndrome (Green *et al.* 1989).

The associations between HHV-6 and AIL and Sjögren's syndrome described above could, therefore, reflect stimulation of herpesvirus replication due to an underlying retrovirus infection.

4.4.6. The involvement of HHV-6 in HD.

The hypothesis that HD has an infectious aetiology has been discussed in Chapter 1 (section 1.3.2). The data are most convincing for HD occurring in younger age groups, and have led to the suggestion that HD in younger persons may arise as an unusual host response to a common transmissable agent acquired relatively late in life.

Members of the human herpesvirus family have been identified as candidate infectious agents. Many studies have shown an association between elevated antibody titres to EBV antigens and HD (Henle and Henle 1973; Hesse *et al.* 1977; Evans and Gutensohn 1984). A number of serological studies have reported a higher antibody prevalence to HHV-6 in patients with HD (Ablashi *et al.* 1988; Biberfeld *et al.* 1988). In a recent case-control study performed by Clark *et al.* (1990) higher antibody titres were found in HD patients compared to controls. In addition HHV-6 seroprevalence and antibody titres were found to be greatest in HD patients aged 15-34 years who lacked siblings.

Forty-seven cases of HD were analysed for the presence of HHV-6 genomes in this study, all were negative. Over 23 cases of HD occurring in persons less than 35 years old were included in the analysis. These data do not support a role for HHV-6 as a candidate transmissable agent responsible for HD in younger persons, and do not support a direct role for HHV-6 in the pathogenesis of HD in any age group. The possibility that HHV-6 infection could play an indirect role in the development of HD remains. An alternative explanation proposed by Clark *et al.* (1990) for their serological data is that the higher antibody prevalence and titres to HHV-6 detected in young HD patients lacking siblings are purely a marker of a lack of early social contact. Late infection with other common viruses, one or more of which may be involved in the aetiology of HD, is also likely to have occurred in this group.

4.4.7. Conclusions.

HHV-6-specific sequences were detected in 2/135 samples from patients with a variety of lymphoproliferative disorders and non-lymphoid neoplasms. The HHV-6 positive samples were from a patient with AIL-like lymphoma and a patient with B-cell NHL occuring in the context of Sjögren's syndrome. Both of these conditions have been previously associated with HHV-6. Precise cellular localization of the viral DNA in these samples would be of considerable value in assessing the potential role of HHV-6 in the aetiology of these lymphomas.

The data described here suggest that HHV-6 is present in some non-tumour cell type, but do not exclude the possibility that the virus is also present in the tumour cells. The detection of HHV-6 DNA in tumour and non-tumour tissues in both patients suggests that HHV-6 did not play a direct role in the pathogenesis of these lymphomas. The possibility remains that HHV-6 may be indirectly involved in the lymphoproliferative processes underlying both AIL and Sjögren's syndrome. Alternatively the detection of HHV-6 DNA sequences in the biopsy material may reflect reactivation and dissemination of herpesvirus infection occurring as a consequence of the severe immunosuppression seen in these two patients. A final possibility is that HHV-6 reactivation is occurring due to the presence of an underlying retroviral infection. The two latter alternatives do not exclude the possibility that HHV-6 is contributing to disease progression in these patients.

CHAPTER FIVE.

IDENTIFICATION OF THE IMMUNOREACTIVE B-CELL EPITOPES OF THE P19 CORE PROTEIN OF HTLV-I.

5.1. INTRODUCTION.

Following the isolation of the first oncogenic human retrovirus, HTLV-I, and the identification of this virus as the aetiological agent of adult T-cell leukaemia/lymphoma (ATL), the search for aetiological associations between retroviruses and other human haematological malignancies has intensified. The use of molecular and serological approaches for searching for viruses related to known leukaemogenic viruses was discussed in Chapter 1 (section 1.5). A variety of techniques have been utilized and/or described for searching for retroviruses related to HTLV-I. These include low stringency DNA hybridization using probes derived from HTLV-I sequences (Manzari *et al.* 1987), amplification of viral sequences using conserved DNA sequences as primers in the PCR (Bangham *et al.* 1988) and screening serum samples using a variety of assays for the detection of antibodies that react with HTLV-I proteins (Ranki and Krohn 1987; Sanders *et al.* 1990; Srivastava *et al.* 1990).

A number of studies have reported the detection of antibodies that react with HTLV-I core proteins in Western blots (WB) in sera from persons who do not appear to be infected with HTLV-I (Koprowski *et al.* 1985; Ohta *et al.* 1986; Ranki and Krohn 1987; Starkebaum *et al.* 1987; Schupbach *et al.* 1988; Sanders *et al.* 1990; Srivastava *et al.* 1990). In these studies, antibodies that cross-react with HTLV-I core proteins were identified in serum samples from healthy persons and persons with a variety of diseases. The authors of these reports have suggested that these persons may be infected with retroviruses related to HTLV-I.

In section 5.1.2 the evidence in favour of this suggestion and possible alternative explanations for the data are discussed. An experimental approach to the investigation of the origin and significance of cross-reactivity with HTLV-I proteins in HTLV-I negative sera was described in Chapter 1 (section 1.5.3). This chapter describes the results of experiments to identify the regions of HTLV-I core proteins that bind to antibodies present in cross-reactive sera. The data obtained could be used to test the hypothesis that persons whose sera show cross-reactivity with HTLV-I core proteins are infected with HTLV-I related retroviruses.

The assays that are currently available for the detection of HTLV-I antibodies are not sufficiently sensitive and specific to be used in isolation. The problems encountered in the serological diagnosis of HTLV-I infection and the requirement for more specific

assays are discussed in the following sections. This chapter describes the results of preliminary experiments that may be of value in the rational design of second generation assays for the detection of HTLV-I-specific antibodies.

5.1.1. Serological diagnosis of HTLV-I infection.

A variety of assays have been used for the detection of antibody to HTLV-I. Many "in house" assays have been developed and have been used for seroepidemiological studies. The variation in the sensitivity and specificity of such assays causes difficulties when comparing and collating studies from different laboratories. Recently a number of diagnostic assays have become commercially available though none of these is suitable for use in isolation.

In areas of Japan endemic for HTLV-I infection, screening programs for the detection of HTLV antibodies in donated blood have been in place for some years. Initially immunofluorescence (IF) using HTLV-I producing cell lines was employed (Hinuma *et al.* 1981), more recently a tiered approach has been adopted (Inaba *et al.* 1989). Samples are screened with a sensitive gelatin particle agglutination (GPA) method which uses gelatin particles coated with virus antigens (Serodia-ATLA, Fujirebio Inc.). Positive sera are tested by IF, and GPA positive, IF negative sera are also tested by WB. The use of WB permits the identification of antibodies to specific viral proteins, as illustrated in Figure 5.1.

Screening of blood donations for HTLV antibody is now also performed routinely in the USA. The US Food and Drug administration (FDA) have recommended a tiered approach to testing (US Public Health Service Working Group 1988). The assays used differ from those employed in Japan in that ELISAs employing whole virus as the target antigen are used for screening. Serum samples which show reactivity greater than a predefined cutoff are retested in duplicate and if positive in either of the duplicates are defined as repeatedly reactive (RR). RR samples are then analysed using more specific serological tests capable of identifying antibody to specific viral proteins.

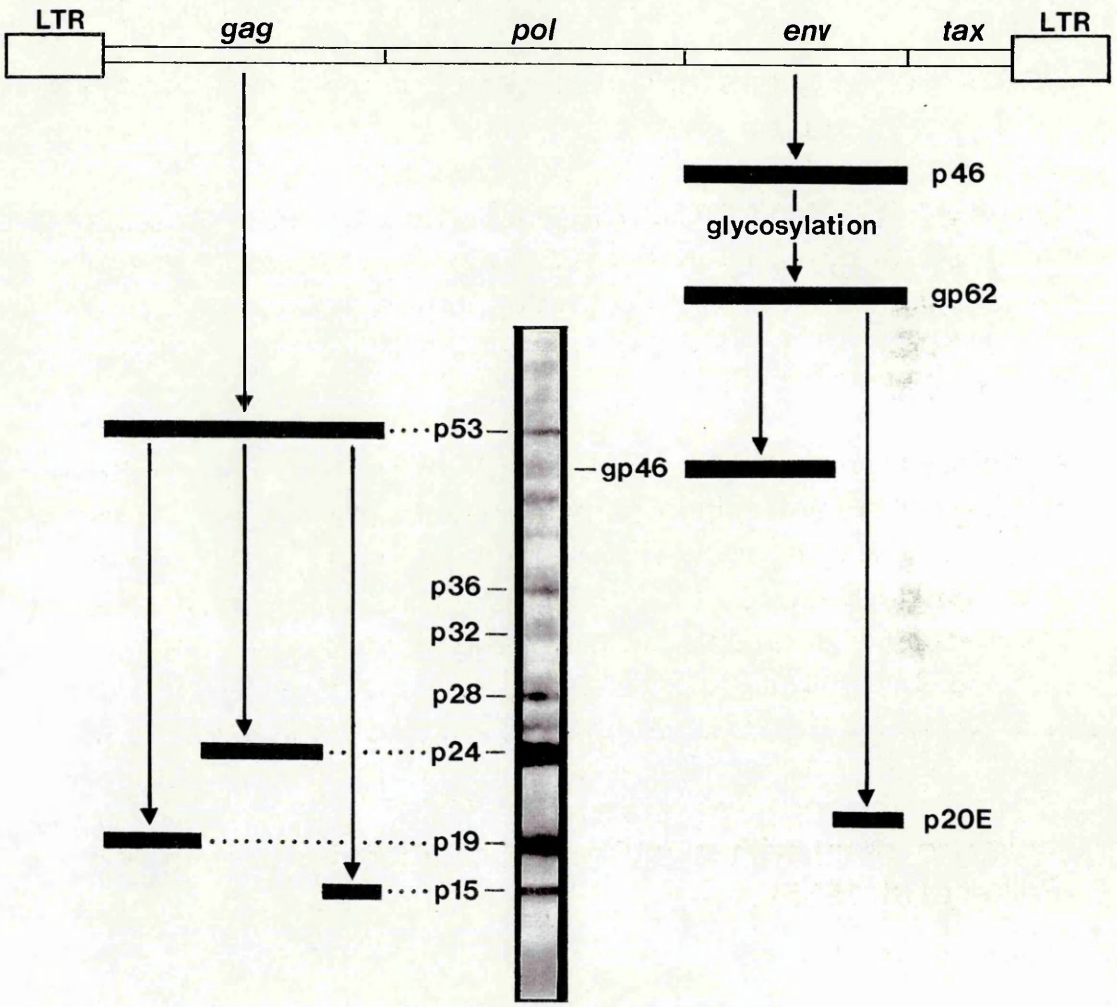
Suitable confirmatory tests include WB and radioimmunoprecipitation assays (RIPA) using whole disrupted virus as the target antigen. According to the confirmatory criteria adopted by the FDA "a specimen must demonstrate immunoreactivity to the gag gene product p24 and to an env gene product (gp46 and/or gp61/68) to be considered

Figure 5.1. The proteins that react with HTLV-I antibody-positive sera on western blot (WB) analysis (central panel) are gag or env gene products, as indicated. P53 is the gag protein precursor and is cleaved to give the viral structural proteins p19, p24 and p15 (Yoshida et al. 1985). These correspond to MA, CA and NC respectively, in the nomenclature of Leis et al. (1988). Antibodies to all these proteins are readily detected by HTLV-I WB analysis.

The env gene codes for a 46kDalton precursor (p46) which is glycosylated to give gp62. The glycoprotein is then cleaved to produce the transmembrane protein (p20E) and the envelope glycoprotein (gp46). The two latter proteins correspond to TM and SU respectively, according to the nomenclature of Leis et al. (1988). Antibodies to envelope proteins are only weakly detected on WB analysis, as shown (see section 5.1.1 for additional discussion).

Additional proteins on HTLV-I WB strips also react with HTLV-I antibody-positive sera. A protein corresponding to p36 has been shown to react with a p19-specific monoclonal antibody (Palker et al. 1985), however Tanaka et al. (1986) reported evidence that a p36 was a gag protein precursor, containing p24 and p15. P32 has been identified as a possible degradation product of p53 and reacts with both p24- and p19-specific monoclonal antibodies (Schupbach and Kalyanaraman, 1989). P28 has been shown to react with a p19-specific monoclonal antibody (Palker et al. 1985) .

Figure 5.1. Detection of HTLV-I-specific antibodies by western blot analysis.



"positive". Serum specimens not satisfying these criteria but having immunoreactivities to at least one suspected HTLV-I gene product (such as p19 only, p19 and p28, or p19 and env) are designated "indeterminate". Serum specimens with no immunoreactivity to any HTLV-I gene products in additional, more specific tests are designated "negative".

The tiered approach adopted in both screening programs is necessitated by the relatively low specificity of the screening assays and the complexity and expense of the confirmatory assays. The three ELISAs licensed by the FDA were estimated to provide a sensitivity of 97.3-100% (p=0.05) and a specificity of 99.3-99.9% (p=0.05) (US Public Health Service Working Group 1988). When used in populations with a low incidence of HTLV infection, however, tests with this degree of specificity may have a low positive predictive value. Large scale studies of blood donors in populations with a low incidence of HTLV-I associated disease have found that between 32% and 37% of RR samples confirm as HTLV positive using RIPA and/or WB (Fang *et al.* 1989; Hartley *et al.* 1990). Indeterminate results (IR) are obtained in 30%-37% of RR sera and negative results in 30%-32%.

Anderson *et al.* (1989) have assessed the relative sensitivities of WB and RIPA for the detection of antibody to specific viral gene products. They found WB to be more sensitive than RIPA for the detection of antibody to the core proteins, p24 and p19, though the difference was not statistically significant for p24. RIPA was significantly more sensitive than WB for the detection of *env*-encoded proteins. Similarly results obtained during mass screening studies indicate that for approximately two thirds of RR sera RIPA is required in addition to WB to obtain a reliable result (Fang *et al.* 1989; Hartley *et al.*. 1990, Lee *et al.* 1990).

The US Public Health Service Working Group (1988) have recommended that donors whose sera are RR but not HTLV positive on more than one occasion should be deferred indefinitely and informed that they have inconclusive test results. Thus the lack of specificity of available screening assays has deleterious consequences in terms of both the loss of HTLV negative units of blood and the difficulty of counselling persons with inconclusive test results. Two additional problems encountered as a result of the testing protocols used are the interpretation of IR and the inability to distinguish between HTLV-I and HTLV-II infection.

5.1.2 Indeterminate reactivities (IR).

Significant numbers of sera give IR on HTLV-I testing. The significance and origin of these reactivities is at present unclear. Seroepidemiological studies are difficult to compare as IR have been defined differently depending on the serological assays that have been used. Many studies have used only WB for the confirmatory analysis of RR sera. This has probably resulted in the classification of some HTLV positive sera as indeterminate. The definition of an IR that has been used throughout this text is a serum sample which shows reactivity to one or more of the core proteins of HTLV-I on WB but which does not confirm as being HTLV-I positive when analysed with one or more of the following confirmatory assays; RIPA, vesicular stomatitis virus (HTLV-I) pseudotype neutralization or competition ELISA.

There are a number of possible explanations for sera with IR.

(a) Early seroconversion or low titre antibody to HTLV.

Few incidences of seroconversion to HTLV-1 have been documented. Following HIV infection anti-core protein antibodies are the first to appear and may precede full seroconversion by several months (Ranki *et al.* 1987). It is possible that a similar sequence might follow infection by HTLV-I. Gout *et al.* (1990) detected an indeterminate pattern of reactivity by WB in serum from a woman following (probable) sexual transmission of HTLV-I. IgG antibody to p19 and related proteins (Pr53, p36, p28, p26) appeared 10 months before antibody to p24. A more recent study by Chen *et al.* (1990a) found that 8 HTLV-I infected individuals had antibodies to both *gag*- and *env*- encoded proteins within 1-3 years of seroconversion. Further studies are required to determine whether these findings can be confirmed in more recently seroconverted persons.

Low viral load has been suggested as a possible explanation for IR by Kwok *et al.* (1990b), who detected HTLV-II DNA using PCR in uncultured PBMCs of a patient whose sera was ELISA, RIPA and IF negative but reacted with p24 on HTLV-I WB.

Other evidence suggests that early seroconversion or low viral load are likely to be infrequent explanations of IR. Ssequential testing of sera from persons with IR failed to show subsequent appearance of antibody to other viral proteins (Hartley *et al.* 1990)

and PCR analysis of PBMCs from the majority of persons with IR failed to detect HTLV-I or -II DNA (Kwok *et al.* 1990a; 1990b). A study of recipients of blood products from persons whose sera gave IR failed to show seroconversion to HTLV-I, providing indirect evidence that the donors were not infected with the virus (van der Poel *et al.* 1989). However Inaba *et al.* (1989) have suggested that blood with low titre HTLV-I antibody may contain low viral loads and is likely to be of low infectivity.

(b) HTLV-II infection.

IR have been reported in some individuals in which HTLV-II and not HTLV-I infection has been identified by amplification of viral DNA using PCR (Lee *et al.* 1989; Kwok *et al.* 1990a). This is likely to be an infrequent explanation for IR as the majority of HTLV-II positive sera do show reactivity with HTLV-I gag and env proteins on WB and/or RIPA (Anderson *et al.* 1989; Lee *et al.* 1989).

(c) Non-specific reactivity.

Non specific reactions caused by the presence of autoantibodies, circulating immune complexes or high levels of Ig have been suggested as a cause of IR (Agius *et al.* 1988). However there appears to be no direct evidence to support any of these suggestions. Immune complex levels in serum and antibody titre to *P. falciparum* correlated with reactivity in HTLV-I and HIV ELISAs in an African population (Biggar *et al.* 1984; 1985). However the positive control used in the immune complex assay and sera from other areas endemic for malaria did not react in the ELISAs (Biggar *et al.* 1985). Thus the high levels of reactivity detected in the African sera were not considered to result from the presence of immune complexes or high Ig levels in the samples. The authors suggest that polyclonal B-cell proliferation due to *P. falciparum* infection could enhance detection of true retrovirus positivity, or that unknown serum factors could be responsible for nonspecific reactions in these sera.

The non-confirmed reactivities observed using HTLV-I ELISAs in these populations may be unrelated to IR obtained on WB, though evidence suggests that sera reactive on ELISA show an high incidence of apparent reactivity with HTLV-I proteins on WB (Schupbach *et al.* 1988; Bolton *et al.* 1989).

No studies appear to have specifically examined the correlation between HTLV-I IR on WB and serum levels of immune complexes, Ig or autoantibodies. However studies of patients with a variety of autoimmune diseases have failed to detect antibodies to HTLV proteins using HTLV-I and/or HTLV-II WB (McDougal *et al.* 1985; Starkebaum *et al.* 1987; Agius *et al.* 1988). These studies do not, therefore, support a link between autoantibody or immune complex levels and IR.

(d) Reactivity with non-viral proteins present on WB strips.

Cellular antigens in the viral lysates used for WB strips may react with antibodies in test sera, producing bands that may be confused with virus-specific bands. Henderson *et al.* (1987) have described the presence of human leukocyte antigens (HLA) in HIV viral lysates. HLA are also likely to copurify with HTLV virions. Based on the known sizes of HLA, reactions on WB may give rise to bands at 34-36kD, 30-32kD and 25kD (Henderson *et al.* 1987; Kwok *et al.* 1990b). Reactivity with these antigens should not be confused with reactivity to viral core proteins, with the possible exception of p36 and p32. The relationship of these proteins to the other *gag*-encoded proteins of HTLV-I has been discussed in the legend to Figure 5.1. Other cell surface proteins present in the viral lysates could migrate with viral core proteins and react with autoantibodies in test sera. There is however no direct evidence to support this possibility.

(e) Cross reactivity between viral and non-viral proteins.

HTLV-I proteins may share immunogenic epitopes with non-viral proteins. Antibodies directed against such non-viral proteins could be responsible for IR in virus-negative sera.

Some monoclonal antibodies to HTLV-I p19 cross react with tissues not infected with HTLV-I indicating that p19 shares an epitope or epitopes with one or more cellular proteins. A monoclonal antibody (MoAb), 12/1-2, which reacts with p19 (Robert-Guroff *et al.* 1981) has been shown to bind to a thymic epithelial cell antigen acquired during ontogeny and to the basal layer of tonsillar, adenoidal and oesophageal squamous epithelium (Haynes *et al.* 1983). Reactivity with fresh thymic epithelial cells

occurred with a cell surface rim pattern. Suni *et al.* (1984) found that MoAb 12/1-2 reacted with syncytiotrophoblasts from first trimester pregnancies and placental tumours, producing a diffuse cytoplasmic staining pattern. Immunoblots using proteins extracted from two choriocarcinoma cell lines identified a 28kD polypeptide as the target antigen.

Ralfkier *et al.* (1986) reported staining of the cytoplasm of basal keratinocytes of normal epithelia, benign dermatoses and CTCL by MoAb 12/1-2. The antibody also reacted in dot blots with conventionally extracted keratin proteins. The distribution of 12/1-2 reactivity in epithelia was different to that of antibodies against a variety of keratin classes thus the authors suggest that 12/1-2 may be reacting with an associated molecule rather than directly with intermediate filaments.

Two additional monoclonal antibodies directed against HTLV-I p19, αHTLV-2 and αHTLV-4 have been shown to react with non-virus-infected tissues and cell lines. αHTLV-2 reacted with the majority of tissues tested, including thymus, kidney tubules, liver, pancreas, prostate adenocarcinoma, melanoma, breast carcinoma and a variety of HTLV-uninfected T- and B-cell lines. αHTLV-4 reacted with kidney glomeruli, prostate adenocarcinoma, melanoma and breast carcinoma (Palker *et al.* 1985).

These results suggest that at least three p19 epitopes may cross react with epitopes present on cellular antigens as the patterns of cross reactivity seen with MoAbs 12/1-2, αHTLV-2 and αHTLV-4 are different. In addition MoAb 12/1-2 reacts with p22 of HTLV-II on WB, while αHTLV-2 and αHTLV-4 do not react with HTLV-II proteins on WB or RIPA (Palker *et al.* 1985), indicating that these MoAbs are reacting with different epitopes on p19.

Cross reactivity with malarial antigens has been considered a possible explanation for the correlation between antibody titres to *P. falciparum* and reactivity with HTLV ELISAs in African sera (Biggar *et al.* 1984; 1985). However this was considered an unlikely explanation as HTLV reactivity by ELISA did not correlate with seroprevalence of *P. falciparum* in this population. Studies in Papua New Guinea where malaria is also endemic have not detected a relationship between reactivity in HTLV-I screening assays and malarial prevalence or antibody titre (Kazura *et al.* 1987; Babona and Nurse 1988; Weber *et al.* 1989).

Sanders *et al.* (1990) found no correlation between the detection of IR using WB and RIPA and Plasmodium species parasitaemia in sera from persons living in Papua New Guinea. Reactivity with HTLV-I proteins on WB has been detected in sera from Papua New Guinea in a number of additional studies (Asher *et al.* 1988; Hardy *et al.* 1989; Re *et al.* 1989; Weber *et al.* 1989). The possibility that the results were due to cross reactivity between HTLV-I and *P. falciparum* antigens was not investigated in any of these studies, but in three of the reports reactivity was apparently detected to more than one viral core protein. Thus two immunoreactive epitopes on *P. falciparum* antigens would have to cross react with epitopes on separate HTLV-I core proteins in order to fully explain the recorded reactivities.

(f) Cross reactivity with endogenous viral proteins.

The human genome is known to contain a number of endogenous retrovirus-related sequences (HERV) many of which are defective (e.g. Steele *et al.* 1984; Callahan *et al.* 1985; Kroger and Horak 1987; Mager and Freeman 1987; Bangham *et al.* 1988; Perl *et al.* 1989). Some of the families of HERVs are related on the basis of sequence homology to the type C oncoviruses and homology with the HTLVs has been reported for a small number of HERV (Mager and Freeman 1987; Perl *et al.* 1989). It is possible that antibodies directed against HERV antigens with homology to HTLV-I proteins could give rise to IR in HTLV-I negative sera.

Mager and Freeman (1987) described a family of HERV which have a histidine tRNA primer binding site (RTVL-H) and found that one member of the family (RTVL-H2) contained sequences with homology to the HTLV *gag* genes. Translating the nucleotide sequence of the homologous region of RTVL-H2 gave 55% homology with HTLV-I p15 and 61% with the p12 NC protein of BLV. However no evidence for expression of this sequence was reported and the homologous coding sequence of RTVL-H2 is interrupted by a termination codon.

Perl *et al.* (1989) identified a transcriptionally active HTLV-related endogenous sequence, HRES-1/1, which contained sequences with homology to the LTRs and *gag* genes of HTLV-I and -II. An open reading frame in HRES-1/1 which could encode a 25kD protein (HRES-1/1 p25) showed regions of homology to HTLV-I p19 and p24. Translation of the coding sequences identified a region of HRES-1/1 p25 with 32% homology to amino acids 92-122 near the C-terminus of HTLV-I p19. A different

region of HRES-1/1 p25 showed 25% homology with HTLV-I p24 amino acid sequences. Transcription of the mRNA encoding HRES-1/1 p25 was detected in leukaemic cell lines, in EBV-transformed human peripheral blood lymphocytes and in human placenta. This last observation is of particular interest in relation to the cross-reactivity of the p19-specific MoAb 12/1-2 with human placental tissues and precipitation of a 28kD protein from choriocarcinoma cell lines by the MoAb (Suni *et al.* 1984). Confirmation that the HRES-1/1 p25 protein is itself expressed and shows serological cross-reactivity with HTLV-I core proteins would permit further evaluation of the possibility that antibodies to HRES-1/1 p25 may be a cause of IR.

(g) Infection with a novel HTLV related retrovirus.

Infection with a novel retrovirus related to HTLV has been advanced as a possible explanation for the presence of antibodies to HTLV-I core proteins in sera from patients with CTCL (Ranki and Krohn 1987; Srivastava *et al.* 1990) multiple sclerosis (Koprowski *et al.* 1985; Ohta *et al.* 1986), and large granular lymphocytic leukemia (Starkebaum *et al.* 1987). The finding by Schupbach *et al.* (1988) that 30-40% of Swiss blood donor sera showed reactivity with one or more HTLV-I core proteins on WB casts some doubt on the significance of these reports. However the relatively high concentration of sera used in this study makes comparison with data from other groups difficult, and could have increased the possibility of non specific reactions. In the following sections these data are discussed in the context of any additional or conflicting evidence for the involvement of retroviruses in these diseases.

Cutaneous T-cell lymphoma (CTCL)

The possibility that a retrovirus related to HTLV-I might be involved in the aetiology of CTCL was discussed in Chapter 1 (section 1.3.4). Serological evidence supporting this notion has been reported by a number of groups.

Ranki and Krohn (1987) studied Finnish patients with MF and large plaque parapsoriasis (PPs) using HTLV-I WB. They detected reactivity with HTLV-I p19 or p24 in 15/27 sera from patients with MF or PPs, 4/42 control sera from patients with dermatological disease and 0/33 control sera from healthy persons. The authors suggest that their results may indicate involvement of a retrovirus related to HTLV-I in MF.

Srivastava *et al.* (1990) also considered that infection with an HTLV-I related virus could be responsible for the reactions with multiple HTLV-I proteins on WB observed in sera from 3/6 MF patients.

Lange-Wantzin *et al.* (1986) detected antibodies to HTLV-I by ELISA in 11.4% of CTCL patients from Scandinavia and west Germany. In this study a competitive ELISA and absorption with HTLV-I infected cells were used to confirm the positive results. The low titre of antibodies detected and their presence in a small proportion of patients was suggested to indicate infection with a related retrovirus rather than HTLV-I (Saxinger *et al.* 1985; Lange-Wantzin *et al.* 1986).

Additional supportive evidence for the involvement of retroviruses in CTCL has come from ultrastructural analyses. A number of groups have reported the detection of retroviral like particles in the cytoplasm of Langerhans cells and related cells of patients with MF, PPs and other premycotic lesions (van der Loo *et al.* 1979; Fullbrandt *et al.* 1983; Slater *et al.* 1985). Saal *et al.* (1989) have also reported the detection of C-type retroviral particles in cultures of PBMCs and lymph node obtained from patients with CTCL. Two studies have reported the detection of reverse transcriptase activity associated with particles of similar density to retroviruses (van der Loo *et al.* 1979; Saal *et al.* 1989). However electron microscopic evidence may be difficult to interpret and most reports have described only intracytoplasmic particles, not typical of mammalian C-type retroviruses.

Manzari *et al.* (1987) have reported the isolation of a novel retrovirus related to HTLV-I, from a patient with CTCL. The virus, named HTLV-V, was isolated from cultured B-cells derived from the peripheral blood of a leukaemic patient with CD4+, CD25- CTCL. Hybridization to HTLV-I probes was detected under low stringency conditions, while HTLV-II and HIV probes did not hybridize. Hybridization at high stringency was observed using a probe derived from a virus present in a patient with T-cell leukemia. This virus was originally described as HTLV-Ic (Pandolfi *et al.* 1985). HTLV-Ic differed from HTLV-I in lacking an internal BamHI site, but otherwise appeared to be closely related by hybridization analysis and restriction enzyme mapping (Pandolfi *et al.* 1985; Manzari *et al.* 1984). Related sequences were detected by Manzari *et al.* (1987) in another seven patients with CD4+, CD8-, CD25- CTCL.

A number of features differentiated these cases from typical ATL cases. They did not all appear to have aggressive disease, the neoplastic cells did not express CD25 and patient sera showed only borderline positivity in HTLV-I ELISAs and gave faint reactions with HTLV-I p24 on WB. However it should be noted that HTLV-I associated malignancies do not always run an aggressive course, as discussed in Chapter 1 (section 1.3.4). In addition restriction enzyme site variation has been previously reported in HTLV-I isolates (Fukasawa *et al.* 1987).

This report has a number of confusing features and little additional information about HTLV-V has been published. At present the potential role of HTLV-V in CTCL remains to be established.

Multiple Sclerosis (MS)

The association between MS and an HTLV-I related retrovirus is controversial. Koprowski *et al.* (1985) reported increased levels of antibodies to HTLV-I p24 in the serum and CSF of MS patients, relative to patients with other neurological disease (OND) and healthy controls. Ohta *et al.* (1986) detected low titre antibodies to HTLV-I core proteins by WB in 11/46 sera from MS patients. Eleven sera from healthy controls and nine sera from patients with OND were negative. A number of groups found that MS sera gave negative results in assays for HTLV-I antibody (Hauser *et al.* 1986; Karpas *et al.* 1986; Ohta *et al.* 1986; Koike *et al.* 1988). However Ohta *et al.* (1986) and Koprowski *et al.* (1986) suggest that the negative results obtained with MS sera may reflect the low sensitivity of the assays employed in some of these studies.

Koprowski *et al.* (1985) detected HTLV-I-related RNA sequences in cells from the CSF of MS patients by in situ hybridization but these results could not be confirmed by Hauser *et al.* (1986). Recently Reddy *et al.* (1989) used the polymerase chain reaction (PCR) to amplify HTLV-I nucleotide sequences from DNA extracted from the PBMCs of 6 patients with MS. However the detection of a similar sequence in one of their control samples and the similarity between the detected sequence and the sequence of the virus infecting the MT-2 cell line used as a positive control raises the possibility that these results were due to contamination of sample material. Other groups have failed to confirm these results (Bangham *et al.* 1989; Richardson *et al.* 1989), thus these data remain controversial.

While a number of features of MS suggest that a viral aetiology is likely, and the association between retroviruses and neurological disease in animals and humans identifies them as candidate viruses, the evidence for the involvement of a retrovirus related to HTLV-I in MS is conflicting and inconclusive.

Large granular lymphocytic leukemia (LGLL)

The evidence for involvement of an HTLV related retrovirus in LGLL comes from two reports. Starkebaum *et al.* (1987) detected antibodies to p19 or p24 (and in one case additional proteins) of HTLV-I by WB in 6/12 sera from LGLL patients. Control sera from patients with Felty's syndrome, rheumatoid arthritis, other connective tissue disorders and healthy individuals were negative. Pandolfi *et al.* (1987) analysed sera from 27 patients with LGLL; 4 were positive in an HTLV-I ELISA, while antibodies to HTLV-I core proteins were detected by WB in 7 sera. These results were not confirmed by Imamura *et al.* (1988) who obtained negative results with sera from 11 patients with LGLL analysed by GPA, IF, ELISA and WB for antibodies to HTLV-I.

Papua New Guinea.

Infection with a retrovirus related to HTLV-I has also been suggested as an explanation for IR detected in sera from Papua New Guinea (PNG) (Weber *et al.* 1989; Sanders *et al.* 1990). Studies of sera from a number of populations in PNG have detected a variable incidence (0-45%) of antibodies to HTLV-I by ELISA (Kazura *et al.* 1987; Asher *et al.* 1988; Re *et al.* 1989).

Kazura *et al.* (1987) detected very high antibody titres (>1:900) in nearly half the sera titrated using a commercial ELISA. The HTLV-I specificity of the reactions obtained in 44/47 of their positive sera was confirmed using a competitive ELISA employing sheep polyclonal antisera to compete out specific antibody. The specificity of this competitive ELISA has been questioned (Weber *et al.* 1989). Data indicating that different species produce antibodies of differing specificity when inoculated with the same antigens (Getzoff *et al.* 1988) suggest that the use of heterologous antisera in competition assays may not be justified. Re *et al.* (1989) analysed the specificity of 72

ELISA positive sera by preadsorption with a commercial virus preparation and confirmed their positive results in 71 of the samples. When tested with WB these sera showed a variety of patterns of reactivity, from p19 only to p19, p24, and multiple gag protein precursors. Both Re *et al.* (1989) and Kazura *et al.* (1987) consider that their data indicate the presence of HTLV-I in the populations tested.

In support of this possibility, Yanagihara *et al.* (1990a) recently reported the serological confirmation of HTLV-I infection in 16/61 members of a PNG population. The positive sera contained antibodies reactive to *gag* encoded proteins and to a recombinant transmembrane protein in WB. Additional confirmation that the serological data indicated true HTLV-I infection was obtained following the isolation of HTLV-I from the peripheral blood lymphocytes of a member of the same population group, the Hagahai (Yanagihara *et al.* 1990b). In addition to the sera in which HTLV-I infection was confirmed, i.e. those in which antibodies to both *env* and *gag* encoded proteins were present, high numbers of sera with IR were detected. The authors suggest that these may indicate early seroconversion, or may be due to infection with an HTLV-I related retrovirus.

Evidence from other studies suggests that the antibodies detected in PNG sera using HTLV-I assays may not be to HTLV-I itself but rather to a related retrovirus. Asher *et al.* (1988) found that 24/49 of their sera showed reactivity to HTLV-I p19, p24 and p53 on WB and some sera reacted weakly to gp46 and gp68. However when thirty of these sera were tested in HTLV-I/VSV pseudotype neutralization assays none showed neutralization, leading the authors to suggest that these individuals might be infected by strains of HTLV-I with antigenically variant envelope proteins.

Weber *et al.* (1989) studied sera from four populations in PNG using a variety of assays, including GPA, competitive ELISA, an "in house" IgG antigen capture radioimmunoassay, IF and WB, and failed to find convincing evidence for infection with HTLV-I. Thirty five of three hundred and fifty seven sera were positive in a commercial ELISA (DuPont), but these samples were negative in a competitive ELISA and did not neutralize vesicular stomatitis virus (HTLV-I) pseudotypes. Three of the thirty five ELISA positive sera showed weak reactivity with HTLV-I core proteins on WB. The authors suggest their data indicate that high levels of false positive results may be obtained when using ELISAs to detect HTLV-I antibody in sera obtained from populations in PNG. They suggest that the weak reactivity with core proteins detected in some sera may indicate cross reactions with an HTLV-related retrovirus.

Sanders *et al.* (1990) used ELISA, WB and RIPA to analyse sera from villagers living in PNG. Reactivity to p19 and/or p24 was detected using HTLV-I WB in 14/15 sera that had shown evidence of reactivity with HTLV-I proteins in ELISA or IFA. One sample reacted in addition to a recombinant transmembrane protein. Reactivity to the gp46 envelope protein was not detected in any sera, thus the authors suggest that infection by a variant strain of HTLV-I may give rise to the IR in these sera.

ATL and TSP have not been reported in PNG (Babona and Nurse, 1988). Although it is possible that these diseases could be under-reported this does suggest that HTLV-I is unlikely to be prevalent in this area at the levels suggested by Kazura *et al.* (1987) and Re *et al.* (1989).

5.1.3. Distinguishing between HTLV-I and HTLV-II infection.

HTLV-II shares approximately 57% DNA sequence homology with HTLV-I, while the structural proteins show 55-85% amino acid sequence homology (Seiki *et al.* 1983; Shimotohno *et al.* 1985). Sera from HTLV-II infected persons show cross reactivity with HTLV-I antigens and will give positive results with HTLV-1 screening assays. Confirmatory assays are also unable to distinguish between antibodies to the two viruses. HTLV-II positive sera react with HTLV-I gag proteins on WB and with HTLV-I gag and env encoded proteins in RIPA (Hartley *et al.* 1990).

For the counselling of HTLV positive persons identified during testing accurate identification of the specific infecting virus is important, as HTLV-II has not been identified as an aetiological agent of disease. Seroepidemiological studies of HTLV infection have also been complicated by the lack of distinction between HTLV-I and HTLV-II.

A number of assays able to distinguish between infection with HTLV-I and HTLV-II have been developed. A virus neutralization assay using vesicular stomatitis virus pseudotypes utilizes differences in the immune response to HTLV-I and -II proteins (Clapham *et al.* 1984), but is technically complex and therefore not suitable for widespread application. A variety of competition ELISAs have been developed. Tedder *et al.* (1984) used a known HTLV-1 positive human serum standard to compete out reactivity to disrupted whole virus, in a study which identified IVDA as a population

with increased risk of HTLV-II infections. Saxinger *et al.* (1984) have described a similar assay which uses sheep anti-HTLV antisera, this assay has also been used by Lange-Wantzin *et al.* (1986) and Kazura *et al.* (1987). The problems associated with the use of antisera from heterologous species for confirmation of antibody specificity were discussed in the previous section. Robert-Guroff *et al.* (1986) used antigens from HTLV-I and HTLV-II producing cells to compete out reactivity to HTLV-I p24. The discriminatory ability of this assay is questionable, as HTLV-I p24 shows approximately 85% homology to the equivalent protein of HTLV-II and HTLV-II positive sera show strong reactivity with HTLV-I p24 in Western blots (Shimotohno *et al.* 1985; Wiktor *et al.* 1990). A competition ELISA does offer the potential for widespread use, however none of these assays is at present commercially available.

Molecular analysis of the virus present in PBMCs provides an alternative to serological assays. HTLV-I and HTLV-II may be distinguished by Southern hybridization with viral probes but this is time consuming and requires relatively large numbers of virus infected cells. DNA amplification using PCR permits rapid analysis of large numbers of samples and requires only small numbers of cells. A number of groups have developed PCR strategies for distinguishing between HTLV-I and HTLV-II. Sequences conserved between the two viruses have been used as PCR primers and discriminatory probes selected from divergent intervening sequences (R. Jarrett, unpublished data). Alternatively a restriction enzyme site difference in the intervening sequence has been used to differentiate HTLV-I from HTLV-II (Lee *et al.* 1989). Kwok *et al.* (1988) have used a different approach, selecting virus specific primers and probes from sequences poorly conserved between the two viruses. The use of PCR to distinguish between HTLV-I and HTLV-II is highly specific. A number of studies using the technique have shown that IVDA in the USA and Italy are more likely to be infected with HTLV-II than HTLV-I (Ehrlich *et al.* 1989; Lee *et al.* 1989; Kwok *et al.* 1990a; Zella *et al.* 1990), confirming the earlier observation of Tedder *et al.* (1984). However the sensitivity of PCR can result in considerable problems with false positives due to sample contamination and sophisticated laboratory equipment and technique are required.

Wiktor *et al.* (1990) have proposed a WB based algorithm for distinguishing between HTLV-I and HTLV-II antibody positive sera. Samples with p19 reactivity of greater than or equal to the intensity of reactivity seen with p24 were scored as HTLV-I positive, while samples with p24 reactivity greater than the reactivity seen with p19 were scored as HTLV-II positive. This system gave 100% accuracy when tested with 57

HTLV-I positive sera and 40 HTLV-II positive sera from persons in whom the identity of the infecting virus had been previously determined by PCR. A recombinant p21E was included with the viral lysate used in the WB strips, to increase the sensitivity with which antibodies to envelope proteins were detected. This technique would therefore appear to provide a useful approach for use at present, though its subjectivity and dependence on the consistency of viral preparations used in the WB may prove to limit its reliability. The commercially available WB kits are, in addition, relatively expensive.

There is therefore a need for a simple, inexpensive serological assay capable of distinguishing between infection with HTLV-I and HTLV-II. The problems associated with the detection of IR in HTLV-I negative sera, as described earlier, also emphasize the need for more specific assays for the diagnosis of HTLV-I infection. It is important for the development of more specific assays that the reasons for non-specific or indeterminate reactivity in HTLV-I negative sera are identified. If the indeterminate reactivities with HTLV-I core proteins represent cross reactivity with shared epitopes this will limit the specificity of assays using recombinant forms of these proteins as target antigens. Alternatively if the epitopes reactive with indeterminate sera are different to those reactive with HTLV-I positive sera, the use of incomplete recombinant proteins or appropriate peptides as targets could eliminate this problem.

5.1.4. Experimental aims.

Two of the major problems associated with the serological methods available for the diagnosis of HTLV-I infection are the interpretation of IR and the distinction between HTLV-I and HTLV-II infection.

IR involving p19 reactivity on WB could be due to autoreactivity to cellular antigens as p19 has been shown to share one or more antigenic epitopes with human tissues. There is evidence to support the hypothesis that a retrovirus related to HTLV-I may be responsible for some IR. This appears to be most convincing for populations identified in PNG though considerable differences in results have been obtained by different laboratories. Other possible explanations for IR include non-specific reactions with viral proteins, reactivity with co-migrating cellular proteins on HTLV-I WB, cross reactivity with antigens on other infectious agents or cross reactivity with endogenous retrovirus antigens.

(I) Investigation of the origin of IR.

The main aim of this study was to investigate the origin of IR involving reactivity with p19 on WB. In order to analyse the specificity of IR, HTLV-I p19 was expressed as a fusion protein in bacteria and used as a target antigen in immunoblots with indeterminate sera.

Antibodies in indeterminate sera which are reacting with viral protein are likely to be directed against linear epitopes rather than discontinuous or conformational epitopes, as the target proteins in WB are denatured. Identification of the linear epitopes reactive with indeterminate sera therefore offers a method of investigating the specificity and origin of IR.

The epitope mapping technique developed by Geysen *et al.* (1987) was used to determine the epitopes of HTLV-I p19 reactive with HTLV-I positive sera and with indeterminate sera. Two groups of sera with IR were studied, sera from two Caucasian patients with dermatological disease and sera from nine individuals from PNG. Four monoclonal antibodies reactive with p19 were also analysed.

(II) Identification of the linear epitopes of HTLV-I p19 reactive with HTLV-II-positive sera.

The identification of epitopes on HTLV-I proteins which react with HTLV-I positive sera but do not cross react significantly with HTLV-II positive sera would be useful for the development of assays to distinguish between infection with the two viruses.

A group of five HTLV-II positive sera were analysed in order to determine the epitopes on HTLV-I p19 cross reactive with HTLV-II positive sera.

5.2. MATERIALS AND METHODS.

Some of the methods employed in the experiments described in this chapter have been described in Chapter 2. Methods that were not described earlier are detailed in the following sections. The sources of some of the materials that were used in the following experiments are given in Table II.I or Table V.I. The sources of materials that are not mentioned in the tables are described in the text.

5.2.1. Origin and characterization of sera.

Monoclonal antibodies.

MoAb 12/1-2 ascites fluid was a gift from Dr. M. Robert-Guroff. MoAb 12/1-2 reacts with HTLV-I p19 and precursor proteins and also cross reacts with HTLV-II p22 and a variety of normal and malignant human tissues, as described in section 5.1.2 (Robert-Guroff *et al.* 1981; Haynes *et al.* 1983; Suni *et al.* 1984). MoAbs 12G4, 9G10, and 13B12 were a gift from Dr. T. Palker and were all obtained as ascites fluid. MoAb 12G4 appears to be specific for HTLV-I p19 and has not been shown to cross react with human tissues (Palker *et al.* 1985). MoAb 12G4 was also obtained from Seralab as MAS 197c, the MoAb from either source is referred to as 12G4 in the following text. MoAb 13B12 reacts with HTLV-I p19 and p19 precursor proteins, but not with HTLV-II p22, and does not appear to cross react with human tissues (Palker *et al.* 1986). MoAb 9G10 reacts with HTLV-I p19 but is not well characterized (Palker *et al.* 1986).

Polyclonal sera.

HTLV-I positive sera were obtained from a variety of sources and were characterized using a number of different assays. The origins of the HTLV-I positive sera used for epitope mapping experiments and the results obtained in HTLV-I assays are given in Table V.II. The origins and results of serological analysis of additional HTLV-I positive sera used in peptide ELISAs are given in Table V.III. For the purposes of this study all

Table V.I. Sources of chemicals

Chemical	Source
Acetic anhydride	Sigma
Dichloromethane	BDH
Diethanolamine buffer	Biorad
Diisopropylethylamine	Sigma
Dimethylformamide (DMF)	Rathburn Chemicals Ltd. (RCL)
Methanol	BDH
Novo Biolab wash buffer	Dr. J. Fuller (Novo Biolabs Ltd.)
Piperidine	RCL
P-nitro-phenol phosphate	Biorad
Trifluoroacetic acid (TFA)	RCL
Tween 20	BDH

Table V.II. ATL, adult T-cell leukaemia; ELISA, enzyme linked immunoassay (DuPont); C-ELISA, competitive ELISA (a modification of the assay described by Tedder et al. 1984); GPA, gel particle agglutination assay (Serodia ATLA, Fujirebio); IF, immunofluorescence; TSP, tropical spastic paraparesis; WB, western blot (DuPont); U.K. sera were characterized by S. Crae; Japanese sera were obtained from Dr. B.C. Dow; serum samples 796 and 797 were obtained from Dr. R.S. Tedder.

Table V.II. Origin and characterization of HTLV-I antibody-positive sera used in epitope mapping experiments.

Sample No.	Country of origin	Disease	Results of serological analysis				
			GPA	IF	ELISA	WB	C-ELISA
374	U.K.	ATL			+	+	+
628	U.K.	ATL			+	+	+
716	U.K.	ATL			+	+	+
731	Japan	Unknown	+	+		+	+
733	Japan	Unknown	+	+		+	+
737	Japan	Unknown	+	+		+	+
739	Japan	Unknown	+	+		+	+
741	Japan	Unknown	+	+		+	+
796	Brazil	TSP	+			+	+
797	Monserrat	TSP	+			+	+

Table V.III. W.I., West Indies; U.K. (Imm-W.I.), immigrant from W.I. now living in the U.K.; 1/2+, only one of the two samples positive; no., number; other abbreviations are defined in the legend to Table V.II. Serum samples were characterized by S. Crae (c) or by the senders of the samples, as follows; (a), Dr. R.S. Tedder; (b), Dr. B.C. Dow; (d), Dr. U. Desselberger.

Table V.III. Origins and characterization of additional HTLV-I positive sera.

No. of samples (sender)		Country of origin	Disease associations	Results of serological assays GPA	IF	ELISA	WB	C-ELISA
1	(a)	W.I.	TSP	+			+	+
12	(b)	Japan	Unknown	+	+			+
2	(c)	U.K.	Relative of ATL patient				+	+
5	(a)	Unknown	Unknown	+				+
5	(d)	U.K. (Imm-W.I.)	TSP	+	+	+		+
2	(d)	U.K. (Imm-W.I.)	Relative of TSP patient	+	1/2+	1/2+		+
2	(d)	U.K. (Imm-W.I.)	Unknown	+	+	+		+
1	(d)	U.K. (Imm-W.I.)	Low lymphocyte no.s					+

sera were confirmed as HTLV-I-positive using a competitive ELISA (J. Garson, personal communication). The HTLV-I-specific competitive ELISA was a modification of the ELISA described by Tedder *et al.* (1984).

Indeterminate sera from two Caucasian patients were identified during the screening of patients with dermatological disease for antibodies to HTLV-I.

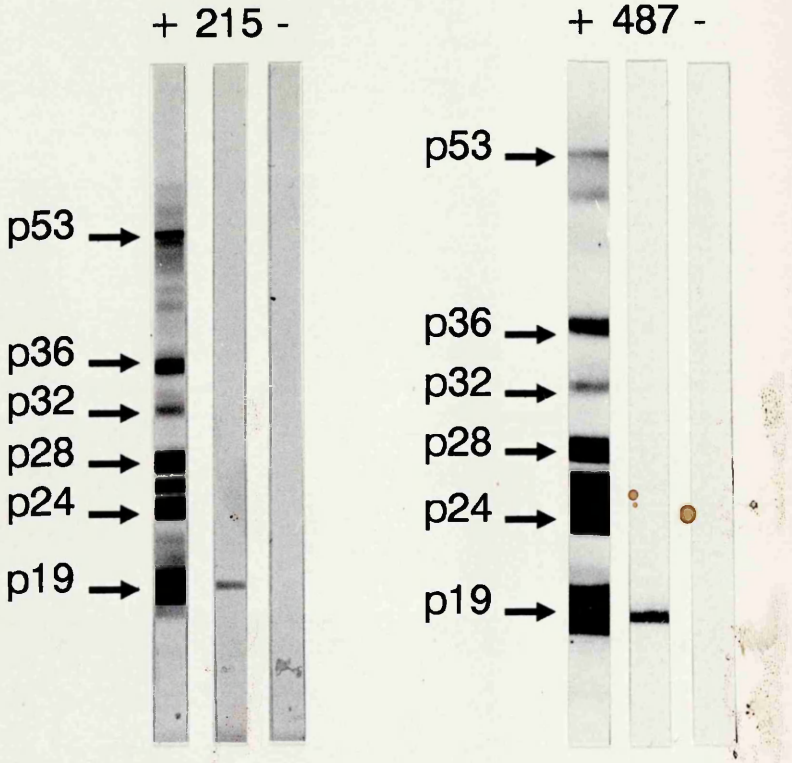
Patient 215 was initially a member of a control group, but subsequent questions revealed that he had chronic acne. The serum from this patient was negative on HTLV-I ELISA (DuPont U.K. Ltd.) according to the manufacturers cutoff value, but the O.D. readings obtained in duplicate assays were high relative to those obtained with other HTLV-I negative sera. When tested in HTLV-I WB reactivity to p19 was detected (Figure 5.2).

The second patient (487) had SS with typical skin lesions. Serum from this patient was negative on HTLV-I ELISA according to the manufacturers recommended cutoff but the O.D. reading was high relative to that obtained with other HTLV-I negative sera. This sample showed reactivity with p19, p26 and p28 on HTLV-I WB (Figure 5.2).

Nine indeterminate sera from PNG were obtained from Professor J. Weber. The results obtained in WB analyses of these sera are shown in Table V.IV. The sera were negative for HTLV-I-specific antibodies in "in house" IgG antigen capture radioimmunoassays and competitive radioimmunoassays performed at the Central Public Health Laboratory (J. Tosswill, personal communication). The results obtained from the analysis of these sera in an HTLV-I-specific competitive ELISA are shown in Table V.IV (J. Garson, personal communication). Eight serum samples gave borderline results, one sample (number 42) was weakly positive.

HTLV-II positive sera were obtained from Dr. R. Tedder. Three sera were of American origin and were identified during the screening of blood donations. Serum sample 854 was obtained from an Italian IVDA and serum sample 806 was obtained from a U.K. IVDA. All five sera were confirmed as HTLV-II positive and HTLV-I negative using the PCR (J. Garson, personal communication). Three sera, 806, 877 and 878, showed strong reactivity with HTLV-I p19 on WB (DuPont); two sera, 854 and 876 reacted weakly to p19 (J. Garson, personal communication, Figure 5.3).

Figure 5.2. Reactivity of serum samples 215 and 487 in HTLV-I WB.



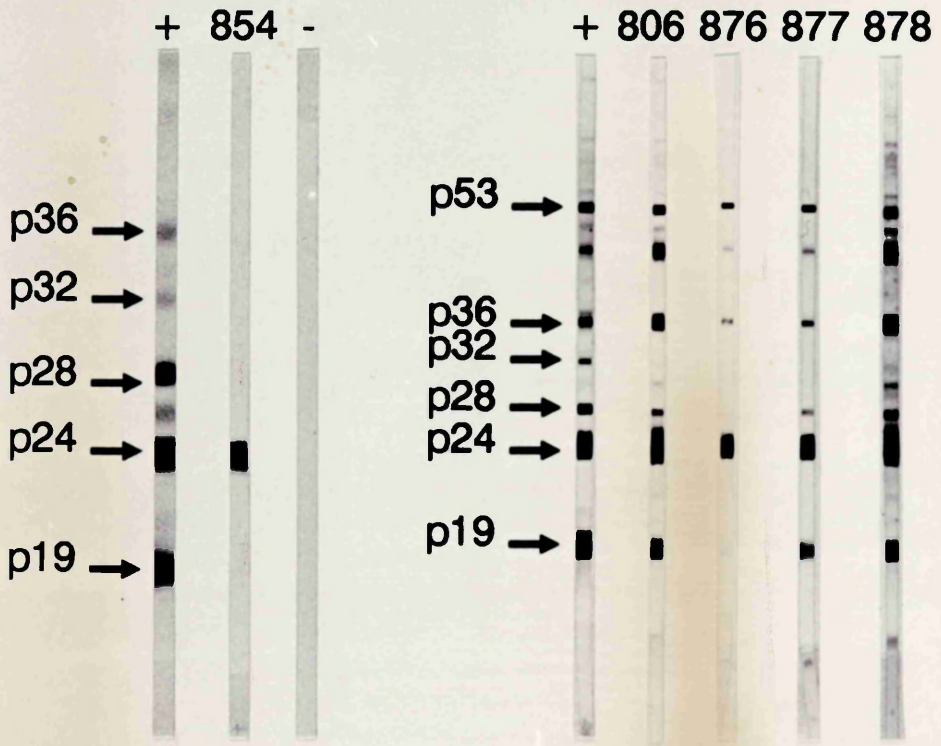
The nomenclature of HTLV-I proteins has been described in the legend to Figure 5.1. (-), HTLV-I antibody-negative control serum; (+), HTLV-I antibody-positive control serum. WB analyses were performed by S. Crae.

Table V.IV. WB, western blot. WB analyses were performed by J. Tosswill using DuPont WB strips. *, the results obtained in competitive ELISA's were communicated by Dr. J. Garson. The results are the mean of duplicate determinations of the extent to which the test serum competed with a standard HTLV-I antibody-positive serum for binding to viral antigen. The data are expressed as a percentage of the competition obtained with a known HTLV-I antibody-positive control serum.

Table V.IV. Results obtained from the analysis of PNG sera in HTLV-I WB and competitive ELISA (C-ELISA).

Sample No.	Mobility of reactive proteins on HTLV-I WB (relative molecular weight x 1000)									C-ELISA % inhibition *
	15	19	21	24	26	28	32	36	53	
884		+			+	+	+		+	79
885		+	+		+	+	+		+	43
886		+			+	+	+		+	50
887		+			+	+	+		+	46
888		+			+	+		+	+	58
889	+	+	+		+	+	+	+	+	55
890		+	+	+	+	+	+	+	+	51
891		+			+	+	+	+	+	63
892		+			+	+	+	+	+	38

Figure 5.3. Reactivity of HTLV-II antibody-positive serum samples in HTLV-I WB.



The nomenclature of HTLV-I proteins has been described in the legend to Figure 5.1. (-), HTLV-I negative control serum; (+), HTLV-I positive control serum. WB analyses were performed by P. Tuke.

Control sera used in the epitope mapping experiments were obtained from a patient with mycosis fungoides (serum 400) and from healthy individuals (samples 1 and 2). All sera were screened by HTLV-I WB and showed no reactivity with viral proteins. Control sera used in other experiments were negative on HTLV-I ELISA (DuPont) and were obtained from healthy individuals (4 sera) and patients with autoimmune disorders (4 sera), haematological malignancies (31 sera) or idiopathic thrombocytopaenic purpura (3 sera).

5.2.2. PGEX-3X vector

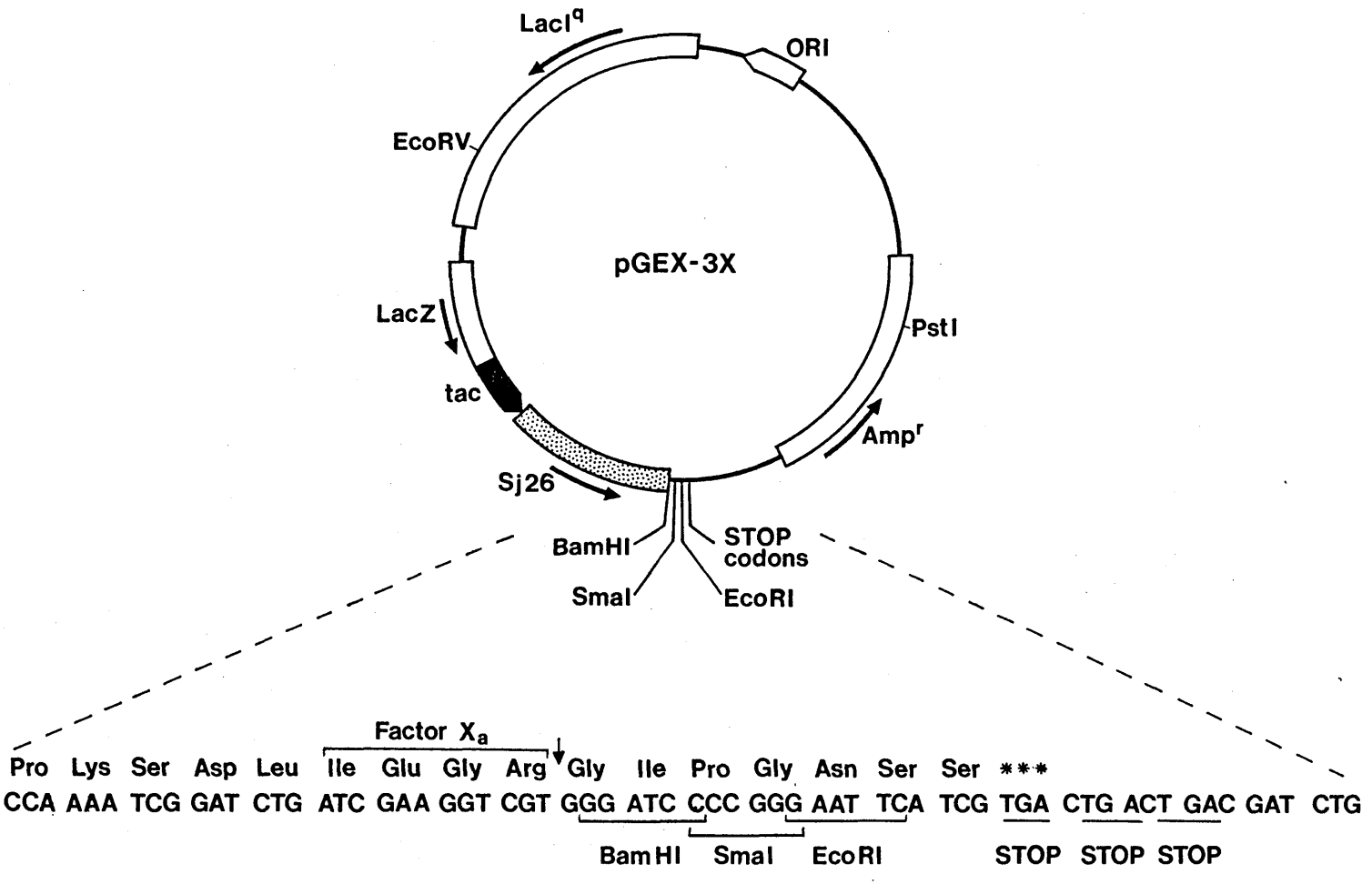
The pGEX-3X vector for the expression of recombinant p19 in bacterial cells was obtained from Amrad Corporation Limited and was first described by Smith and Johnson (1988). The vector is constructed to permit the expression of foreign polypeptides as fusion proteins with a 26-kDa glutathione-S-transferase (GST) originating from *Schistosoma japonicum*. The expressed fusion protein can be purified under non-denaturing conditions by affinity chromatography with glutathione linked to agarose beads. The structure of pGEX-3X is shown in Figure 5.4. The DNA sequence encoding the foreign polypeptide may be cloned into BamHI, SmaI or EcoRI restriction enzyme sites. The 3' vector sequences contain translation termination codons in all three reading frames. Fusion protein expression is under the control of the *tac* promoter, and is therefore inducible with IPTG (de Boer *et al.* 1983). PGEX-3X also contains coding sequences for a recognition site for the protease, factor X_a, in a 5' position relative to the cloning site. Thus the foreign polypeptide may be cleaved from GST using factor X_a following expression of the fusion protein.

5.2.3. Cloning and sequencing of p19.

The p19 coding sequences of an HTLV-1 provirus integrated as a single copy in the leukaemic cells of a patient with ATL (patient 374) were cloned into the pGEM-7Zf(+) vector (Promega Corporation). The PCR was used to specifically amplify p19 coding sequences, while incorporating restriction enzyme sites for cloning at 5' and 3' ends.

Figure 5.4. Amp^r, ampicillin resistance gene; $LacI^q$, the q mutation of the gene encoding the lac repressor ensures more efficient repression of the synthesis of proteins under the control of the tac promoter; lacZ, a portion of the gene encoding beta-galactosidase; ori, origin of plasmid DNA replication; Sj26, gene encoding the glutathione-S-transferase of Schistosoma japonicum; tac, the hybrid trp and lac (tac) promoter (de Boer et al. 1983). The nucleotide sequence of the plasmid at the C-terminus of Sj26 is shown, indicating the location of stop codons, restriction enzyme cleavage sites and the cleavage site for Factor Xa. The location of the unique PstI and EcoRV sites are shown. The structure of pGEX-3X is derived from Smith and Johnson (1988).

Figure 5.4. Structure of the pGEX-3X expression vector.



The PCR was performed as described in Chapter 2, section 2.3.4. The primers used for the amplification, P-19/5' and P-19/3' are shown in Figure 5.5. The amplified product was purified by PAGE as described in Chapter 2, sections 2.2.5-2.2.6, digested with BamHI and separated from the small terminal fragments by PAGE. The p19 fragment was then ligated into the BamHI site of pGEM-7Zf(+) as illustrated in Figure 5.6. The products of ligation reactions were transformed into *E. coli*, strain DH5α, and recombinant plasmids were selected by α-complementation, as described in section 2.5.3.

Plasmid pG7Zf19.5, which contained the p19 coding sequences in the orientation illustrated in Figure 5.6, was selected for nucleotide sequence analysis. The sequencing strategy is illustrated in Figure 5.7. The p19 insert was sequenced on both strands using T7 and SP6 primers (Promega Corporation) to prime DNA synthesis from flanking plasmid sequences. Two primers, P1 and P2, derived from sequences within the insert were obtained from an "in house" oligonucleotide synthesis facility and used to complete the nucleotide sequencing as shown in Figure 5.7.

Primer P1 was a 17-mer oligonucleotide, ACTACTCCCTCCTAGCC, corresponding to the upper strand nucleotides 1014-1030 of lambda-ATK-1 (Seiki *et al.* 1983). Primer P2 was a 17-mer oligonucleotide, ATTTCATTCACCCGGCC, corresponding to the lower strand nucleotides 1071-1055 of lambda-ATK-1 (Seiki *et al.* 1983). One hundred nanograms of each of the primers were included in the appropriate sequencing reactions. This quantity was calculated to give a molar ratio of primer to template of 10:1.

Expression of rp19.

HTLV-1 p19 was expressed in *E.Coli* as a fusion protein with GST using the pGEX-3X expression vector (Smith and Johnson 1988). The pG7Zf19.5 plasmid (Figure 5.6) was digested with BamHI and EcoRI and the p19 insert subcloned into the BamHI and EcoRI sites of pGEX-3X to create the plasmid pGEX-3X19. *E. Coli* strains JM105, JM109 and HB101 were transformed with the recombinant plasmid and the relative efficiencies of expression of rp19 in each strain assessed.

Figure 5.5. The nucleotide sequences of the primers used for amplification of the p19 coding sequences of the HTLV-I provirus present in the leukaemic cell DNA of patient 374. The extent to which the primers 19/5' and 19/3' base pair with the lower and upper strands of HTLV-I DNA is shown. The nucleotide sequence of HTLV-I is based on that published by Seiki et al. (1983). The regions of the oligonucleotide primers that do not pair with HTLV-I DNA sequences generate restriction sites for BamHI at either end and a single restriction site for EcoRI at the 3' end of the product obtained following amplification, as illustrated at the base of the diagram. The locations of the codons for the initiating methionine (met^{I}) and the C-terminal leucine (leu) residue are shown.

Figure 5.5. Amplification of the p19 coding region of HTLV-I.

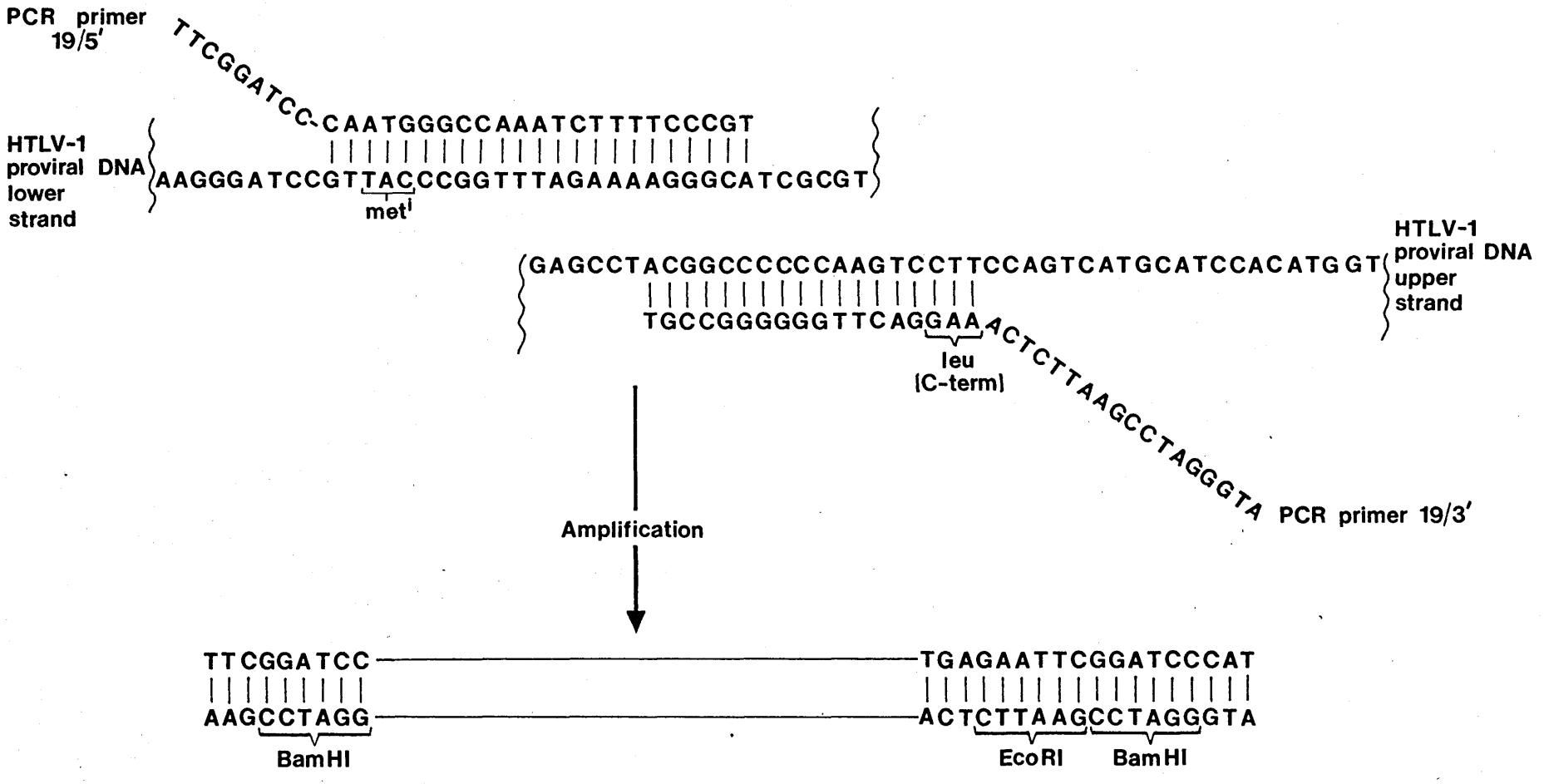


Figure 5.6. (a) The structure of the cloning vector, pGEM-7Zf(+). Amp , ampicillin resistance gene; f1ori, bacteriophage f1 origin of replication; lacZ alpha-peptide, gene coding for the alpha-peptide of ß-galactosidase, the presence of this gene permits the selection of recombinant plasmids by alpha-complementation (see section 2.5.3); MCS, multiple cloning site; ori, plasmid origin of replication; SP6, promoter for SP6 RNA polymerase; T7 promoter for T7 RNA polymerase. Following amplification of the p19 coding region of the HTLV-I provirus integrated in the DNA obtained from the leukaemic cells of patient 374 (b), the amplified product (c) and the pGEM-7Zf(+) vector were cleaved with BamHI, purified on polyacrylamide and agarose gels respectively and ligated. Recombinant plasmids were obtained with the p19 coding region inserted in either orientation, only one of which is illustrated (d). The orientation of the insert was determined by digestion with the restriction enzyme EcoRI, as cleavage sites for this enzyme are located at the 3' end of the p19 insert and between the BamHI and T7 polymerase sites in the vector.

Figure 5.6. Cloning of the amplified p19 coding region into pGEM-7Zf(+).

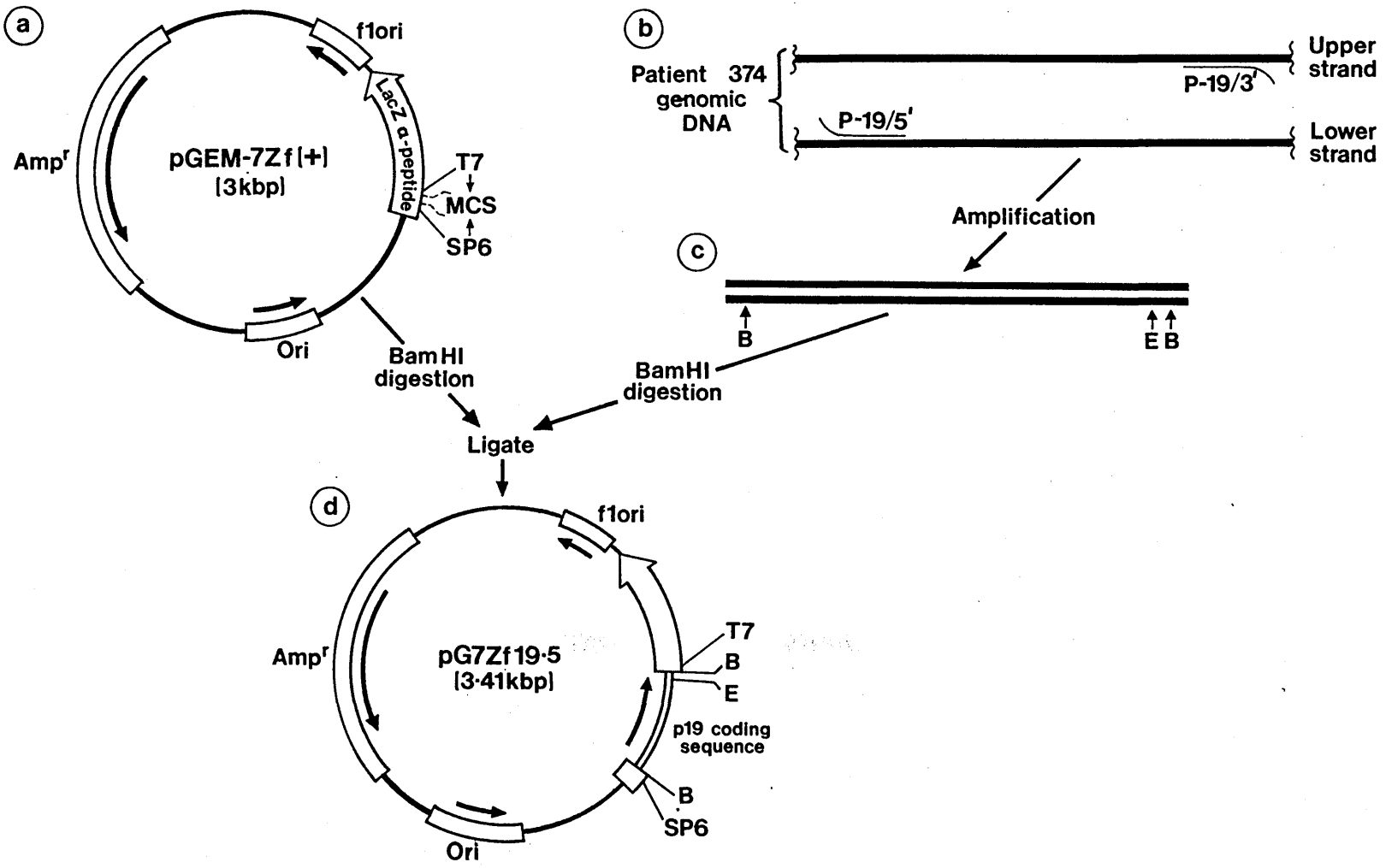
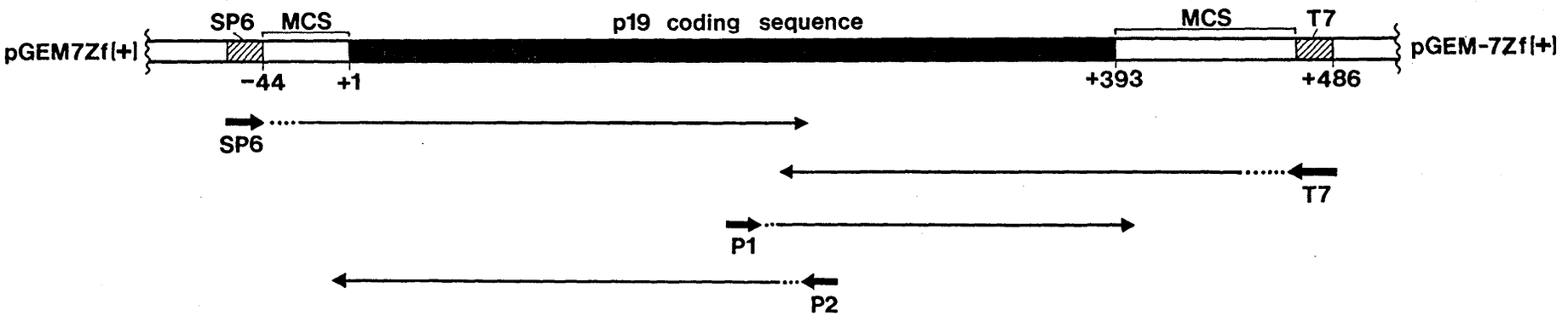


Figure 5.7. The structure of the p19 insert and the adjacent vector sequences in the plasmid pG7Zf19.5 is shown at the top of the figure. The nucleotides are numbered with the A of the methionine initiation codon numbered as +1. The nucleotide sequence of the p19 insert cloned into the pGEM-7Zf(+) vector was determined using primers complementary to the SP6 polymerase promoter (SP6), the T7 polymerase promoter (T7) and sequences internal to the p19 insert (P1 and P2). The primers are represented on the diagram by thick arrows. The extent of the DNA sequence determined using each of these primers is shown with a thin arrow. The dotted regions indicate sequences 3' to the primer which were not determined in sequencing reactions using that primer. MCS, multiple cloning site.

Figure 5.7. DNA sequence analysis of the p19 insert cloned into PGEM-7Zf(+).



Overnight cultures of *E. coli* transformed with pGEX-3X or the recombinant plasmid pGEX-3X19 were diluted 1:100 in 100mls of LB-medium (*E. coli* strain HB101) or minimal medium (*E. coli* strains JM105 and JM109) containing 100μg/ml of ampicillin and grown at 37°C to an OD_{600nm} of 0.5. Expression of the recombinant or non-recombinant protein was induced by the addition of IPTG to a final concentration of 0.5mM and the cultures were incubated at 37°C with shaking. The optimal induction time for fusion protein expression following the addition of IPTG was determined in time course experiments, using induction times of 0, 30, 60, 90, 120, 150 and 180 minutes. Optimal expression was obtained 120 minutes after the commencement of induction.

5.2.4. Preparation of cell extracts.

Small scale preparations of cell extracts were made using 2mls of the induced bacterial cultures. Cells were pelleted at 13000rpm for 1 minute, washed in filtered water and lysed in 200μl of SDS-PAGE sample buffer (Table V.V) by vigorous pipetting. Cell debris was removed by centrifugation at 13000rpm for 5 minutes and 20μl of the supernatant analysed for the presence of fusion protein by SDS-PAGE.

Large scale preparations of cell extracts were used for the purification of rp19 and were performed by Shauna Crae as follows. Bacterial cells containing the recombinant plasmid were cultured in 500mls of LB-medium. Following centrifugation at 7000rpm for 10 minutes at 4°C, the bacteria were washed in 50mls of TNE then resuspended in 10mls of TNE. The bacteria were lysed using Triton X-100 and lysozyme and bacterial debris removed by centrifugation.

The supernatant was mixed with 2mls of 50% glutathione-agarose beads (sulphur linkage, Sigma) in mouse-tonicity phosphate buffered saline (MTPBS, Table V.VI). Glutathione-agarose beads were preswollen in MTPBS, washed twice and stored as a 50% solution in MTPBS. The fusion protein was adsorbed onto the beads by mixing gently for 10 minutes. The beads were pelleted by centrifugation at 1000 rpm for 3 minutes, and washed three times in MTPBS. The fusion protein was eluted from the beads by competition with reduced glutathione as follows. Two millilitres of freshly

Table V.V. Buffers and solutions used for SDS-PAGE.

Coomassie blue staining solution.

Coomassie blue	0.05%
methanol	30%
glacial acetic acid	10%

Destaining solution

methanol	30%
glacial acetic acid	10%

SDS-PAGE running buffer

glycine	192mM
Tris pH8.8	25mM
SDS	0.1%

SDS-PAGE running gel

acrylamide	10%
methylene-bis-acrylamide	0.33%
Tris pH8.8	0.56M
SDS	0.1%
ammonium persulphate	0.025%
TEMED	0.07%

SDS-PAGE sample buffer

SDS	2.5%
beta-mercaptoethanol	2.5%
Tris pH6.8	100mM
glycerol	10%
bromophenol blue	0.01%

SDS-PAGE stacking gel

acrylamide	5%
methylene-bis-acrylamide	0.17%
Tris HCL pH6.8	0.12M
SDS	0.1%
ammonium persulphate	0.075%
TEMED	0.07%

Table V.VI. Buffers used in immunoassays.

Blocking buffer

sodium phosphate, monobasic	9mM
sodium phosphate, dibasic	2.75mM
sodium chloride	145mM
adjusted to pH8.0 with phosphoric acid	
Tween 20	0.1%
ovalbumin	1%
bovine serum albumin	1%
sodium azide	0.05%

Carbonate buffer

sodium hydrogen carbonate	10mM
EGTA	1mM
adjusted to pH9.5 with sodium hydroxide	

Disruption buffer

sodium phosphate, monobasic	0.1M
2-mercaptoethanol	0.1%
SDS	1%
adjusted to pH7.2 with sodium hydroxide	

Mouse tonicity phosphate buffered saline (MTPBS)

sodium chloride	150mM
sodium phosphate, dibasic	16mM
sodium phosphate, monobasic	4mM
adjusted to pH7.3 with sodium hydroxide	

Phosphate buffered saline (PBS)

sodium phosphate, dibasic	9mM
sodium phosphate, monobasic	2.75mM
sodium chloride	145mM
adjusted to pH7.2 with phosphoric acid	

Substrate buffer

sodium phosphate, dibasic	0.1M
citric acid	0.08M
adjusted to pH4.0 with phosphoric acid	

Table V.VI Buffers used in immunoassays (continued).

Tris-buffered saline (TBS)

sodium chloride	144mM
Tris pH7.4	25mM

Tris-Marvel-Tween 20 (TMT)

sodium chloride	144mM
Tris pH7.4	5mM
dried milk (Marvel)	2%
Tween 20	0.5%

WB buffer

glycine	192mM
Tris pH8.8	25mM
SDS	0.1%
methanol	20%

prepared reduced glutathione (Sigma) in 50mM Tris HCl (pH 8.0) was added to the beads and mixed gently for 10 minutes. The beads were pelleted, the supernatant removed and a second elution performed. The eluates were combined and the concentration of protein determined by S. Crae using a kit manufactured by Biorad.

SDS-PAGE.

SDS-PAGE was used as described by Laemmli (1970) in order to fractionate bacterial cell extracts. Gels were run on a vertical gel set (Gibco-BRL) in SDS-PAGE running buffer using 10% acrylamide in the running gel and 5% in the stacking gel (Table V.V). The running gel was poured, leaving a 4cm space above, and allowed to polymerise for 30 minutes under 100µl of H_2O saturated butan-2-ol. The butan-2-ol was rinsed away with dH_2O, the stacking gel poured and allowed to polymerise for 30 minutes with the well-forming comb in place. Samples were boiled in SDS-PAGE sample buffer for 3 minutes, loaded onto the gel and electrophoresed at a constant voltage of 200V for 3-4 hours at room temperature. Protein bands were visualized by gentle agitation in Coomassie blue staining solution for 2 hours followed by destaining solution for 4 x 45 minutes (Table V.V).

5.2.5. WB analysis of serum reactivity with rp19.

(a) Preparation of WB strips.

Reactivity of rp19 with HTLV-1 positive and indeterminate sera was evaluated by WB analysis. Purified GST or GST fusion proteins were loaded on SDS-PAGE gels at 2.55µg per well and electrophoresed as described above.

After electrophoresis gels were transferred to WB buffer (Table V.VI) for 10 minutes. Nitrocellulose membranes were cut to size and presoaked in WB buffer. Gels were placed onto the nitrocellulose membrane and inserted between sheets of 3MM Whatman paper, which were presoaked for 10 minutes in WB buffer. The gel sandwich was placed between scotchbrite pads and clamped between the plates of a vertical electroblotting apparatus (Biorad). Protein transfer was effected by applying a constant voltage of 20V across the plates for 16 hours at 4°C.

The nitrocellulose membrane was removed after protein transfer, agitated gently in 200mls of tris-buffered saline (TBS, Table V.VI) containing 2% marvel for 3-4 hours, then washed for 2 hours in TBS containing 0.05% Tween 20. The membrane was dried at room temperature and stored until use at -20°C.

(b) Detection of antibody to rp19.

WB analyses of sera for the detection of antibodies reactive with GST or the GST-rp19 fusion protein were performed by Shauna Crae, using WB strips prepared as described above. Sera were analysed at a 1:25 dilution.

5.2.6.Solid phase synthesis of octapeptides.

The materials and reagents used for the solid phase synthesis of multiple peptides were obtained from Cambridge Research Biochemicals. These included:

Chemically modified polyethylene pins embedded in polypropylene blocks in a 12 x 8 microtitre plate format.

Polypropylene trays with wells arranged in a 12 x 8 microtitre plate format.

Side chain protected fluorenylmethoxycarbonyl- (Fmoc-) L-amino acid active esters.

1-hydroxybenzotriazole (HOBt).

Monoclonal antibody specific for an epitope on sperm-whale myoglobin (MoAbMyo).

Computer software (copyright, Commonwealth Serum Laboratories Commission, Parkville, Australia) obtained from Cambridge Research Biochemicals was used for the construction of peptide synthesis schedules and for the analysis of data.

The published sequence of the lambda-ATK-1 clone of HTLV-I (Seiki *et al.* 1983) was used to derive the amino acid sequence of the p19 core protein. One hundred and twenty two overlapping octapeptides based on this sequence were synthesized in duplicate. The 5' methionine of p19 was numbered as amino acid 1, in order to conform to the numbering sequences used in previously published reports. However this residue is removed from the protein when myristylation occurs (Ootsuyama *et al.* 1985) and so was not included in an octapeptide. The first octapeptide to be synthesized was therefore numbered 2. The peptides were synthesized in situ on chemically modified polyethylene pins arranged in a microtitre plate format using a modification of the method described by Geysen *et al.* (1987), as recommended by the manufacturer. The synthesis uses mild base deprotection of Fmoc-L amino acids prior to amino acid coupling, in contrast to the strongly acidic conditions needed for deprotection of the t-butoxycarbonyl- (Boc-) amino acids used by Geysen *et al.* (1987).

F-moc deprotection of the Fmoc-β-alanine group pre-attached to the polyethylene pins was effected by immersing the pins up to half their height in a bath of 20% (volume/volume) piperidine in dimethylformamide (DMF) for 30 minutes at room temperature. The pins were removed and washed in DMF (5 minutes) then methanol (4 x 2 minutes), air dried for 10 minutes and washed in DMF (5 minutes). Fresh solvent was used for each wash.

Octapeptides were synthesized by the sequential coupling of preformed active ester derivatives of side chain protected amino acids onto the alanine residues. Synthesis commenced with the most C-terminal amino acid of each octapeptide.

Fmoc-amino acid active esters were dissolved to form 3M solutions in an equimolar solution of HOBt in DMF immediately prior to dispensing. One hundred microlitre aliquots of the amino acid solutions were dispensed into the wells of polypropylene trays, the deprotected pins inserted into the wells and the blocks of pins and trays sealed in plastic bags to reduce evaporative losses. The coupling reactions were allowed to proceed overnight and then the pins were washed in DMF (2 minutes), methanol (4 x 2 minutes) and finally in DMF (2 minutes).

Cleavage of the Fmoc protecting group from the added amino acid was effected using the deprotection protocol described above. Coupling, washing and deprotection steps were repeated until synthesis of the octapeptides was completed. The final amino acid was acetylated after deprotection and washing as follows. The pins were inserted into

wells containing DMF/acetic anhydride/triethylamine 5:2:1 (volume/volume/volume), the blocks and trays sealed in plastic bags and incubated at room temperature for 90 minutes. Pins were then washed in DMF (2 minutes), methanol (4 x 2 minutes) and air dried for 10 minutes.

Side chain protecting groups were removed by incubating the pins with a mixture of TFA/phenol/ethanedithiol 95/2.5/2.5 (volume/weight/volume) at room temperature for 4 hours. The pins were washed in dichloromethane (2 x 2 minutes), 5% diisopropylethylamine in dichloromethane (2 x 5 minutes), dichloromethane (5 minutes), air dried for 10 minutes, washed in H_2O (2 minutes) and finally washed in methanol for 18 hours. The blocks of pins were dried under vacuum over silica gel overnight and then stored at room temperature in polythene boxes containing silica gel, in sealed plastic bags.

Control tetrapeptides were synthesized simultaneously to check the efficacy of synthesis. Two tetrapeptides were synthesized in duplicate on each polypropylene block. One of the peptides, with the amino acid sequence proline-leucine-alanine-glutamine is known to react with MoAbMyo. The other peptide, for which the amino acid sequence is glycine-leucine-alanine-glutamine, does not react with MoAbMyo. Reactivity of these peptides with MoAbMyo was assayed for each block of peptide-linked pins, when each set of peptides was first analysed and on a number of subsequent occasions.

5.2.7. Analysis of serum reactivity with octapeptides.

Sera were tested for reactivity with the peptides using an ELISA. Before use the pins were immersed in disruption buffer (Table V.VI) preheated to 60°C, and sonicated at 35kHz (2 x 600 Watts per cycle) in a Transonic ultrasonic water bath (T780/H, Camlab) for 20 minutes.

Horse radish peroxidase (HRPO) ELISA.

Initially sera were analysed using the method recommended by Cambridge Research Biochemicals. All incubations and washes were performed with agitation. Non specific reactivity was blocked by incubating the peptide-coated pins and microtitre plates

(Dynatech) in 200µl of blocking buffer (Table V.VI) containing 0.05% sodium azide for 2 hours at room temperature. The peptide-coated pins were then immersed in 175µl of sera diluted in blocking buffer containing 0.05% sodium azide and incubated overnight at 4°C. Unbound antibody was removed by washing (4 x 10 minutes) in phosphate buffered saline (PBS, Table V.VI) containing 0.05% Tween 20 (PBST) at room temperature. The peptide coated pins were then incubated with 175µl of a 1/1000 dilution of HRPO-conjugated goat anti-murine IgG (Sigma) or HRPO-conjugated goat anti-human IgG (Sigma) in blocking buffer at room temperature for 1 hour. Unbound conjugated antibody was removed by washing (4 x 10 minutes) in PBST at room temperature. Bound conjugate was detected by immersing the pins in 150µl per well of substrate buffer (Table V.VI) containing 0.5mg/ml 2,2'-azinobis(3-ethylbenzthiazoline-sulfonic acid) (ABTS, Sigma) and 0.3µl/ml of hydrogen peroxide. The substrate incubation was continued for 30 minutes at room temperature in the dark. Colour development was stopped by removing the pins and the absorbances read at 405nm in a Titertek plate reader (Flow Laboratories).

Before reusing the pins, the antibody-peptide interactions were disrupted using ultrasonication in disruption buffer as described above.

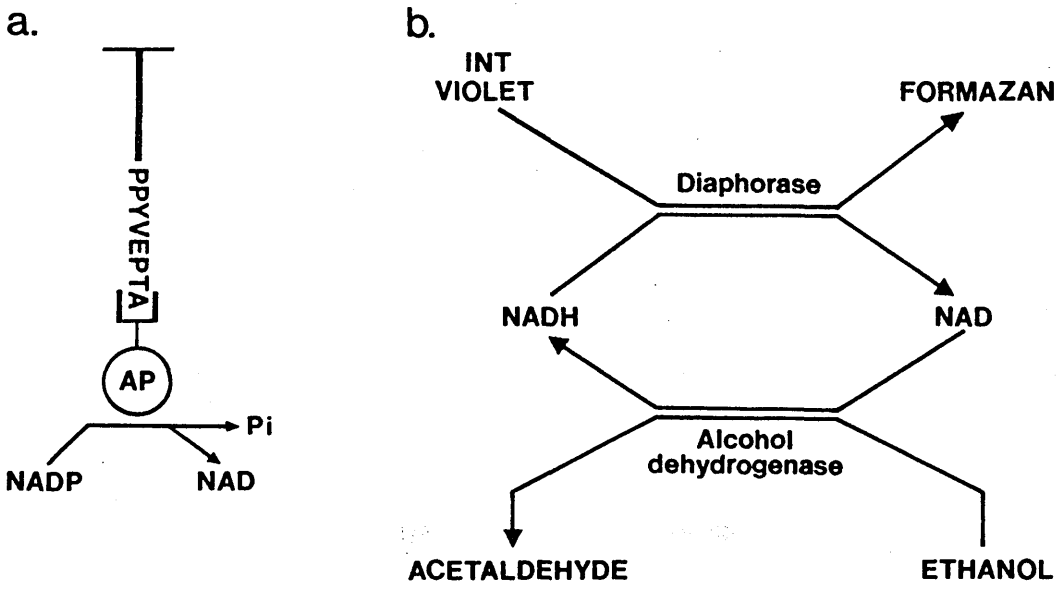
Amplified ELISA.

To increase the sensitivity of the assay an ELISA using alkaline phosphatase as the conjugated enzyme and a subsequent amplification step (AMPAK, IQ [Bio] Ltd.) was used (Figure 5.8). All polyclonal sera and MoAb 12/1-2 were assayed using the amplified ELISA.

The method was essentially similar to that described above for the HRPO ELISA, with the following alterations. One hundred and fifty microlitres of Tris-Marvel-Tween 20 with 20% goat serum (TMT/GS, Table V.VI) were used for blocking nonspecific reactivity with the peptides. Serum samples and alkaline phosphatase-conjugated antibodies were diluted into a final volume of 125µl of TMT/GS. Novo Biolabs wash buffer was used in all washing steps. The secondary antibody conjugates used were alkaline phosphotase-conjugated goat anti-human IgG, gamma-heavy chain-specific (Seralab), used at a 1/1000 dilution and goat anti-murine IgG (Biorad), used at a 1/4000 dilution. After removal of unbound enzyme-conjugated antibody by washing, the pins were incubated in 100µl of AMPAK substrate solution for 10-30 minutes. The pins

Figure 5.8. (a) The diagram shows a representation of a peptide-coated polyethylene pin following incubation with the primary antibody and with the alkaline phosphatase (AP)-conjugated secondary antibody. The AMPAK (IQ [Bio]) substrate, which contains nicotinamide adenine dinucleotide phosphate (NADP), is then added. The NADP is dephosphorylated to form nicotinamide adenine dinucleotide (NAD) by the action of alkaline phosphatase. NAD continues to accumulate until the peptide-coated pin is removed from the substrate solution. (b) The AMPAK amplifier solution is added, this contains the enzymes alcohol dehydrogenase and diaphorase and p-iodonitro-tetrazolium violet, (INT-violet). The presence of NAD then activates a cyclic reaction, in which alcohol dehydrogenase catalyzes the reduction of NAD by ethanol. The NADH formed in the reaction then reduces INT-violet to produce a red formazan dye and regenerate NAD. The latter reaction is driven by the enzyme diaphorase. The rate of formation of the coloured dye is proportional to the amount of NAD present following the substrate incubation step (Stanley et al. 1985).

Figure 5.8. The amplification step in the alkaline phosphatase ELISA.



were removed and an equal volume of AMPAK amplifier solution was added and incubation continued for 10-30 minutes until colour development was optimal. The reactions were stopped by the addition of 0.3M sulphuric acid and the absorbances were measured at 495nm.

For polyclonal sera reactivity with an octapeptide was considered significant if the absorbance on each set of pins exceeded the mean plus three standard deviations of the lowest 90% of values. All sera were assayed on duplicate sets of pins. Some sera were analysed on multiple occasions at varying dilutions. Results for these sera are given for assays performed using the lowest dilution tested. If tested at this dilution on multiple occasions, only the octapeptides giving significant reactivities on each occasion were considered to be positive.

Comparison between HRPO and amplified ELISAs.

The sensitivity of the HRPO and amplified ELISAs was compared using the control tetrapeptides derived from sperm whale myoglobin that were synthesized as a control for the efficiency of synthesis (see section 5.2.6). Dilutions of MoAbMyo were assayed for reactivity with the tetrapeptides using the HRPO and the amplified ELISAs as described above. For the amplified ELISA, a substrate incubation time of 1 hour and an amplification time of 10 minutes were used.

MoAb 12/1-2 and four HTLV-I positive sera were assayed with both the HRPO ELISA and the amplified ELISA.

5.2.8. Confirmatory ELISAs.

Fifteen or twenty two amino acid long peptides were selected for additional experiments on the basis of results obtained in the PEPSCAN ELISAs. The amino acid sequences of these peptides are shown in Table V.VII. Peptides are numbered according to the positions of the amino-terminal and carboxy-terminal amino acids in the protein sequence of p19. Approximately 0.1mmole of a 22 amino acid long C-terminal peptide, P109-130, was obtained from Alta Bioscience (Birmingham University). P109-130 was synthesized using Fmoc chemistry and the amino acid composition analysed to confirm

Table V.VII. The amino acid sequence of HTLV-I p19 was derived from the nucleotide sequence of HTLV-I reported by Seiki et al. (1983). Amino acids are numbered with the initiating methionine of p19 as 1. The amino acid sequence of HTLV-II p22 is derived from the nucleotide sequence of HTLV-II reported by Shimotohno et al. (1985). Amino acids are numbered with the initiating methionine of p22 as 1. The amino acid sequence of HRES-1/1 p25 ORF is derived from the nucleotide sequence of HRES-1/1 reported by Perl et al. (1989). The overlapping amino acid sequences given in the table correspond to amino acids numbered 21-40 in Perl et al. (1989).

Table V.VII Sequences of 22 and 15 amino acid long peptides.

Origin of Peptide	Designation	Amino acid sequence
HTLV-I p19	P109-130	DPPDSDPQIPPPYVEPTAPQVL
	P2-16	GQI FSRSASPIPRPP
	P7-21	RSASPEPRPPRGLAA
	P32-45	YRLEPGPSSYDFHQL
	P67-81	LLASLLPKGYPGRVN
	P97-111	RPAPPPPSSPTHDPP
	P102-116	PPSSPTHDPPDSDPQ
	P107-121	THDPPDSDPQIPPPY
	P112-126	DSDPQI PPPYVEPTA
	P116-130	QI PPPYVEPTAPQVL
HTLV-II p22	P(II)2-16	GQI HGLSPTPIPKAP
	P(II)7-21	LSPTPIPKAPRGLST
	P(II)67-81	YSLLASLIPKGYPGR
	P(II)118-133	SPEAHVPPPYVEPTT
HRES-1/1 p25 ORF	PA	PRPPSRSQAQTPPRS
	PB	RSQAQTPPRSVPRLR

the accuracy of the synthesis. Fifteen peptides, fifteen amino acids in length, were also obtained from Alto Bioscience. These peptides were synthesized in approximately 5mg quantities using a BT7400 peptide synthesizer (Biotech Instruments).

Nine peptides derived from HTLV-I p19 were chosen to overlap octapeptides reactive in the PEPSCAN ELISA. Four peptides were derived from homologous sequences in HTLV-II p22. Two peptides, overlapping in sequence by 10 amino acids, were derived from the translation of the nucleotide sequence of an HERV with homology to the C-terminus of HTLV-I p19 (Perl *et al.* 1989).

Serum reactivity with 15 and 22 amino acid long peptides was detected using an ELISA. Optimal conditions for performing the assays were established following comparisons between Immulon I, Immulon II, Immulon IV and microtitre plates (Dynatech). Time course experiments using P109-130 were used to select the serum incubation time required to maximise binding to the peptide (90-120 minutes).

All incubations were performed with agitation. Microtitre plate (Dynatech) wells were coated with 100µl of peptide in carbonate buffer (Table V.VI) for 16 hours at room temperature. Peptide 109-130 was used at 1ng/µl, all other peptides were used at 10ng/µl. After washing the wells three times in Tris-buffered saline (TBS, Table V.VI) containing 1mM magnesium chloride and 0.1% Tween 20 (TBST), nonspecific binding was blocked by incubation with 200µl of TBST with 2% Marvel and 20% goat serum (TBMT/GS) for 2 hours at room temperature followed by washing as before. Sera were added at appropriate dilutions in 100 µl of TBMT/GS and incubated for 2 hours at room temperature, before washing the wells 6 times in TBST. Bound antibody was detected by incubation with 100µl per well of alkaline phosphatase-conjugated goat antibody to human IgG, gamma-heavy chain-specific (Seralab) diluted 1/1000 in TBMT/GS for 1 hour at room temperature. Wells were washed 6 times in TBST then 100µl of p-nitrophenol-phosphate in diethanolamine buffer was added. Colour development was stopped after 30 minutes with 0.4M sodium hydroxide and absorbances read at 405nm.

Sera were assayed at multiple dilutions from 1/10-1/3000 except where stated otherwise. Results were defined as positive if the absorbance value exceeded the mean plus three standard deviations of the absorbance values obtained with negative control sera and was greater than 0.1 O.D. units. A minimum of four negative control sera were included in each experiment.

5.2.9 Adsorption of p19 specific antibody.

In order to confirm the specificity of antibody binding to p19-derived peptides, four HTLV-I positive and four PNG sera were preadsorbed with rp19 before assaying in the P109-130 ELISA. Serial ten-fold dilutions of rp19, were made from stock solutions of 3ng/µl to 0.3pg/µl and/or 10ng/µl to 0.1pg/µl in TBMT/GS. Serum samples were diluted in 100µl of the rp19 solutions and preincubated for 2 hours at room temperature. Sera were used at a dilution which gave 50% of the maximum absorbance in the p109-130 ELISA, as determined from titration curves. The p109-130 ELISA was performed as described above. Identical experiments using GST in place of rp19 were performed as controls.

5.2.10. Secondary structure predictions.

The secondary structures and antigenic indices of HTLV-I p19 and HTLV-II p22 were predicted and plotted using the programs "Peptidestructure" and "Plotstructure" from the Genetics Computer Group Sequence Analysis software package, version 4.7, on a MicroVAX 3600 computer. Secondary structure predictions are based on the algorithms of Chou and Fasman (1978). The antigenic index is based on weighted values for hydrophilicity (Hopp and Woods 1981), surface probability (Emini *et al.* 1985), flexibility (Karplus and Schultz 1985), and secondary structure (Chou and Fasman 1978; Garnier *et al.* 1978), as described by Jameson and Wolf (1988).

5.2.11. Data base searches.

Computerized protein and nucleotide sequence databases were searched using the Genetics Computer Group Sequence Analysis software package, version 4.7.

A search of the NBRF protein sequence data bank for homology with the sequences of the 4 octapeptides reactive with MoAb 12/1-2 was performed using the program "Find". The search allowed 3 mismatches out of 8 amino acids.

A search of the nucleotide sequence data banks for homology to the 10 amino acid sequence spanned by the three octapeptides reactive with 7/9 PNG sera was performed using the program "TFastA". This program translates all nucleotide sequences (upper and lower strands) containing open reading frames in all 3 reading frames and searches the translated peptide sequences for homology with a target sequence. The best 40 matches from the Genbank (updated 1989), EMBL (updated September 1990) and NewEMBL (updated October 1990) data banks were examined.

An additional search of the NBRF protein sequence databank (updated July 1990) was performed using the program "Wordsearch" to look for protein sequences with homology to the C-terminal 30 amino acids of p19.

5.3.RESULTS.

5.3.1.Sequencing of the p19 insert in pG7Zf19.5.

The sequence of the p19 coding sequences amplified from the HTLV-I provirus present in the PBMCs of patient 374 and cloned into pGEM-7ZF(+) is shown in Figure 5.9. An autoradiograph of representative results is illustrated in Figure 5.10. Seven nucleotide changes from the prototype lambda-ATK-1 sequence determined by Seiki *et al.* (1983) were identified. Translation of the nucleotide sequence identifies alterations to three amino acids, compared to the translated lambda-ATK-I sequence. A non-conservative change from arginine to glutamine was identified at position 17. A conservative change from alanine to valine was identified at position 58 and a non-conservative change from arginine to tryptophan was identified at position 59.

5.3.2.Expression of rp19.

IPTG-induction of protein expression in bacteria containing the pGEX-3X19 plasmid resulted in increased expression of a protein which showed a relative molecular mass of 41000 on analysis by SDS-PAGE. A higher level of expression was detected in extracts from the transformed *E.coli* strain HB101 compared to strains JM105 and JM109 (Figure 5.11). *E.Coli* strain HB101 was subsequently used for large scale expression of rp19.

WB analysis using MoAb 12/1-2, confirmed that a protein of relative molecular weight 41000, that was induced by IPTG in cultures of bacteria containing pGEX-3X19, reacted with MoAb 12/1-2 (Figure 5.11, panel B). MoAb 12/1-2 did not react with any proteins present in extracts of IPTG-induced cultures of bacteria containing pGEX-3X.

Figure 5.9. (A), nucleotide sequence of p-19 coding region of the lambda-ATK-1 clone of HTLV-I (Seiki et al. 1983); (B), nucleotide sequence of the p19-coding region cloned into PGEM-7Zf(+), a dash indicates that the sequence was identical to that of lambda-ATK-1, letters indicate nucleotide changes relative to lambda-ATK-1; (C), amino acid sequence of p19 translated from the lambda-ATK-1 nucleotide sequence (single letter code); (D), amino acid changes from the protein sequence given in (C) as predicted from the nucleotide sequence shown in (B). The nucleotide sequences included within the primers used for PCR, 19/5' and 19/3', are indicated with continuous lines.

Figure 5.9. Nucleotide sequence of the HTLV-I p19-coding region cloned into pGEM-7Zf(+)

```
                                          30                                                    60
A   ATG GGC CAA ATC TTT TCC CGT AGC GCT AGC CCT ATT CCG CGA CCG CCC CGG GGG CTG GCC
B   --- --- --- --- --- --- --- --- --- --- --- --- --- --G --- --- -A- --- --- ---
C    M   G   Q   I   F   S   R   S   A   S   P   I   P   R   P   P   R   G   L   A
D                                                                    Q

                                          90                                                   120
A   GCT CAT CAC TGG CTT AAC TTC CTC CAG GCG GCA TAT CGC CTA GAA CCC GGT CCC TCC AGT
B   --- --- --- --- --- --- --- --- --A --- --- --- --- --- --- --- --- --- --- ---
C    A   H   H   W   L   N   F   L   Q   A   A   Y   R   L   E   P   G   P   S   S
D

                                         150                                                   180
A   TAC GAT TTC CAC CAG TTA AAA AAA TTT CTT AAA ATA GCT TTA GAA ACA CCG GCT CGG ATC
B   --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- -TC T-- ---
C    Y   D   F   H   Q   L   K   K   F   L   K   I   A   L   E   T   P   A   R   I
D                                                                        V   W

                                         210                                                   240
A   TGT CCC ATT AAC TAC TCC CTC CTA GCC AGC CTA CTC CCA AAA GGA TAC CCC GGC CGG GTG
B   --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
C    C   P   I   N   Y   S   L   L   A   S   L   L   P   K   G   Y   P   G   R   V
D

                                         270                                                   300
A   AAT GAA ATT TTA CAC ATA CTC ATC CAA ACC CAA GCC CAG ATC CCG TCC CGT CCC GCG CCA
B   --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
C    N   E   I   L   H   I   L   I   Q   T   Q   A   Q   I   P   S   R   P   A   P
D

                                         330                                                   360
A   CCG CCG CCG TCA TCC CCC ACC CAC GAC CCC CCG GAT TCT GAT CCA CAA ATC CCC CCT CCC
B   --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
C    P   P   P   S   S   P   T   H   D   P   P   D   S   D   P   Q   I   P   P   P
D

                                         390
A   TAT GTT GAG CCT ACG GCC CCC CAA GTC CTT
B   --- --C --- --- --- --- --- --- --- ---
C    Y   V   E   P   T   A   P   Q   V   L
D
```

Figure 5.10. Representative results obtained from the analysis of the nucleotide sequence of pG7Zf19.5 using the SP6 primer. Nucleotides 31-118 of the upper strand of the p19-coding region are illustrated (nucleotides are numbered from the A of the initiation codon). Differences from the published nucleotide sequence of the lambda-ATK-1 clone of HTLV-I (Seiki et al. 1983) are underlined.

Figure 5.10. Nucleotide sequence analysis of pG7Zf19.5.

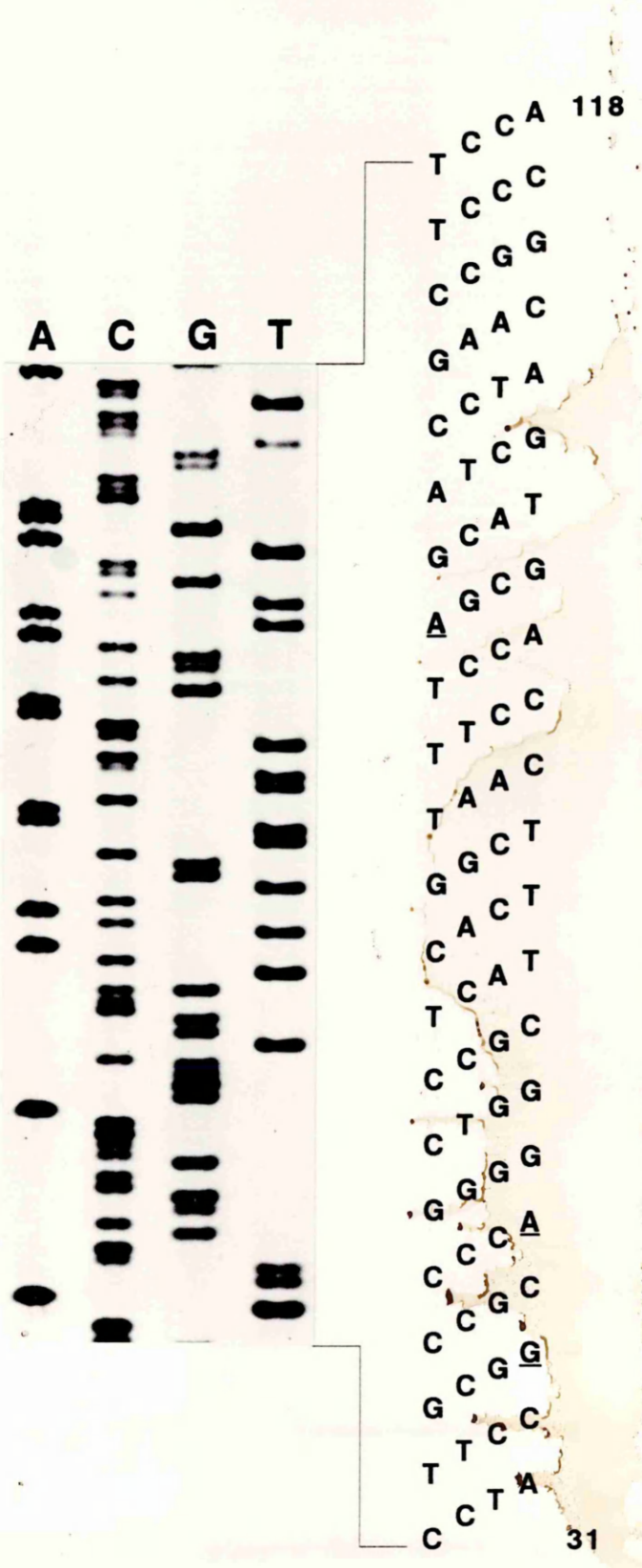
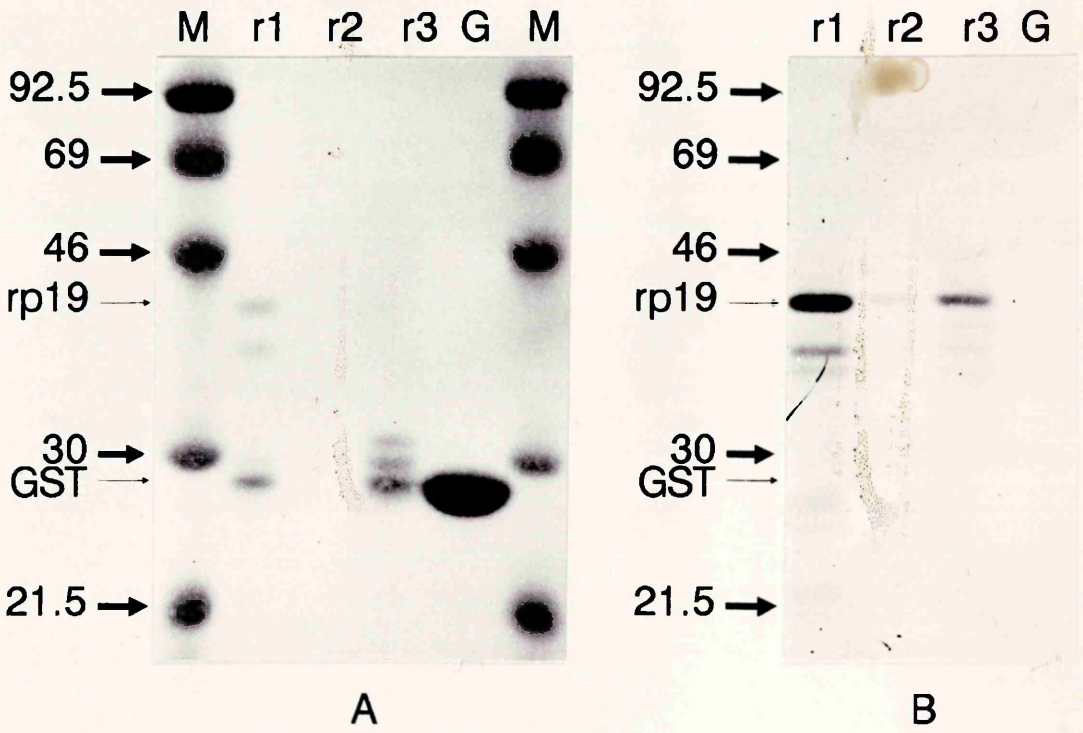


Figure 5.11. Proteins were purified from bacterial cell extracts using glutathione-agarose beads, as described in section 5.2.4 and separated on SDS-PAGE. Cell extracts were made from IPTG-induced cultures of E. Coli strains HB101 (r1), JM105 (r2) and JM109 (r3) containing the recombinant plasmid, PGEX-3X19; and JM109 containing the non-recombinant plasmid pGEX-3X (G). The thick arrows show the migration distances of protein molecular weight markers (Amersham International plc), the relative molecular weights (x1000) of the markers are indicated alongside.

Panel A, Coomassie blue-stained gel. The protein with the highest relative molecular weight (41000) seen in lane r1 was taken to be the recombinant fusion protein, rp19, as indicated by the thin arrow. The proteins of lower relative molecular weight are probably breakdown products of rp19 (see below). The non-recombinant GST protein is seen in lane G, migrating with a relative molecular weight of 27000.

Panel B, the reactivity of MoAb 12/1-2 with the purified proteins was analysed by WB after transferring a replica of the gel shown in panel A onto nitrocellulose membrane. MoAb 12/1-2 bound to proteins of relative molecular weight 41000 in extracts of all E. Coli strains containing the recombinant plasmid, supporting the identification of this protein as the rp19 fusion protein. The reactivity of MoAb 12/1-2 with multiple lower molecular weight proteins in lanes r1, r2 and r3, indicates that these proteins are breakdown products of rp19.

Figure 5.11. Expression of the rp19 fusion protein in bacteria and reactivity of rp19 with MoAb 12/1-2.



5.3.3. Reactivity of HTLV-I-positive and indeterminate sera with rp19.

HTLV-I positive serum 374 showed reactivity with the rp19 fusion protein and did not react with any proteins on WB strips made using non-recombinant GST (Figure 5.12). Two indeterminate sera from PNG were analysed for reactivity with rp19 by WB. Both samples showed reactivity with the rp19 fusion protein (Figure 5.12). The PNG sera reacted in addition with two proteins of higher relative molecular weight in WB strips. Reactivity with these two proteins was detected in WB strips made from purified extracts of cultured bacteria containing the non-recombinant plasmid in addition to extracts from bacteria containing the recombinant plasmid. It is therefore likely that these reactivities represent antibody binding to bacterial proteins that are copurified with GST and the GST-fusion protein.

5.3.4. Epitope mapping.

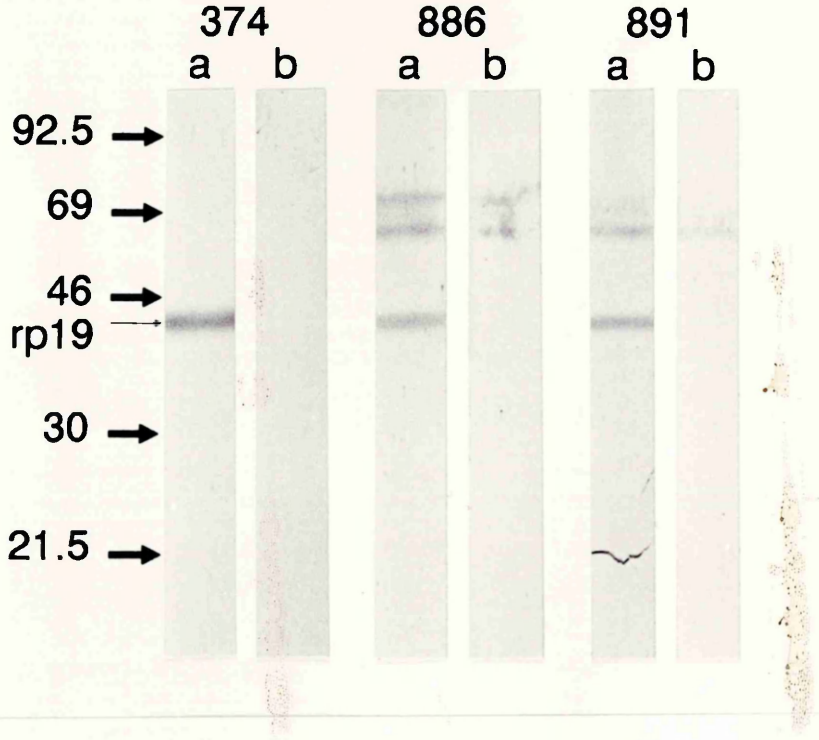
The results obtained from the comparison between the HRPO and amplified ELISAs are shown in Figure 5.13. Use of the amplified ELISA produced at least a ten-fold increase in sensitivity, though the ratio of the positive to the negative signals was similar.

Analysis of monoclonal and polyclonal sera with the p19 octapeptides using the HRPO and amplified ELISAs gave qualitatively similar, though not completely identical, results. Experiments to determine the relative sensitivity of the two techniques for the analysis of sera with the p19 octapeptides were not performed.

Monoclonal antibodies.

MoAb 12/1-2 (1:1000 dilution of ascites) reacted with four adjacent octapeptides, numbers 116-119, near the C-terminus of p19 in duplicate assays (Figures 5.14 and 5.15). These octapeptides share the common sequence PPYVE, which is completely conserved in the p22 core protein of HTLV-II. Analysis of MoAb 12/1-2 with the HRPO and amplified ELISAs gave qualitatively similar results (Figures 5.14 and 5.15).

Figure 5.12. Reactivity of HTLV-I antibody-positive and PNG sera with the rp19 fusion protein.



HTLV-I antibody-positive serum sample 374 and PNG serum samples 886 and 891 were analysed for reactivity with the glutathione S-transferase (GST)-p19 fusion protein (rp19), (a), and for reactivity with GST, (b), as described in section 5.2.5. The thick arrows show the migration distances of protein molecular weight markers (Amersham International plc), the relative molecular weights (x1000) of the markers are indicated alongside. Rp19 migrates with a relative molecular weight of 41000 (thin arrow).

Figure 5.13. A monoclonal antibody (MoAbMyo), known to react with the tetrapeptide proline-leucine-alanine-glutamine (PLAQ) in sperm whale myoglobin, was analysed for reactivity with the tetrapeptides PLAQ and glycine-leucine-alanine-glutamine GLAQ (see sections 5.2.6 and 5.2.7). MoAbMyo was assayed at dilutions of 1, 1/5, 1/10 and 1/50. The four curves show the reactivity of the MoAb with PLAQ and GLAQ determined using the amplified alkaline phosphatase (AMPAK) and horse-radish peroxidase (HRPO) detection systems as detailed in the key on the right of the panel.

Figure 5.13. Comparison of the amplified alkaline phosphatase and the horse-radish peroxidase ELISAs.

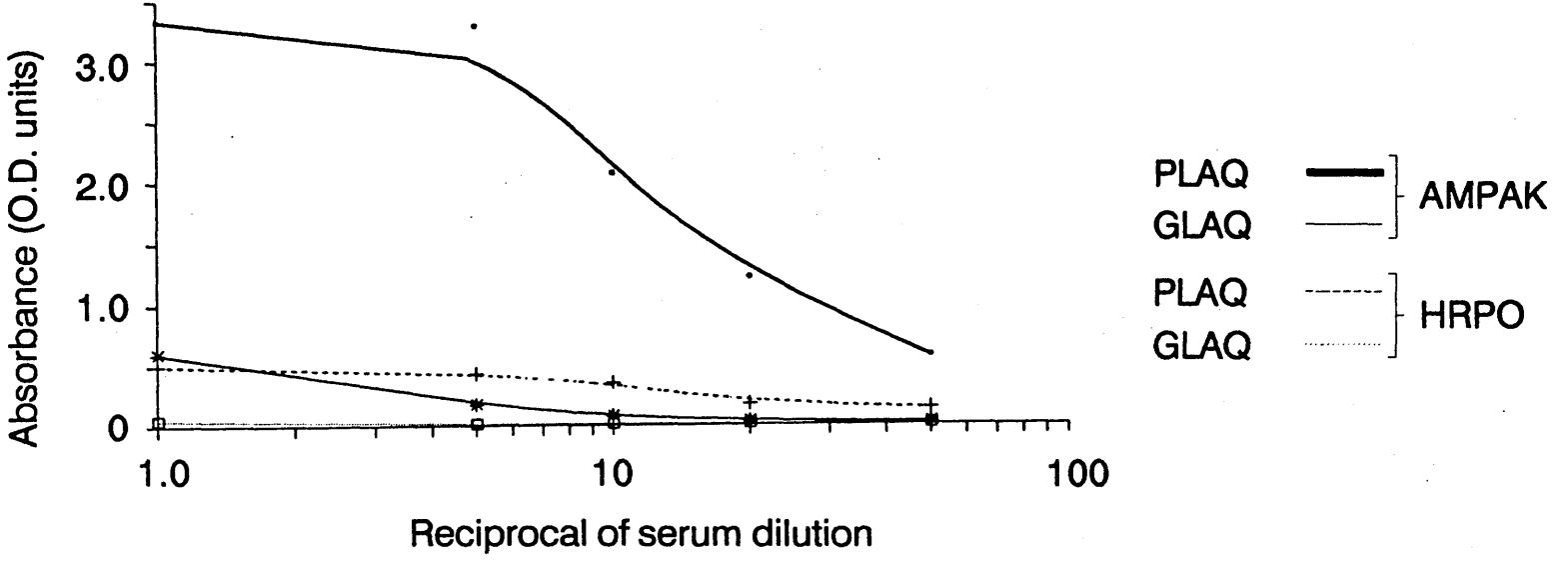


Figure 5.14. The graphs show the results obtained from the analysis of MoAb 12/1-2 for reactivity with the p19 octapeptides using the horse-radish peroxidase (HRPO) ELISA. The heights of individual bars indicate the absorbance readings obtained for individual octapeptides in the ELISA. Panels A and B show the results of ELISAs performed on duplicate sets of octapeptides. MoAb 12/1-2 was analysed at a dilution of 1/1000.

Figure 5.15. The graphs show the results obtained from a similar analysis of MoAb 12/1-2 using the amplified alkaline phosphatase (AMPAK) ELISA. The scale on the y-axis extends to 3.0 O.D. units, compared with 0.6 O.D. units in Figure 5.14, thus the signal-to-noise ratio obtained with the amplified ELISA is higher than that obtained with the HRPO ELISA.

Figure 5.14. Reactivity of MoAb 12/1-2 with p19 octapeptides: HRPO ELISA.

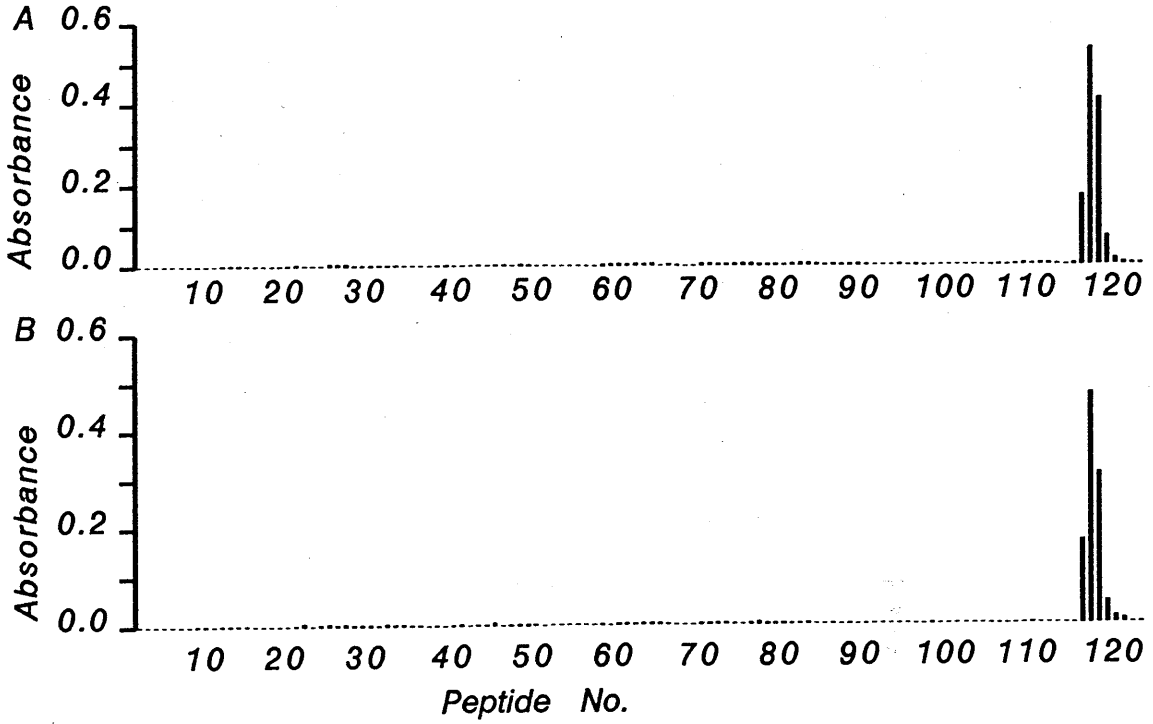
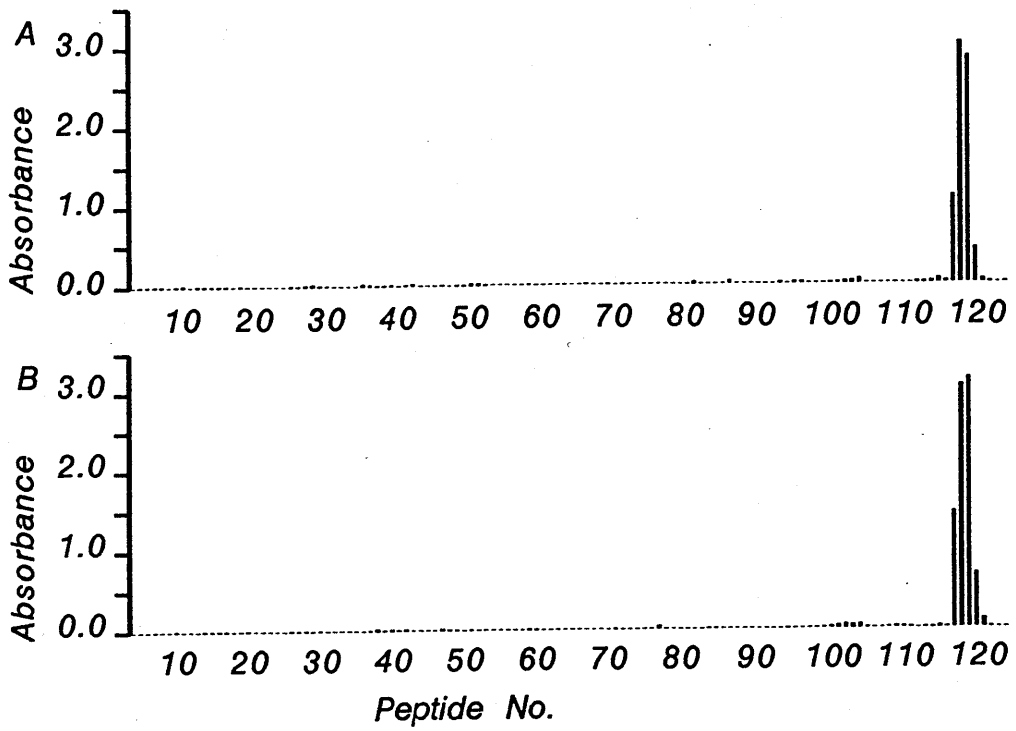


Figure 5.15. Reactivity of MoAb 12/1-2 with p19 octapeptides: amplified ELISA



In the amplified ELISA the absorbance readings obtained with non-reactive octapeptides were lower relative to those obtained with reactive peptides when compared with the readings obtained in the HRPO ELISA.

MoAb 12G4 (1/200 dilution of MAS 197c) was assayed three times using the AMPAK detection system on duplicate sets of pins. No consistent reactivity with any octapeptide or group of octapeptides was identified. Negative results were also obtained with MoAbs 13B12 and 9G10. MoAb 13B12 was assayed at a dilution of 1/80 (recommended working dilution 1/50-1/100) using the HRPO detection system only, due to the limited amounts of antibody available. MoAb 9G10 was assayed using the AMPAK detection system at a dilution of 1/160. This is below the recommended working dilution of 1/50-1/100 but was limited by sample availability.

HTLV-I positive sera.

Ten HTLV-I positive sera were analysed in the PEPSCAN ELISA at dilutions that varied from 1/200 to 1/800. The dilution used was selected on the basis of serum availability and the titre of HTLV-I antibody detected by ELISA (DuPont) or by GPA (ATLA, Serodia).

The patterns of reactivity of the ten HTLV-I positive sera with duplicate sets of octapeptides are shown in Figures 5.16-5.25. A summary of the data is shown in Figure 5.26. Nine of the ten sera showed reactivity with one or more groups of 2-8 adjacent peptides derived from the C-terminal 21 amino acids of p19 (octapeptides 110-123). Three of these sera, numbers 628, 796 and 797, also reacted with groups of 2-5 adjacent peptides between octapeptide 102 and octapeptide 108. None of the HTLV-I positive sera reacted with octapeptide 109. Serum 716 showed reactivity with isolated octapeptides only.

Reactivity with various single peptides in duplicate sets of pins was seen with a number of other sera. No groups of 2 or more octapeptides other than those derived from the C-terminal 29 amino acids of p19 showed reactivity with the HTLV-I positive sera.

Figures 5.16-5.25. The graphs show the results obtained from the analysis of HTLV-I antibody-positive serum samples in the PEPSCAN ELISA. The heights of the bars indicate the absorbance readings obtained for individual octapeptides in the ELISA. Panels A and B show the results of ELISAs performed on duplicate sets of octapeptides. Sera were analysed at dilutions of 1/200 (sample 731), 1/400 (samples 628, 733, 737, 739, 741, and 796), 1/500 (samples 374 and 716) or 1/800 (sample 797).

Figure 5.16. Reactivity of HTLV-I antibody-positive serum 374 with p19 octapeptides.

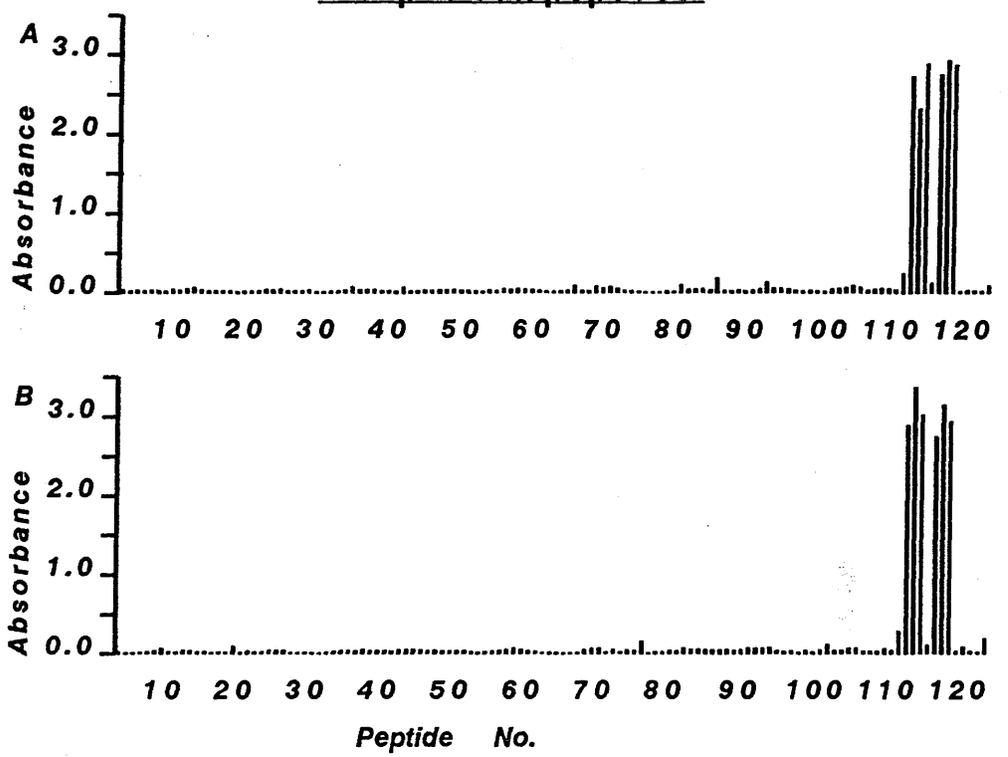


Figure 5.17. Reactivity of HTLV-I antibody-positive serum 628 with p19 octapeptides.

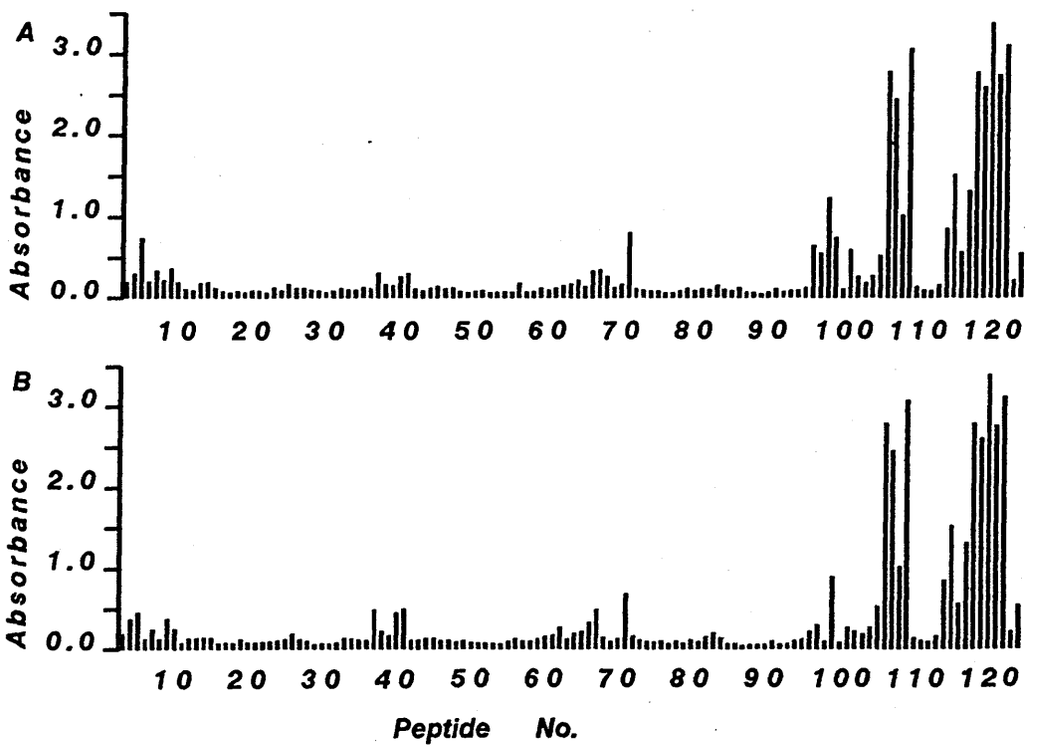


Figure 5.18. Reactivity of HTLV-I antibody-positive serum 716 with p19 octapeptides.

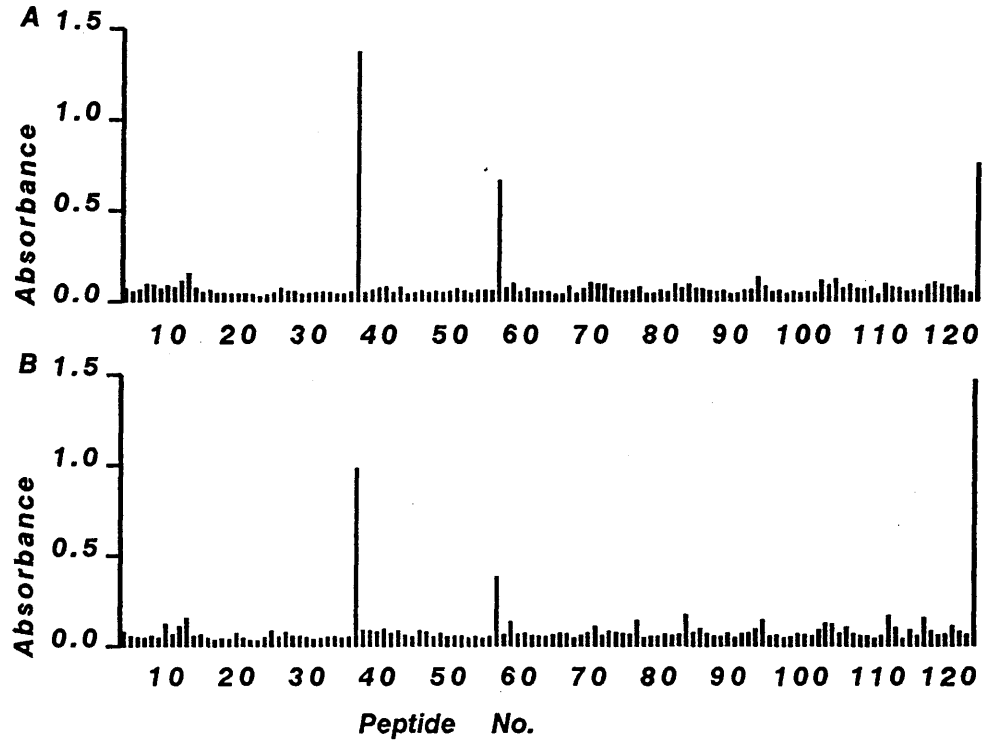


Figure 5.19. Reactivity of HTLV-I antibody-positive serum 731 with p19 octapeptides.

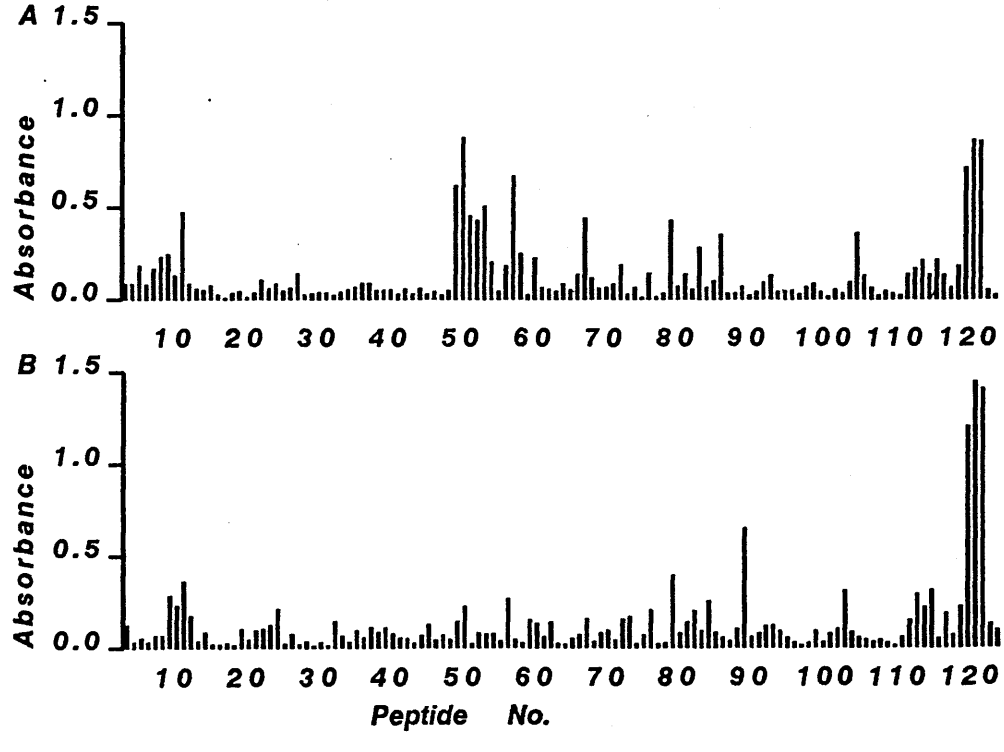


Figure 5.20. Reactivity of HTLV-I antibody-positive serum 733 with p19 octapeptides.

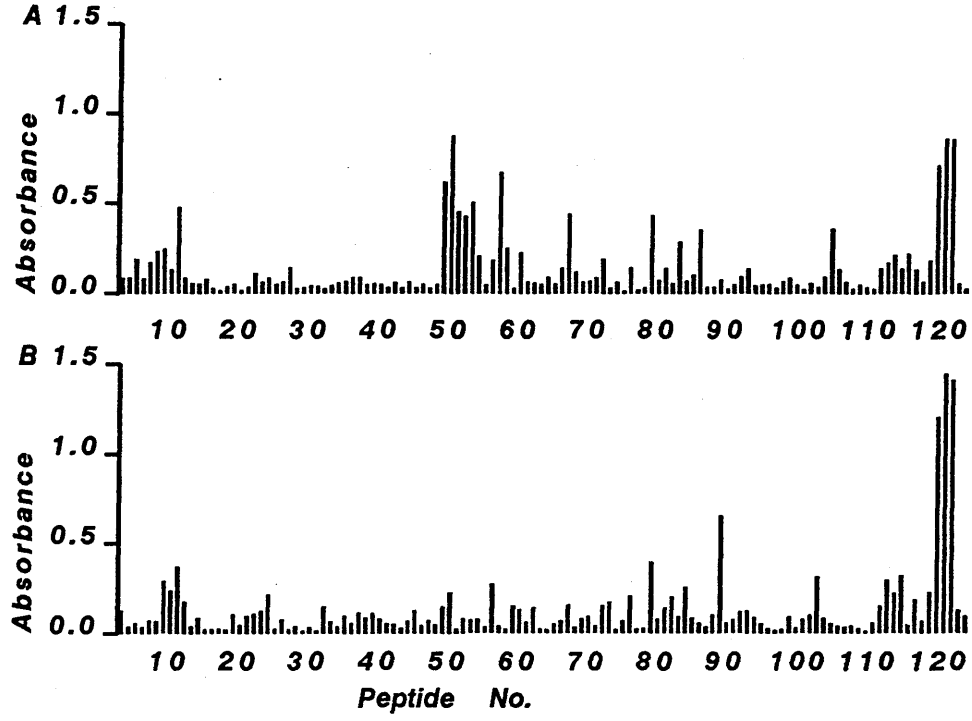


Figure 5.21. Reactivity of HTLV-I antibody-positive serum 737 with p19 octapeptides.

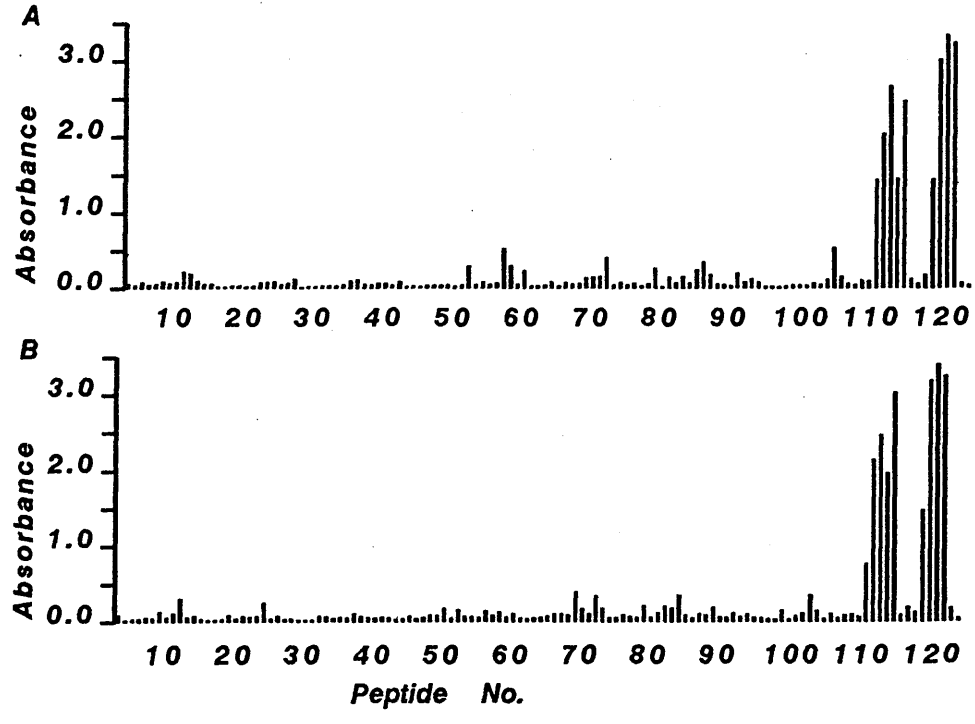


Figure 5.22. Reactivity of HTLV-I antibody-positive serum 739 with p19 octapeptides.

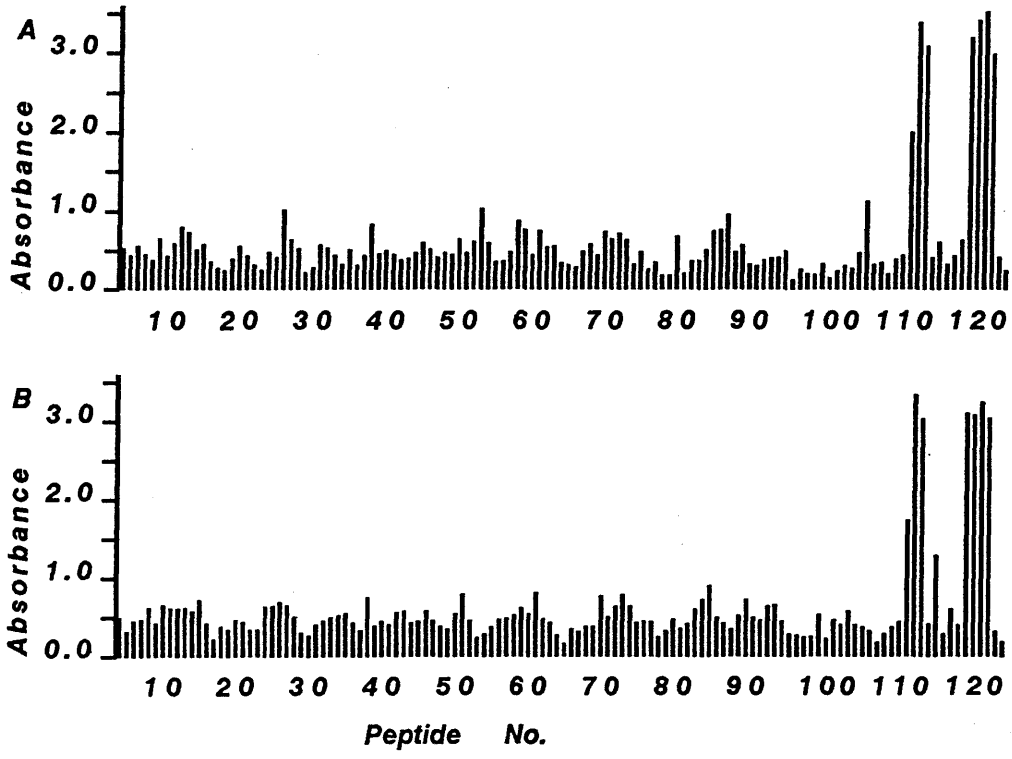


Figure 5.23. Reactivity of HTLV-I antibody-positive serum 741 with p19 octapeptides.

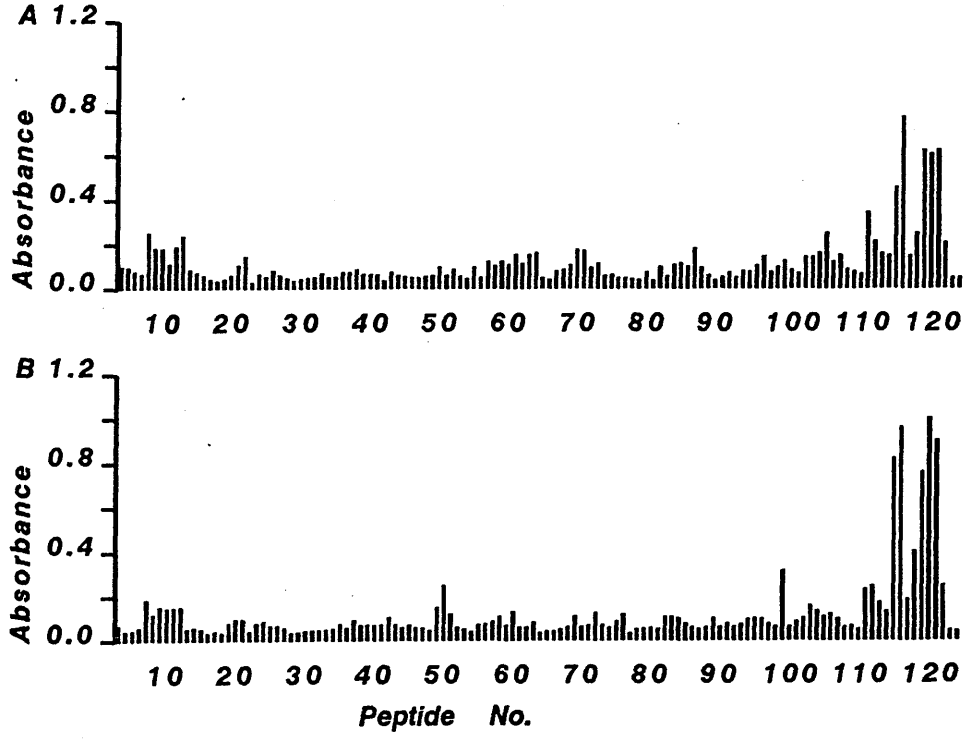


Figure 5.24. Reactivity of HTLV-I antibody-positive serum 796 with p19 octapeptides.

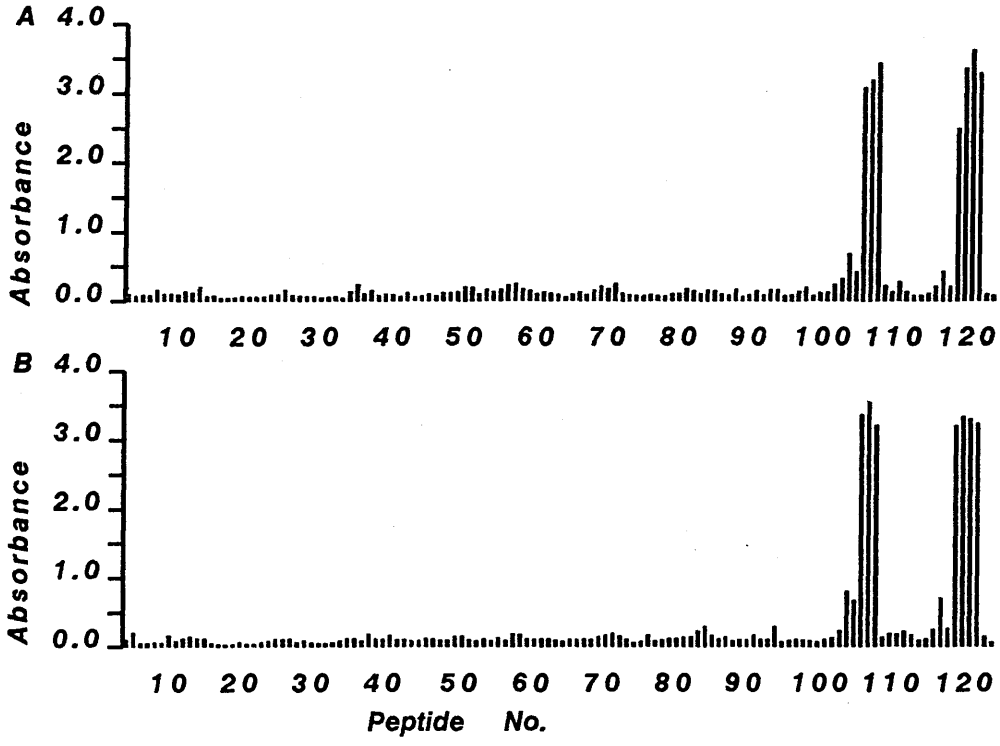


Figure 5.25. Reactivity of HTLV-I antibody-positive serum 797 with p19 octapeptides.

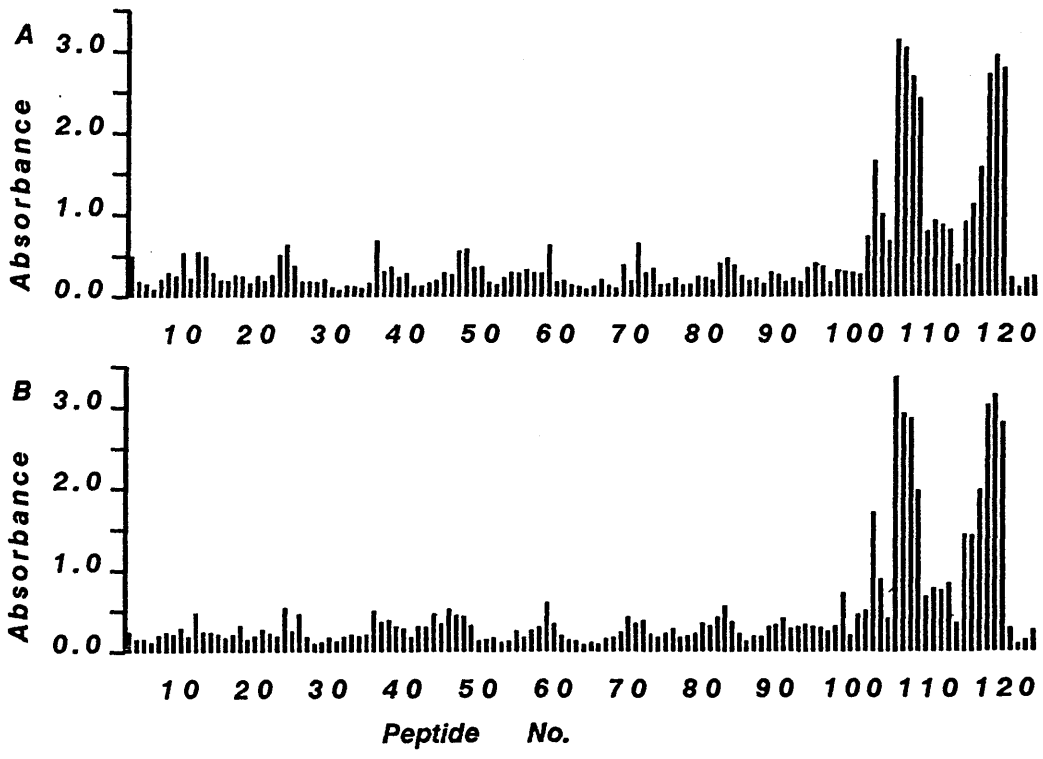
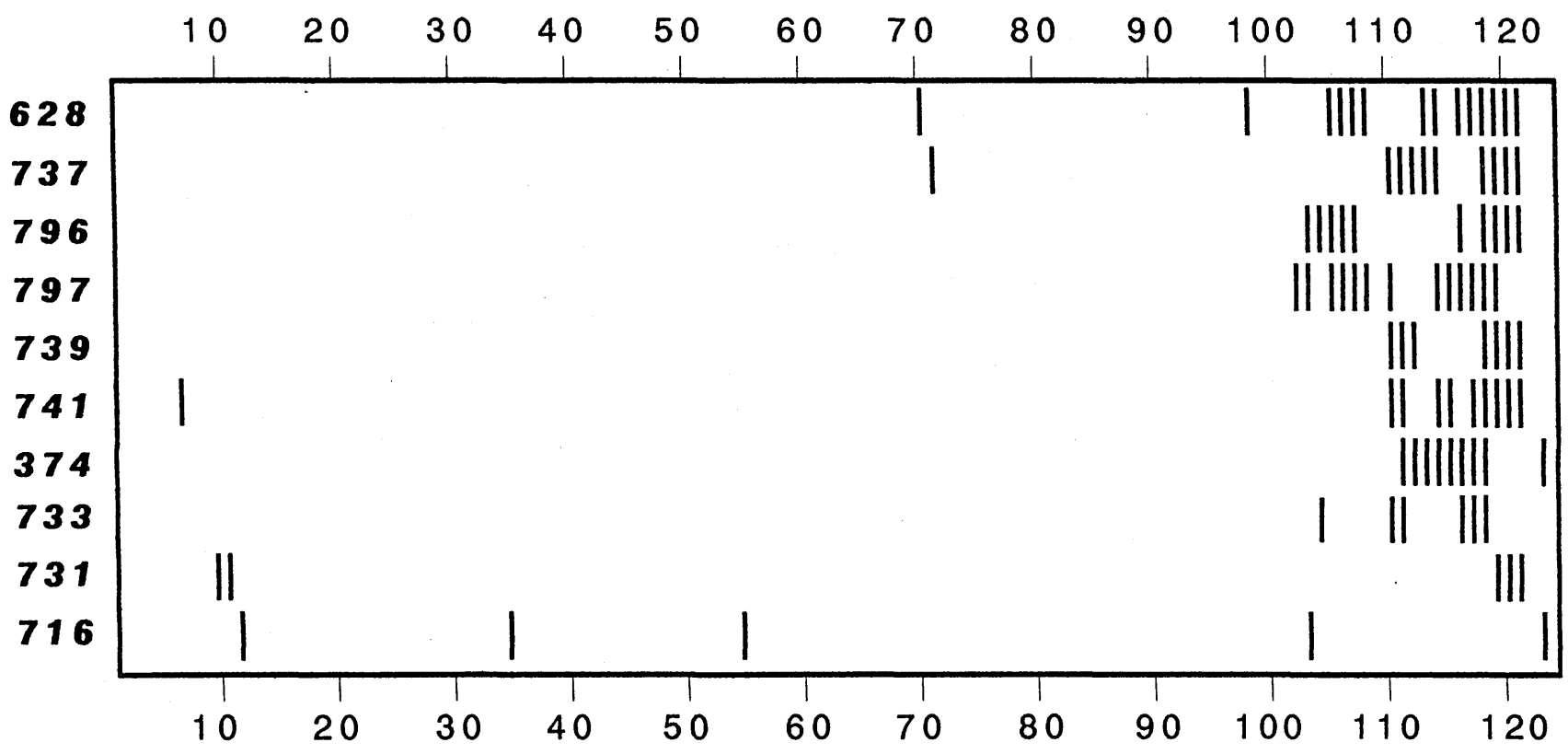


Figure 5.26. The numbers above and below the panel indicate the octapeptide number. Octapeptides showing significant reactivity with a serum sample are shown with a vertical bar opposite the sample number, which is given on the left of the panel.

Figure 5.26. Summary of the results obtained with HTLV-I antibody-positive serum samples in the PEPSCAN ELISA.



Reactivity of these sera with the C-terminal region of p19 was confirmed with an ELISA using P109-130. All ten HTLV-I positive sera reacted with this peptide. The highest serum dilutions which gave a positive result varied from 1/300-1/3000 (Table V.VIII).

In order to investigate the significance of the reactivities detected with isolated octapeptides, selected sera were analysed in ELISAs using 15 amino acid long peptides overlapping the reactive octapeptides. The results of these experiments are given in Table V.IX. None of the sera which had reacted with isolated octapeptides showed reactivity with 15 amino acid long peptides that included the same sequence.

The lack of reactivity detected in these experiments was not solely due to the use of peptides of a different length and from a different source, as three fifteen amino acid long peptides derived from the C-terminal 29 amino acids of p19 did show reactivity with one or more of serum samples 374, 737 and 739 (Table V.IX). The 15 amino acid long peptides that were reactive with these sera were derived from regions of p19 that corresponded to groups of reactive octapeptides.

For serum sample 716 antibody binding to a longer peptide corresponding to an isolated reactive octapeptide was detected. This serum sample reacted with the 22 amino acid long peptide, P109-130, at dilutions up to 1/3000 (Table V.VIII). Serum sample 716 was not tested for reactivity with a fifteen amino acid long peptide derived from this region.

Indeterminate sera.

(a) Sera from caucasian patients.

The specificity of the indeterminate reactions observed with sera from two caucasian patients, numbers 215 and 487, was assessed using two approaches.

To determine whether factors in serum other than Ig were contributing to binding to p19, IgG was purified from serum and used in HTLV-I WB (DuPont) at a concentration equivalent to a 1/100 serum dilution. No change in the pattern of reactivity was observed with either sample (S. Crae, unpublished results).

Table V.VIII. Reactivity of HTLV-I antibody-positive, HTLV-II antibody-positive and indeterminate sera with P109-130.

HTLV-I + sera		HTLV-II + sera		Indeterminate sera PNG		Caucasian	
No.	Titre	No.	Titre	No.	Titre	No.	Titre
374	3000	806	-	884	+50*	215	-
628	3000	854	-	885	1000	487	-
716	3000	876	-	886	+50*		
731	300	877	10	887	100		
733	1000	878	30	888	100		
737	3000			889	3000		
739	3000			890	3000		
741	1000			891	3000		
796	3000			892	1000		
797	3000						

Sera were tested at dilutions of 1/10, 1/30, 1/100 etc. up to 1/3000. ELISAs were performed as described in section 5.2.8. The titre indicates the highest dilution giving a positive result. No., number; PNG, Papua New Guinea; HTLV-I+, HTLV-I antibody-positive; HTLV-II+, HTLV-II antibody-positive; -, negative; *, there was insufficient sample material to assay these sera at multiple dilutions, both sera were positive when tested at a dilution of 1/50.

Table V.IX. Sera were tested at dilutions of 1/10, 1/30, 1/100 etc. to 1/3000 in ELISAs with 15 amino acid (aa) long peptides that overlapped with octapeptides reactive in the PEPSCAN ELISA. Experiments were performed as described in section 5.2.8. HTLV-I +, HTLV-I antibody-positive; HTLV-II+, HTLV-II antibody-positive; No., number; -, negative.

Table V.IX. Reactivity of selected sera with 15 amino acid long peptides.

Sample No.	Sample group	Reactive octapeptides	Corresponding 15 aa peptide	Result
716	HTLV-I+	35	P32-45	-
737	HTLV-I+	71	P67-81	-
806	HTLV-II+	5,7,8	P2-16	-
			P7-21	-
			P(II)2-16	-
			P(II)7-21	-
854	HTLV-II+	11	P2-16	-
			P7-21	-
			P(II)2-16	-
			P(II)7-21	-
876	HTLV-II+	70	P67-81	-
			P(II)67-81	-
215	Indeterminate	99,100,	P97-111	-
		103-108,	P102-116	-
		117,118	P107-121	-
			P112-126	-

To determine whether serum binding to p19 was due to interaction with a specific epitope on the protein, purified IgG from samples 215 and 487 were analysed in the PEPSCAN ELISA. The IgG samples were analysed at concentrations equivalent to serum dilutions of 1/100. Sample 215 showed strongest reactivity with a group of 6 adjacent octapeptides numbers 102-107 and weaker reactivity with two pairs of octapeptides, numbers 99-100 and 117-118 (Figure 5.27). Sample 487 reacted with one pair of octapeptides, numbers 112-113 and with two additional isolated octapeptides, 39 and 115 (Figure 5.28).

When tested in ELISAs using longer peptides overlapping the regions reactive in the PEPSCAN ELISA, both sera gave negative results (Tables V.VIII and V.IX). Serum 215 did not react with P97-111, P102-116, P107-121, P111-125 or with P109-130. Serum 487 did not react with P109-130.

Serum samples 215 and 487 were also analysed in ELISAs using the 15 amino acid long peptides derived from the endogenous retrovirus, HRES-1/1 (Table V.VII). Neither of the samples showed evidence of reactivity with these peptides.

(b) Sera from residents of Papua New Guinea.

Nine sera from Papua New Guinea which reacted with p19 and related proteins on HTLV-I WB were tested in the PEPSCAN ELISA. Sera were analysed at dilutions which varied from 1/400 to 1/800, as limited by serum availability. The results obtained are illustrated in Figures 5.29-5.37 and summarized in Figure 5.38. Seven of the sera showed reactivity with a group of 3 adjacent octapeptides, numbers 109-111, one of these sera (sample 891) reacted in addition with octapeptide 112. Serum 887 reacted with two of these octapeptides, 110 and 111. Serum 886 did not show significant reactivity with any group of octapeptides when tested at a dilution of 1/800. All nine sera showed reactivity with isolated octapeptides elsewhere in p19.

Figures 5.27-5.28. The graphs show the results obtained from the analysis of IgG purified from serum samples obtained from two Caucasian patients in the PEPSCAN ELISA. The heights of the bars indicate the absorbance readings obtained for individual octapeptides in the ELISA. Panels A and B show the results obtained in ELISAs performed on duplicate sets of octapeptides. Purified IgG was diluted to a final concentration of 0.035mg/ml for analysis; this was equivalent to serum dilutions of approximately 1/100.

Figure 5.27. Reactivity of indeterminate serum 215 with p19 octapeptides.

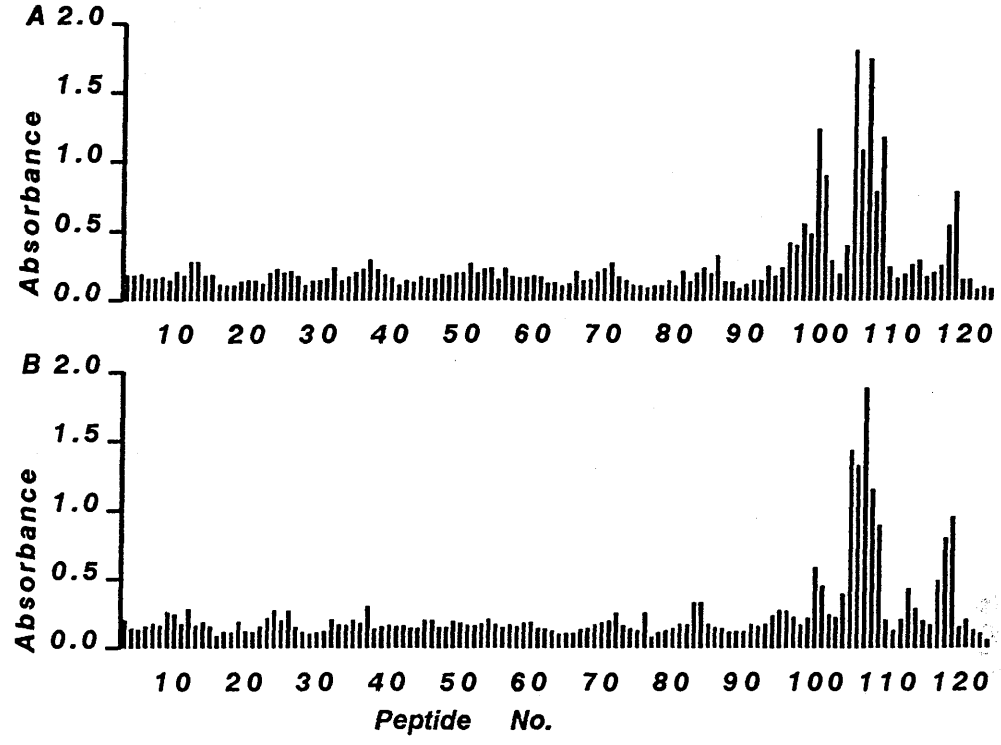
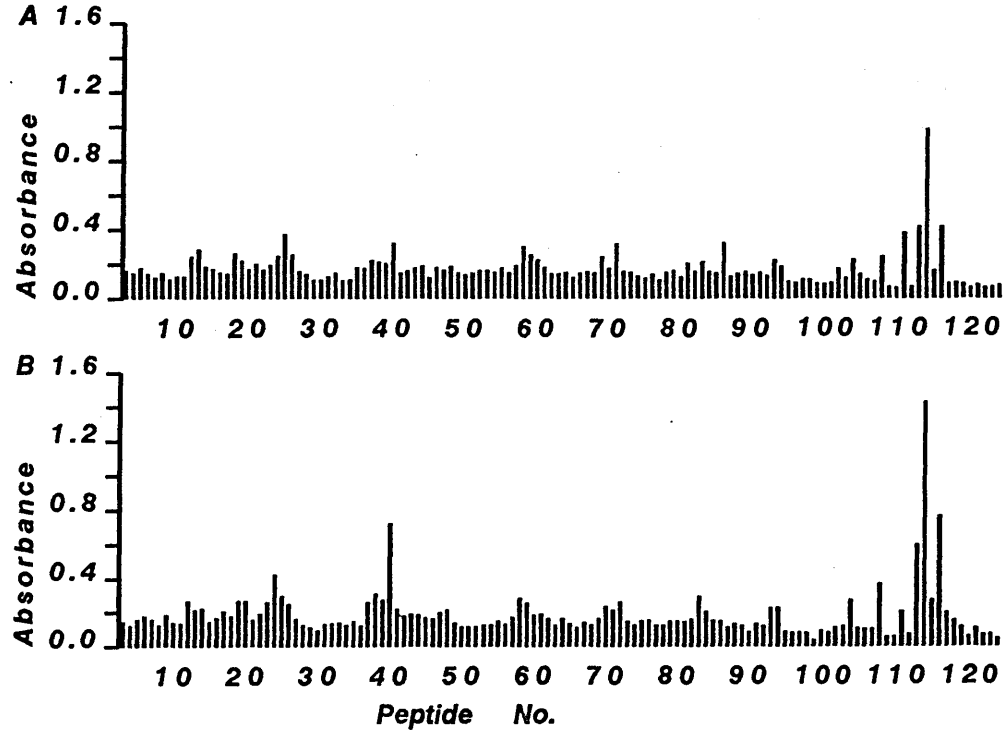


Figure 5.28. Reactivity of indeterminate serum 487 with p19 octapeptides.



Figures 5.29-5.37. The graphs show the results obtained from the analysis of serum samples from residents of Papua New Guinea (PNG) in the PEPSCAN ELISA. The heights of the bars indicate the absorbance readings obtained for individual octapeptides in the ELISA. Panels A and B show the results of ELISAs performed on duplicate sets of octapeptides.Sera were analysed at dilutions of 1/400 (samples 887 and 884) or 1/800.

Figure 5.29. Reactivity of PNG serum 884 with p19 octapeptides.

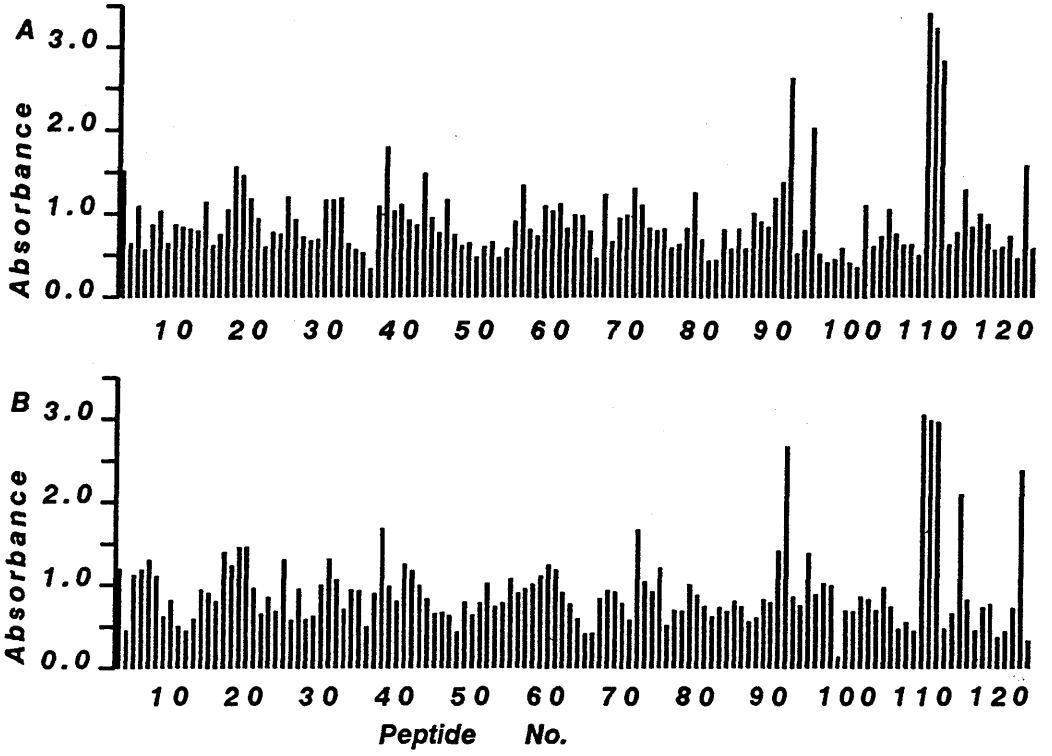


Figure 5.30. Reactivity of PNG serum 885 with p19 octapeptides.

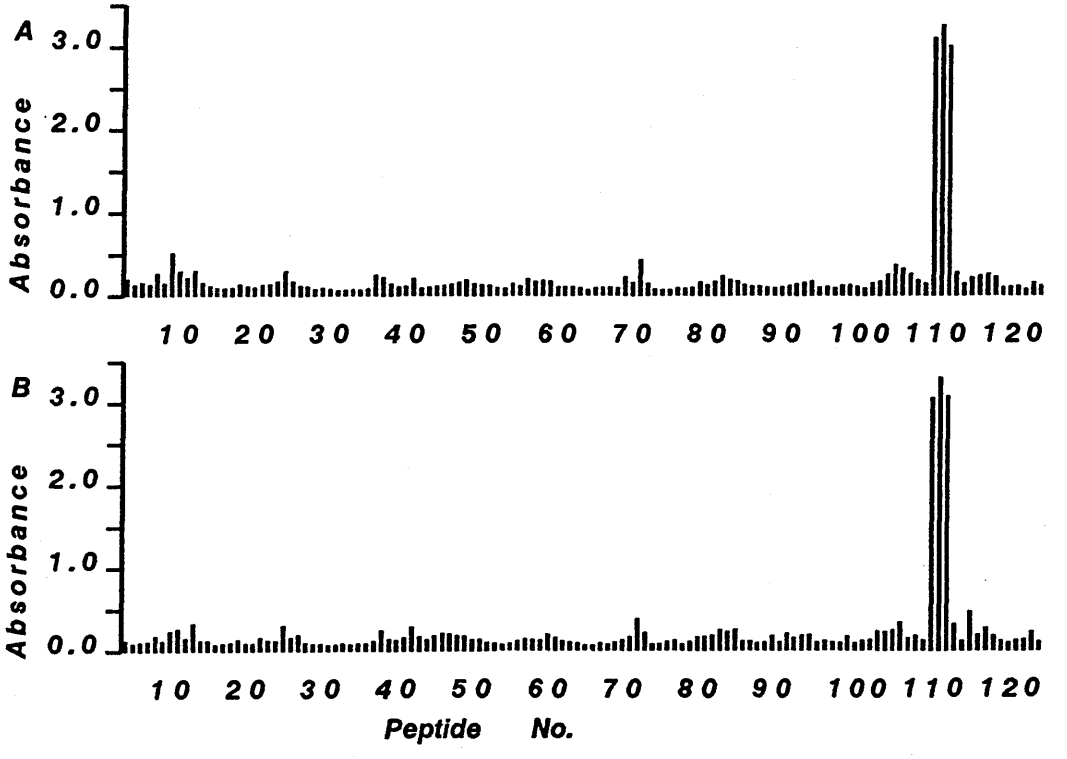


Figure 5.31. Reactivity of PNG serum 886 with p19 octapeptides.

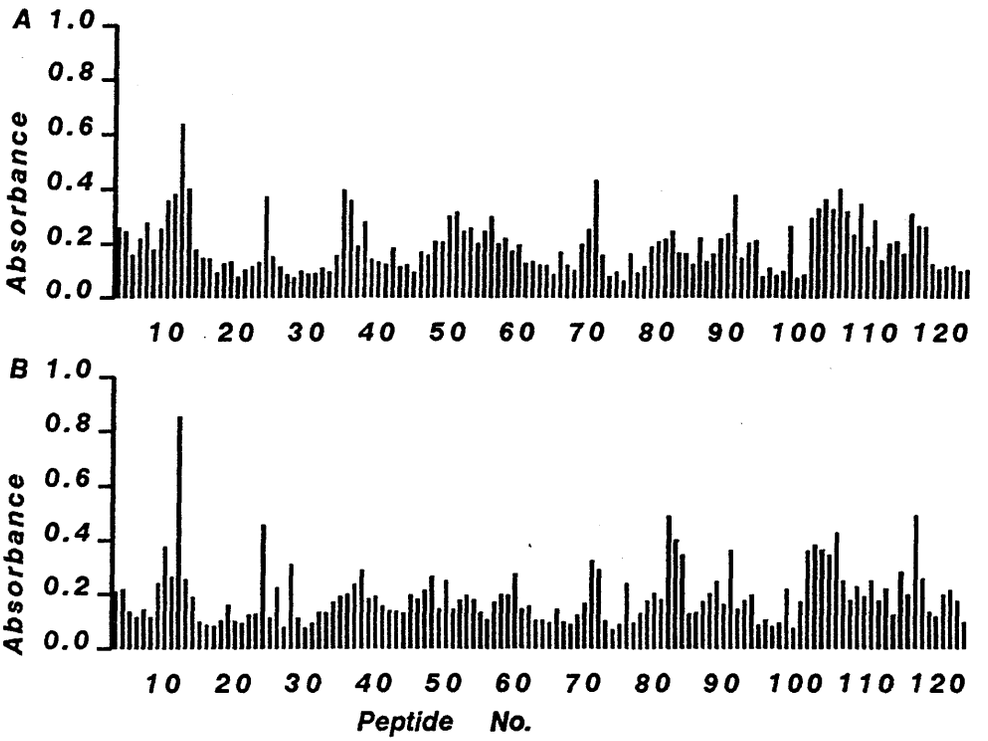


Figure 5.32. Reactivity of PNG serum 887 with p19 octapeptides.

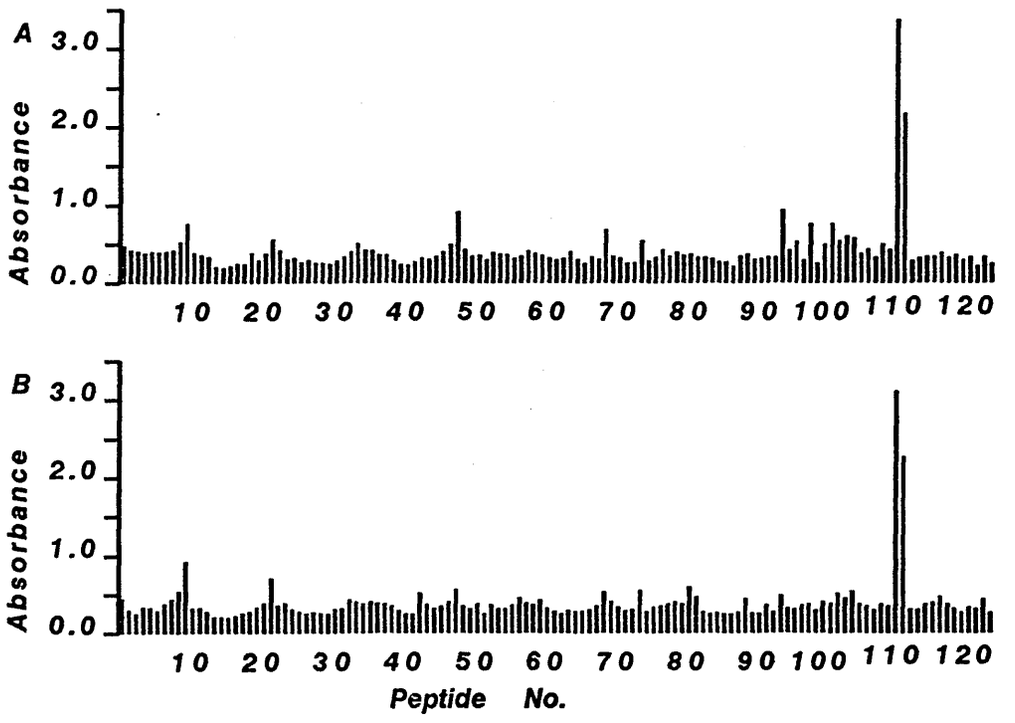


Figure 5.33. Reactivity of PNG serum 888 with p19 octapeptides.

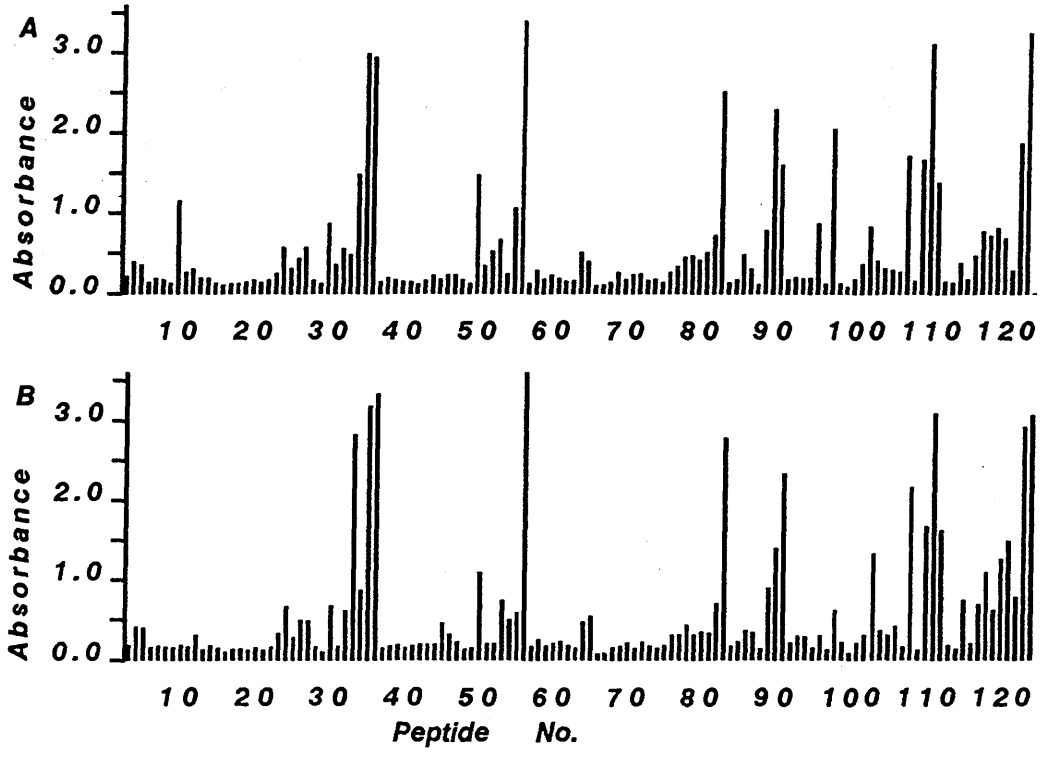


Figure 5.34. Reactivity of PNG serum 889 with p19 octapeptides.

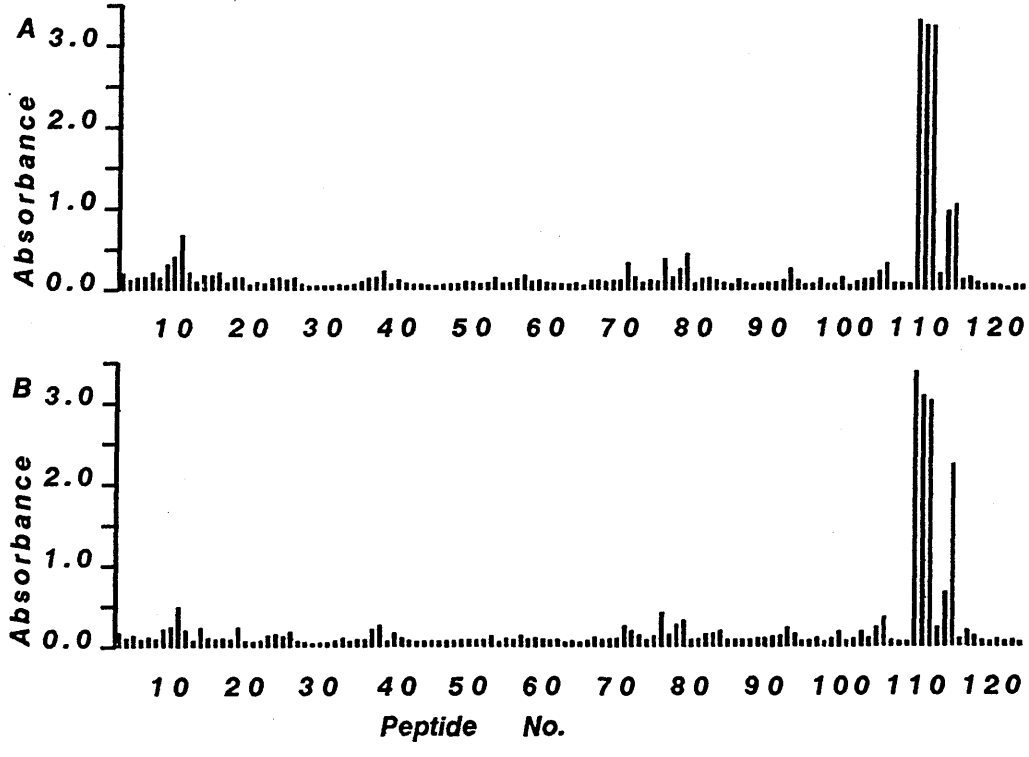


Figure 5.35. Reactivity of PNG serum 890 with p19 octapeptides.

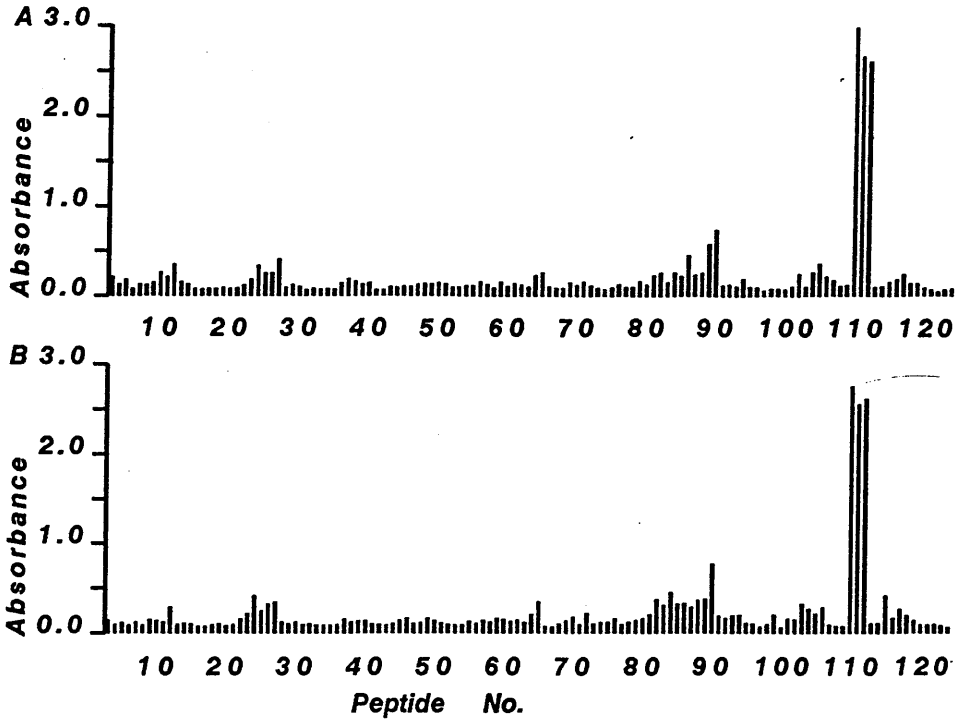


Figure 5.36. Reactivity of PNG serum 891 with p19 octapeptides.

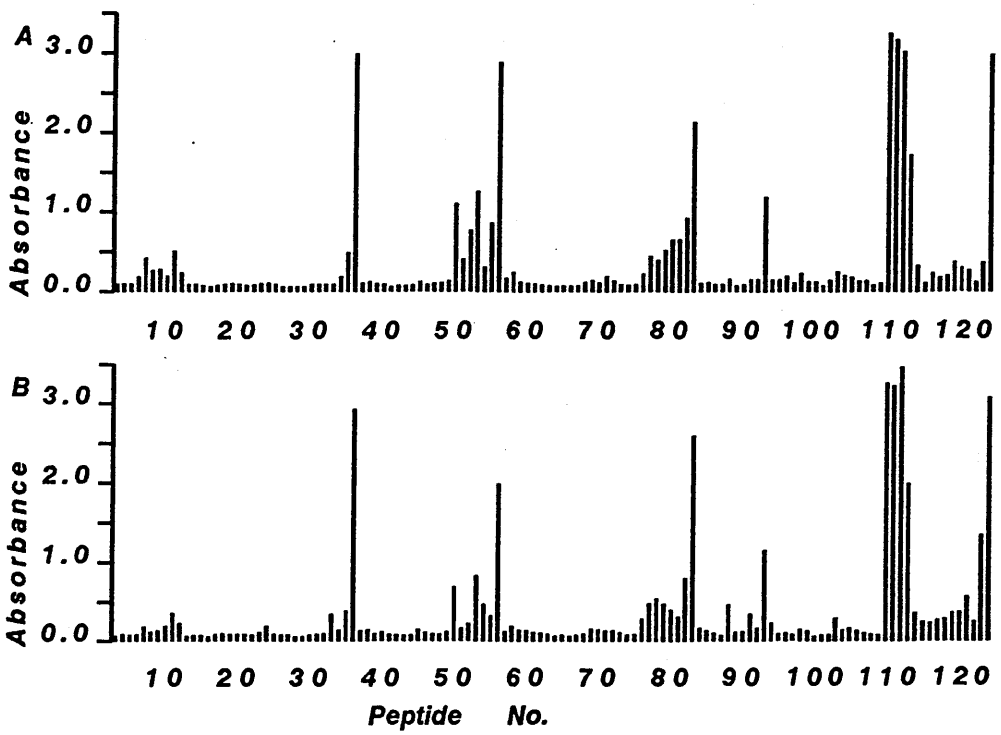


Figure 5.37. Reactivity of PNG serum 892 with p19 octapeptides.

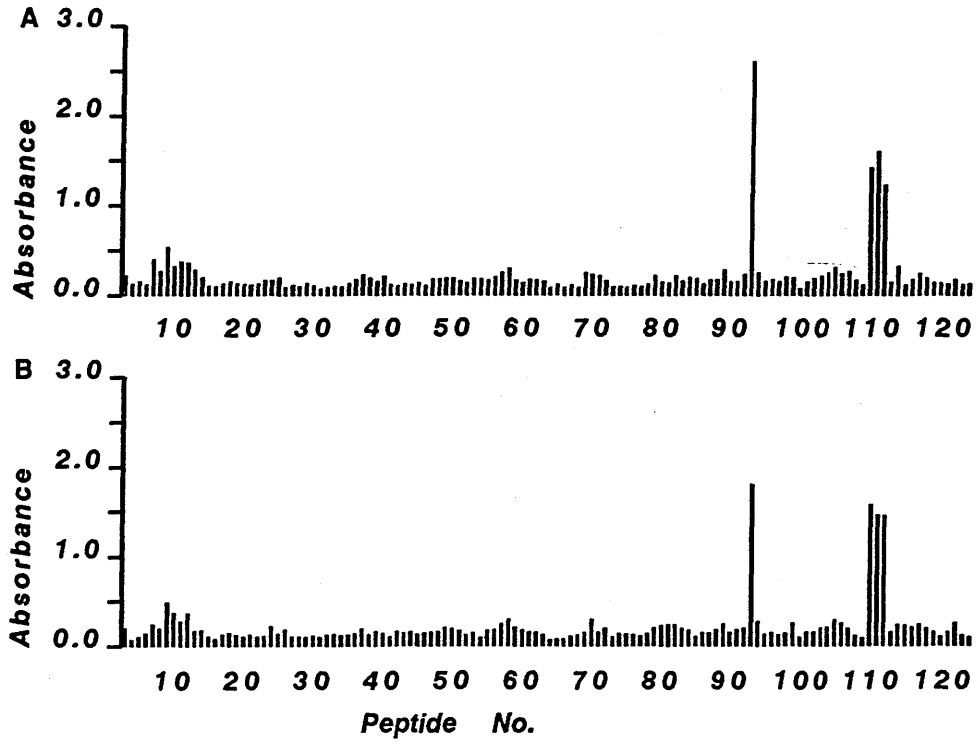
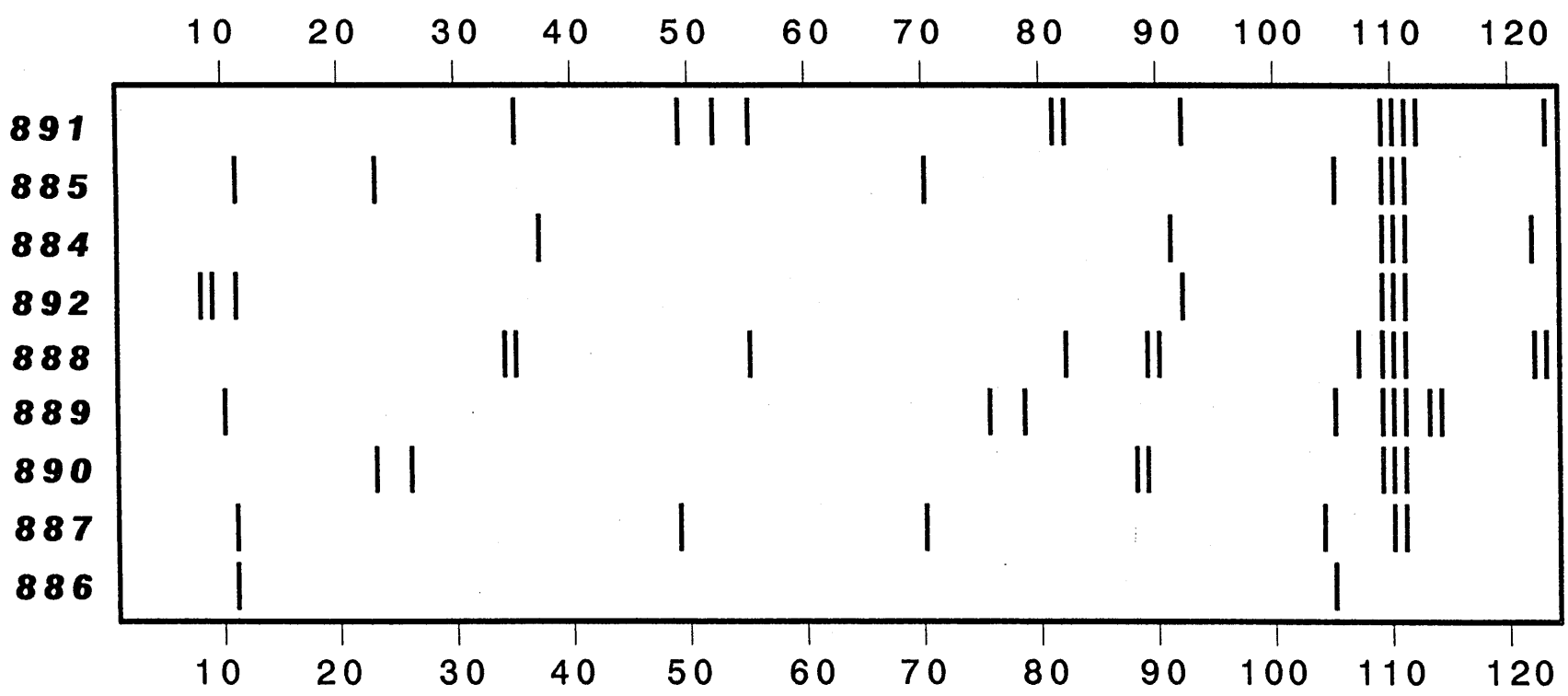


Figure 5.38. The numbers above and below the panel indicate the octapeptide number. Octapeptides showing significant reactivity with a serum sample are shown with a vertical bar opposite the sample number, which is given on the left of the panel.

Figure 5.38. Summary of the results obtained from the analysis of serum samples from residents of Papua New Guinea in the PEPSCAN ELISA.



Seven of the sera were tested at multiple dilutions in the P109-130 ELISA, there was insufficient serum to perform titrations of samples 884 and 886. The seven sera tested were positive at dilutions from 1/100-1/3000 (Table V.VIII). Titration curves for the sera are shown in Figure 5.39. The results obtained for five of the seven sera are not distinguishable from titration curves obtained with the HTLV-I positive sera (Figure 5.40). The results obtained with the remaining two PNG sera (samples 887 and 888) are more similar to the titration curve obtained for the HTLV-II positive sample 878 (Figure 5.39, panel B).

HTLV-II positive sera.

Five HTLV-II positive sera were analysed at dilutions that varied from 1/200 to 1/400 with the PEPSCAN ELISA. The dilution used depended on serum availability. HTLV-II positive sera showed a less consistent pattern of reactivity than HTLV-I positive sera (Figures 5.41-5.45). A summary of the data is shown in Figure 5.46.

HTLV-II-positive sera showed significant binding to single octapeptides or pairs of octapeptides only. Two sera, 877 and 878, reacted with isolated octapeptides within the epitope reactive with MoAb 12/1-2.

ELISAs using 15 and 22 amino acid long peptides derived from HTLV-I p19 sequences were used to further investigate the reactivities observed with HTLV-II positive sera in the PEPSCAN ELISA. Fifteen amino acid long peptides derived from regions of HTLV-II p22 that were homologous to the reactive p19 octapeptides were also used in ELISAs. The results of these experiments are summarized in Table V.IX. Samples 806 and 854 were negative when assayed with P2-16 and P7-21. These samples were also tested for reactivity with peptides from homologous regions of HTLV-II p22 and again gave negative results. Serum 876 was negative when tested in ELISAs using P67-81 and the homologous HTLV-II peptide, P(II)67-81. All five HTLV-II positive sera were tested for reactivity with P109-130, serum samples 877 and 878 were weakly reactive, the other sera were negative (Table V.VIII). There was insufficient serum available from samples 877 and 878 for testing with P116-130 or the homologous HTLV-II peptide, P(II)118-133.

Figure 5.39. Serum samples were tested at dilutions of 1/10, 1/30,1/100 etc. to 1/3000. Serum reactivity in the P109-130 ELISA was determined as described in section 5.2.8. The results are shown on two seperate graphs for clarity. Panel A shows the results obtained from the analysis of four serum samples obtained from residents of Papua New Guinea (PNG); Panel B shows the results obtained from the analysis of three PNG serum samples (solid lines) and two HTLV-II antibody-positive serum samples (dashed lines). Individual curves indicate the results obtained from the analysis of the samples shown on the right of each panel.

Figure 5.39. Reactivity of PNG sera and of HTLV-II antibody-positive sera in the P109-130 ELISA: serum titrations.

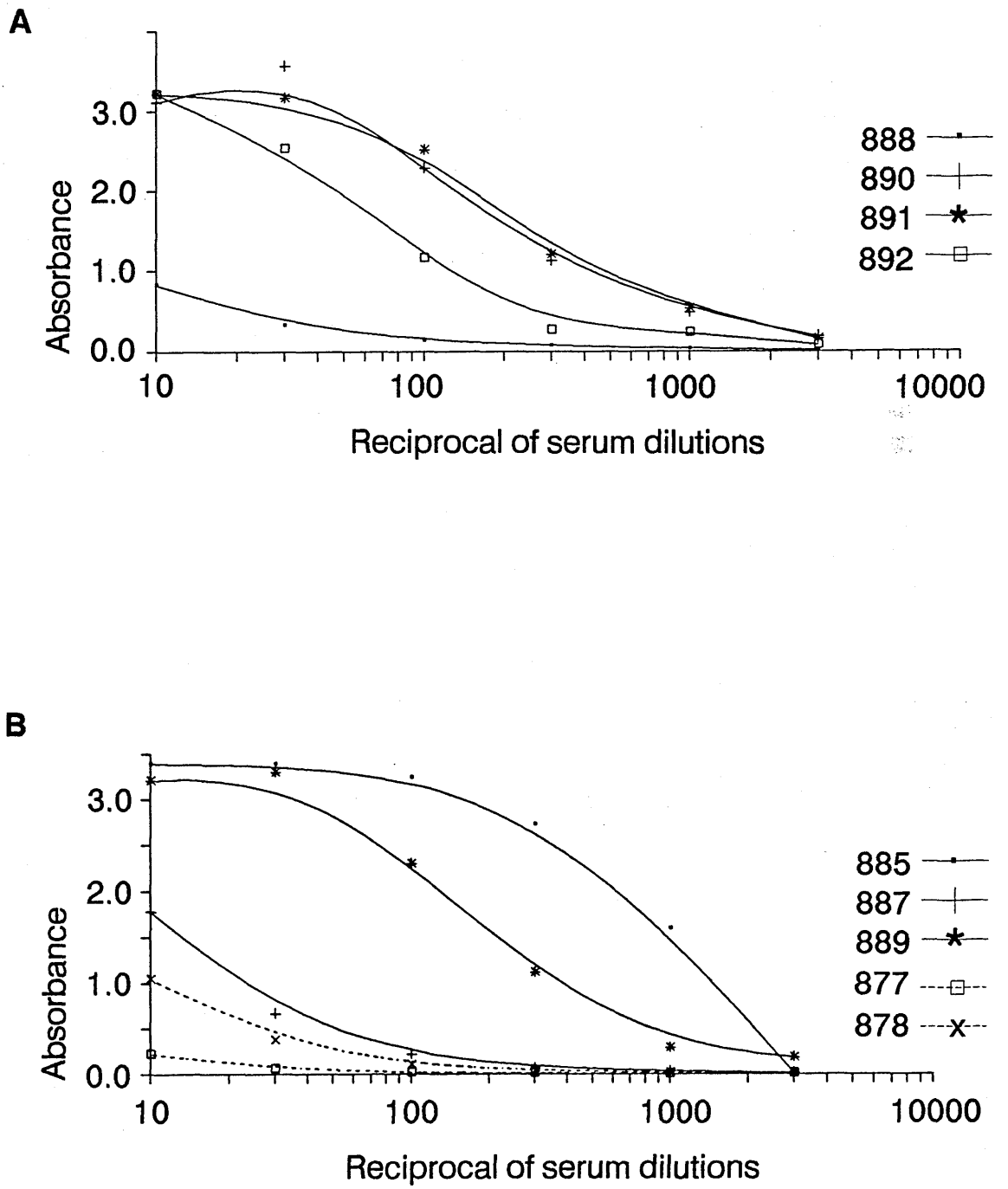
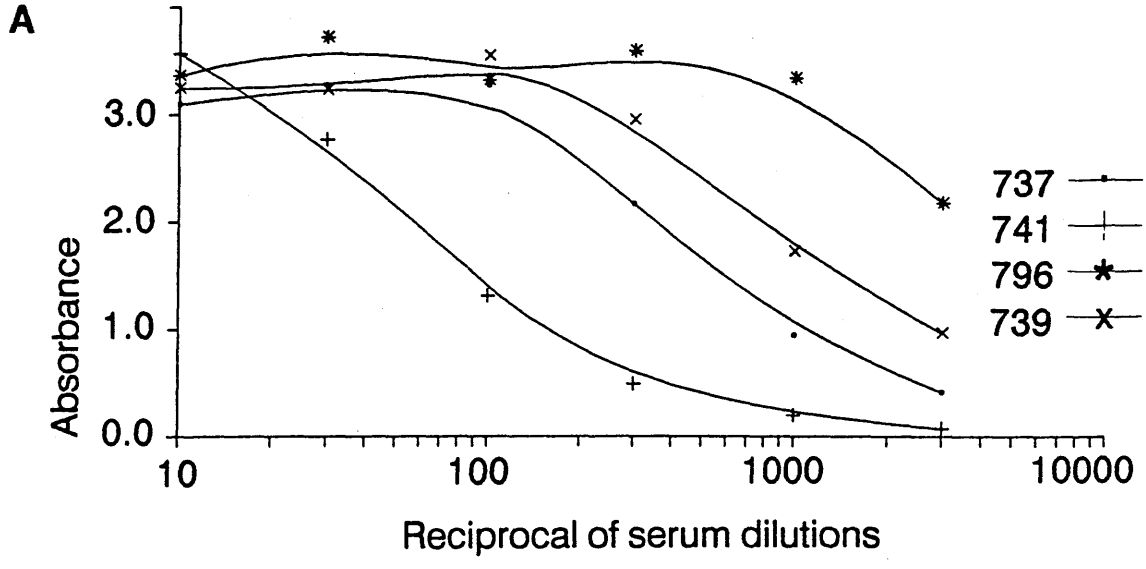
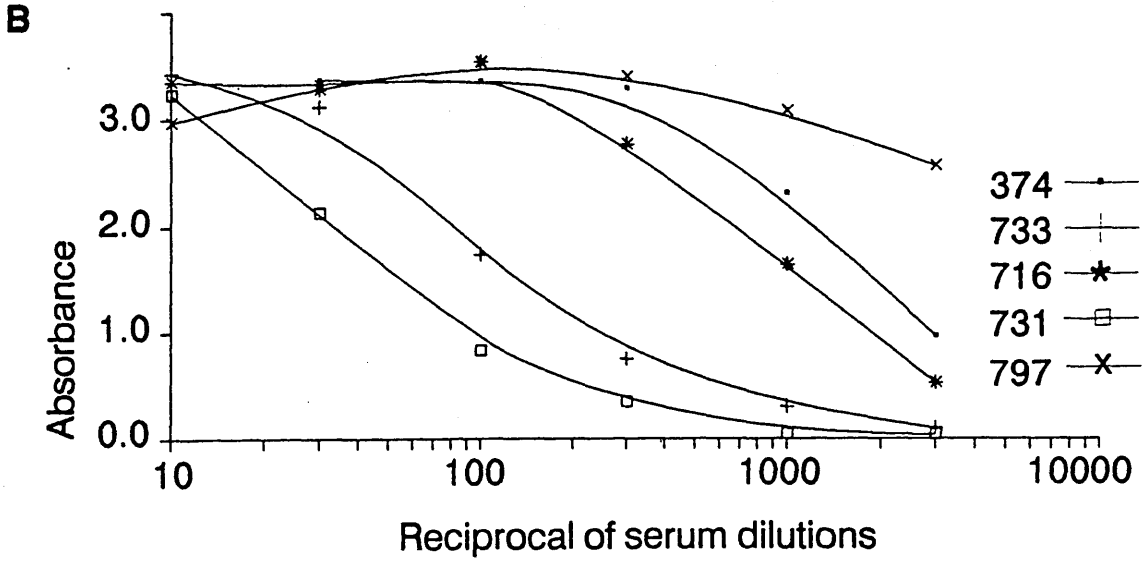


Figure 5.40. Serum samples were tested at dilutions of 1/10, 1/30,1/100 etc. to 1/3000. Serum reactivity in the P109-130 ELISA was determined as described in section 5.2.8. The results are shown on two seperate graphs for clarity. Panels A and B show the results obtained from the analysis of nine HTLV-I antibody-positive serum samples. Individual curves indicate the results obtained from the analysis of the samples shown on the right of each panel.

Figure 5.40. Reactivity of HTLV-I antibody-positive sera in the P109-130 ELISA: serum titrations.





Figures 5.41-5.45. The graphs show the results obtained from the analysis of HTLV-II antibody-positive serum samples in the PEPSCAN ELISA. The heights of the bars indicate the absorbance readings obtained for individual octapeptides in the ELISA. Panels A and B show the results of ELISAs performed on duplicate sets of octapeptides. Sera were analysed at dilutions of 1/200 (samples 876, 877 and 878) or 1/400.

Figure 5.41. Reactivity of HTLV-II antibody-positive serum 806 with p19 octapeptides.

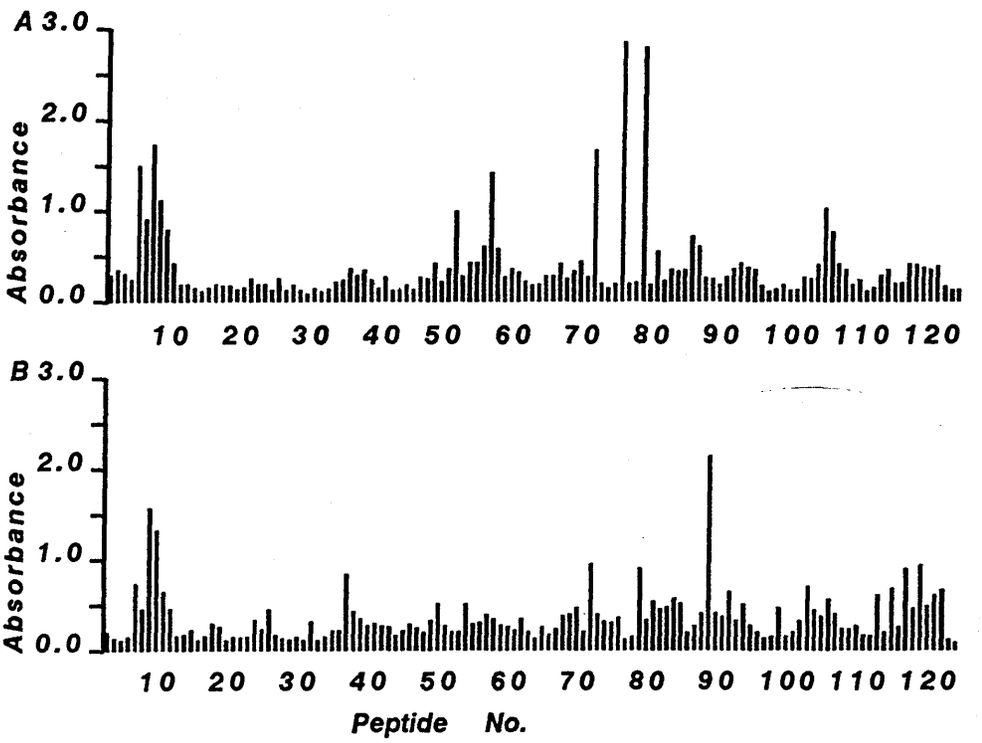


Figure 5.42. Reactivity of HTLV-II antibody-positive serum 854 with p19 octapeptides.

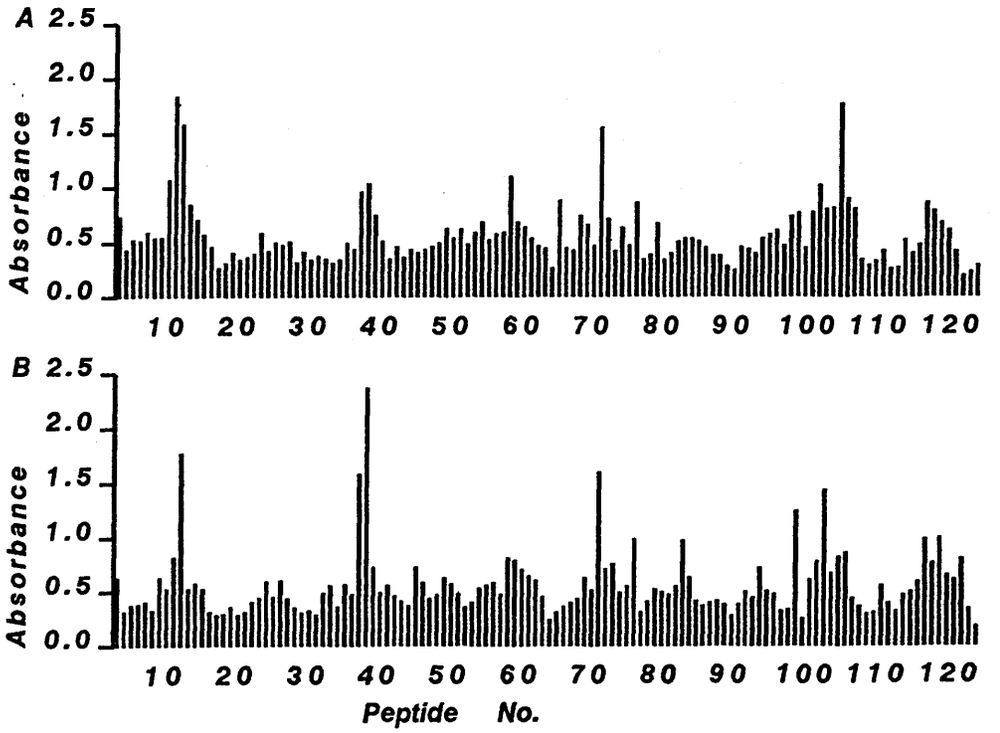


Figure 5.43. Reactivity of HTLV-II antibody-positive serum 876 with p19 octapeptides.

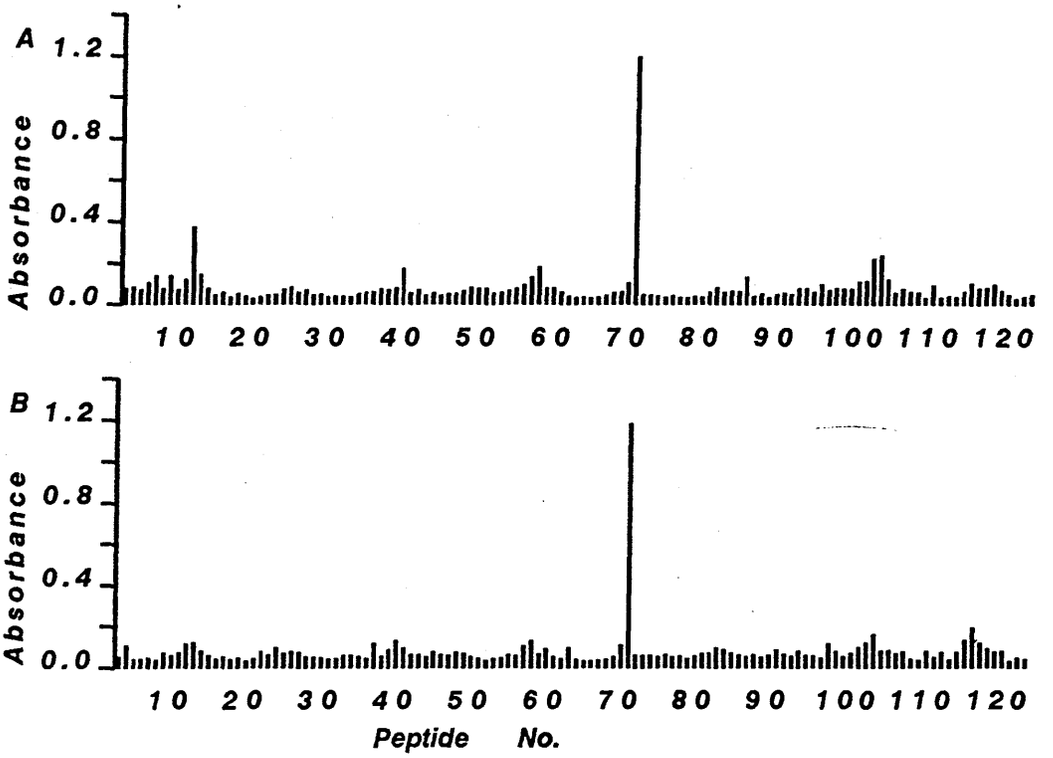


Figure 5.44. Reactivity of HTLV-II antibody-positive serum 877 with p19 octapeptides.

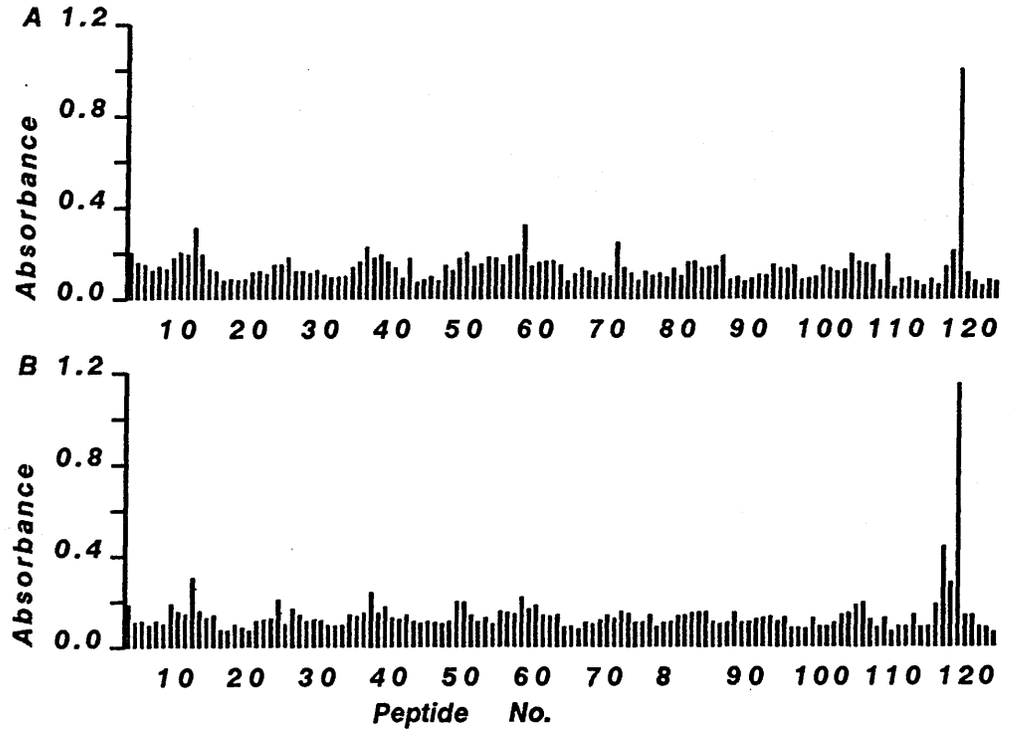


Figure 5.45. Reactivity of HTLV-II antibody-positive serum 878 with p19 octapeptides.

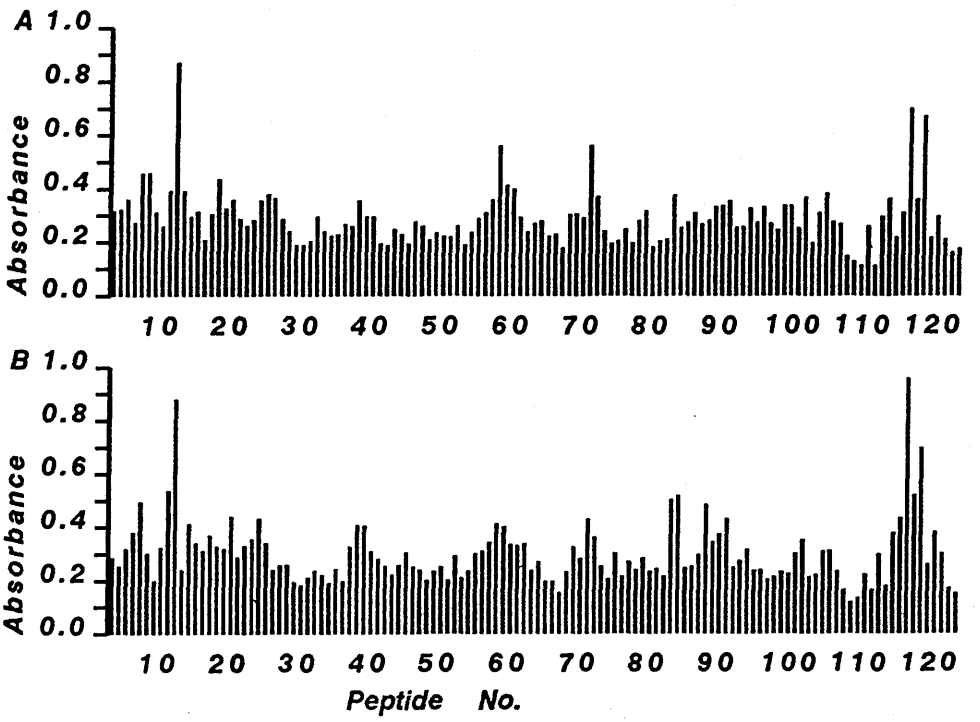
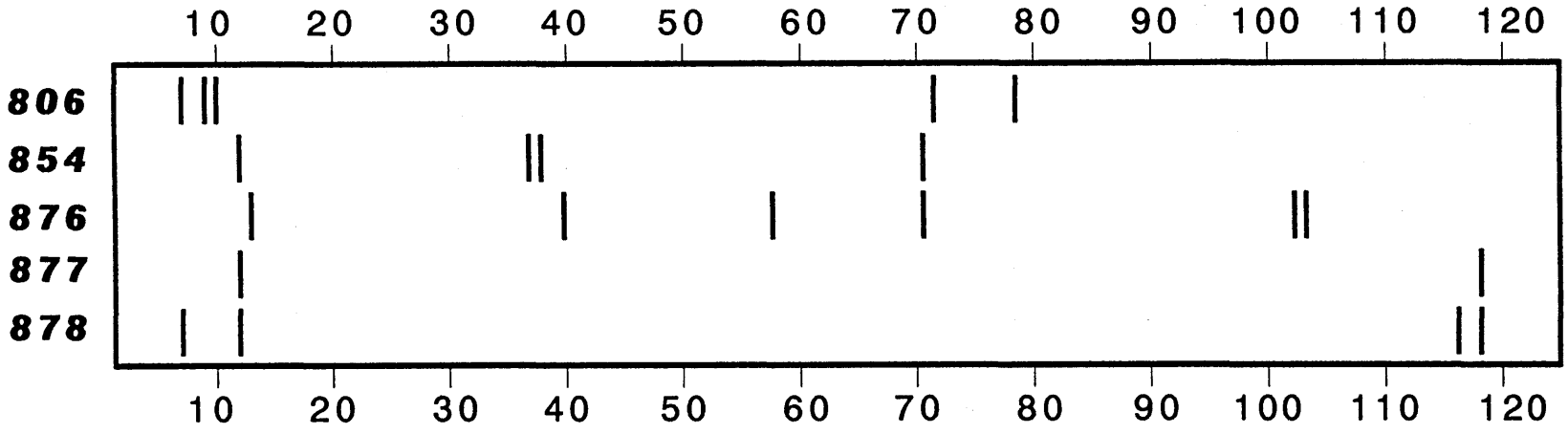


Figure 5.46. The numbers above and below the panel indicate the octapeptide number. Octapeptides showing significant reactivity with a serum sample are shown with a vertical bar opposite the sample number, which is given on the left of the panel.

Figure 5.46. Summary of the results obtained with HTLV-II antibody-positive serum samples in the PEPSCAN ELISA.



HTLV-negative sera.

Three sera which gave negative results in HTLV-I ELISA (DuPont) and HTLV-I WB (DuPont) were analysed in the PEPSCAN ELISA. All sera were analysed at a dilution of 1/200. The results of these analyses are shown in Figures 5.47-5.49. All three serum samples showed reactivity with isolated octapeptides in duplicate assays. Sample 2 showed reactivity with a group of 3 adjacent octapeptides, numbers 101-103. The absorbance values obtained in the ELISAs with these three octapeptides were low relative to the background readings, when compared to the peak reactivities seen in HTLV-I-positive sera (Figure 5.48).

5.3.5. Reactivity of HTLV-I-positive and PNG sera in P109-130 ELISA following preadsorption with rp19.

To investigate further the p19 specificity and the relative affinity of the reactions seen with P109-130, 4 PNG sera and 4 HTLV-I positive sera were preadsorbed with rp19 before testing in the ELISA. Preadsorption with rp19 resulted in a concentration dependent reduction in antibody binding to P109-130 in the 4 PNG sera (Figure 5.50). Similar results were obtained with HTLV-I positive sera 733, 737 and 739 (Figure 5.50). The reactivity of sample 716 in the P109-130 ELISA was less efficiently reduced by preadsorption with rp19 (Figure 5.50, panel a).

5.3.6. P109-130 ELISA.

In order to assess whether the C-terminal region of p19 showed consistent reactivity with HTLV-I positive sera, an additional 32 sera that were positive in the HTLV-I competitive ELISA, were analysed in the P109-130 ELISA. Nine of the HTLV-I-positive sera tested in the PEPSCAN ELISA were also analysed. Sera were tested in four replicate assays a dilution of 1/50, with the exception of two samples which were tested once in duplicate. The results were compared to those obtained with 45 sera that gave negative results in HTLV-I ELISA (DuPont). The results obtained are shown in Figure 5.51. The two groups of sera could be readily distinguished using a cut-off value equal to an O.D. of 0.29, as shown in the figure.

Figures 5.47-5.49. The graphs show the results obtained from the analysis of HTLV-I antibody-negative serum samples for reactivity with HTLV-I p19 octapeptides in the PEPSCAN ELISA. The heights of the bars indicate the absorbance readings obtained for individual octapeptides in the ELISA. Panels A and B show the results of ELISAs performed on duplicate sets of octapeptides. Sera were analysed at dilutions of 1/200.

Figure 5.47. Reactivity of HTLV-I antibody-negative serum 1 with p19 octapeptides.

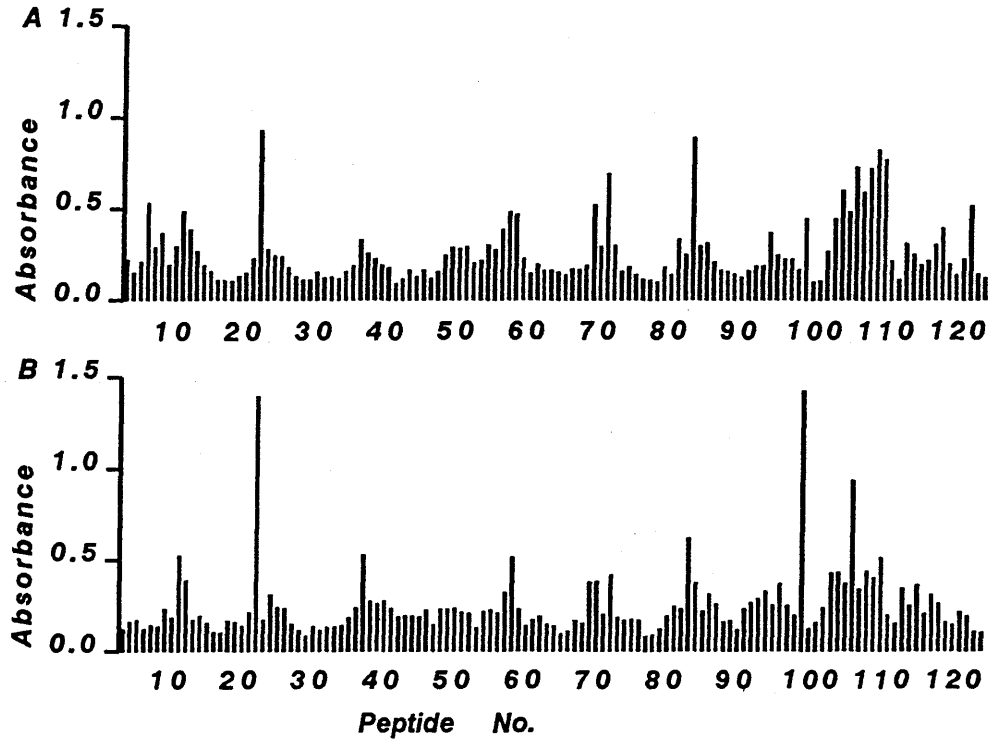


Figure 5.48. Reactivity of HTLV-I antibody-negative serum 2 with p19 octapeptides.

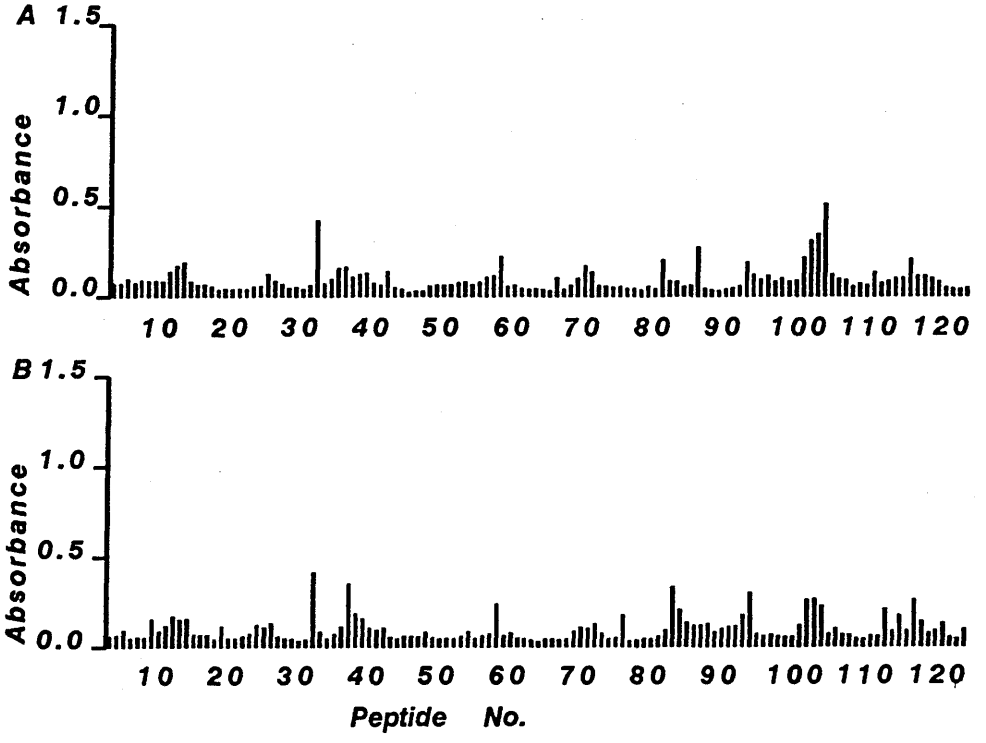


Figure 5.49. Reactivity of HTLV-I antibody-negative serum 400 with p19 octapeptides.

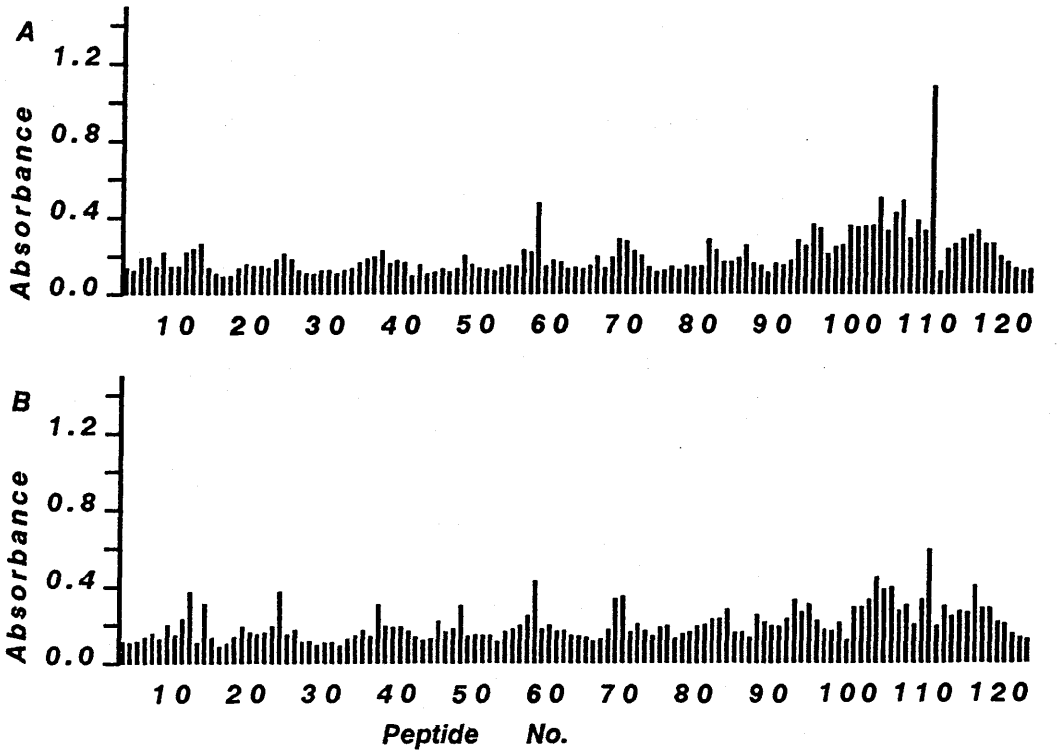


Figure 5.50. The reactivity with P109-130 observed following preabsorption of serum samples with the recombinant p19 fusion protein (rp19) is expressed as a percentage of the result (absorbance of light at 495nm) obtained in an identical ELISA using unabsorbed sera. The results are shown on two graphs for clarity. Solid lines show the results obtained when serum samples were preabsorbed with rp19. Dashed and dotted lines show the the results obtained in control experiments following preabsorption with glutathione-S-transferase. The results are the mean values obtained in duplicate experiments. A, HTLV-I antibody-positive serum samples 716 (—&---) and 733 (-+-&-+-), PNG serum samples 885 (-✱-&-✱-) and 889 (-⊟-&-⊟-); B, HTLV-I antibody-positive serum samples 737 (—&---) and 739 (-+-&-+-), PNG serum samples 890 (-✱-&-✱-) and 891 (-⊟-&-⊟-).

Figure 5.50. Reactivity of HTLV-I antibody-positive and PNG sera in the P109-130 ELISA following preadsorption with rp19.

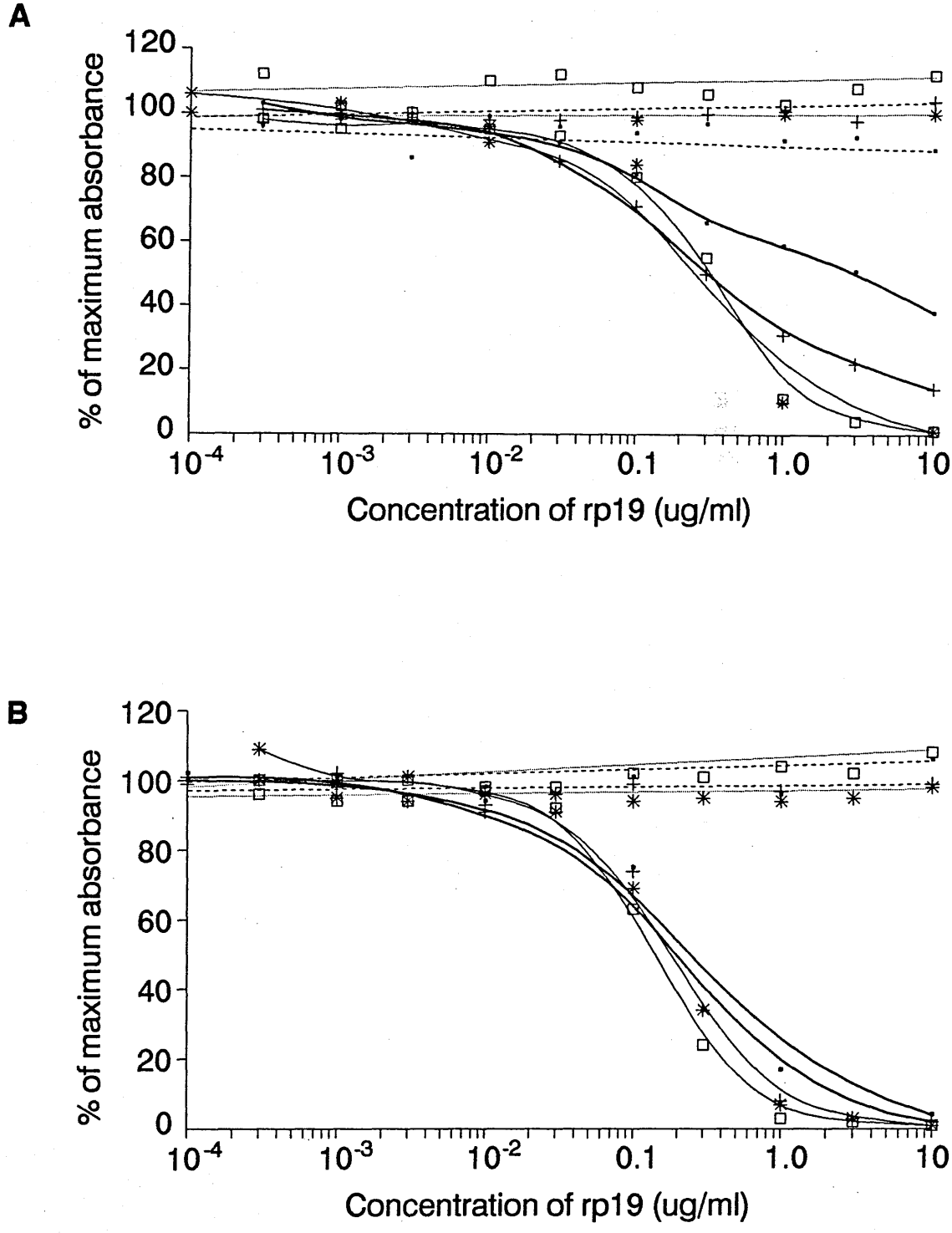
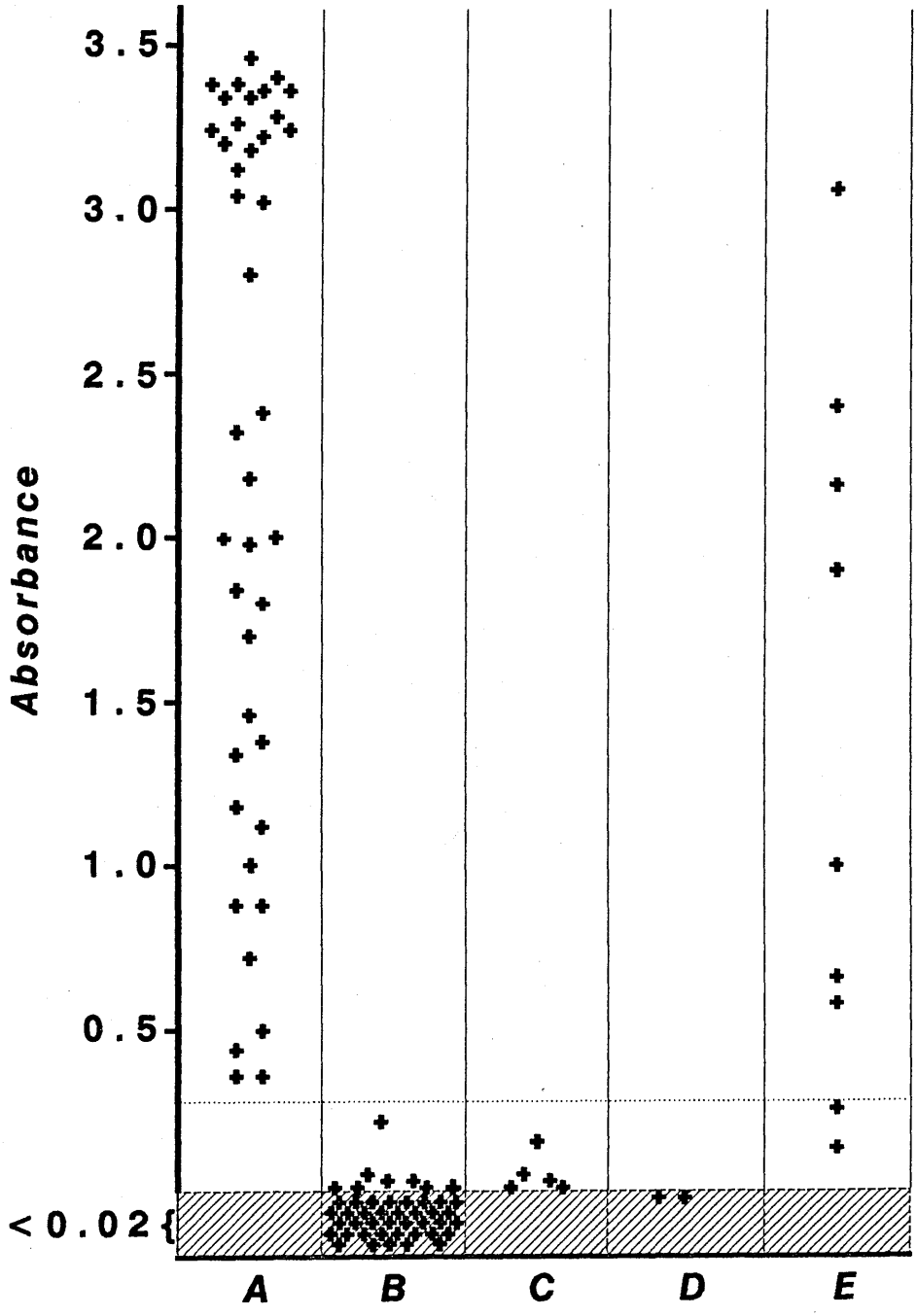


Figure 5.51. Panel A, HTLV-I antibody-positive sera; panel B, HTLV-I antibody-negative sera; panel C, HTLV-II antibody-positive sera; panel D, sera from Caucasian patients showing reactivity with HTLV-I p19 on WB; panel E, sera from residents of Papua New Guinea (PNG) showing reactivity with HTLV-I p19 on WB. The results shown are the means of four determinations with the following exceptions; the PNG sera and two of the HTLV-I antibody-negative sera were assayed in duplicate owing to limited sample availability. The shaded area at the base of the graph, delineated by dashed lines, indicates a region that is not drawn to scale, but represents absorbance readings of less than 0.02 O.D. units. The dotted line at 0.29 O.D. units indicates the cut-off value that discriminates between the HTLV-I antibody-positive and HTLV-I antibody-negative samples in this assay.

Figure 5.51. Reactivity of serum samples in the P109-130 ELISA.



Three samples were analysed that gave discordant results in other assays. The results obtained with these sera are shown in Table V.X. All three samples were negative in competitive ELISA. One of the samples was positive (i.e. above the cut-off value) in the P109-130 ELISA, while the other two were negative.

The HTLV-II positive and indeterminate sera were also tested in the P109-130 ELISA under the conditions described above. The results, as the mean of duplicate determinations, are shown in Figure 5.51. HTLV-II positive sera gave O.D. readings within the range of results obtained with negative control sera. The two indeterminate sera from Caucasian patients also gave results within the range obtained with negative control sera. Indeterminate sera from PNG gave O.D. readings within the range of results obtained with HTLV-I positive sera, with the exception of samples 888 and 887, which gave results below the cutoff value.

5.3.7 Secondary structure predictions.

The predictions for hydrophilicity, surface probability, flexibility, antigenic index and secondary structure for HTLV-I p19 are shown as one-dimensional curves in Figure 5.52. Figure 5.53 shows a two-dimensional plot of the predicted secondary structure of p19, according to the modified Chou-Fasman method described in the reference manual for Wisconsin GCG. The region of p19 most strongly predicted to be antigenic according to the algorithm of Jameson and Wolf (Jameson and Wolf 1988) includes amino acids 111-117. This overlaps the region predicted to be an antigenic determinant according to the algorithm of Hopp and Woods (1981), which lies between amino acids 109-116. Additional regions with a high antigenic index are located between amino acids 15-18 and 35-42.

Figures 5.54 and 5.55 show the results obtained from similar analyses of HTLV-II p22. The regions of HTLV-II p22 most strongly predicted to be antigenic according to the algorithm of Jameson and Wolf are located between amino acids 15-18, 35-42 and 129-132. Use of Hopp and Woods' algorithm indicates that the region of p22 between amino acids 36-42 is likely to be an antigenic determinant.

Table V.X. Origins and characterization of serum samples that gave discordant results in HTLV-I serological assays.

Sample No. (sender)	*Country of origin*	*Disease associations*	*Results of serological assays*				*P109-130 ELISA O.D reading**
			GPA	*IF*	*ELISA*	*C-ELISA*	
746 (a)	*Japan*	*Unknown*	+	-		-	*0.123+/-0.012*
835 (b)	*U.K.*	*Relative of TSP patient*	*1/32*	*1/16*	*1/32*	-	*0.977+/-0.155*
840 (b)	*U.K.*	*Relative of TSP patient*	*1/32*	*1/8*	*1/16*	-	*0.177+/-0.02*

ELISA, enzyme-linked immunoassay (DuPont); C-ELISA, competitive ELISA (a modification of the assay described by Tedder et. al 1984); GPA, gel particle agglutination asssay (Serodia ATLA, Fujirebio; IF, immunofluorescence; TSP, tropical spastic paraparesis; +, positive (serum titre not available); -, negative. . Serum sample 746 was obtained from Dr. B.C. Dow; serum samples 835 and 840 were obtained from Dr. U. Desselberger. The HTLV-I-specific antibody titres of the serum samples 835 and 840 obtained in the GPA, IF and ELISA are taken from Mowbray et al. (1989). The results obtained in the competitive ELISAs were communicated by Dr. J. Garson. The O.D. readings for the P109-130 ELISA are the mean (+/- standard deviation) of four determinations.

Figure 5.52. The curves are one-dimensional representations of the secondary structure of the p19 core protein of HTLV-I, obtained using the programs "PeptideStructure" and "PlotStructure", as described in section 5.2.10. Amino acid residues are numbered on the x-axis. Attributes of the sequence are shown as continuous curves. The top four panels indicate hydrophilicity calculated according to the algorithm of Hopp and Woods (1981) (HW), surface probability (Emini et al. 1985), flexibility (Karplus and Schulz 1985) and antigenic index calculated according to the algorithm of Jameson and Wolf (1988), respectively. The next three panels show the locations of turns, alpha helices and beta sheets as predicted by the algorithm of Chou and Fasman (1978) (CF). The bottom three panels show the predicted sites of turns, alpha helices and beta sheets according to the algorithm of Garnier et al. (1978) (GOR).

Figure 5.52. Predicted features and secondary structure of the p19 core protein of HTLV-I.

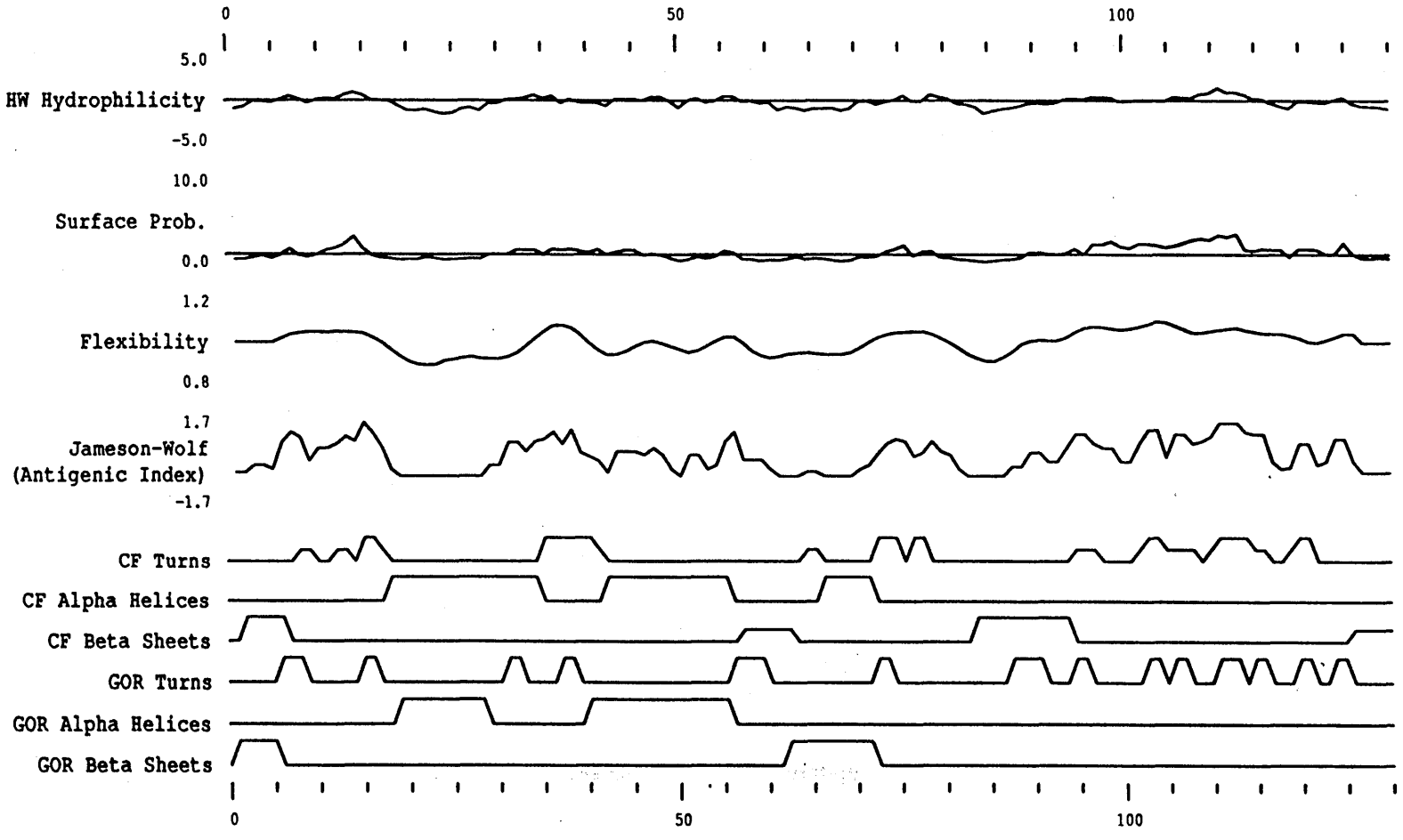


Figure 5.53. The predicted secondary structure and antigenic index of p19 were obtained using the programs "PeptideStructure" and PlotStructure", as described in section 5.2.10. The structure shown was derived using the algorithm of Chou and Fasman (1978). Alpha helices are shown with a sine wave, beta sheets with a sharp saw-tooth wave, coils with a dull saw-tooth wave and turns with 180° changes in line direction. Superimposed on the curve are octagons indicating sites where the antigenic index exceeds a value of 1.2. The size of each octagon is proportional to the value of the antigenic index. The amino-terminal (NH2) and carboxy-terminal (COOH) ends of the protein are indicated.

Figure 5.53. Predicted secondary structure and antigenic index of the p19 core protein of HTLV-I

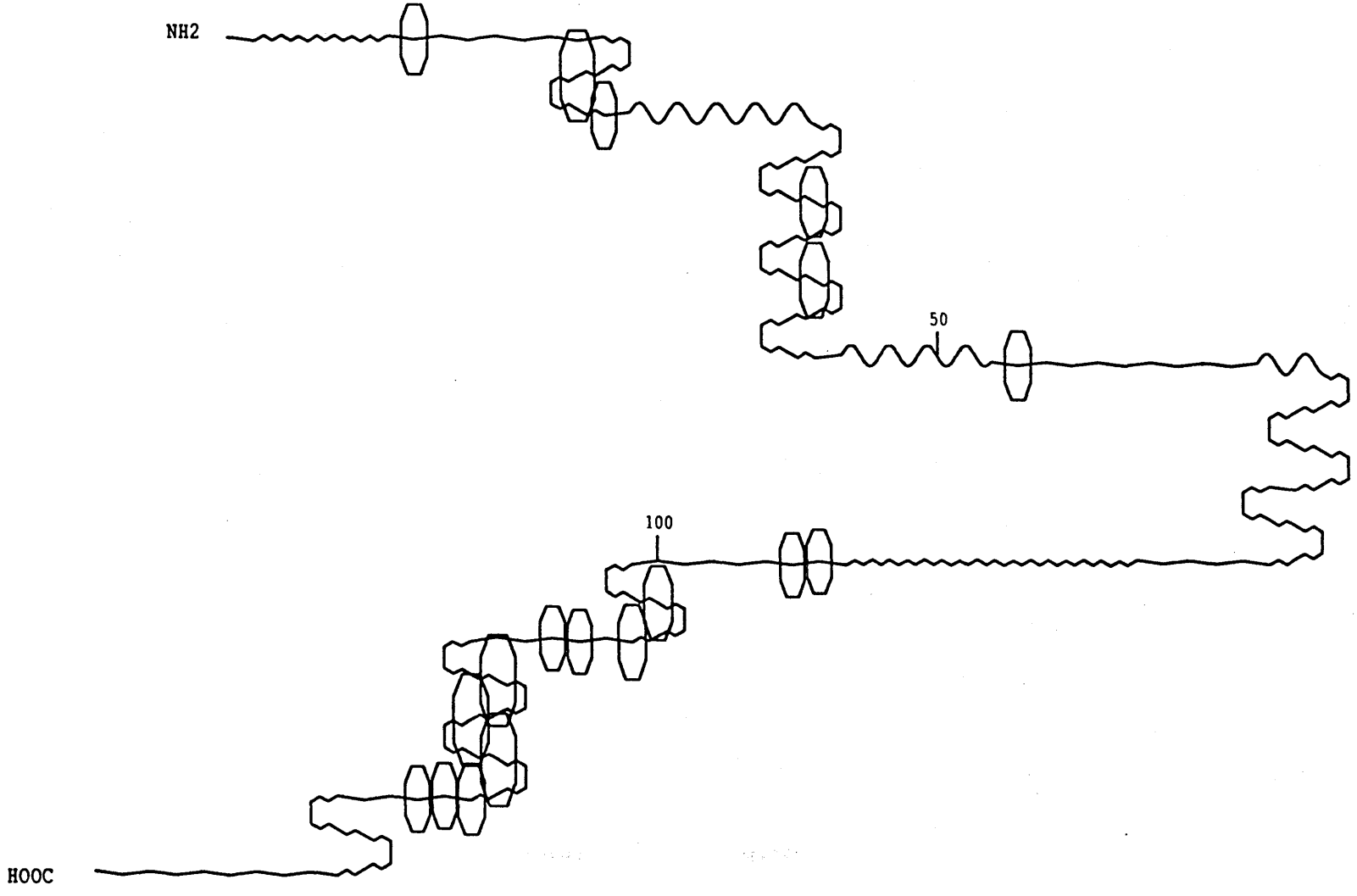


Figure 5.54. The curves are one-dimensional representations of the secondary structure of the p22 core protein of HTLV-II, obtained using the programs "PeptideStructure" and "PlotStructure", as described in section 5.2.10. Amino acid residues are numbered on the x-axis. Attributes of the sequence are shown as continuous curves. The top four panels indicate hydrophilicity calculated according to the algorithm of Hopp and Woods (1981) (HW), surface probability (Emini et al. 1985), flexibility (Karplus and Schulz 1985) and antigenic index calculated according to the algorithm of Jameson and Wolf (1988), respectively. The next three panels show the locations of turns, alpha helices and beta sheets as predicted by the algorithm of Chou and Fasman (1978) (CF). The bottom three panels show the predicted sites of turns, alpha helices and beta sheets according to the algorithm of Garnier et al. (1978) (GOR).

Figure 5.54. Predicted features and secondary structure of the p22 core protein of HTLV-II

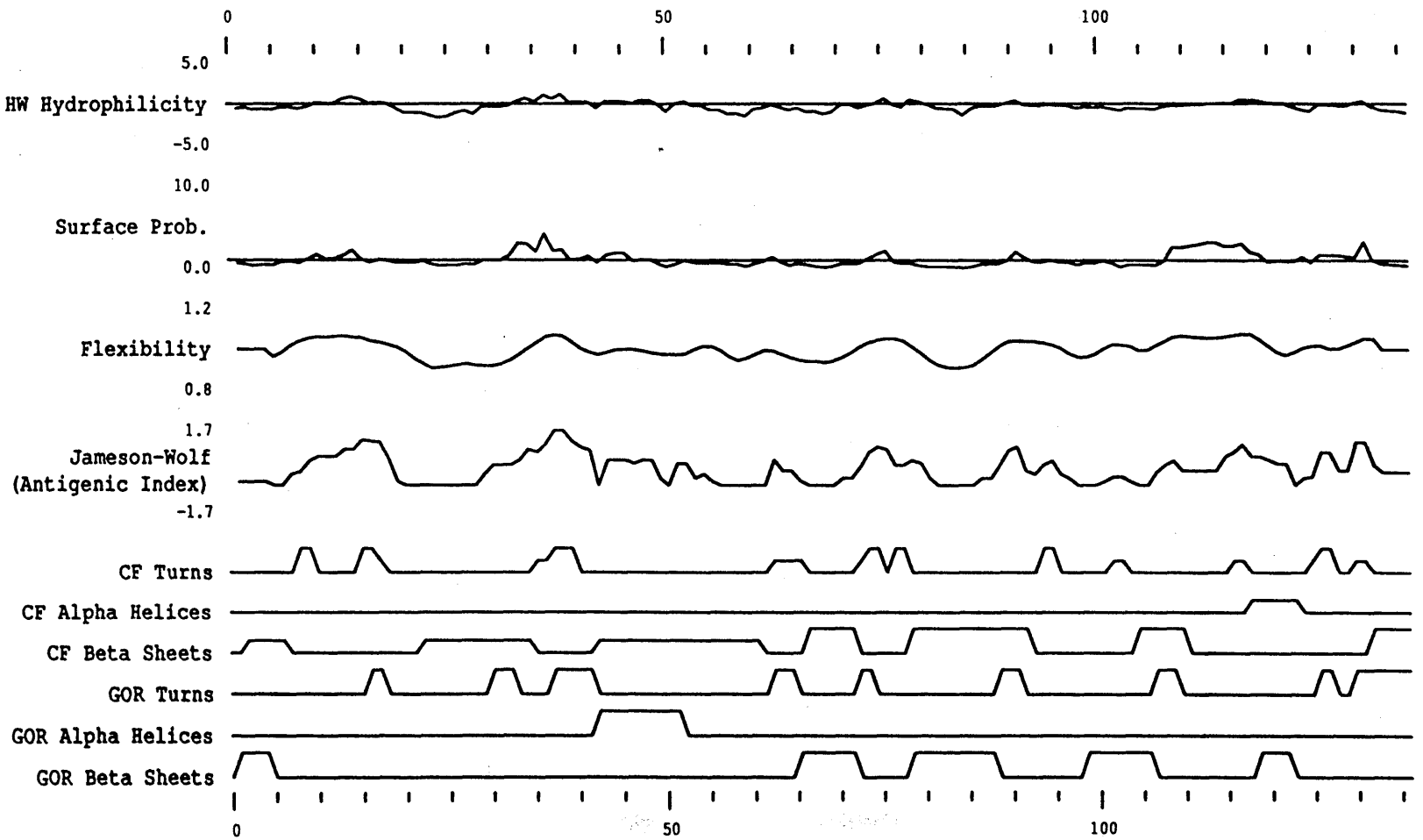
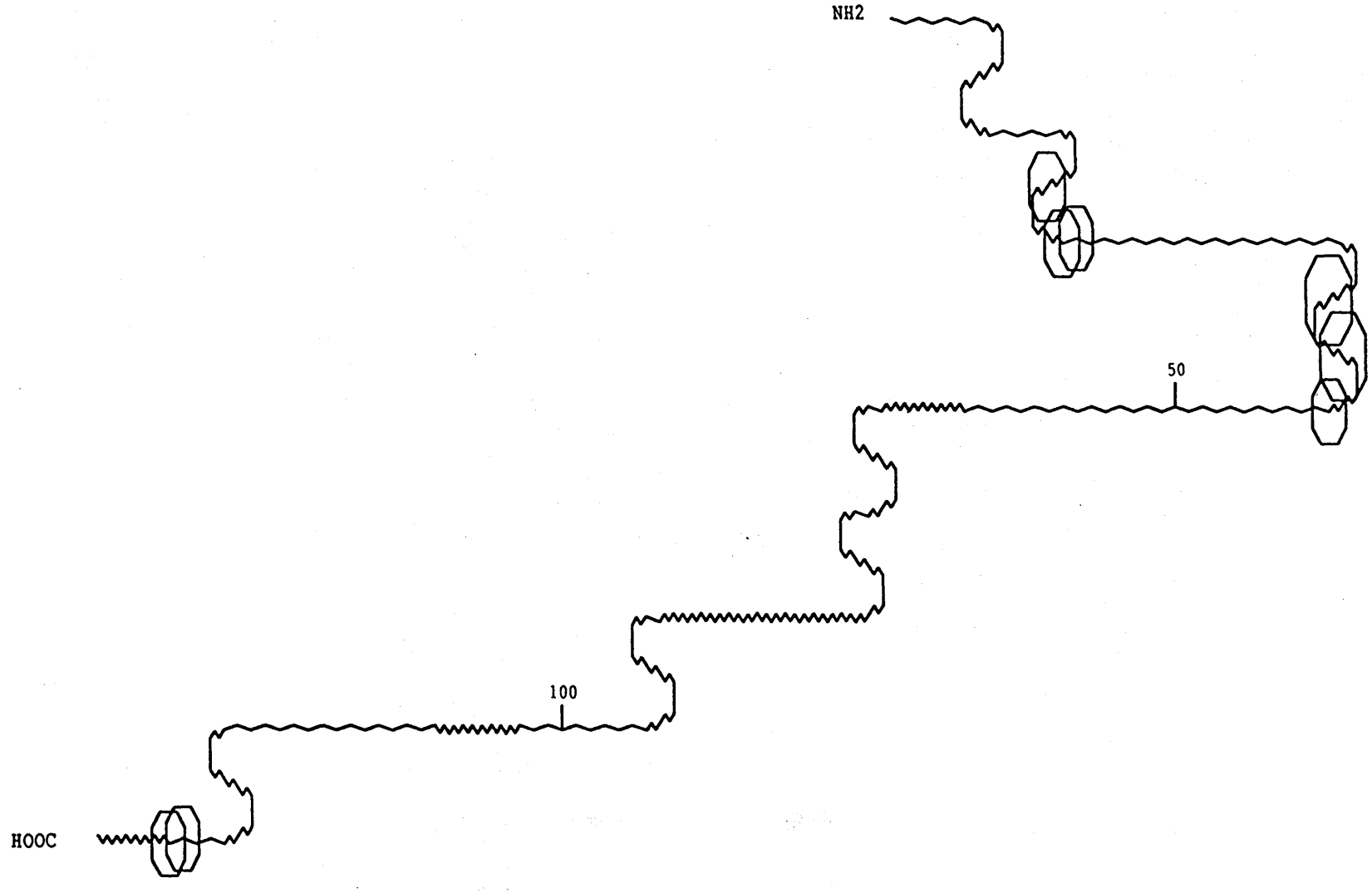


Figure 5.55. The predicted secondary structure and antigenic index of p22 were obtained using the programs "PeptideStructure" and PlotStructure", as described in section 5.2.10. The structure shown was derived using the algorithm of Chou and Fasman (1978). Alpha helices are shown with a sine wave, beta sheets with a sharp saw-tooth wave, coils with a dull saw-tooth wave and turns with 180° changes in line direction. Superimposed on the curve are octagons indicating sites where the antigenic index exceeds a value of 1.2. The size of each octagon is proportional to the value of the antigenic index. The amino-terminal (NH2) and carboxy-terminal (COOH) ends of the protein are indicated.

Figure 5.55. Predicted secondary structure and antigenic index of the p22 core protein of HTLV-II



5.3.8. Data bank searches.

(a) The NBRF protein sequence data bank was searched for protein sequences with homology to the amino acid sequences of octapeptides 116-119 which showed reactivity to MoAb 12/1-2. The amino acid sequence of octapeptide 120 was also included in the search as this octapeptide showed a low level of reactivity with MoAb 12/1-2 in the HRPO ELISA.

A total of 493 sequences were identified which shared 5/8 amino acids in common with one or more of the five octapeptides. A complete match with the sequence PPYVE, the sequence shared between the four most strongly reactive octapeptides, was identified for a number of proteins. Homology was identified with amino acids 129-133 of the alpha chains of the DNA-directed RNA polymerases of spinach chloroplast (Sijben Müller *et al.* 1986) and tobacco chloroplast (Shinozaki *et al.* 1986). Amino acids 563-567 of the precursor protein of the major surface antigen of Leishmania tropica also showed complete homology with this sequence (Button and McMaster 1988). The hypothetical protein, F-215, of Vaccinia virus contains the sequence PPYVE at positions 12-16 (Niles *et al.* 1986).

(b) The Genbank, EMBL and NewEMBL data banks were searched for nucleotide sequences which would code for amino acids contained in the 10 amino acid long sequence of P19 that showed reactivity with PNG sera. No sequences were identified that would code for a region with more than four consecutive amino acids identical with the target sequence of DPPDSDPQIP. The maximum match with the target sequence obtained was 6/8 amino acids and this was obtained with numerous protein sequences. The majority of matches obtained were with proline rich sequences.

(c) A search for homology with the C-terminal 30 amino acids of HTLV-I p19 revealed limited homology with a range of proteins. In most cases the homology was due to the presence of multiple proline residues in protein sequences.

5.4.DISCUSSION.

5.4.1.HTLV-I positive sera.

This study has identified the C-terminal 30 amino acids of the p19 gag protein of HTLV-I as the location of the linear B-cell epitopes reactive with HTLV-I positive sera. Within this region a number of patterns of reactivity were observed, with no one octapeptide or group of octapeptides showing reactivity with all sera. Octapeptide 118 was most frequently reactive, forming part of a reactive group of peptides in 8/10 sera.

These results are in agreement with previous data suggesting that amino acids 100-130 of p19 are highly immunogenic in man and may form at least two antigenic regions (Palker *et al.* 1986; Kuroda *et al.* 1990). Data from both these studies suggested that peptides including the 11 or 12 most C-terminal amino acids of p19 were reactive with the majority of HTLV-I positive sera. Palker *et al.* (1986) found that 16/18 HTLV-I positive sera reacted with peptide SP71, corresponding to amino acids 120-130, while 2/9 sera reacted in addition to peptide SP61 (amino acids 102-115). Kuroda *et al.* (1990) detected reactivity to P19-100II (amino acids 100-130) in 94/94 HTLV-I positive sera, while fewer of the sera, 80/94, reacted with P19-100I (amino acids 100-119).

Our data indicate that the C-terminal 30 amino acids of p19 do include more than one antigenic region. The data shown in Figure 5.26 identifies these regions as sequences included within octapeptides 102-108 and octapeptides 110-121. In addition, serum 716 appears to react with a distinct linear antigenic determinant within the C-terminal 8 amino acids of p19. Reactivity with the antigenic region spanned by octapeptides 110-121 was more consistently found in HTLV-I positive sera (9/10 of sera tested) than reactivity with the region spanned by octapeptides 102-108 (3/10 sera tested).

The variation in the patterns of reactivity seen within these regions, indicates that each region includes a number of overlapping epitopes. These regions may therefore be considered to represent "antigenic sites", as suggested by Schoofs *et al.* (1988). The variation observed in different sera may be a result of genetic variation in antibody repertoires. Other studies performed with polyclonal sera directed against heterologous antigens have also shown that a number of overlapping epitopes may be identified within an antigenic site (Schoofs *et al.* 1988).

Kuroda *et al.* (1990) suggest that a peptide containing amino acids 100-130 of p19 could be used reliably as a target antigen in assays for the detection of HTLV-I antibodies. Our epitope mapping data suggest that it may be possible to use a smaller peptide containing amino acids 110-130 without loss of sensitivity. We used a 22 amino acid long peptide, P109-130 as a target antigen in an ELISA to test this hypothesis and were able to clearly distinguish 46 HTLV-I positive from 42 HTLV-I negative sera.

It is of interest that Horal *et al.* (1990) found that 1/21 HTLV-I positive sera did not react in an ELISA using a slightly smaller peptide containing the C-terminal 19 amino acids of p19. In this report it is not clear how the HTLV-I positive sera were defined. This may be important, as HTLV-I and HTLV-II positive sera are difficult to distinguish using routine assays, as discussed earlier. However the result we obtained from epitope mapping serum 733 suggest that the difference in length between our 22 amino acid long peptide and the 19 amino acid long peptide used by Horal *et al.* (1990) may have been significant. Serum 733 reacted most strongly with octapeptides 110 and 111 in the PEPSCAN ELISA while no significant reactivity was observed with octapeptide 112 which corresponds to the amino terminal portion of the peptide used by Horal *et al.* (1990). Figure 5.26 shows that serum 733 did show significant reactivity with octapeptides 116-118. However antibody binding to these octapeptides was weak relative to the reactivity with octapeptides 110 and 111 (Figure 5.20). The results of epitope mapping would therefore predict that this serum might not react with a peptide corresponding to the C-terminal 19 amino acids of p19.

5.4.2. Monoclonal antibody 12/1-2.

The four adjacent octapeptides reactive with MoAb 12/1-2 in the PEPSCAN ELISA share the sequence PPYVE. This sequence, therefore, includes the essential residues for interaction between the antibody and p19. This amino acid sequence is completely conserved in HTLV-II p22 (residues 125-129). The identification of this sequence as the epitope reactive with MoAb 12/1-2 is consistent with the known reactivity of this MoAb with the p22 protein of HTLV-II (Palker *et al.* 1985).

An epitope reactive with MoAb 12/1-2 is known to be present on one or more cellular antigens (see previous discussion, section 5.1.2). A search of the NBRF-protein sequence data bank did not reveal homology with any proteins likely to produce the patterns of cross reactivity that have been observed with this MoAb.

However, it may not be possible to detect a cross reactive epitope on a different protein on the basis of sequence homology with the actual epitope on p19. The residues that are essential for interaction with antibodies have been identified in various epitopes using peptides in which each amino acid is replaced in turn (Schoofs *et al.* 1988; Scopes *et al.* 1990). These studies have shown that in some epitopes all residues are essential for the interaction with antibody, while in others as few as 2/6 or 3/7 residues may be essential. If only 2 amino acids in the epitope sequence are essential for interaction with the MoAb it would not be possible to identify candidate cross-reacting proteins on the basis of sequence homology.

5.4.3. Indeterminate sera.

We used two approaches to study IR detected in 2 HTLV-I negative sera obtained from Caucasian patients. The possibility that unknown factors in serum were contributing to non-specific binding to viral proteins was addressed by testing purified IgG on HTLV-I WB. Identical patterns of reactivity with p19 and related proteins were detected. While this does not exclude the possibilities that high levels of IgG or immune complexes may be the cause of non-specific binding, the results do show that the reactions observed with p19 are IgG mediated.

Analysis of purified IgG from serum samples 215 and 487 in the PEPSCAN ELISA detected reactivity with different groups of p19 octapeptides near the C-terminus of the protein. A significant level of binding to octapeptides reactive with MoAb 12/1-2 was detected in sample 215, though the level of reactivity was low relative to that detected to octapeptides 103-108 (Figure 5.27). Sample 487 did not react with octapeptides recognized by MoAb 12/1-2. Thus the indeterminate reactivities detected in these two samples do not appear to be due to autoantibodies directed against the cellular antigen(s) recognized by MoAb 12/1-2.

Neither of these sera reacted in ELISAs using longer peptides from the regions reactive on the PEPSCAN ELISAs. This may be due to low antibody titres or low antibody affinity as the epitope density and quantity in the peptide ELISAs is low compared to the density and quantity of peptide that should be present on the pins (Geysen *et al.* 1987). The negative results obtained in ELISAs using peptides from the HERV sequence HRES-1/1 are difficult to interpret for similar reasons. Autoantibodies to endogenous antigens are likely to be of low affinity and low titre, and therefore might not be detectable under the assay conditions used.

It is unlikely that the results obtained in the PEPSCAN ELISAs represent spurious reactivities, as for both samples reactivity with groups of adjacent peptides was detected, and similar patterns of reactivity were not detected in negative control sera. Experiments using appropriate peptides for preadsorption prior to either WB or PEPSCAN ELISA would be useful to confirm the results obtained in this study.

Other MoAbs to p19 show cross reactivity with human tissues (Palker *et al.* 1985) and the data suggest that p19 shares more than one epitope with cellular antigens, as discussed earlier. Analysis of these MoAbs with the PEPSCAN ELISA was unsuccessful. This have have been due to the use of suboptimal serum concentrations in the assays. Alternatively these MoAbs may be directed against conformational epitopes and thus might not react with the octapeptides. It is possible that the reactivities detected with p19 octapeptides in samples 215 and 487 might be due to autoantibodies directed to epitopes shared with cellular antigens, other than that identified with MoAb 12/1-2.

The analysis of indeterminate sera from PNG produced very different results to those obtained with indeterminate serum samples 215 and 487. Eight of the nine PNG sera showed reactivity with a consistent group of peptides near the C-terminus of p19 and these sera gave high O.D. readings in an ELISA using a longer peptide spanning this region. The data therefore show that the indeterminate results obtained with the PNG sera are due to reactions with a specific antigenic site on p19. In some of the PNG sera, the affinity and titre of antibodies reacting with the p19 peptide, as estimated by serum titrations and preadsorptions with rp19, could not be distinguished from results obtained with HTLV-I positive sera. These results suggest that the antibodies in the PNG sera are directed against an epitope with an high degree of homology with the reactive region of p19.

These data offer support for the hypothesis that the IR detected in PNG sera are due to infection with a retrovirus related to HTLV-I. As recent reports have indicated that HTLV-I infection is present in PNG, it is necessary to consider the possibility that these sera may be derived from HTLV-I infected persons. However the results of other serological assays suggest that these sera are distinct from HTLV-I positive sera. These sera do not react with *env* encoded proteins in HTLV-I WB, and with one exception do not react with p24 in HTLV-I WB. They also give negative results in IgG antigen-capture radioimmunoassays and competitive radioimmunoassays, "in house" assays developed at the Virus Reference Laboratory (J. Tosswill personal communication).

The results of our experiments suggest that the antigens eliciting the p19 reactive antibodies in the two groups of sera are not identical though are likely to be highly related. As a group the PNG sera showed a more consistent pattern of reactivity to p19 octapeptides than the HTLV-I positive sera. In addition there were differences in the octapeptides most frequently reactive with the two groups of sera. Although the peptides reactive with the PNG sera overlapped the more C-terminal antigenic site identified with HTLV-I positive sera, seven of the samples reacted with octapeptide 109, which did not react with any HTLV-I positive sera. It should be noted, however, that the results obtained with HTLV-I positive sample 733 and PNG sample 887 in the PEPSCAN ELISA are very similar. The results obtained in the PEPSCAN ELISA could not, therefore, be used to distinguish between the two groups of sera.

An alternative explanation for the data is that the antibody response to HTLV-I proteins detected in the sera from residents of PNG varies from that seen in the HTLV-I-infected persons because of genetic variation in antibody repertoire. This could give rise to differences in the pattern of antibody response to an identical antigen. If this interpretation of the data is correct it is necessary to argue that these individuals are unable, in most cases, to mount an immune response to the p24 core protein of HTLV-I.

The possibility remains that the reactivity of the PNG sera with the p19 antigenic site is due to cross reactivity with an antigen present on some other infectious agent, e.g. *P. falciparum*. It is difficult to exclude this possibility, but searches of the NBRF-protein databank and the GenBank and EMBL databanks have failed to identify candidate nucleotide or protein sequences with homology to the region of p19 identified in our experiments. No *P. falciparum* proteins were identified in any of the data bank searches.

The results of the epitope mapping experiments provide a potential approach to testing the hypothesis that a retrovirus related to HTLV-I is present in these individuals. The peptide sequence common to the reactive octapeptides could be used to derive potential coding sequences, and these used to synthesize redundant primers for PCR, as discussed in Chapter 1 (section 1.5.3). It would then be possible to attempt to amplify viral DNA using, for example, a predicted primer binding site sequence for the 5' PCR primer and the above 3' primer.

5.4.4.HTLV-II positive sera.

The identification of antigenic epitopes on HTLV-I proteins that are reactive with HTLV-II positive sera offers the potential for the development of serological assays capable of distinguishing between infection with these two viruses. The epitope reactive with MoAb 12/1-2, PPYVE, is completely conserved between the two viral proteins. However only two of the HTLV-II positive sera showed reactivity to octapeptides within this region when analysed in the PEPSCAN ELISA. In these two samples reactivity was detected to 1 or 2 isolated octapeptides (115 and/or 117) only. Both sera reacted weakly in ELISAs using a longer peptide overlapping this region, P109-130, suggesting that the reactivities detected with these isolated octapeptides may represent low titre or low affinity antibodies.

The significance of the reactivities with isolated or paired octapeptides elsewhere in p19 detected in the other 3 HTLV-II positive sera is more difficult to assess, as the sera did not react in ELISAs using longer peptides overlapping some of the reactive octapeptides. Possible explanations for the detection of reactivity with isolated octapeptides in the PEPSCAN ELISAs are discussed below.

5.4.5.P109-130 ELISA.

The results obtained from the analysis of HTLV-I-positive, HTLV-II-positive and HTLV-I-negative control sera suggest that an ELISA using P109-130 may have diagnostic potential. Figure 5.51 shows that sera that are positive in the HTLV-I competitive ELISA may be clearly distinguished from negative control sera.

The results obtained from the analysis of three sera that give discordant results on other assays are intriguing. As false positive results may frequently be obtained with the GPA (Matsumoto *et al.* 1990), it is likely that the results for sample 746 reflect lack of HTLV-I-specific antibody. For samples 835 and 840, the negative results obtained in the competitive ELISA and the P109-130 ELISA may reflect the relative lack of sensitivity of these assays compared to the GPA, ELISA and IF assays. Both these patients were relatives of TSP patients, and thus were at increased risk of HTLV-I infection (Mowbray *et al.* 1989). However the possibility that the low titre reactivities detected in these two samples using the GPA, ELISA and IF may represent false positive results cannot be eliminated in the absence of additional confirmatory testing.

It was possible to distinguish the 5 HTLV-II positive sera from HTLV-I positive sera using the P109-130 ELISA. Although only a very limited number of HTLV-II positive sera have been analysed, the data indicate that testing of additional sera would be worthwhile, in order to establish the potential of the assay for distinguishing HTLV-I and HTLV-II positive sera.

Two indeterminate sera from Caucasian patients gave similar results to HTLV-I negative sera in the p109-130 ELISA. However 7 of the 9 indeterminate sera from PNG gave results within the range of those obtained with HTLV-I positive sera. Thus if, as suggested above, the PNG sera are from persons infected with a retrovirus related to HTLV-I, infection with this putative retrovirus could not be distinguished from infection with HTLV-I using this assay.

Lipka *et al.* (1990) have recently described the identification of an HTLV-I specific epitope present on the gp46 envelope glycoprotein. Chen *et al.* (1990b) have also described recombinant proteins containing HTLV-I and HTLV-II envelope protein sequences that were used in WB to distinguish between HTLV-I-positive and HTLV-II-positive sera. However a significant proportion of HTLV-I positive sera showed cross-reactivity with the HTLV-II derived recombinant protein in the latter study. It is likely that assays capable of distinguishing reliably between HTLV-I-positive and HTLV-II-positive sera will need to include a number of different, virus-specific epitopes. The C-terminal p19 peptide identified in the experiments described in this chapter may therefore be useful in combination with other antigenic epitopes for the distinction between HTLV-I-positive and HTLV-II-positive sera.

5.4.6. Comparison with secondary structure predictions.

The results of PEPSCAN analysis of p19 are in good agreement with the prediction of antigenic index, based on secondary structure, hydrophilicity, surface probability and flexibility shown in Figures 5.52-53. The region most strongly predicted to be antigenic is included within the site which was most frequently reactive with HTLV-I positive sera.

The region of HTLV-II p22 most strongly predicted to be antigenic lies between amino acids 36-42. Homology between HTLV-I p19 and HTLV-II p22 in this region is relatively low, only 4/7 amino acids are identical. Reactivity to octapeptides from this region of p19 was detected in two HTLV-II positive sera, samples 854 and 876, though in sample 876 reactivity was with an isolated octapeptide only. Additional experiments using peptides from this region of HTLV-I p19 or HTLV-II p22 were not performed, thus assessment of the significance of the result obtained with samples 854 and 876 is difficult.

5.4.7. Reactivity with isolated octapeptides.

The reactivity of serum 716 with the C-terminal octapeptide was confirmed in experiments using a longer, 22 amino acid C-terminal peptide. The reactivity of sample 716 with an isolated octapeptide is likely to be due to the C-terminal location of the reactive epitope, as discussed below. HTLV-II positive samples 877 and 878, which reacted with octapeptides 115 and/or 117 also reacted weakly with a longer, overlapping C-terminal peptide. However the reactivity observed in these and other HTLV-I and HTLV-II positive serum samples with isolated peptides elsewhere in p19 could not be confirmed in ELISAs using overlapping, longer peptides. In addition reactivity to isolated octapeptides was observed in HTLV-I negative control sera.

These data suggest that reactivities to isolated octapeptides usually represent spurious nonspecific binding. This may occur due to coincidence between the sequence and/or confirmation of the octapeptide and some other antigenic epitope that is not preserved in adjacent peptides. Alternatively antibody may be binding to synthetic by-products, such as partially deleted or prematurely terminated peptides, present on the same pins. However this latter explanation seems unlikely where reactivity with an isolated octapeptide is detected on duplicate pins.

Shi *et al.* (1984) found that non-specific binding to solid-phase-bound peptides was associated with the presence of lysine near the amino terminus. This effect was enhanced by the presence of adjacent hydrophobic residues and reduced by the presence of adjacent acidic residues. These authors suggest that small peptides with an overall cationic and hydrophobic character are likely to bind many antibody types. The isolated reactivities seen in the PEPSCAN ELISA involved 33 of the 122 octapeptides. Two of the three octapeptides that were most frequently reactive, numbers 11 and 70, contain cationic and hydrophobic residues. However the third most frequently reactive peptide, number 105, has a number of polar and acidic residues.

An alternative explanation for binding of sera to isolated octapeptides is that amino acids essential for interaction with antibody occur at either end of the octapeptide. It has been suggested that sequential epitopes are from 5-8 residues in length (Kabat 1970; Schechter 1971). As discussed above, only a few of the residues making up a defined epitope may be essential for interaction with the relevant antibody. Essential residues in defined epitopes have been shown to be separated by from 2-4 amino acids (Geysen *et al.* 1984; Schoofs *et al.* 1988; Scopes *et al.* 1990). Thus reactivity with an isolated octapeptide could arise if an epitope contained two residues essential for interaction with antibody that were separated by 6 amino acids. However the results of experiments using longer peptides overlapping isolated reactive octapeptides do not support this interpretation.

The reactivity of serum 716 with octapeptide 123 and not with adjacent octapeptides suggests that the C-terminal leucine residue is an essential component of the epitope reacting with this serum. In this case reactivity with an isolated octapeptide could be due to lack of any adjacent octapeptides C-terminal to this one. If the C-terminal leucine is an essential residue for interaction with serum 716, this would be consistent with the result obtained in the P109-130 ELISA following preadsorption with rp19. The recombinant protein did not appear to adsorb out P109-130 specific antibodies from this serum as effectively as from the other HTLV-I positive sera. Foreign proteins expressed in bacteria are frequently degraded at their C-termini (Carroll and Laughon 1987) and loss of the C-terminal leucine residue could result in a significant loss of antibody affinity for rp19 if this residue is essential for interaction with antibodies in this serum.

5.4.8. Conclusions.

This study has identified a region at the C-terminus of the p19 gag protein of HTLV-I that contains the linear B-cell epitopes reactive with HTLV-I positive sera. These epitopes appear to form at least two antigenic sites located within the C-terminal 30 amino acids of HTLV-I p19.

The epitope reactive with a MoAb, 12/1-2 which reacts with HTLV-I p19 and cross-reacts with HTLV-II p22 and a number of normal and malignant human tissues was identified as amino acids 119-123 of p19.

The reactivity with p19 in HTLV-I WB detected in sera from two Caucasians appeared to involve antibody binding to different C-terminal regions of p19. These reactive regions did not coincide with the epitope reactive with MoAb 12/1-2. The IR detected in these serum samples could not, therefore, be explained by the presence of antibodies directed against the endogenous, cellular antigen(s) recognized by MoAb 12/1-2.

The reactivity with p19 in HTLV-I WB detected in sera from PNG was shown to be due to specific reactivity with a defined epitope in the C-terminal region of HTLV-I p19. The antibodies to the C-terminus of p19 that were present in four of the PNG sera were of similar titre and affinity to those present in HTLV-I positive sera. The data provide some support for the hypothesis that IR in these sera may be due to infection with a retrovirus related to HTLV-I.

Preliminary experiments suggest that a peptide derived from the C-terminal 22 amino acids of p19 has potential as a diagnostic reagent. This peptide should be useful for the serological diagnosis of HTLV-I infection and may also be useful for distinguishing between HTLV-I and HTLV-II positive sera.

CHAPTER SIX.

GENERAL DISCUSSION:

CONCLUSIONS AND PROSPECTS FOR FURTHER STUDY.

6.1. THE CLASSIFICATION OF HD.

6.1.1. The detection of Ig gene rearrangements in tumour biopsies.

The clinical and epidemiological features of HD, described in Chapter 1 (section 1.3.2) indicate that this disease is heterogeneous. There is therefore a need for further characterization of HD cases and possibly for novel classification systems, as has been discussed in Chapters 1 and 3. The data described in Chapter 3 suggest that HD cases may also vary in terms of the presence or absence of clonal Ig gene rearrangements in the HRS cells. It is of considerable interest to determine whether there are any clinical or biological differences in HD cases which differ in this respect.

The analysis of tumour tissues by Southern hybridization, the technique employed in this study, is however likely to be of limited use for the further investigation of this question. The small numbers of malignant cells in HD tumour tissues and the difficulty of identifying conclusively the cell types containing the rearrangements limit the number of cases likely to provide useful information. A number of other experimental approaches may be of value. Southern blot analysis of purified HRS cell populations, derived using cell-separation techniques such as those described by Sundeen *et al.* (1987) and Sitar *et al.* (1989) may permit the conclusive identification of the cells containing the rearrangements. However it may be difficult to purify HRS cells from other cell types (Sundeen *et al.* 1987) and it may be difficult to obtain sufficient numbers of HRS cells for analysis.

Recently described techniques for the detection of Ig and TCR gene rearrangements using the PCR (d'Auriol *et al.* 1989; McCarthy *et al.* 1990) offer the potential to surmount the problem of small cell numbers. In addition Haase *et al.* have recently described the analysis of tissue sections for Maedi Visna virus using the PCR in situ (personal communication). The use of this method for the detection of clonal Ig gene rearrangements in HD tissues could show conclusively whether or not the HRS cells contain the rearrangements.

6.1.2. The relationship between the detection of Ig gene rearrangements and of EBV genomes.

The HD biopsies that were charaterized in the experiments described in Chapter 3 were also analysed for the presence of EBV genomes. The results obtained showed that the detection of EBV genomes did not correlate with the detection of Ig gene rearrangements, though only a small number of cases contained sufficient HRS cells for assessment (Gledhill *et al.* in press). Similar results have been obtained by others (Weiss *et al.* 1987a; Herbst *et al.* 1989). Possible explanations for these findings have been discussed in Chapter 3.

Although it was not possible to relate the presence or absence of clonal Ig gene rearrangements in HD cases to biological difference between cases, the detection of EBV genomes did show a relationship with patient age. Analysis of over 90 cases of HD has shown that HD patients aged 50 years or over and paediatric patients are more likely to be EBV positive than patients 15-34 years old (Jarrett *et al.* manuscript submitted).

The distribution of EBV-positive HD cases is related more closely to the epidemiological subgroups of HD identified by MacMahon (1966) than to the histological subtypes more commonly used to classify HD (see section 1.3.2). These data support the suggestion made in section 1.3.2, that there is a requirement for more detailed characterization of HD and possibly for alternative classification systems for this disease.

6.2. THE INVOLVEMENT OF HHV-6 IN LYMPHOPROLIFERATIVE DISORDERS.

The results of a study to investigate the role of HHV-6 in lymphoproliferative disorders have been described in Chapter 4. HHV-6 genomes were detected in tumour and non-tumour tissues from two patients with lymphomas. The assessment of the significance of a molecular association between a virus and a malignancy was discussed in Chapter 1 (section 1.4.3). The two methods of assessment described were (a) the determination of the clonality of the tumour cells and virus-infected cells and (b) the determination of the consistency of the association.

Both of the lymphomas that were positive for HHV-6 DNA were shown to be clonal in origin. However it could not be conclusively shown that the viral DNA sequences were present in the tumour cells. Nor was it possible to determine whether the virus-infected cells had undergone clonal expansion. The lack of suitable material for in situ hybridization precludes further investigation of the former issue. Further characterization of the HHV-6 genome may permit the latter question to be addressed, possibly by a method similar to that used to assess the clonality of EBV-infected cells (Raab-Traub and Flynn 1986).

HHV-6 DNA sequences were identified in an AIL-like T-cell lymphoma and in a B-cell lymphoma occurring in a patient with a history of Sjögren's syndrome. HHV-6 genomes were not consistently identified in tissues from patients with either AIL-like lymphoma or lymphomas occurring in the context of Sjögren's syndrome.

The significance of the molecular association between HHV-6 and the these lymphomas therefore appears questionable. However although the majority of lymphomas occurring in patients with Sjögren's syndrome are negative for HHV-6 sequences (Josephs *et al.* 1988b; R.F. Jarrett unpublished data), there are a statistically significant excess of HHV-6-positive cases in comparison to other types of lymphoma.

The possibility that lack of a consistent association between a virus and a tumour could result from inappropriate disease classification was discussed in Chapter 1 (section 1.4.3). Thus one possible explanation for these data is that lymphomas occurring in Sjögren's syndrome patients may include more that one distinct disease entity. The alternative possibility discussed in Chapter 1, that a phenotypically identical tumour could arise as a result of a distinct pathogenic mechanism offers a more plausible explanation. The possible involvement of herpesviruses in addition to HHV-6 in the pathogenesis of lymphomas occurring in the context of Sjögren's syndrome was discussed in Chapter 4. If this speculation is correct, only a subset of such lymphomas would show a molecular association with HHV-6.

The molecular similarity between HHV-6 and CMV (Lawrence *et al.* 1990) suggests that it is unlikely that HHV-6 is directly involved in lymphomagenesis. Evidence that regions of HHV-6 DNA may contribute to cell transformation in vitro (Razzaque 1990) is of uncertain significance, as similar data have been reported for CMV (Galloway *et al.* 1986; Sugden 1986) which does not appear to be oncogenic in vivo.

The data reported in Chapter 4 are more consistent with the hypothesis that HHV-6 is playing an indirect role in lymphomagenesis in Sjögren's syndrome and/or AIL. Indirect involvement of viruses in lymphoid malignancy may be difficult to establish using a molecular approach, as discussed previously (section 1.4.2). Serological studies have supported an association between HHV-6 and Sjögren's syndrome (Ablashi *et al.* 1988). However there is a need for case-control studies to confirm this association.

The possibility that HHV-6 and other herpesviruses could be involved in the pathogenesis of Sjögren's syndrome itself is intriguing and warrants further investigation. Chronic immune stimulation due to viral antigen expression or altered expression of cellular antigens by virus-infected cells could result both in autoimmune phenomena and in lymphomagenesis.

6.3. THE USE OF EPITOPE MAPPING IN THE SEARCH FOR RETROVIRUSES RELATED TO HTLV-I.

Chapter 5 describes the results of experiments that form part of a novel approach to the identification of viruses related to known leukaemogenic viruses. The data provide evidence to support the notion that residents of PNG whose sera react with HTLV-I proteins on WB are infected with a retrovirus related to HTLV-I. Specific epitopes on HTLV-I p19 that are reactive with these sera have been identified. These data may now be used to predict a coding sequence for this region, assuming that the amino acid sequence is conserved in the putative related virus. Various techniques for using amino acid sequences to generate oligonucleotide primers for use in the PCR have been described. These include the synthesis and use of mixtures of oligonucleotides which have all four alternative nucleotides present at every third base (Compton 1990). Alternatively, the inclusion of inosine residues at every third base has been shown to permit base-pairing with any nucleotide at this position (Knoth *et al.* 1988).

The choice of an additional 5' or 3' primer remains a problem. For most of the sera analysed in this study, the reactivity with HTLV-I proteins can largely be explained by reactivity with an epitope on p19 alone (see Table V.IV and Figure 5.1), thus analysis of additional HTLV-I proteins may not reveal additional conserved epitopes. The tRNA used to prime synthesis of negative strand DNA during retroviral replication is conserved between the related retroviruses HTLV-I, HTLV-II and BLV; all use $tRNA_{Pro}$ as a primer (Seiki *et al.* 1982; Sagata *et al.* 1985; Shimotohno *et al.* 1985). The sequence of the $tRNA_{Pro}$ binding site could therefore be used to derive a 5' primer for use in the PCR.

Although evidence for the presence of HTLV-I itself in PNG has recently been reported (Yanagihara *et al.* 1990a; Yanagihara *et al.* 1990b), HTLV-I related diseases have not been described. This could be due to significant under-reporting of disease as many areas of PNG are remote and some populations have had little contact with outsiders. In addition the low incidence of HTLV-I related disease could reflect the short life-expectancy of persons from this region, as the majority of cases of ATL and TSP occur in persons over 50 years old (Yamaguchi *et al.* 1990).

This region has not been reported to show a high incidence of other haematological malignancies, that could be associated with other leukaemogenic retroviruses. This could be a result of disease under-reporting and short life-expectancy, as discussed above. However retroviruses closely related to HTLV-I may not necessarily be oncogenic in vivo, as illustrated by the lack of association between HTLV-II infection and malignant disease. The isolation of such retroviruses is still of considerable value. The comparative study of closely related viruses is likely to provide insights into the features of HTLV-I that are important in leukaemogenesis.

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