HUMAN HERPESVIRUS 8 AND NEW PATHOLOGICAL ASSOCIATIONS

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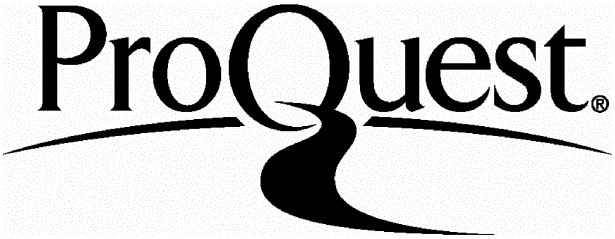

ABSTRACT

The prevalence, diversity and disease association of human herpesvirus 8 (HHV-8) may be more extensive than previously reported. HHV-8 subgenomic sequences in tissues representing a broad range of pathologies were investigated. DNA from the $KS330_{233}$ region of open reading frame (ORF) 26 in various oral lesions in HIV-infected and HIV-seronegative individuals was first sought. HHV-8 DNA sequences were amplified in 12 out of 16 of non-KS oral lesions of HIV-positive but none of the HIV-negative controls. A diversity of sequences was observed. The presence of HHV-8 DNA in two groups of granulomatous diseases - sarcoidosis and oral granulomatous disorders - was then investigated. Thirty-eight out of 39 (97.4%) sarcoid tissues and all 17 oral granulomatous tissues were positive for HHV-8 DNA in ORFs25 and 26. A diversity of ORF26 sequences was again observed. ORF26 sequences from tissues of the granulomatous disorders and from a range of non-granulomatous diseases (Kaposi's sarcoma, B-cell lymphoma, Kikuchi's disease and other lymphadenitides) were compared with sequences reported by other laboratories. The sequences could be categorised into at least 8 groups based on hot-spot mutations in codons 134, 141, 152, 167 and 169 of the ORF26 protein. Intra-host variability of HHV-8 was then investigated in a selection of neoplastic and non-neoplastic tissues. Wide intra-host and intra-lesional hypervariability were found, particularly in patients with sarcoidosis and Kikuchi's disease. Two mechanisms for genotyping such a wide range of variability were postulated: existence of HHV-8 as quasispecies and co-infection by multiple HHV-8 variants.

It is concluded that: 1. HHV-8 DNA ORF26 is commonly found in oral lesions of HIV-infected patients, in sarcoid tissues and in oral granulomatous tissues; 2. the virus may be classified into at least eight genetic groups based on sequence variations in ORF26; and 3. the inter-host, intra-host and intra-lesional sequence variability of ORF26 can be considerable.

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ABBREVIATIONS

AIDS	acquired immunodeficiency syndrome
APS	ammonium persulphate
BCBL	body cavity based lymphoma
bp	base pair
BSA	bovine serum albumin
CMV	cytomegalovirus
DNA	deoxyribonucleic acid
dNTPs	deoxynucleotide triphosphates
ds	double stranded
EBV	Epstein-Barr virus
EDTA	disodium ethylenediaminetetra-acetate
ELISA	enzyme linked immunosorbant assay
GRC	G protein coupled receptor
HHV-6	human herpes virus 6
HHV-7	human herpes virus 7
HHV-8	human herpes virus 8
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
HSV-1	herpes simplex virus 1
HSV-2	herpes simplex virus 2
HVS	herpes virus saimiri
IFA	immunofluorescence assay
IL-6	interleukin 6
IL-8	interleukin 8
IgE	immunoglobulin class E
IgG	immunoglobulin class G
IgM	immunoglobulin class M
IPTG	isopropylthiogalactoside
kb	kilobase pair
KS	Kaposi's sarcoma

MIP	macrophage inflammatory protein
MGUS	monoclonal gammopathy of undetermined significance
MM	multiple myeloma
mRNA	messenger RNA
MRS	Melkersson-Rosenthal syndrome
nt	nucleotide(s)
OFG	orofacial granulomatosis
ORF	open reading frame
PBC	peripheral blood cells
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEFF	paraffin embedded formalin fixed
RE	restriction enzyme
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
RT	reverse transcription
SSCP	single stranded conformation polymorphism
ss	single stranded
TBE	tris-borate-EDTA
TE	tris-EDTA
x-gal	5-bromo-4-chloro-3-indolyl-β-D-galactosidase
VZV	varicella-zoster virus

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1. KAPOSI'S SARCOMA AND THE DISCOVERY OF HUMAN HERPESVIRUS 8

1.1. Kaposi's sarcoma

In 1872 Moritz Kaposi detailed unusual skin tumours in five men in their sixth and seventh decades of life (Kaposi, 1872). He described the disorder as "nodules ranging in size from a peppercorn to that of a pea or hazelnut, and brownish-red or bluish-red in colour; smooth; elastic; singly or in plaque-like groups; developing first on the sole or dorsum of the foot, thereafter spreading rapidly to the hands. The nodules sometimes atrophy and may ulcerate at the later stage. The histologic structure consists of foci of fusiform spindle cells and a rich new growth of capillaries. The syndrome is a multiple idiopathic pigmented sarcoma of the skin" (Kaposi, 1872).

The sarcoma described by Kaposi, is now called Kaposi's sarcoma (KS), and is of the "classic" type. It has since been mainly recognised as a benign tumour common in southern Mediterranean and Jewish geriatric populations. Despite initial impressions that KS was rare among Africans, subsequent studies revealed that KS was actually more common in African blacks than any other population in the world (Kaposi, 1872; McHardy et al., 1984; Oettle, 1962; Slavin et al., 1969).

With the introduction of renal transplantation in the 1970s and the coming of the AIDS pandemic, additional cases of KS began to be observed. These cases did not fit into the definition of classic KS or African KS, prompting a search for a common aetiologic agent. KS may now be classified into four distinct clinical varieties - classic, endemic (African), iatrogenic (immunosuppression-associated) (Gonzales et al., 1997), and AIDS-associated (epidemic) (Martin et al., 1993).

The histopathological features of KS are well defined and are the same in all clinical forms. This lesion consists of interweaving bands of spindle-shaped endothelial cells, with atypical vascular channels enmeshed in reticular and collagen fibres. Histologically, KS patterns may be defined as occurring in two stages. The early stage, clinically corresponding to the flat presentation, consists of focal proliferation of thin-walled vessels that often show plump endothelial cells. In the late or neoplastic stage, the endothelial cells group as prominent nodules in which are found numerous extravasated erythrocytes and siderophages (Green et al., 1984).

A variety of clinical presentations have been seen in KS. In patients with AIDS, the lesion begins as a multicentric neoplastic process that manifests as multiple red/purple (vascular appearing) macules, and in more advanced stages, as nodules occurring on the skin or mucosal areas (Safai et al., 1985). The lesions may enlarge, become darker, or coalesce or form clusters. The tip of the nose is a peculiar and frequent location for these lesions. Facial, scalp, periorbital, and conjunctival involvement is also seen (Martin et al., 1993). For endemic KS, the clinical behaviour is more unpredictable. It can be a slow-growing and indolent, or became very aggressive with rapid visceral involvement.

1.1.1. AIDS-associated KS

The sudden appearance of KS among male homosexuals in the early 1980s further pointed to immunosuppression as an underlying condition for KS development. Simultaneous reports from different parts of United States of

America (USA) describing the first disseminated and aggressive cases of KS in homosexuals were the starting points of numerous lists of case reports of KS, so much so that it is considered as one of the most common manifestations of the acquired immunodeficiency syndrome (AIDS).

KS is also the most common neoplasm associated with human immunodeficiency virus (HIV)-infection. AIDS-associated KS almost exclusively manifests in patients infected with HIV-1 whose risk factor for HIV-infection is homosexual male activity. It is the most aggressive form of KS, with lesions occurring both on the skin and in the viscera, and with variable disease progression (Wahman et al., 1991). In 50% or more of patients with AIDS-related mucocutaneous KS, oral or perioral involvement is an initial or early manifestation of severe HIV disease (Porter and Scully, 1994).

AIDS-KS shows a preferential distribution for head, neck and truncal regions (Safai et al., 1985). Cutaneous lesions usually appear as faint, asymptomatic macules in the oropharyngeal mucosa, and at the postauricular area of the scalp, ear lobes and tip of the nose (Ficarra et al., 1988). The palate and the gingiva are the most common sites of KS in the oropharyngeal mucosa in homosexual/bisexual men (Ficarra et al., 1988). Two unusual sites, the masseter muscle and parotid gland, have also been reported to be involved (Ficarra et al., 1988). In one series of patients with oral/pharyngeal KS, 22% presented with oral lesions as the sole manifestation of HIV disease, and 45% had oral lesions that developed concomitantly with skin and visceral lesions. (Ficarra et al., 1988).

1.1.2. Classic KS

As Kaposi first observed, this typically arises in elderly men of Mediterranean or eastern European Jewish decent. It is an indolent disease with paranodular skin lesions but little or no visceral involvement (Oettle, 1962). The male to female ratio is 10-15:1, and the majority of patients are between 50 and 80 years old at the onset of the disease (Rothman et al., 1962; Safai et al., 1985). The lesions may appear simultaneously at various sites but usually involve the lower legs, taking on the appearance of purplish or violaceous patches or confluent macules. Oedema and interference with lymphatic drainage are frequent problems that may cause pain, difficulty in mobility and skin ulceration.

1.1.3. African (endemic) KS

African (endemic) KS is widespread in southern equatorial Africa among young black adult males and prepubescent children (Martin et al. 1993). Systemic involvement can occur and disease progression is often rapid; in certain regions of sub-Saharan central Africa, KS represents up to 9% of all cancers (Oettle, 1962). Four different forms of endemic African KS have been described on the basis of clinical features (Table 1.1); histologically these forms are indistinguishable from classic KS. They include: a benign nodular form, with a course resembling classic KS; an aggressive form, with large exophytic lesions invading and destroying underlying tissues; a florid form, which is highly aggressive; and a

Table 1-1 CLINICAL CHARACTERISTICS OF KAPOSI'S SARCOMA

Type	Appearance	Mucocutaneous distribution	Lymph node involvement	Viscreal involvement	Behaviour
Classic	Patches, plaques and nodules.	Lower extremities, may disseminate lesions	Rare	Uncommon	Indolent. Often associated with lymphedema,; visceral lesions occur late; survival 10-15 years
Endemic African					
1.Benign nodular	Papules and nodules	Multiple; localized into lower extremities	Rare	Rare	Indolent. Survival 8-10 years
2.Aggressive	Large exophytic nodules and fungating lesions	Lower extremities	Rare	Uncommon	Presence of invasive and destructive lesions on underlying sucutaneous tissues and bone. Survival 5-8 years
3.Florid	Nodules	Widely disseminated	Uncommon	Uncommon	Rapidly progressive, aggressive and invasive. Early visceral involvement. Survival 3-5 years.
4.Lymphade-nopathic	Lymphade-nopathy	Rare	Always	Frequent	Rapidly progressive. Survival 2-3 years.
Iatrogenic Immunosoppressi on	Patches, nodules and plaques	Extremities; rarely disseminates	Rare	Uncommon	Indolent. Regression after therapy is discontinued
AIDS-associated	Patches, nodules; fusiforms and irregular.	Multifocal and widely disseminated. Frequent oral lesions	Frequent	Frequent	Rapidly progressive. Survival 2 months to 5 years (median 18 months)

lymphadenopathic form, mostly present in adolescent children with lesions in the lymph nodes and with visceral dissemination. The last mentioned form is highly malignant and leads to fatality within 1-2 years.

1.1.4. Iatrogenic KS

Iatrogenic (immunosuppression-associated) KS includes corticosteroid-induced KS and post-transplant KS (Fife and Brewer, 1996; Gotti et al., 1997). It may occasionally lead to systemic involvement. Case reports have associated it with systemic lupus erythematosus (Klein et al., 1974), temporal arteritis (Leung et al., 1981) and lymphoproliferative disorders for which treatment with immunosuppressive drugs were given (Klepp et al., 1978; Gange et al., 1978; Kapadia et al., 1977). In the literature it also possible to find reports describing an excess of HLA-B5, HLA-B8, HLA-B18, and HLA-DR5 and a decreased frequency of HLA-A1 and HLA-B7 haplotypes in Italian, Greek, Jewish and Arabic individuals affected by this form of KS (Brunson et al., 1990).

1.2. Epidemiological aspects of KS

Epidemiological evidence suggests that all forms of KS have an infectious aetiology (Wahman et al. 1991). In particular, the frequency of KS in HIV disease is strikingly higher in homosexual or bisexual males than patients with haemophilia, transfusion recipients or injecting drug users (Hermans et al., 1996, Beral et al.,

1990). KS is also more common in HIV-infected female sexual partners of bisexual men than those of heterosexual injecting drug users (Beral et al., 1990). HIV-related KS in homosexual males can occur in distinct geographic locations, suggesting a clustering of disease (Beral et al. 1990). Disease akin to classic KS may be more common in non-HIV-infected homosexual men than the general population suggesting transmission of an infectious aetiological agent, possibly via sexual routes or close faecal contact (Beral et al., 1992).

The rise in the frequency of classic KS in Sweden in the 25 years prior to 1982 (Dictor and Attewell, 1988) and the fall in the prevalence of HIV-related KS in the US (Katz et al. 1994) and Europe over the last ten years (Hermans et al., 1996) also point to an infectious aetiology of KS. The reasons for these observed changes probably relate to differences in sexual behaviour, recent moderation in sexual activity and/or increased use of barrier-type contraception, leading to decreased transmission of the possible infectious agent of KS (Fife and Bower, 1996).

1.3. Detection of the herpesvirus 8 genome in KS lesions

Several candidate infectious agents have been proposed as etiologic agents of KS: including cytomegalovirus (CMV) (Giraldo et al., 1980), HIV (Nakamura et al., 1988) and human T-cell lymphotrophic virus type 1 (HTLV-1) (Lebbe et al., 1997; Warmuth et al., 1997). When representational difference analysis was employed to examine for pathogen specific genomic nucleotide sequences in

AIDS-associated KS lesions (Chang et al., 1994), sequences homologous to Epstein-Barr virus (EBV) and herpesvirus saimiri (HVS) of the gamma-herpesviridae subfamily (Roizman 1990) were identified. These sequences have since been reproducibly amplified by many other groups from KS lesions of the AIDS-related (Ambroziak et al., 1995; Huang et al., 1995; De Lellis et al., 1995; Moore and Chang, 1995; Noel, 1995; Roizman, 1995; Schalling et al., 1995; Su et al., 1995; Cathomas et al., 1996; Noel, 1996; Corbellino et al., 1996; Ganed et al., 1996; O'Neill et al., 1996; Sosa et al., 1996; Lebbe et al., 1997), classic (Boshoff et al., 1995; Dupin et al., 1995; Huang et al., 1995; Lebbe et al., 1995; De Lellis et al., 1995; Rady et al., 1995; Buonaguro et al., 1996), endemic (Huang et al., 1995; Lebbe et al., 1995; Lellis et al., 1995; Schalling et al., 1995; Buonaguro et al., 1996; Cathomas et al., 1996; Chang et al., 1996; Chuck et al., 1996; Eto et al. 1996) and iatrogenic (Gluckman et al., 1995; Lebbe et al., 1995; Buonaguro et al., 1996; Henghold et al., 1997) varieties. By contrast, most other angiogenic lesions are negative for these sequences (Chang et al., 1994; Jin et al., 1996). The virus that bears the unique sequences has been called Kaposi's sarcoma herpesvirus or human herpes virus 8 (HHV-8). Sequencing of a 20.7-kb clone from a KS library provided further evidence that HHV-8 is a gamma-2 herpesvirus (Moore et al., 1996). HHV-8 is therefore the first member of the genus Rhadinovirus known to infect humans. Further evidence of the close association between HHV-8 and KS has since been found. HHV-8 sub-genomic sequences and transcripts (Huang et al., 1996; Staskus et al., 1997) have also been localised in the nuclei of endothelial and spindle cells of all types of KS lesions (Boshoff et al., 1995; Aluigi et al., 1996; Li

et al., 1996; Sun et al. 1996; Zhong et al. 1996; Zhong et al. 1997) and in circulating KS-like spindle cells (Sirianni et al., 1997).

1.4. HHV-8 in mucocutaneous lesions other than KS

HHV-8 DNA is present in normal skin of HIV-infected patients with KS (Dupin et al., 1995; Gaidano et al., 1996; Lebbe et al., 1997); and more rarely, in the normal skin of patients with classic KS, endemic KS (Lebbe et al., 1995; Ambroziak et al. 1995; Uthman et al., 1995; Boshoff et al., 1996; Monini et al., 1996), or iatrogenic KS (Dictor et al., 1996), and lesional tissue of patients with squamous cell or basal cell carcinomas (Rady et al., 1995; Tyring et al., 1996; Inadi et al., 1996). The HHV-8 infection load appears to be lower in the normal skin and the other cutaneous lesions than in lesional KS tissue (Dupin et al., 1995; Rady et al., 1995).

HHV-8 sequences were detected in one sample of skin with scabies and in a glomerulonephritis lesion from immunosuppressed patients with KS (Noel et al., 1996), a patient with pemphigus vulgaris without HIV infection or KS, (Memar et al., 1997) and a patient with mycosis fungoides (Uccini et al., 1997). HHV-8 sequences were also detected in occasional angiosarcomas (Gyulai et al., 1996; Kizumi et al., 1996) and tissue affected by angiolymphoid hyperplasia with eosinophilia (Gyulai et al., 1996). However, HHV-8 DNA was not detected in immunosuppression-associated dermatofibromas, despite sharing many histologic similarities with AIDS-related KS lesions (Foreman et al., 1997).

1.5. HHV-8 and lymphoproliferative disorders

HHV-8 DNA sequences have been amplified from body cavity-associated B cell lymphomas (BCBLs) in HIV-infected (Cesarman et al., 1995; Carbone et al., 1996; Gaidano et al., 1996) and some non-HIV-infected patients (Nador et al., 1995; Cesarman et al., 1996; Carbone et al., 1996; Strauchen et al., 1996; Weiss, 1996). These BCBLs, sometimes also termed primary effusion lymphomas (Nador et al., 1996), exclusively involve the pleural, pericardial and peritoneal cavities (Knowles et al., 1989; Feiner et al., 1989; Walts et al., 1990; Karcher et al., 1992; Chadburn et al., 1993; Green et al., 1995; Ansari et al., 1996), and tend to present as lymphomatous effusions with no identifiable tumour mass. Unlike other AIDS-related non-Hodgkin's lymphomas, the BCBL tumour cells exhibit indeterminate immunophenotypes, are usually associated with Epstein-Barr virus (EBV), and consistently lack rearrangements of the c-MYC genes (Knowles et al., 1989; Cesarman et al., 1995; Carbone et al., 1996). Of note is the content of the HHV-8 genome in BCBL tissues that is much higher than those in KS lesions (Cesarman et al., 1995).

HHV-8 sequences have also been detected in peripheral blood mononuclear cells (PBMCs) (Dupin et al., 1995), in lymph node tissue (Soulier et al., 1995; Tirelli et al., 1996) of HIV-infected patients with Castleman's disease, and in lymph node tissue of non-HIV infected patients with localised (Barozzi et al., 1996) or multicentric Castleman's disease (Soulier et al., 1995; Gessain et al., 1996; Karcher et al., 1995; McDonagh et al., 1996). Castleman's disease was firstly described as a benign mediastinal lymphoid mass originally termed

mediastinal lymph node hyperplasia. These lesions may occur in any part of the body that contain lymphoid tissue, although 70 per cent are found in the anterior mediastinum. Most frequent is the localised *hyaline vascular type,* which appear as a solitary asymptomatic, tumour-like mass in the mediastinum. The rarer *plasma-cell type* of Castleman's disease occurs as localised and generalised forms. The *localised* form presents as a solitary tumour-like mass similar to the hyaline type; the *generalised* or *multicentric* form presents as widespread lymphadenopathy and the POEMS syndrome (*p*olyneuropathy, *o*rganomegaly, *e*ndocrine abnormalities, *m*onoclonal gammopathy and *s*kin rashes). All forms are accompanied by fever and anaemia.

HHV-8 sequences have also been proposed in some angioimmunoblastic lymphadenopathies in non-HIV-infected patients, in particular a distinct benign, non-HIV-related, lymphadenopathy histologically characterised by a predominantly follicular lesion with giant germinal centre hyperplasia and increased vascularity (Luppi et al., 1996). A similar disease entity with an identical histological appearance has been reported in one HIV-infected patient (Soulier et al., 1995). Furthermore HHV-8 sequences have been detected in lung nodules of a non-HIV infected Italian patient with interstitial pneumonitis (Luppi and Torelli, 1996).

HHV-8 DNA has also been detected, albeit rarely, in other lymphoproliferative disorders, including non-Hodgkin's lymphoma, Hodgkin's disease, reactive lymphadenopathies (Bigoni et al., 1996) and cutaneous lymphoma in AIDS (Corbellino et al., 1996). The viral load is significantly higher in lymphoid tissue from HIV-infected persons as compared to HIV-seronegative individuals

(Bigoni et al., 1996), although it is still lower than in splenic tissue or peripheral blood cells (PBCs) from the same patients. This suggests that the presence of HHV-8 in these lesions may be a reflection of HHV-8 carriage by non-neoplastic B cells (Corbellino et al., 1996). Detection of HHV-8 in mature T-cell lymphoproliferative disorders has been reported (Sander et al., 1996), but this has not been confirmed by others (Cesarman et al., 1995; Pastore et al., 1995; Pawson et al., 1996).

1.6. HHV-8 in B-cell lines: the virus visualised and characterised

Cell lines derived from BCBL effusion cells frequently contain HHV-8 genomes and their study has provided insights into the biological properties of HHV-8. The BC-1 line harbours HHV-8 but not EBV DNA, while another line, BC-2, harbours both viruses (Cesarman et al., 1995). Treatment of BC-1 with phorbol esters rapidly induces lytic growth of HHV-8 and progeny virus are then shed into the supporting medium (Renne et al., 1996). The induced B cells can be observed to contain 110 nm intranuclear herpesvirus-like nucleocapsids and complete cytoplasmic virions (Said et al., 1996). The length of genome of the virus is estimated to be similar (e.g. 160-170 kb) to other gammaherpesviruses. The HHV-8 genome is, like that of EBV, maintained in latently infected B cells as extrachromosomal, episomal, monomeric circles, with induction from latency leading to the selective accumulation of linear genomic forms (Renne et al., 1996). By contrast, only covalently closed, circular episomes of HHV-8 are identified in

KS tissue, while linear forms, arising from of viral replication, are additionally found in PBCs of KS patients (Decker et al., 1996). While uninduced BCBL lines have not yet been shown to permit propagation of HHV-8, the virus can be cultured from skin lesions of patients with AIDS-associated KS using the human embryonal-kidney epithelioid line 293 (Foreman et al., 1997), thus providing evidence that the virus is able to replicate vegetatively in vitro.

Sequencing of a 12.3-kb HHV-8 clone obtained from a genomic library derived from BC-1 revealed homology between HHV-8 with parts of the EBV genome. The sequences of some ORFs of HHV-8 are homologous to EBV-membrane antigen p140, HVS p160, cellular type D cyclins, and HVS and cellular G protein coupled receptors (Cesarman et al., 1996). Furthermore, transcription of these 4 ORFs can be demonstrated in BC-1 (Moore et al., 1996). A novel abundant 1.2-kb RNA, polyadenylated nuclear RNA (called PAN RNA) has also been identified from the BC-1 line; it appears speckled in the nuclei by immunofluorescence and may be a viral lytic early transcript (Sun et al., 1996; Zhong et al., 1996; Zhong et al., 1997).

The BC-1 cell line was used to create a cosmid and phage genomic library, allowing the full characterisation of the HHV-8 nucleotide sequence, except for a 3 kb region at the right end of the genome (Russo et al., 1996). The BC-1 HHV-8 genome is 140.5-kb long, with a unique coding region flanked by multiple 801-bp terminal repeat sequences. A genomic duplication that apparently arose in the parental tumour is present in this cell culture-derived strain. At least 81 ORFs and 5 internal repeat regions are present in the long unique region. In addition to viral

structural and metabolic proteins, the virus encodes homologues to complement-binding proteins, three cytokines (two macrophage inflammatory proteins, MIP-1α, MIP-1β, and interleukin-6, IL-6), dihydrofolate reductase, *bcl-2*, interferon regulatory factors, interleukin 8 receptor, neural cell adhesion molecule-like adhesin and a D-type cyclin, (Russo et al., 1996). A subsequent study which sequenced a 17-kb segment of HHV-8 between ORFs 11 and 17 confirmed that the viral genome contains a single 13-kb divergent locus wherein are nine ORFs that are homologous with, or related to, cellular proteins (Nicholas et al., 1997). A fourth potential cytokine gene, BCK, was also identified, in addition to a viral thymidylate synthetase gene, the T1.1 abundant lytic cycle nuclear RNA gene, and two genes related to the immediate-early protein of the gamma-2 class herpesvirus bovine herpesvirus type 4: 1E1-A and 1E1-B (Nicholas et al., 1997).

The herpes viral-like particles produced from BC-1 cells can further infect PBC-derived CD19+ B cells. This suggests that HHV-8 is transmissible and B-lymphotropic (Mesri et al., 1996).

More recently, another BCBL-derived cell line has been established and characterised, BC-3 which does not contain EBV DNA (Arvanitikas et al., 1996). The structure of the HHV-8 gene in this cell line has not yet been reported.

1.7. HHV-8 in the gastrointestinal tract

Early reports suggested that HHV-8 DNA is rarely detected in the throat and sputum of HIV-infected patients with KS (Whitby et al., 1995), and that HHV-8 DNA is not present in saliva of patients with HIV-related KS (Ambroziak et al., 1995). Subsequent workers found HHV-8 DNA in the saliva of up to 33% of HIV-infected persons, although not in HIV-negative individuals (Boldogh et al., 1996). Vieira and colleagues (1997) demonstrated the potential infectivity of HHV-8 in saliva, showing that infectious HHV-8 can be present in saliva, as evidenced by the persistence of HHV-8 DNA in 293 cell cultures inoculated with cell-free saliva fluid, and by the induction of viral-specific RNAs in 293 cells inoculated with cell-free saliva fluid. While the virus has been identified in oral KS lesions (Jin et al., 1996; Flaitz et al., 1997), it is not known if HHV-8 is present in the normal oral mucosa of such individuals. HHV-8 DNA has also been amplified from duodenal aspirates and intestinal biopsy tissue of HIV-infected individuals (Thomas et al., 1996). Stool samples were rarely found to be HHV-8 DNA positive in one study (Whitby et al., 1996).

1.8. HHV-8 in neural tissue

KS can present as a varicelliform distribution in immunocompromised patients, adopting a dermatomal distribution in sites of previous cutaneous herpes zoster (Niedt et al., 1988). Likewise HHV-8 has been described in paravertebral

ganglia of patients with AIDS and KS (Corbellino et al., 1996), pointing to neurotropism of the virus, which may account for the clinical distribution of the cutaneous lesions.

1.9. HHV-8 in peripheral blood

HHV-8 DNA has been detected in peripheral blood, plasma and serum of HIV-infected patients with and without AIDS-related KS (Collandre et al., 1995; De Milito et al., 1995; Whitby et al., 1995; Flamand et al., 1996; Marchioli et al., 1996; Harrington et al., 1996) and, occasionally, allograft recipients (Aluigi et al., 1996; Decker et al., 1996), non-HIV infected homosexual males (Milito et al., 1995), and healthy blood donors (Bigoni et al., 1996; Decker et al., 1996; Blackbourn et al., 1997). HHV-8 DNA could be amplified from CD19+/CD20+ peripheral blood B cells (Ambroziak et al., 1995; Flamand et al., 1996; Harrington et al., 1996) and CD14+ monocytes (Flamand et al., 1996) of patients with KS, CD34+ endothelial cells of pleural effusions associated with KS, and to a lesser extent, in CD3+ T cells (Harrington et al., 1996).

The frequency of detection of HHV-8 in PBCs in some (Whitby et al., 1995) but not all studies (Humphrey et al., 1996) may relate to the extent of HIV-related immunosuppression, being higher in patients with advanced KS lesions (Lebbe et al., 1997). Indeed it may be possible to predict clinical involvement of KS in HIV disease by the presence of HHV-8 in PBCs (Shingadia et al., 1995; Whitby et al., 1995; Brambilla et al., 1996; Lefrere et al., 1996; Moore et al.,

1996). Of note, however, is the presence of HHV-8 in PBCs of patients receiving intravenous foscarnet, ganciclovir therapy or both (Humphrey et al., 1996). Interestingly, HHV-8 infection of cord blood mononuclear cells can be blocked by foscarnet, suggesting that transmission of HHV-8 requires biologically active, replicating virus (Mesri et al., 1996).

Virally-infected CD19+ cells of one healthy blood donor were found to express HHV-8 RNA in infected target cells, demonstrating that HHV-8 is potentially infectious (Blackbourn et al., 1997). The finding that HHV-8 was not detectable in the PBCs of relatives of HHV-8-positive sporadic KS patients suggests that HHV-8 is a poorly transmittable virus, or is eliminated by the relatives' intact immune system (Uccini et al., 1997).

1.10. HHV-8 in genital tissue and fluids

HHV-8 was reported not to be found in testicular tissue from HIV-infected males with KS (Corbellino et al., 1996). Initial studies suggested that HHV-8 was rarely present in semen (Ambroziak et al., 1995; Marchioli et al., 1996). However, in a large cohort of Italian men from the Po valley of unknown HIV status, without KS, up to 44% of prostate specimens and 91% of ejaculates contained HHV-8 DNA; positivity was not associated with the spermatic heads but the cellular fraction containing urethral and other cells (Monini et al., 1996). Furthermore 22% of samples of glands penis or male foreskins were HHV-8 DNA positive, in comparison to only 6.5% of samples of female genital tract (Monini et al., 1996).

While these data would suggest that HHV-8 may be frequently found in the genital tract, other studies have not found HHV-8 positivity in the prostate tissue of Italian injecting drug users with AIDS Italian or of American HIV-non-infected patients without KS (Corbellino et al., 1996), or in sperm samples of healthy HIV-negative healthy males (Corbellino et al., 1996; Howard et al., 1997). A more recent study failed to confirm the initially reported high rate of HHV-8 detection in the sperm of HIV-positive American homosexual men (14% c.f. 91%), and did not demonstrate any direct relationship between the detection of HHV-8 and the sampling time relative to the development of KS (Gupta et al., 1996). However, an HHV-8 transcript encoding a small membrane protein was recently identified by in situ hybridisation in prostate cells of American HIV-infected and uninfected individuals (Staskus et al., 1997). In most instances the hybridisation-positive cells were localised to the glandular epithelium. It would thus appear that these cells do have the potential to transmit HHV-8 infection in prostatic secretions.

HHV-8 has not so far been found in vulval mucosa or cervical malignancies of females without a history of HIV disease (Tasaka et al., 1996).

1.11. HHV-8 and bone marrow

Multiple myeloma (MM) is the second most frequent haematological malignancy in the USA. It is characterised by the accumulation of malignant plasma cells in the bone marrow and the presence of a monoclonal immunoglobulin produced by the malignant plasma cells. Monoclonal gammopathy of

underdetermined significance (MGUS) is recognised as a precursor of MM and is characterised by a monoclonal immunoglobulin in the serum or urine and an increase of monoclonal plasma cells in the bone marrow. Rettig and colleagues (1997) found all of 15 patients with MM and 2 of 8 patients with MGUS to be HHV-8 DNA positive. In situ hybridisation also revealed nuclear and cytoplasmatic staining specific to HHV-8 DNA sequences in the myeloma bone marrow cells. Rettig and colleagues (1997) also demonstrated the presence of virally-derived IL-6 (vIL-6) in the myeloma bone marrow stromal cell samples, but not in normal healthy controls. It was thus hypothesised that vIL-6 may contribute to a mechanism whereby bone marrow stromal cells infected with HHV-8 promote myeloma growth (Rettig et al., 1997).

1.12. Serological detection of HHV-8

Serological assays have been developed that confirm, and further define the epidemiology of HHV-8. Initial assays were based upon usage of BC-1 cells as the source of viral antigen. Immunoblotting of BC-1 proteins revealed a doublet of high molecular weight nuclear antigens, p226/p234, distinct from known EBV proteins, which could be specifically detected by serum from KS patients; 80% of HIV-related KS patients had antibodies to these antigens, while only 18% of HIV-infected homosexuals without KS, and none of 122 blood donors or 20 HIV-infected men with haemophilia had such antibodies (Kedes et al., 1996; Gao et al., 1996). Conversion to seropositivity to the HHV-8 antigens usually occurred before

the clinical appearance of KS. Likewise, antibodies to another antigen (p40), a lytic cycle antigen, expressed after n-butyrate stimulation of BC-1 cells, were detected in 67% of patients with AIDS-related KS and 7% of HIV-infected patients without KS (Miller et al., 1996). Further assays have been developed based on other BCBL lines: the BCBL-1 and BCP-1 cell lines. These lines do not express EBV, and thus the problem of cross-reactivity between EBV and HHV-8 derived antigens is circumvented. Immunofluorescent assays (IFA) based upon these cells showed that IgG antibodies to HHV-8-specific antigens are present in almost all patients with AIDS-related KS tested, and up to 30% of HIV-infected homosexual men without KS (Kedes et al., 1996; Gao et al., 1996). Furthermore, all patients with African endemic KS and 96% of American patients with AIDS-associated KS were seropositive for lytic and latent HHV-8 antigens (Leanette et al., 1996), while about 25% of healthy adults and 2-8% of childrens were seropositive (Leanette et al., 1996).

Enzyme linked immunoabsorbent assays (EIAs) detecting IgG antibodies to a capsid-related truncated recombinant protein encoded by HHV-8 ORF65 have also been developed (Simpson et al., 1996); 84% of patients with HIV-related KS were found to have detectable antibodies while few blood donors from the US or UK were seropositive, although seropositivity was more common in healthy persons from Uganda than the UK or USA (Simpson et al., 1996). Of concern is that only 80 to 90% of patients with KS were seropositive to one or more HHV-8 derived antigens, yet all were HHV-8-infected. Thus current estimates of HHV-8 prevalence based upon serological studies may have to be revised as assay

sensitivity and specificity improve (Rickinson, 1996; Gompels et al., 1996; Mocarski et al., 1997; Parry et al., 1997).

1.13. Possible mechanisms of HHV-8 in the pathogenesis of KS

As detailed previously, analysis of the putative translation products of the HHV-8 ORFs revealed homology between a number of known human cellular receptors, cell cycle enzymes and chemokines important in a variety of cellular and immunological homeostatic mechanisms. The putative translation product of ORF74, for example, is a G protein coupled receptor (GCR). These receptors are important in cellular growth and differentiation, and some GCRs are involved in malignant transformation (Arvanitakis et al., 1997). The closest cellular homologue to the putative HHV-8 GCR are the IL-8 receptors A and B, and the closest viral homologue is the HVS *ECRF3* gene, which encodes a functional IL-8 receptor (Ahuja et al., 1993). The putative product of HHV-8 ORF 72 shows homology to mammalian cyclin D proteins (Li et al., 1997). Cyclins are required for cellular division (Peters, 1994), and cyclin D proteins in particular are regulatory subunits that activate cellular kinases to phosphorylate checkpoint molecules. The HHV-8 cyclin protein may be associated with kinase activity in phosphorylating (and thus inactivating) the checkpoint molecule retinoblastoma-tumour suppressor protein. Thus, HHV-8 has the potential to overcome cell-cycle arrest, thereby increasing the likelihood of tumour development (Chang and Moore, 1996; Murphy, 1997).

As mentioned earlier, other ORFs of HHV-8 have been found to encode

proteins similar to two MIPs (encoded by ORF K6 and ORF K4), vIL-6 (by ORF K2) and interferon regulatory factor (IRF) (by ORF K9).

The virally-derived IL-6 (vIL-6) was found to be expressed in HHV-8 infected BCP-1 cells, ascitic lymphoma cells from HIV-negative PEL and the B-cell rich areas of lymph node tissue from a patient with AIDS-related KS. It has also been detected in KS lesions, initially in CD34+ (endothelial cells) or CD45+ (leukocytes) (Moore et al., 1996), and more recently by the generation of a genomic library from KS tissue derived DNA extract (Neipel et al., 1997). Viral MIP-1s have been found to inhibit non-syncytial inducing (NCI) HIV-1 entry via the CCR5 chemokine receptor, suggesting that MIP-1s are functional in CCR5 binding and may contribute to interactions between HHV-8 and HIV-1. Hence HHV-8 may have some inhibitory action in HIV-1 infection, which may explain why patients with AIDS and KS have a better prognosis than patients with AIDS but not KS (Morecroft et al., 1997). MIP-1s are β-chemokines, being produced by T cells, B cells, macrophages, Langherans cells and neutrophils. They are chemoattractants for monocytes, eosinophils, B cells and killer cells and additionally inhibit stem cells replication. MIP-1s also promote accumulation of CD4+ T cells. The relevance of viral MIP-1s to the pathogenesis of KS is not yet understood.

HHV-8 also possesses genes similar to *bcl*-2, complement-binding proteins similar to CD21/CR2 (from ORF4) and an NCAM-like adhesion molecule (from ORF K14), which may interfere in immune surveillance. The precise functions of viral products encoded by these genes have not been reported.

Typical cellular strategies against viral infection include cell cycle arrest, induction of apoptosis and induction of cell mediated immunity. It would appear that HHV-8 has the means to overcome these to enable its persistence in the host. Indeed, vIL-6, vIRF and vbcl-2 can inhibit apoptosis, while vIRF can interfere with interferon-induced MHC antigen presentation and cell-mediated responses (Moore et al., 1996). I will discuss later (chapter 4) the possible role of these effector molecules in provoking the granulomatous inflammatory response.

2. METHODS

2.1. Specimens

Sample were obtained from tissue libraries and clinics from several countries, particularly England and Italy. All tissue sections had previously been paraffin embedded, formalin fixed (PEFF) in the course of routine processing for histological evaluation. A complete list of samples is detailed in Table 2.1

BC-1 and BCP-1 cell lines (Cesarman et al., 1995; Gao et al., 1996) were obtained from the American Type Culture Collection.

2.2. Deparaffization of PEFF tissues

PEFF tissues (30-50 μm thickness) were incubated at room temperature for 30 min with 1 ml of xylene in Eppendorf tubes to permit the paraffin to dissolve. Following vortexing and centrifugation in a fixed angled Eppendorf microfuge (30 s at 12,000g) the xylene was aspirated. A second xylene step of 15 min was taken to further ensure that the paraffin had dissolved. The samples were then incubated at room temperature for 10 min with 100% ethanol to remove the xylene, vortexed and centrifuged. This step was repeated twice. The pellets were left on the bench for 10 min to allow ethanol to evaporate.

Table 2-1 Histology, origin and number of tissue specimens and patients tested

Histologic feature	Origin	Total specimens	Total patients
Oral Squamous Carcinoma	Brazil	9	9
Oral Squamous Carcinoma	Australia	3	3
Oral Squamous Carcinoma	Italy	2	2
Mediterranean Kaposi's sarcoma	Italy	18	16
Iatrogenic Kaposi's sarcoma	Israel	11	11
HIV+ PBMC	UK	15	15
HIV+ Kaposi's sarcoma	Italy	10	10
HIV+ Kaposi's sarcoma	UK	11	11
HIV+ Kaposi's sarcoma	Australia	4	4
HIV+ Kaposi's sarcoma	Canada	10	10
HIV+ Kaposi's sarcoma	Germany	8	8
HIV+ Oral ulcerations	UK	10	10
HIV+ Oral ulcerations	Germany	5	5
HIV+ Oral ulcerations	Canada	5	2
HIV+ Oral ulcerations	Australia	2	2
HIV+ Oral Lesions	UK	6	6
HIV+ Oral hairy leukoplakia	Germany	5	5
HIV+ Oral hairy leukoplakia	Italy	5	5
HIV+ Oral hairy leukoplakia	Canada	1	1
HIV+ non-Hodgkin's disease	Italy	6	6
Kikuchi disease	Singapore	14	14
Kikuchi disease	Italy	1	1
Sarcoidosis	Italy	39	17
Reactive Lymphadenitis	Italy	17	17
Granulomatous Lymphadenitis	Singapore	6	6
B Cell lymphoma	Singapore	5	5
Non-Hodgkin's lymphoma	Italy	12	12
Lung cancer	Italy	22	22
Skin basocellular epitelioma	Italy	17	17
Oral healthy mucosa	UK	10	10
Oral lesions	UK	11	11
Orofacial granulomatous disorders	UK	17	17
Miscellaneous Disorders	Italy	11	11
Lung inflammatory disorders	Italy	32	32
		360	336

2.3. Extraction of DNA from samples

DNA was extracted from all the samples using Geneclean Kit III (BIO 101, La Jolla, CA). This procedure is based on the lysing and nuclease-inactivating properties of sodium iodide (NaI) together with the nucleic acid binding properties of silica particles (EZ-Glassmilk). The procedure was performed as follows: cut PEFF tissue section was mixed with 500 µl of lysis buffer 6M NaI, and 20 µl of silica particles (Appendix 1). The reaction tubes were vortexed and incubated at room temperature for 10 min with constant agitation. During this period, all nucleic acids are presumed to have bound to the silica particles. The vessels were vortexed again, then centrifuged in a fixed angled Eppendorf microfuge (30 s at 12,000g); the supernatant was removed by suction leaving the pellet intact. The pellets then underwent three washes with 500 µl of the washing buffer NewWash (BIO 101, La Jolla, CA) (Appendix 1). After removal of NewWash the vessels were placed at 56^{o}C with open lids in an Eppendorf heating block for 5 min to allow the pellets to dry. The nucleic acid was eluted twice by resuspending the pellets in 40 µl of distilled water, then vortexed and incubated at 56^{o}C for a further 10 min. Following another vortexing and centrifugation step, the supernatant was removed for use as template in the first round of PCR. The remaining extract was then stored at -20^{o}C.

2.4. Amplification of herpesviral and mycobacterial DNA

2.4.1. Routine amplification of HHV-8 sub-genomic DNA

Amplification of the DNA was carried out using a modification of the method described by Chang et al. (1994). The primary reactions were carried out in a volume of 25 µl containing sterile water, *Taq* polymerase buffer (Gibco BRL, Paisley, Glasgow, Appendix 1), 15 mM $MgCl_2$, 200 µM of each of the 4 deoxynucleoside triphosphates (dNTPs) (Boehringer Mannheim, Lewes, East Sussex), 20 pmol of each outer primer (Sequence of primer are given in Table 2.2), 1 unit of *Taq* polymerase (Gibco BRL, Paisley, Glasgow) and 2.5 µl of HHV-8 DNA template. A PTC-100 thermal cycler (MJ Research, Inc., GRI Ltd, Dunmow, Essex) was programmed to perform 1 cycle of 5 min at 94^{o}C followed by 30 cycles of 94^{o}C for 1 min (denaturation), 60^{o}C for 1 min (annealing) and 72^{o}C for 1 min (elongation). The secondary reactions were also carried out in a volume of 25 µl, containing sterile water, *Taq* polymerase buffer, 15 mM $MgCl_2$, 200 µM dNTPs, 20 pmol of each inner primer, 1 unit of *Taq* polymerase and 1 µl of primary PCR product. The amplifications were carried out under the same cycling conditions as the primary reaction. The sensitivity of the nested PCR procedure, as estimated by the amplification of serial dilutions of plasmid HHV-8 DNA, was equivalent to the DNA content of 1 copy.

Table 2-2 Primer sequences for amplifying HHV-8, EBV and 16s RNA mycobacterial DNA

<u>ORF26 HHV-8</u>		
Sense primer	KS1	5'AGCCGAAAGGATTCCACCAT3'
Anti-sense primer	KS2	5'TCCGTGTTGTCTACGTCCAG3'
Sense primer	Ksin1	5'TTCCACCATTGTGCTCGAAT3'
Anti-sense primer	Ksin2	5'TACGTCCAGACGATATGTGC3'.
<u>ORF25 HHV-8</u>		
Sense primer I	25.1	5'CATGGATACATTGTCAGGACCTC3'
Anti-sense primer	25.2	5'GTCACATCTGACGTTGCCT3'
Sense primer II	25.3	5'GGCAACCTGCGAGATAATTCC3'
<u>EBV</u>		
Sense primer	K01	5'TGATGGAGGCAGGCGCAAAAAAG3'
Anti-sense primer	KO2	5'GAAACCAGGGAGGCAAATCTACT3'
Sense primer	KI1	5'CGCAAAAAAGGAGGGTGGTTT3'
Anti-sense primer	KI2	5'CATCGTCAAAGCTGCACACAG3'
<u>Mycobacterial 16s RNA</u>		
Sense primer I	P1	5'GCGTGCTTAACACATGCAA3'
Anti-sense primer	P2	5'CGCTCACAGTTAAGCCGT3'
Sense primer II	P1-2	5'CTGGCTCAGGACGAACGCT3'

2.4.2. DNA amplification of EBV subgenomic DNA

Samples were also subjected to nested PCR procedures to amplify a 470 bp DNA sequence within the *Bam* HI K fragment, (from coordinates 109311 to 109780) (Baer et al., 1984). The composition of the PCR mix was identical to that used for HHV-8 DNA amplification. A PTC-100 thermal cycler was used. Thirty cycles of 94^{O}C for 1 min (denaturation), 58^{O}C for 40s (annealing) and 72^{O}C for 1 min (elongation) were carried out. The primer sequences used for this amplification are listed in Table 2.2.

2.4.3. DNA amplification of CMV, HHV-6 and HHV-7 subgenomic DNA

For CMV, a nested approach was used to amplify a 245-bp segment in the glycoprotein B-coding region, corresponding to nt 1285 to 1529. For HHV 7, primer sequences which were flanking a 124-bp region within the genome and a nested PCR were used. Heminested PCR was used to amplify a 176-bp segment of the HHV 6 genome in the *Bam*HI S region, which is within the large tegument protein-coding region. Primer sequences are listed in Table 2.3.

Table 2-3 Primer sequences for CMV, HHV-6 and HHV-7

CMV

Sense primer	5'GGAAACGTGTCCGTCTTCGA3'
Antisense primer	5'GAAACGCGCGCGGCAATCGG3'
Sense primer	5'GTGTTCTGGCAAGGCATCAA3'
Antisense primer	5'CGTTGATCCACACACCAGGC3'

HHV-7

Sense primer	5'TATCCCAGCTGTTTTCATATAGTAAC3'
Antisense primer	5'GCCTTGCGGTAGCACTAGATTTTTTG3'
Sense Primer	5'CAGAAATGATAGACAGATGTTGG3'
Antisense primer	5'TAGATTTTTTGAAAAAGATTTAATAAC3'

HHV-6

Sense primer	5'GATCCGACGCCTACAAACAC3'
Antisense primer I	5'TACCGACATCCTTGACATATTAC
Antisense primer II	5'GGCTGATTAGGATTAATAGGAGA3'

2.4.4. Mycobacterium 16sRNA amplification

To amplify mycobacterial 16sRNA DNA, a heminested PCR procedure was used. Primers which anneal specifically to sequences in the 16S rRNA gene common to 38 species of mycobacteria and which flank species-specific sequences that were chosen (Rogall et al., 1990). The procedure yields products whose lengths vary from 565 to 590 bps, depending on the mycobacterial species. First-round PCR was carried out in 50 µl volumes, each containing of tissue DNA, 10 mM Tris-HCl pH 8.8, 50 mM KCl, 2.5 mM $MgCl_2$, 0.1% Triton X-100, 200 µM each of the dNTPs, 0.1 µM of each primer, and 1.5 units of *Taq* DNA polymerase. Amplification was performed over 35 cycles, each consisting of 93°C for 30 s, 55°C for 30 s and 72°C for 60 s. One µl of the product was transferred for second-round amplification, which was carried out as in the first round except that primer P1-2 was used instead of P2. The primers sequences are reported in Table 2.2. The sensitivity of the heminested PCR procedure, as estimated by the amplification of serial dilutions of *M. tuberculosis* (strain H37Rv) chromosomal DNA, was equivalent to the DNA content of 3 bacilli (S. Patel, unpublished data).

2.5. Detection of PCR products

Ten µl of PCR product was mixed with 2 µl of loading buffer (Tris-disodium ethylenediaminetetracetate (EDTA), pH 8.0, containing 40% sucrose and 0.25% bromophenol blue) and electrophoresed through a 2% composite agarose

gel (3:1, NuSieve:Seakem, Flowgen, Lichfield, Staffs) in 1 x Tris-borate-EDTA-buffer (TBE, Appendix 1). A 1 kb ladder molecular weight marker (Gibco BRL, Paisley, Glasgow) was run either side of the test samples to assess the size of the amplified product. Gels were then stained in a ethidium bromide solution (concentration of 5 μg/ml). The DNA fragments were visualised using a short wave uv transilluminator and photographed using an instant Polaroid camera.

2.6. Polymerase chain reaction-single stranded conformation polymorphism assay

When studying genetic polymorphism involving large numbers of specimens, the polymerase chain reaction-single stranded conformation polymorphism (PCR-SSCP) assay is a discriminatory procedure for scanning sequence polymorphisms within amplicons (Yusof et al., 1994). The PCR-SSCP assay is based on single stranded (ss) DNAs forming tertiary structures whose conformations are dependent upon their unique DNA sequences. When ssDNAs are electrophoresed through a non-denaturing gel they migrate at a rate determined by their conformation. For the PCR-SSCP assay, DNA extracts were first submitted to a primary PCR amplification process as previously described in section 2.4.1. The secondary PCR was then carried out in a 20 μl volume containing sterile water, *Taq* polymerase buffer, 15 mM $MgCl_2$, 70 μM dNTPs, 20 pmol of each outer primer, 2 μCi of ^{32}P-deoxycytosine 5'-phosphate (Amersham

International, Amersham, Bucks), 1 unit of *Taq* polymerase and 2 µl of primary PCR product.

2.6.1. Preparation of SSCP assay plates

Two plates were required, a thermostatic plate (25 cm x 61 cm) and a glass plate (21 cm x 55 cm). The thermostatic plate was placed on an LKB2010-100 macromould and wiped over with 100% ethanol. Then 10 ml of "repel-silane" (2% solution of dimethyldichlorosilane in 1,1,1-trichloroethane, BDH Limited, Poole, Dorset) was spread over the plate and left for 5 min. The remaining fluid was removed and the process repeated. Before using the plate it was wiped over again with ethanol and left to dry. Repel-silane is an effective repellent, but highly toxic. An alternative, Gel Slick (Hydrolink, Flowgen, Lichfield, Staffs) (which requires only one treatment of 5 ml) was used in later studies.

The glass plate was wiped over with 100% ethanol and then covered with 10 ml of "bind-silane" mix (20 ml absolute ethanol, 600 µl 10 % acetic acid v/v, and 60 µl of gamma-methacryloxypropyltrimethoxy silane). After leaving for 5 min, the remaining fluid removed and the procedure repeated. As with the thermostatic plate, it was wiped over with ethanol before use.

The spacers were placed on the thermal plate. Then the glass plate placed over it, making sure that the treated sides were facing inwards. The plates and spacers were clamped together, after which the gel was poured.

2.6.2. Preparation of SSCP assay gel

To 51.2 ml of distilled water, 20 ml of MDE gel (Hydrolink, Flowgen, Lichfield, Staffs), 4.8 ml of 10 x Tris-borate-EDTA-buffer (TBE) and 4.0 ml of glycerol were added and mixed thoroughly. Just before the gel was poured, 360 µl of 10% ammonium persulphate and 36 µl of N,N,N',N'-tetramethulethylenediamine (TEMED) (Gibco BRL, Paisley, Glasgow) were added. The mixture was carefully transferred to a 50 ml syringe and the gel mixture introduced, by capillary action, in the space between the plates. The comb was then placed and firmly secured with a clamp into the top of the gel, that was left to set for a minimum of 1 hr.

2.6.3. Loading the gel

The PCR products were mixed in a 1:1 ratio with loading buffer (98% deionised formamide, 0.05% bromophenol blue, 0.05% xylene cyanol and 20 mM EDTA). The diluted PCR products were denatured in a heating block at 95^{o}C for 5 min to yield ssDNA. The samples were then snap cooled on ice and left until ready for loading on the gel. They were loaded and electrophoresed through the non-denaturing polyacryamide gel at 8 W for 18 hr at 15^{o}C in a Macrophor sequencing apparatus, the temperature being maintained using a water jacketed thermostatic plate connected to a thermostatic circulator (Pharmacia LKB Biotechnology, Uppsala, Sweden). After electrophoresis, the gel was covered with

Saran wrap and exposed to autoradiographic film (Hyperfilm-MP, Amersham International, Amersham, Bucks).

2.6.4. Development of the autoradiograph

The autoradiograph was placed in a tray of developer (4:1 ratio water to developer concentrate, Kodak, Hemel Hempstead, Herts) so that it was completely submerged, and agitated for 2 min then rinsed thoroughly in water. The film was then immersed in fixative (4:1 ratio water to fixer concentrate, Kodak, Hemel Hempstead, Herts), rinsed thoroughly and left to dry. Once dry, it was examined on an X-ray viewer.

2.7. Purification of PCR products for sequencing

The PCR products were purified using Geneclean (Bio101, Vista, CA). This kit is based on the lysing and nuclease-inactivating of NaI together with the nucleic acid binding properties of silica particles (as glass milk). The PCR products were loaded onto a 2% composite agarose gel, as described earlier (section2.5), in alternate wells to allow enough room for cutting out the product from the gel without risk of contamination from other products. The products were then electrophoresed and detected using ethidium bromide as described previously. The products were viewed using long wave uv light and each band cut with a scalpel and transferred to separate 1.5 ml Eppendorfs. The gel was then photographed in

the presence of shortwave uv light to check that all the DNA had been removed. Each sample was weighed and TE added to ensure that the Eppendorfs were of roughly equal weight. The agarose was then melted at 56°C in the presence of 6M NaI and TBE modifier (the latter reagent is required to correct the pH of the solution to enable nucleic acids to bind to the silica matrix after the agarose has melted). Once the agarose had melted, 5 µl of glass milk was added to each reaction vessel and vortexed. This was left on the bench for 5 min. The tubes were vortexed once more, then spun briefly (12,000 g) to pellet the glass milk. The supernatant was removed and the pellets underwent 3 washes using New Wash (Bio101, La Jolla, CA) with centrifugation (5 s at 12,000 g) and removal of the supernatant between each wash. The pellet was left to dry, then resuspended in sterile tissue culture water, heated for 5 min at 56°C, and the DNA from the glass milk eluted. After elution the vessels were centrifuged (30 s at 12,000 g) and the supernatant removed and placed in a 0.5 ml Eppendorf tube. To confirm that the cleaning had been successful and to roughly estimate the quantity of DNA recovered, 1 µl of this supernatant was taken and run on a 2% agarose gel, stained and visualised.

2.8. Automated sequencing of PCR products

Sequencing of the PCR products was done by cycle sequencing using the PRISM™ Ready Reaction DyeDeoxy™ Terminator Cycle Sequencing Kit with AmpliTaq™ FS DNA polymerase (Perkin Elmer, Forest City, CA). In each case, both

strands of the PCR product were sequenced. The 20 µl sequencing reaction consisted of 8 µl of the sequencing mix, 3.2 pmol of one of the primers and purified DNA at approximately 100 ng for the HHV-8 fragment (to calculate required amounts of DNA refer to Appendix 1). The reaction vessels underwent 25 cycles of 30 s at 95°C, 30 s at 50°C and 4 min at 72°C in a PTC-100 thermal cycler. These were held at 4°C for a further 10 min. The reaction vessels were then placed on ice and the DNA precipitated (Appendix 1).The sequencing pellets were then electrophoresed by the sequencing laboratory facilities in the CPHL site. Following this, the raw sequence data was processed and analysed using the programmes "Analysis", "SeqEd" (Applied Biosystems, Foster City, CA), and "Lasergene Navigator" (DNASTAR, Madison) (Appendix 1).

2.9. T/A cloning

This was done using the LigATor kit (R&D Systems, Minneapolis, MN). It is based on T/A cloning (TAC), which assumes that PCR products generated by *Taq* polymerase possess a single adenosine overhang at each 3' end, made possible by the template-independent terminal transferase activity of *Taq* polymerase (Clark, 1988; Holten et al., 1991; Marchuk et al., 1991). The procedure involves freshly amplified DNA fragments being ligated to a vector with single 5' thymidine overhangs (pTAg), and 1 µl of the ligated products were then transformed into competent *E. coli* cells (Stratagene, La Jolla, CA). The plasmid DNA is added to the competent cells (Appendix 1), mixed gently, and stored on ice for 30 min; the

cells were then heat shocked at 42°C for 2 min and snap cooled in ice; finally, 80 µl of S.O.C media (Appendix 1) is added to the cells followed by incubation at 37°C for 1 hr to allow the cells to start expressing antibiotic resistance. The cells are then plated out onto LB agar plates (Appendix 1) containing 50 mg/ml ampicillin (which allows the selection of bacteria that had taken up the plasmid/vector), 0.5 µM isopropylthiogalactoside (IPTG) and 80 µg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactosidase (x-gal). The latter two compounds are necessary to determine the presence of inserts by insertional inactivation. The cells were grown overnight in an incubator at 37°C following which 20 white colonies were picked. The presence of white colonies indicates that the cloning has been successful and that the PCR products inserted into the vector. The plasmid contains the *Lac Z* gene which produces the enzyme β-galactosidase, which when induced by IPTG leads to breakdown of the substrate x-gal, giving a blue product. Therefore, colonies without inserts are blue, being *Lac Z* positive. However, when DNA is inserted into *Lac Z*, the bacteria can no longer utilise x-gal, so the colony remains white.

2.9.1. Screening colonies for inserts

The colonies were screened for inserts by PCR (Pampfer, 1993). Briefly this was done as follows. White colonies were labelled, picked, and transferred to 50 µl of secondary PCR master mix. The mixture was then submitted to PCR using the inner primers and the products visualised.

2.10. HHV-8 plasmid preparation

A nested PCR following previously described protocols was used to generate a positive aliquot from the BC-1 cell line. The sample was electrophoresed in a 2% agarose gel and stained with ethidium bromide. DNA was extracted and purified from the PCR product using Geneclean Kit III (Bio101, La Jolla, CA) and eluted in 10 µl of sterile water. One µl was electrophoresed in a 2% agarose gel to quantify the amount of the viral DNA present in the aliquot, as earlier described above. The sample was cloned into a pTAg vector using The LigAtor Kit (R&D System Europe, Abingdon) and propagated in L-Broth overnight. The plasmid DNA was purified using Qiagen Plasmid Midi Kit (Qiagen, Surrey) and DNA quantified spectrophotometerally. A 20-fold dilution of the plasmid was made to be used as template to determine the end point sensitivity of the nested PCR.

2.11. Quantification of DNA

This was done by end point dilution PCR. All the samples were compared to a 10-fold dilution series of the plasmid HHV-8. This series was subjected to a first round of PCR using the outer primers. Following agarose gel electrophoresis, the end points of the plasmid and sample dilution series were determined. The virus copy number was then calculated (Appendix 1).

3. HHV-8 VARIANTS IN ORAL TISSUES OF HIV-INFECTED INDIVIDUALS

3.1. Introduction

Oral lesions play an important role in the diagnosis and in the staging of the HIV disease. The presence of lesions such as oral candidosis and oral hairy leukoplakia (OHL) are well recognised indicators of advancing HIV disease. The commonest oral manifestations in HIV disease are oral candidosis, OHL, oral KS, periodontal diseases and aphthous ulcers; less common lesions include granulomas, polyps and keratosis. Oral ulcers in HIV disease are caused by a range of pathogens including herpes simplex viruses and CMV; however, most are idiopathic (Greenspan and Greenspan, 1996). Painful aphthous-like ulceration, often diagnosed as major or herpetiform ulcer types, presenting mainly at the palate or buccal mucosa have also been described in HIV-infection (Silverman et al., 1986; Schiodt et al., 1987; Phelan et al 1987; Pindborg, 1989; MacPhail et al., 1992;). These ulcers may first appear at the time of HIV-seroconversion (Silverman et al., 1986; Robert et al., 1988; Greenspan and Greenspan, 1996) and may be associated with a rash and pharyngeal and/or oesophageal ulcers (Rabeneck et al., 1990; Bach et al., 1990). Other oral lesions are less common in the presence of HIV disease, but still should be considered in the differential diagnosis of viral disorders.

Herpesviruses can be shed in saliva, and EBV, which is closely related to HHV-8, undergoes replication in oropharyngeal epithelial cells (Sixbey et al., 1984). The detection of HHV-8 in saliva has been recently reported in HIV-positive patients with KS, HIV-positive without KS and HIV-negative with KS (Vieira et al., 1997; Boldogh et al., 1996). Although, increased shedding of EBV in

HIV-infected individuals was reported by Alsip et al. (1988), the role of immunosuppression in association with HHV-8 oral shedding has not yet been addressed. On the other hand, in peripheral blood cells of healthy people, HHV-8 would be found at a rate of up to 10% with a similar frequency rate in lymph nodes of HIV-seronegative persons (Bigoni et al., 1996). By analogy with EBV, the lymphoid system could represent a reservoir of latenty infected cells from which the virus may reactivate during immunosuppression. This is further suggested shown by the finding that a higher virus load is found in lymphoid tissue of HIV-infected patients (Bigoni et al., 1996). Furthermore, Harrington et al. (1997) reported HHV-8 DNA and most cellular cytokines may be reactivated by the HIV tat gene, suggesting that the tat gene product upregulates viral replication which then aids in the reactivation of HHV-8 from the latent state. Because in the oral cavity lymphoid cells are widespread in minor and major salivary glands, these structures may act as reservoirs for viruses that could later reactivate in the presence of immunosuppression.

With these considerations, I undertook a study to determine if HHV-8 can infect oral tissues of HIV-positive patients.

3.2. Materials and methods

3.2.1. Specimens

PEFF tissues were obtained from 25 incisional oral biopsy samples taken from 23 patients with HIV infection who attended the oral medicine clinic of a genitourinary medicine department in central London. Eleven had histologic features consistent with KS, 10 non-specific ulceration and 6 other diseases. Also studied were paraffin blocks of 16 skin biopsy samples from Italian patients with classic KS and 20 diagnostic biopsy samples from patients with various oral diseases attending general dental clinics (including 9 with oral squamous cell carcinoma). The HIV antibody status of the patients in the latter 2 groups was unknown. Specimens were cut, deparaffinized and DNA was extracted as described in chapter 2.

3.2.2. DNA amplification and characterisation of HHV-8 diversity

PCR was used to amplify DNA sequences from ORF26 HHV-8, and from EBV, CMV, HHV-6 and HHV-7. Positive amplicons were detected as described in chapter 2. PCR-SSCP, cloning and sequencing of HHV-8 amplicons were performed as described in chapter 2.

3.3. Results

HHV-8 ORF26 DNA could be amplified from 5 of 11 oral KS and 12 of 16 non KS oral tissues from HIV-positive patients, 10 of 16 classic KS tissues, and none of 20 oral samples taken from general dental patients. PCR-SSCP analyses and DNA sequencing demonstrated that the HHV-8 sequences of 11 of the 27 positive tissue samples were unique. A common HHV-8 DNA sequence was identified in 3 oral KS samples, 5 non KS oral samples and 1 cutaneous KS sample. Another sequence was common to 5 KS tissues (2 oral and 3 cutaneous). Figure 3.1 illustrates sequence polymorphism of HHV-8 DNA amplified from all the oral non KS tissue samples and from representative KS samples. EBV, CMV and HHV-6 and 7 DNA were not amplified from any of the oral tissue samples.

From each of the 27 HHV-8-positive tissues, the sequence of the ORF26 segment encompassed by nested primers (171 bp in length, representing 57 codons) was aligned and compared with the others, and with 36 sequences reported from previous studies involving patients from the United States and Africa (Chang et al., 1994; Moore et al., 1995; Huang et al., 1995; Cesarman et al., 1995). Genetic variation within the segment was not, in most instances, random. Using the prototype virus sequence (Chang et al., 1994) as the basis for comparison, there were altogether 109 base changes among the 61 sequences examined. Of these changes, 102 (95%) were confined to 6 positions in 5 codons: 134, 141, 152, 167 and 169 (numbered according to reference Chang et al., 1994). At codons 134 and 167, nucleotide

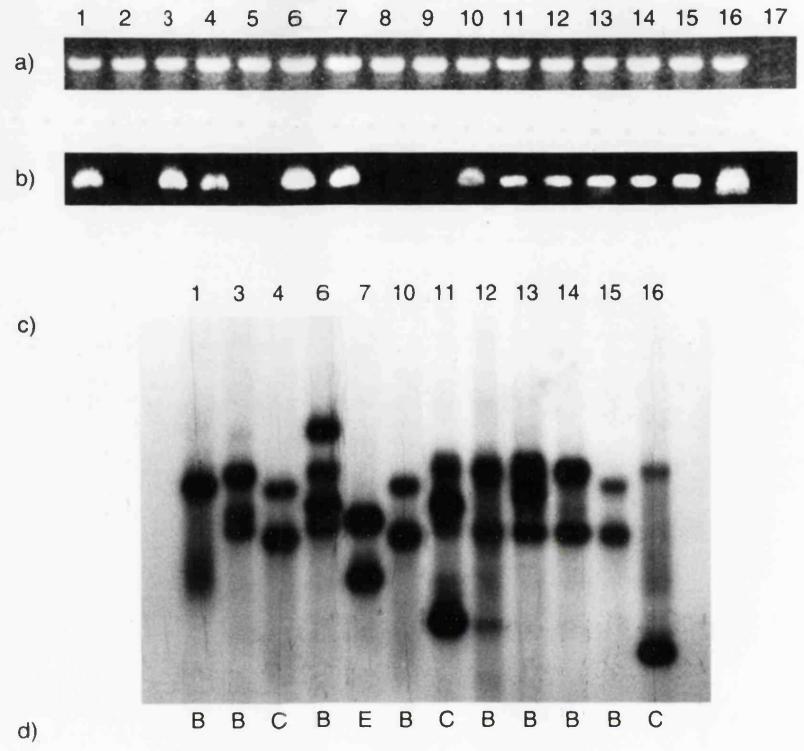

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
a)
b)
1 3 4 6 7 10 11 12 13 14 15 16
c)
B B C B E B C B B B B C
d)

Figure 3-1 ORF26 HHV-8 DNA in non KS oral tissues of HIV carriers.
a. Ethidium bromide-stained bands of electrophoresed 110-bp DNA amplified from the human b-globin gene. b. Ethidium bromide-stained bands of electrophoresed 210-bp DNA amplified from ORF26 of the HHV-8 genome. c. ^{32}P-autoradiographic bands of single-stranded DNA derived from 210-bp HHV-8 DNA PCR products after electrophoresis through a nondenaturing gel. Lane numbers correspond to those of a and b. d. HHV-8 group assignments. Lanes 1-14: non KS oral tissues; lanes 1-10: ulcer tissues (1 and 2: tongue; 3: angle of the mouth; 4 and 5: palate; 6: inner cheek; 7: buccal floor; 8: tongue; 9: buccal sulcus; 10: palate). Lane 11: keratosis (tongue). Lane 12: mucocele (lip). Lane 13: abscess (gingiva). Lane 14: pyogenic granuloma (gingiva). Lane 15: HIV+ KS. Lane 16: cutaneous KS (Mediterranean). Lane 17: water.

Different banding patterns predict differences in nucleotide sequences.

Variant group	Molecular basis for grouping	Distribution of variants UK/Italy	USA/Africa	Total
	130 140 150 160 170 180			
A	NGFD **P** VFPMVV**P**QQLGHAILQQ**L**LVYHIYSKISAGAP **D** D**V**NMAELDYLTTNVSFMGQ ccc gat	0	20	20
B	.... L ..... D ..... ctc gat	16	6	22
C	.... I ..... G ..... atc ggt	6	2	8
D	.... T ..... G ..... acc ggt	0	7	7
E	.... L ..... G ..... ctc gat	0	0	0
others		3	1	4
	(Total)	25	36	61

Table 3-1 Molecular basis for grouping of HHV8 ORF26 sequences and geographical distribution of HHV-8 variants.

The predicted group A peptide sequence, from codon positions 130 to 186, is shown in full, using the single letter code. Amino acid residues whose codons are the sites of 95% of mutations are in bold. Residues that allow classification of variants into groups B, C, D and E are shown in alignment with the group A sequence; dots signify residues that are identical to group A. Codons that specify group-specific amino acid changes are depicted as small-case letters below the corresponding amino acid residues. Not shown are codons in which mutations are silent, and amino acid changes in the group of variants that cannot be classified as A, B, C, D or E.

substitutions occurred at positions that lead to amino acid changes (Table 3.1). Substitutions at codons 141 (G→T at the third base), 152 (C→T at the first base) and 169 (A→C at the third base) are silent. Based on amino acid changes predicted from substitutions at codons 134 and 167, all sequences, including those that were unique and possessed mutations in other codons, could be segregated into 5 main groups (A to E) (Table 3.1).

Table 3.1 also shows the geographical distribution of variants. Group A and D variants were exclusive to tissues of patients from the USA and Africa. Group B and C variants were also identified in American and African tissues (group B: 6/36=17%; group C: 2/36=6%), but were less prevalent than in tissues of London and Italian patients (group B: 16/25=64%; group C: 6/25=24%). Of the 10 oral non KS tissues from the London patients, 70% belonged to group B.

Seven of the 109 base substitutions that did not involve the 5 codons aforementioned were located at codons 130 (A→G at the second base), 138 (A→T at the first base), 142 (A→T at the second base), 146 (G→T at the third base), 163 (C→T at the third base), 164 (G→A at the third base) and 168 (T→C at the third base). Only the substitutions at codons 138 and 142 are predicted to lead to amino acid changes. Tissue extracts whose PCR products yielded these base changes were reamplified and resequenced. The changes remained the same, discounting the possibility that *Taq* polymerase misincorporation had led to the detection of unique sequences.

3.4. Discussion

This study identifies the oral mucosa as another site of HHV-8 infection in people who are HIV-infected. Factors that could lead to false-negative results after PCR amplification for HHV-8 DNA from fixed archived tissues i.e., fragmentation of target DNA (Ya Feldman, 1973), and formaldehyde-mediated cross-linking of nucleic acid polymers to each other and to proteins were taken into consideration (Goelz et al., 1985). A nested approach to detect HHV-8 DNA (to amplify the population of target DNA that had escaped reaction with formalin) was taken, and to adopt $KS330_{233}$ primers as outer primers (to keep the segment of amplifiable DNA short, thereby increasing the chance of detecting nonfragmented target DNA). Nevertheless, 6/11 oral KS and 6/16 cutaneous KS tissues were negative for HHV-8 DNA in our nested PCR assay. This is likely to reflect the combined effects of nucleic acid polymer degradation (Ya Feldman, 1973) and formalin (Goelz et al., 1985) on the specimens. It is noted that most other PCR assays for HHV8 DNA in KS tissues (Chang et al., 1994; Moore et al., 1995; Huang et al., 1995) had used snap-frozen or fresh biopsy specimens.

The overall rate of positive results in this study is, notwithstanding, high. It is unlikely to be due to carryover PCR contamination, because an array of unique sequences was found. Thus the results obtained from the KS samples and, in particular, the oral non KS tissues, arose from HHV-8 DNA that was already present in tissue samples prior to the study.

That HHV-8 may be present in oral non KS, albeit pathological, tissues of people who are HIV infected points to the oral mucosa of such individuals as a site of

HHV-8 infection. The pleiotropism of HHV-8 in the mouth of HIV-positive individuals is similar to that of CMV (Jones et al., 1993) and contrasts with EBV, which preferentially replicates in the tongue (Greenspan et al., 1985). However, unlike CMV and EBV, it is unclear if the oral mucosa can support active HHV-8 replication. Comprehensive PCR studies of saliva, and of viral RNA and protein expression in tissues, will be necessary to establish if saliva is a vehicle of HHV-8 transmission.

HHV-8 DNA was detected exclusively in oral tissues of people with HIV infection but not in those who were presumed to be uninfected. As this study has not defined which cell types in the oral compartment were infected, the possibility that the detection of the viral genome in the oral tissues is due to the presence of HHV-8-infected mononuclear cells (Whitby et al., 1995) cannot be excluded. However, <10% of HIV-positive patients without KS are positive for HHV8 DNA in their peripheral blood mononuclear cells (Whitby et al., 1995); this contrasts with the 70% positivity rate in the oral non KS tissues examined here. It is hence unlikely that the high positivity rate found in the oral tissues has been entirely due to infiltration by HHV-8-infected blood cells. An in situ hybridisation study has showed the presence of HHV-8 in vascular endothelial cells and perivascular spindle-shaped cells of Kaposi's sarcoma lesions (Li et al., 1996) but which cells in the oral mucosa harbour HHV-8 DNAhas yet to be defined precisely

The presence of HHV-8 in oral tissues may be related to the immunosuppressive state that follows HIV infection. That immunosuppression per se may heighten the activity of HHV-8 is suggested by the relatively quick appearance of KS lesions in patients who undergo immunosuppressive therapy and their resolution

after discontinuation of therapy (Martin et al., 1993). However, as HIV-1 proviral DNA can be located in oral epithelial cells (Qureshi et al., 1995), HIV may play a more direct role, e.g., by heterologous (HIV-HHV-8) transactivation, in promoting HHV-8 infection in the oral compartment. To confirm this, an examination of the state of activation of HIV-1 in oral epithelial cells and the precise localisation of HHV-8 to the oral epithelium will be needed.

The absence of EBV DNA in the oral tissues examined was surprising. The small sample size and the consequent susceptibility to sampling errors are probable contributory factors. Furthermore, none of the samples showed histologic changes suggestive of OHL. The absence of DNA from the other herpesviruses known to be shed into saliva is less remarkable, since they are not considered to persist to any significant extent in the oral mucosa of people who are HIV-infected. In contrast, the finding that HHV-8 DNA is found in the majority of this small set of tissues does underscore the prominence of HHV-8, relative to the other herpesviruses, in oral tissues of HIV-positive patients.

Diversity in the HHV-8 DNA sequences derived from the study samples was demonstrated: 11 out of 25 sequences were different from each other. Polymorphism in this region of the viral genome has also been observed in a study of American and African KS patients (Huang et al., 1995), and another study (Cesarman et al., 1995) has demonstrated the hypervariability of $KS330_{233}$ fragment when compared to other regions in the 965-bp ORF. Sequence analysis of this short segment of ORF 26 may allow the molecular epidemiology of HHV-8 to be studied in archived tissues.

4. HHV-8 VARIANTS IN SARCOID AND ORAL GRANULOMATOUS TISSUES

4.1. Introduction

4.1.1. Sarcoidosis

Sarcoidosis has been described in histopathological terms as a systemic disorder characterised by the presence in multiple tissues of non-caseating epithelioid-cell granulomas, which may spontaneously resolve or convert to hyaline connective tissue (Mitchell et al., 1977). In the active phase of the disease, activated macrophages aggregate at several tissue sites and coalesce as giant and epithelioid cells (Thomas et al., 1987). Also accumulating at these sites are $CD4^+$ cells, which belong to the T_H 1 subset (Kawakami et al., 1995) and show restriction in their use of T-cell receptor genes (Silver et al., 1996), indicating stimulation by a limited repertoire of antigens. Clinical expressions of sarcoidosis are variable and can be protean, but radiologically the disease most frequently presents as bilateral hilar lymphadenopathy with or without pulmonary mottling (Scadding et al., 1985; James, 1994).

Sarcoidosis is an enigmatic disease. For many years, the sarcoid granulomatous response has been thought to result from an immune action mounted to sequestrate foreign agents (Thomas et al., 1987; Scadding et al., 1985; James, 1994). That this disease may have an infectious origin can be inferred from observations of space-time clusters of sarcoidosis among nurses in the Isle of Man (Hills et al., 1987) and fire-fighters in Rhode Island (Kern et al., 1993), an outbreak of acute sarcoid arthritis in Norfolk (Jawad et al., 1989), and transmission of sarcoidosis following cardiac (Burke et al., 1990) and bone marrow

transplantation (Heyll et al.,1994). Animal experiments provide further evidence for a transmissible agent. In these, homogenates prepared from sarcoid tissues induced granulomas at sites of injection in the footpads of mice (Mitchell et al., 1969; Taub et al., 1974; Mitchell et al., 1976); tissue supernates that had passed through a 0.2 μm filter also led to granuloma formation, while γ-irradiation and freezing (at -20°C but not -70°C) of the homogenates abolished the effect (Mitchell et al., 1976).

Alveolar macrophages have been shown to play a pivotal role in orchestrating inflammatory cell accumulation, granuloma formation and fibrogenesis (Prior et al., 1996). This activation is largely effected by the release of proinflammatory mediators, such as interleukin (IL)-1, tumour necrosis factor-alpha (TNF-α), IL-6, and granulocyte-macrophage colony-stimulating factor (GM-CSF). Prior et al. (1996) showed in their *in-vitro* study that unstimulated sarcoid PBMCs and broncoalveolar lavage (BAL) cells, from 17 patients with sarcoidosis not ungoing under any drug treatment, produced more IL1β, TNF-α, IL-6 and GM-CSF than PBMCs of the study control population (Prior et al., 1996). Another study showed that IL-6 concentration was expressed at high levels from PBMCs and BAL of patients at the stage of active sarcoidosis (Homolka and Muller-Quernhein, 1993). Zheng et al. (1995) demonstrated that the amount of TNF-α spontaneously released by alveolar macrophages correlated positively with the phenotypic expression of CD4 (a surface marker specific to T helper/inducer cell subset), CD25 (the IL-2 receptor, expressed by activated T cells), CD14 (markers found in monocytes and macrophages), VLA-4, RFD1 and 27E10. VLA-4 is a

member of the beta-1 subfamily of integrins, and serves as one of the receptors for fibronectin, a ligand for vascular-cell adhesion molecule-1 (VCAM-1) expressed in cytokine-activated endothelial cells. RFD1 recognizes a unique MHC Class II antigen that may be specifically expressed by dendritic cells; a previous study showed that patients with sarcoidosis showed higher percentages of macrophage-like cells in PBCs expressing RFD1 than did normal subjects (Ainslie et al., 1989). 27E10 is a marker for macrophages that is frequently expressed in acute inflammatory lesions but largely or completely absent in chronic inflammation (Bhardwaj et al., 1992).

The aetiology of sarcoidosis is puzzling. A wide variety of agents has been implicated as possibly providing the antigens that elicit the sarcoid granulomatous response. Candidates include clay soil, pine tree pollen, mycoplasma and mycobacteria (Sharma et al., 1986; Johnson et al., 1996). The role of *Mycobacterium tuberculosis*, and other mycobacteria, is the subject of many studies and considerable debate (Mangiappan et al., 1995; Almenoff et al., 1996; El Zaatari et al., 1996). Freidig and colleagues studied 419 lymph nodes biopsies with various histopathological diagnosis found 93 with a diagnosis compatible with sarcoidosis but showed that all were negative for any mycobacterial pathogen after lymph node culture (Freidig et al., 1986). Granulomatous formation has been observed to follow infection by herpesviruses like varicella zoster virus and herpes simplex virus type 1 (Packer et al., 1984; Shieder et al., 1986; Fisher et al., 1987; Holbach et al., 1990), and in some cases, sarcoid-type granulomas are seen (Bisaccia et al., 1983; Wright et al., 1989; Redondon et al., 1992; Chang et al.,

1994). Specific associations between granulomatous diseases and herpesviruses are very rare, however.

4.1.2. Oral granulomatous disorders

The term orofacial granulomatosis (OFG) has been used to describe a clinicopathologic disorder characterised by recurrent or persistent swelling of the facial or oral tissue associated with the development of non-caseating granulomas (Wiesenfeld et al., 1985). It unifies previously recognised clinical entities such as Melkersson-Rosenthal syndrome (MRS), Miescher's chronic granulomatosis cheilitis, inflammatory bowel disease localised to the oral cavity (notably Crohn's disease), oral sarcoidosis, and oral mycobacterial infection (Wiesenfeld et al., 1985).

Melkersson-Rosenthal syndrome (MRS) was first described by a Swedish neurologist in 1928 as a triad comprising persistent orofacial swelling, peripheral facial nerve palsy and a fissured tongue. Not all these symptoms are required for a diagnosis, however, as it is uncommon for all symptoms of MRS to occur simultaneously (New et al., 1933; Klaus et al., 1959).

Miescher's granulomatous cheilitis is an isolated non-remissive labial swelling of one or both lips (Tylesley et al., 1979). It is probably oligosymptomatic of MRS (Worsaae et al., 1982; Hornstein, 1973; Klaus et al., 1959) since histopathological features of both conditions are identical, these features being

granulomatous lesions, nonspecific inflammatory changes and dilated lymphatics (Miescher, 1945).

Crohn's disease was first described in 1932 as a chronic, panenteric intestinal inflammatory disease (Tyldesley, 1979). It may have two clinical manifestations: one that is aggressive and perforating, and the other that is indolent and non-perforating. Patients with the perforating variety present with abscesses or perforation or both, and is associated with a higher reoperation rate; in contrast, non-perforating Crohn's disease is associated with obstruction and bleeding (Mishina et al., 1996). Clinical symptoms associated with the non-perforating variety include abdominal pain, diarrhoea, weight loss, vomiting, fever and rectal bleeding.

Granulomatous changes in Crohn's disease are found in the affected gut as part of a transmural inflammatory process (Tylesley et al., 1979). They are not confined to the small intestine but may be found in all parts of the gut from mouth to anus. However oral lesions may antedate bowel lesions by several years (Williams et al., 1991; Carr, 1974; Morgan et al., 1987; Tylesley et al., 1979; Field et al., 1989) and may be the only obvious site of disease (Scully et al., 1982); therefore its detection may lead to an earlier diagnosis (Ivanyi et al., 1993). Oral lesions can also occur later in established Crohn's disease (Croft et al., 1972; Alpert et al., 1974; Carr, 1974; Basu et al., 1975).

Whether OFG can progress to Crohn's disease is controversial. Worsaae and colleagues (1982) did not observe signs of Crohn's disease in his study of 33 patients with OFG, while Field and colleagues (1989) reported that 16% of 62

OFG patients developed Crohn's disease within 10 years; Moreover, Wiesenfeld et al. (1985) found that 10% of 60 patients already had evidence of the disease at the time of presentation. Scully et al. (1982) showed that 37% of his study population with OFG but no gastrointestinal symptoms showed Crohn's-like changes detected after rectal biopsy and radiology (Scully et al., 1982). Similar results were found by Wiesenfeld and colleagues (1985), who reported that 22 out of 60 patients had no gastrointestinal symptoms or evidence of gastrointestinal disease.

Sarcoidosis was found to be associated with OFG in 3% of a study population (Wiesenfeld et al., 1985), involving oral and facial tissues, primarily the lips. In addition to the features of orofacial granulomatosis, patients with sarcoidosis may present with other lesions on the face, such as parotid and submandibular salivary gland enlargement, lacrimal gland enlargement and thickening of nasal mucosae. The characteristic corrugated buccal mucosa in Crohn's disease seems to be uncommon in oral sarcoidosis (Tyldesley, 1979). Nevertheless, histologic changes are similar. Definitive diagnosis depends on presence of non-caseating granulomas in the lungs or other organs.

The aetiology of OFG and of the other granulomatous disorders is unclear and several hypotheses have been suggested. Hornstein (1973) suggested that MRS and cheilitis granulomatosa arise from a hereditary or acquired disposition to disturbances of autonomic nervous system that produces increased permeability of blood vessels in facial skin. The granulomatous reaction in the oedematous tissue is considered to result from an allergic response to unspecified circulating antigens (Hornstein, 1973). In those cases not associated with inflammatory bowel disease

or sarcoidosis, there are a number of reported cases in which parent or siblings are affected (Rosenthal et al., 1931, New et al., 1933, Ekbom, 1950, Levenson et al., 1984, Carr, 1966). Familial disposition with incomplete autosomal dominance and variable penetrance has also been reported (Vistness et al., 1971; Wieselfeld et al., 1985; Levenson et al., 1984; Lygidakis et al., 1979; Worsaae et al., 1982). Smeets et al. (1994) found evidence for autosomal dominance in a mother and child with symptoms and signs of MRS.

There is some evidence for an allergic predisposition in OFG patients. Sixty per cent in one OFG study population associated with definitive or possible Crohn's disease and 59% of patients with no evidence of Crohn's disease or sarcoidosis were atopic, with a history of infantile eczema, allergy or asthma (James et al., 1986). There was a similarly high incidence of atopy in oral Crohn's patients as well as their first degree relatives, compared with a normal population rate of 15 % (James et al., 1986).

A relationship between dietary components and the development of orofacial disorders has also been suggested. (Lamey et al., 1990). Patton et al. (1985) found evidence of intolerance to specific foods in 18 % of their patients with OFG. However, a more recent study found that none of 6 patients with OFG were sensitive to food additives or contactants (Morales et al., 1995). Nevertheless, food intolerance is frequently found in patients with inflammatory bowel disease and the use of low allergen diets has resulted in a significant improvement in some patients (Jones et al., 1985; Hunter et al., 1983; O'Morain et

al., 1984). Furthermore, toothpaste has been implicated in Crohn's disease (Sullivan, 1990; Ben Schlomo et al., 1990; Braegger et al., 1990)

Finally, infection causes have been postulated. Elevated serum IgG antibody levels to mycobacterial stress proteins have been reported in 4/4 patients with Crohn's disease and in 3/6 patients with OFG (Ivanyi et al., 1993). Ten of eleven patients were responsive to penicillin injections, and so evidence of infection with spirochetes has been postulated (Liu et al., 1993).

4.2. Material and Methods

4.2.1. Tissue samples

4.2.1.1. Sarcoidosis

PEFF tissues derived from transbronchial, lymph node and skin biopsies performed to substantiate sarcoidosis in 15 patients attending the University Hospital of Chieti were studied. In addition, an oral biopsy specimen each from 2 patients with oral ulceration presenting to the University Hospital of Bari were examined. All 17 patients had chest X-ray changes suggestive of intrathoracic sarcoidosis and all were Kveim test-positive. Specimens were included for study if histologically they contained multiple granulomas that were predominantly non-necrotizing and were negative for acid-fast bacilli and fungi after Ziel-Neelsen, periodic acid-Schiff and Gomori's methenamine silver staining. As negative controls, PEFF tissues of Italian patients with pathologies other than sarcoidosis, from the lung, lymph node, skin and oral mucosa, were used. PEFF skin biopises of

Italian patients with classic Kaposi's sarcoma and post-nasal space biopsies of patients with nasopharyngeal carcinoma were used as controls for HHV-8 and EBV DNA amplification, respectively. Sputum samples stained positive for acid-fast bacilli referred from London patients with suspected tuberculosis were included as controls for mycobacterial DNA detection.

The study was conducted in 3 phases. In the first, HHV-8 DNA was sought from tissue sections from 37 biopsies of the 15 sarcoidosis patients from Chieti, Italy. When 36 specimens were found positive, another set of sections cut from the same 37 tissue blocks, and 39 control tissues were despatched from Chieti. A technical staff member in Chieti coded this set of specimens without revealing the code (until after testing) to me. The 2 specimens from oral sarcoidosis patients from Bari were also despatched. Sections were examined for HHV-8, mycobacterial and EBV DNA during this second phase. Nucleotide sequences of HHV-8 and mycobacterial amplicons were also characterized at this stage. In the third phase, sections from 10 sarcoid tissues from Chieti (a subset of the 37 originally examined) were batched with a further 61 non-sarcoid tissues, similarly coded and despatched to Prof. SR Porter, who then sent the specimens to me to examine for the presence of HHV-8, mycobacterial and EBV DNA. Also redespatched were coded sections from the 2 oral sarcoid tissues from Bari, together with 13 oral controls. In all, for each pathogen, sarcoid tissues were tested at least 4 times and non-sarcoid tissues twice.

4.2.1.2. Oral granulomatous disorders

PEFF archived tissues of 17 incisional biopsy specimens taken from 17 patients with facial or oral swelling in which non-caseating granulomas were histologically evident were used. As controls, 21 oral biopsy samples from patients with various other, non-granulomatous oral diseases were used. All samples were taken from patients who attended the Department of Oral Medicine of the Eastman Dental Institute for Oral Health Care Science in London. All laboratory testing for HHV-8 was undertaken while blinded to the nature of the study specimens.

4.2.2. DNA extraction

Thirty μm sections were cut from each tissue block and deparaffinized and DNA was extracted as described in chapter 2. DNA from sputa containing acid-fast bacilli taken from patients subsequently confirmed by sputum culture to have been infected with *M. tuberculosis* was extracted using phenol-chloroform.

4.2.3. PCR amplification

A nested PCR approach was used to amplify HHV-8 ORF26 DNA as described in chapter 2. To amplify mycobacterial DNA, we used a heminested PCR procedure, described in chapter 2. Sterile water was used as a negative control in each PCR reaction. As positive controls for HHV-8, mycobacterial and EBV DNA amplification respectively, appropriate dilutions of pHHV-8, chromosomal DNA

from the H37Rv strain of *M. tuberculosis* and DNA extracted from the C15 tumour (Busson et al., 1987) were used in each reaction. Extracts were also subjected to PCR to amplify a 110-bp segment of the β-globin gene (Saiki et al. 1985).

4.2.4. PCR-SSCP assay

HHV-8 and mycobacterial amplicons were processed to the ^{32}P-based SSCP assay, exactly as described in Chapter 2, to scan for sequence differences. Tissue extracts in which DNA sequences were amplified and observed in the PCR-SSCP assay to produce different band patterns were reamplified and analysed in a second PCR-SSCP gel run. This was in order to exclude *Taq* polymerase-induced misincorporation as a cause of the differences observed (Eckert et al., 1991).

4.2.5. DNA sequencing

HHV-8 PCR products that yielded differential banding profiles in the PCR-SSCP assay and representative HHV-8 amplificates that yielded identical band patterns were submitted for DNA sequencing, as described in chapter 2. Mycobacterial amplicons were similarly sequenced, and the sequences of the V2 hypervariable region (Neefs et al., 1991) were compared with those lodged with the University of Illinois rRNA database.

4.2.6. Further analysis of HHV-8 sequences

HHV-8 sequences (171 bp in length, not including the inner primer sequences) were aligned against HHV-8 sequences reported in the literature and analysed for relatedness using the Clustal algorithm in the MEGALIGN program of the LASERGENE System (DNASTAR Inc., Madison, WN). Categorisation of the HHV-8 sequences into genomic groups was also done as in chapter 2.

4.2.7. Statistical analysis

Observations from all study phases of sarcoidosis were combined. Data were analysed with Chi square or Fisher's exact test (2-tailed).

4.3. Results

4.3.1. Sarcoidosis

Characteristics of the patients from whom tissues with sarcoidosis-like changes were obtained and the histologic features of the tissues are summarised in Table 4.1. The majority of the tissues showed no or very minimal necrosis, and the extent of fibrosis was variable. Seventeen (44%) and 15 (38%) contained asteroid and Schaumann bodies, respectively.

The relative PCR detection frequencies of HHV-8 and mycobacterial DNA in sarcoid and non-sarcoid tissues are shown in Table 4.2. For HHV-8, only data from amplification of the ORF26 fragment are presented in this table. Of the 38 specimens found positive for ORF26 DNA, 31 (82%) were also positive for ORF25 DNA. The 12 specimens from 8 patients that were found positive by nested PCR for ORF26 in the second phase again tested positive in the third, blinded phase. Sarcoid tissues from each of the body sites sampled had significantly higher prevalence of HHV-8 ORF26 sequences than non-sarcoid tissues (P values obtained after comparing between pulmonary, lymphoid, dermal and oral tissues were <0.0001, <0.0001, 0.006 and 0.029, respectively). The prevalence of mycobacteria sequences was significantly higher in sarcoid lymph node tissues (P=0.003), while the differences observed with sarcoid and non-sarcoid tissues from other sites were not significant. Overall, the prevalence of HHV-8 ORF26

Patient characteristics					Histologic characteristics					
Patient	**Age**	**Sex**	**Chest X-Ray Staging**		**Specimen Code**	**Site of Biopsy**	**Necrosis**	**Fibrosis**	**Asteroid Cells**	**Schaumann Bodies**
A	44	F	I		A1	Scalene lymph node	N	A	No	Yes
					A2	Scalene lymph node	N	A	No	No
					A3	Mediastinal lymph node	N	B	Yes	No
					A4	Mediastinal lymph node	N	A	Yes	Yes
B	56	F	II		B1	Scalene lymph node	N	N	No	No
					B2	Mediastinal lymph node	N	N	No	Yes
					B3	Lung	A	B	No	No
					B4	Lung	N	N	Yes	No
C	43	M	III		C1	Mediastinal lymph node	N	C	Yes	No
D	48	F	III		D1	Mediastinal lymph node	N	C	No	No
					D2	Retroperitoneal lymph node	B	B	Yes	No
					D3	Mediastinal lymph node	N	A	Yes	No
					D4	Mediastinal lymph node	N	B	Yes	Yes
					D5	Lung	N	C	No	Yes
					D6	Lung	N	C	Yes	No
E	75	F	II		E1	Mediastinal lymph node	A	A	Yes	Yes
					E2	Scalene lymph node	A	A	Yes	Yes
					E3	Mediastinal lymph node	A	A	Yes	Yes
F	63	F	I		F1	Mediastinal lymph node	A	A	No	Yes
G	65	F	I		G1	Skin	N	N	No	No
H	49	F	II		H1	Mediastinal lymph node	A	A	No	Yes
					H2	Mediastinal lymph node	A	A	Yes	Yes
					H3	Scalene lymph node	N	B	No	Yes
I	86	F	I		I1	Mediastinal lymph node	A	N	Yes	Yes
J	60	M	II		J1	Mediastinal lymph node	N	B	No	No
L	58	F	I		L1	Mediastinal lymph node	N	A	No	No
					L2	Scalene lymph node	N	A	Yes	No
M	63	F	III		M1	Lung	A	C	No	No
					M2	Lung	A	N	No	No
N	45	F	I		N1	Skin	N	N	No	No
O	71	F	II		O1	Mediastinal lymph node	A	A	No	No
					O2	Mediastinal lymph node	N	A	Yes	No
					O3	Mediastinal lymph node	A	B	No	Yes
					O4	Scalene lymph node	N	A	No	No
P	30	F	III		P1	Lung	N	B	Yes	No
					P2	Lung	A	C	Yes	Yes
					P3	Mediastinal lymph node	A	C	No	No
Q	36	M	III		Q1	Inner cheek	N	A	No	No
R	44	F	III		R1	Inner cheek	N	A	No	No

Table 4-1 Characteristics of sarcoidosis study patients and their tissue specimens

Extent of fibrosis and necrosis, as determined by microscopic examination (2.5 X magnification) of whole sections, is as follows: N: absence; A: 0-25%; B: 25-50%; B: >50%.

Table 4-2 Frequency of HHV-8, mycobacterial and EBV DNA sequences in sarcoid and non sarcoid tissues.

Type of lesion	Total specimens	Total patients	No. HHV-8 DNA positive	No. mycobacterial DNA positive	No. EBV DNA positive
SARCOID TISSUES					
Pulmonary	8	4	8	3	0
Lymphoid	27	11	26	11	0
Dermal	2	2	2	2	0
Oral	2	2	2	1	0
NON SARCOID TISSUES					
Pulmonary	54	54	0	22	12
Lung cancer	22	22	0	18	2
Non-granulomatous	17	17	0	2	3
alveolitis	2	2	0	0	1
Granulomatous alveolitis	6	6	0	0	0
Emphysema	1	1	0	0	0
Atelectasia	6	6	0	2	6
Normal mucosa					
Lymphoid	29	29	6	1	0
Non-specific lymphadenitis	17	17	5	1	0
B cell lymphoma	12	12	1	0	0
Dermal					
Basal cell carcinoma	17	17	0	15	2
Oral	22	22	1	0	0
Squamous cell carcinoma	9	9	0	0	0
Control tissues	13	13	1	0	0
OTHER CONTROL TISSUES					
Nasopharyngeal carcinoma	3	3	0	1	3
Mediterranean Kaposi's sarcoma	16	16	10	1	0
Tuberculosis sputum samples	6	6	0	6	0

sequences in all sarcoid tissues (38/39) was significantly higher than in non-sarcoid tissues (6/113) ($P<0.0001$); that of mycobacteria-like sequences in the two tissue groups (17/39 vs. 39/113) was not significant.

When patients whose tissues were studied in the blinded phase were included as units of analysis, 8/8 sarcoidosis patients compared to 3/56 controls were HHV-8 ORF 26 DNA-positive ($P<0.0001$). By contrast, 3/8 sarcoidosis patients compared to 4/56 controls were positive for mycobacteria-like sequences ($P=0.0364$).

EBV DNA was amplified in none of sarcoid, 8 of non-sarcoid (6 pulmonary; 2 dermal) and all 3 nasopharyngeal carcinoma tissues.

The 38 HHV-8 ORF26 sequences amplified from sarcoid tissues were diverse. This is illustrated in Figure 4.1, which shows the autoradiographic appearance following an SSCP assay of 10 of the amplified sequences; here, none of samples (except the plasmid controls) gave rise to identical banding patterns, reflecting DNA sequences unique to each. The extent of diversity is depicted in the dendrogram in Figure 4.2, which compares the 38 sarcoid tissue-derived HHV-8 sequences with 71 others reported previously (Cesarman et al., 1995; Huang et al., 1995; Moore et al., 1995; Luppi et al., 1996). The majority of sequences (N=28) were identical to those previously characterised. They bore base changes at certain preferred positions, which allow their assignment into 5 groups based on predicted changes in the amino acid sequence (Table 3.1). The molecular basis of grouping is summarised in Table 3.1, and group assignments are shown in Table 4.3. In 10

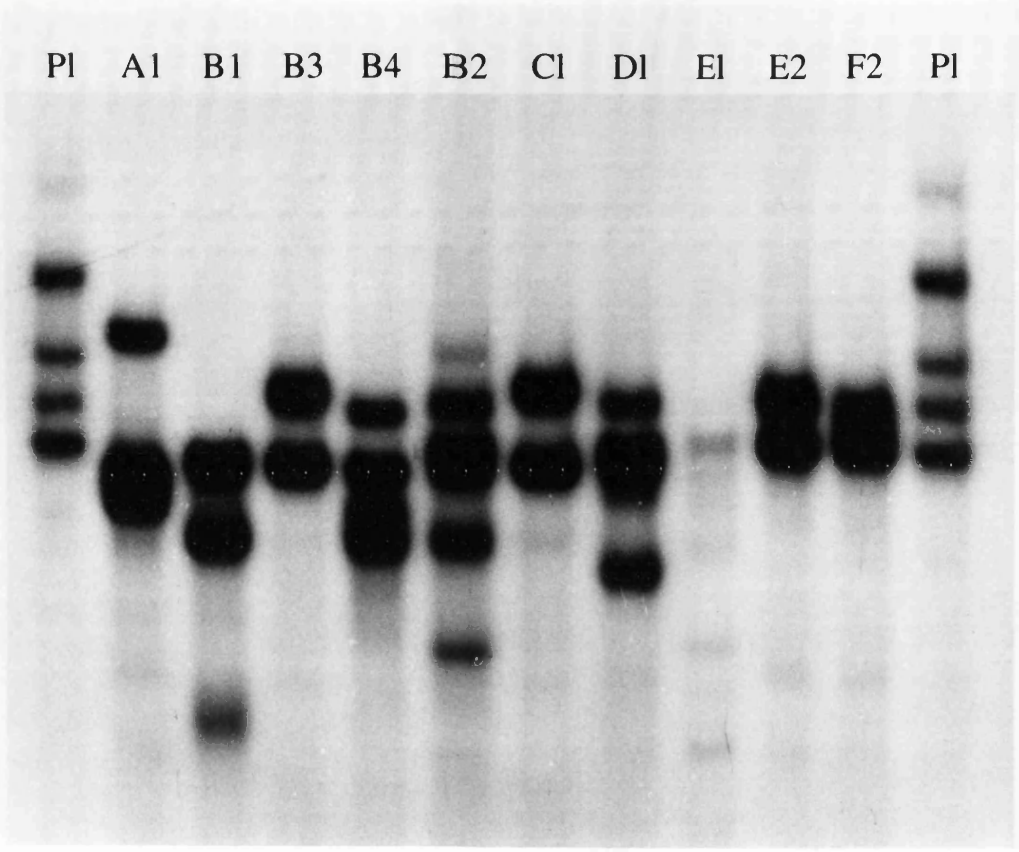


Figure 4-1 SSCP profile of HHV-8 ORF26 DNA amplified from sarcoid tissues.

Autoradiographic banding patterns produced from a representative nondenaturing gel in which was electrophoresed single-stranded PCR products of HHV-8 sequences. Specimen codes are as indicated. Pl denotes plasmid (pHHV-8).

Different banding patterns predict differences in nucleotide sequences.

Table 4-3 Assignment of HHV-8 and mycobacterial DNA sequences into groups and species

SPECIMEN CODE	KSHV GROUP	LOCATION OF BASE SUBSTITUTIONS IN UNIQUE KSHV SEQUENCES	MYCOBACTERIAL SPECIES
A1	B	-	species unidentified
A2	E	-	(negative)
A3	C	-	(negative)
A4	C	-	related to *M. cookii*
B1	A	codon 152 (c→t, 1st position);	
		codon 167 (g→a, 2nd position)	species unidentifie[d]
B2	A	-	(negative)
B3	C	codon 146 (c→g, 2nd position);	
		codon 152 (c→t, 1st position)	species unidentified
B4	C	-	species unidentified
C1	C	codon 169 (g→a, 1st position)	species unidentified
D1	B	codon 140 (g→a, 1st position);	
		codon 175 (t→c, 3rd position)	related to *M.fortuitum*
D2	C	-	(negative)
D3	C	-	(negative)
D4	C	-	species unidentified
D5	C	codon 181 (t→c, 2nd position)	(negative)
D6	C	-	(negative)
E1	B	-	species unidentified
E2	B	-	(negative)
E3	C	-	(negative)
F1	B	-	(negative)
G1	C	-	species unidentified
H1	C	-	(negative)
H2	C	-	(negative)
H3	C	codon 157 (c→g, 3rd position);	
		codon 168 (t→a, 3rd position)	(negative)
I1		-	related to *M. cookii*
J1	C	-	species unidentified
L1	C	-	(negative)
L2	C	-	(negative)
M1	C	-	species unidentified
M2	C	-	(negative)
N1	C	codon 134 (c→t, 3rd position)	species unidentified
O1	E	-	(negative)
O2	C	codon 134 (c→t, 3rd position);	
		codon 168 (t→a, 3rd position)	(negative)
O3	C	-	(negative)
O4	C	-	species unidentified
P1	C	-	(negative)
P2	B	-	(negative)
P3	A	codon 134 (c→a, 1st position)	related to *M.fortuitum*
Q1	B	-	(negative)
R1	B	codon 134 (c→t, 3rd position)	species unidentified

Table 4.3. Assignment of HHV-8 and mycobacterial DNA sequences into groups and species, respectively, and identification of base substitutions in unique HHV-8 species

Dashes denote no changes other than in group-specifying positions (see Fig. 4.3). Small-case single letters refer to nucleotides. The reference HHV-8 nucleotide sequence is that of the prototype (Chang et al., 1994). [a]refers to species with an identical V2 sequence; mycobacterial species amplified from all other positive specimens possess unique V2 sequences.

sequences (amplified from B1, B3, C1, D1, D5, H3, N1, O2, P3 and R1), nucleotide substitutions occurring at locations other than the group-specifying positions were also observed; these base changes are identified in Table 4.3.

Figures 4.1 and 4.2 also show that in 7 of 9 patients from whom multiple tissue sites were biopsied (A, B, D, E, H, O and P), different HHV-8 ORF26 sequences could be recovered from each biopsy. Figure 4.1 illustrates that for patient B, HHV-8 sequences amplified from the scalene and mediastinal lymph nodes, and from the two transbronchial tissues were disparate; for patient E, the sequence amplified from the scalene node was different to that from the mediastinal node. Figure 4.2 displays a fuller representation of the multiplicity of sequences recovered from tissues of all the 7 patients.

A variety of mycobacteria-like 16S rRNA sequences could be identified in 14 of the 18 amplicons derived from sarcoid tissues. A number bore unique sequences in the V2 hypervariable region. For the purpose of this study, a mycobacterial sequence that differed in the V2 region by no more than two nucleotides from the sequence of the most closely related species was considered related to that species. Sequences that diverge by more than two bases were considered to belong to uncharacterised mycobacterial species. Table 4.3 shows the species assignment of the sequences amplified from sarcoid tissues. HHV-8 group assignment is based on sequence differences shown in Table 3.1.

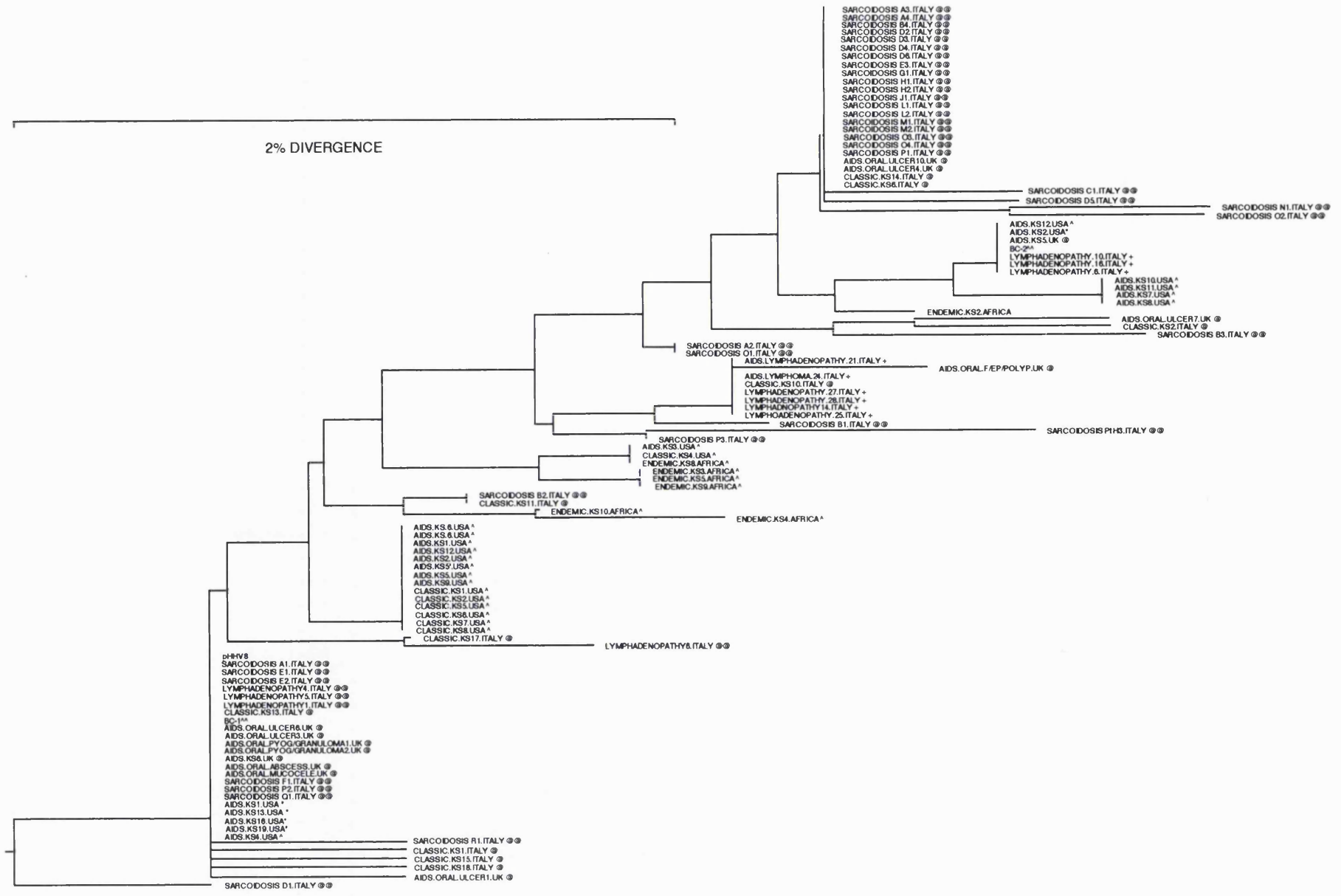

2% DIVERGENCE
SARCOIDOSIS A3.ITALY @@
SARCOIDOSIS A4.ITALY @@
SARCOIDOSIS B4.ITALY @@
SARCOIDOSIS D2.ITALY @@
SARCOIDOSIS D3.ITALY @@
SARCOIDOSIS D4.ITALY @@
SARCOIDOSIS D6.ITALY @@
SARCOIDOSIS E3.ITALY @@
SARCOIDOSIS G1.ITALY @@
SARCOIDOSIS H1.ITALY @@
SARCOIDOSIS H2.ITALY @@
SARCOIDOSIS J1.ITALY @@
SARCOIDOSIS L1.ITALY @@
SARCOIDOSIS L2.ITALY @@
SARCOIDOSIS M1.ITALY @@
SARCOIDOSIS M2.ITALY @@
SARCOIDOSIS O3.ITALY @@
SARCOIDOSIS O4.ITALY @@
SARCOIDOSIS P1.ITALY @@
AIDS.ORAL.ULCER10.UK @
AIDS.ORAL.ULCER4.UK @
CLASSIC.KS14.ITALY @
CLASSIC.KS6.ITALY @
SARCOIDOSIS C1.ITALY @@
SARCOIDOSIS D5.ITALY @@
SARCOIDOSIS N1.ITALY @@
SARCOIDOSIS O2.ITALY @@
AIDS.KS12.USA^
AIDS.KS2.USA*
AIDS.KS5.UK @
BC-2^
LYMPHADENOPATHY.10.ITALY +
LYMPHADENOPATHY.16.ITALY +
LYMPHADENOPATHY.6.ITALY +
AIDS.KS10.USA^
AIDS.KS11.USA^
AIDS.KS7.USA^
AIDS.KS8.USA^
ENDEMIC.KS2.AFRICA
AIDS.ORAL.ULCER7.UK @
CLASSIC.KS2.ITALY @
SARCOIDOSIS B3.ITALY @@
SARCOIDOSIS A2.ITALY @@
SARCOIDOSIS O1.ITALY @@
AIDS.LYMPHADENOPATHY.21.ITALY +
AIDS.ORAL.FÆP/POLYP.UK @
AIDS.LYMPHOMA.24.ITALY +
CLASSIC.KS10.ITALY @
LYMPHADENOPATHY.27.ITALY +
LYMPHADENOPATHY.28.ITALY +
LYMPHADNOPATHY.14.ITALY +
LYMPHOADENOPATHY.25.ITALY +
SARCOIDOSIS B1.ITALY @@
SARCOIDOSIS P1H3.ITALY @@
SARCOIDOSIS P3.ITALY @@
AIDS.KS3.USA^
CLASSIC.KS4.USA^
ENDEMIC.KS8.AFRICA^
ENDEMIC.KS3.AFRICA^
ENDEMIC.KS5.AFRICA^
ENDEMIC.KS9.AFRICA^
SARCOIDOSIS B2.ITALY @@
CLASSIC.KS11.ITALY @
ENDEMIC.KS10.AFRICA^
ENDEMIC.KS4.AFRICA^
AIDS.KS.6.USA^
AIDS.KS.6.USA^
AIDS.KS1.USA^
AIDS.KS12.USA^
AIDS.KS2.USA^
AIDS.KS5*.USA^
AIDS.KS5.USA^
AIDS.KS9.USA^
CLASSIC.KS1.USA^
CLASSIC.KS2.USA^
CLASSIC.KS5.USA^
CLASSIC.KS6.USA^
CLASSIC.KS7.USA^
CLASSIC.KS8.USA^
CLASSIC.KS17.ITALY @
LYMPHADENOPATHY6.ITALY @@
pHHV8
SARCOIDOSIS A1.ITALY @@
SARCOIDOSIS E1.ITALY @@
SARCOIDOSIS E2.ITALY @@
LYMPHADENOPATHY4.ITALY @@
LYMPHADENOPATHY5.ITALY @@
LYMPHADENOPATHY1.ITALY @@
CLASSIC.KS13.ITALY @
BC-1^
AIDS.ORAL.ULCER6.UK @
AIDS.ORAL.ULCER3.UK @
AIDS.ORAL.PYOG/GRANULOMA1.UK @
AIDS.ORAL.PYOG/GRANULOMA2.UK @
AIDS.KS6.UK @
AIDS.ORAL.ABSCESS.UK @
AIDS.ORAL.MUCOCELE.UK @
SARCOIDOSIS F1.ITALY @@
SARCOIDOSIS P2.ITALY @@
SARCOIDOSIS Q1.ITALY @@
AIDS.KS1.USA *
AIDS.KS13.USA *
AIDS.KS16.USA*
AIDS.KS19.USA*
AIDS.KS6.USA^
SARCOIDOSIS R1.ITALY @@
CLASSIC.KS1.ITALY @
CLASSIC.KS15.ITALY @
CLASSIC.KS18.ITALY @
AIDS.ORAL.ULCER1.UK @
SARCOIDOSIS D1.ITALY @@

Figure 4-2 Phylogenetic relationship of HHV-8 ORF26 sequences derived from sarcoid tissues.

This dendrogram shows how HHV-8 sequences recovered in this study diverge from each other and from sequences previously reported. Each specimen is assigned a number, the geographical location of the patient from which the specimen was derived, and the disease state of the patient. Codes of the specimens studied here are coloured; specimens derived from a given patient share the same colour. Superscript markings denote references to this and other studies, as follows: @Di Alberti et al., 1997(appendix 2); @@this study; *Moore and Chang, 1995; +Luppi et al., 1996; ^Huang et al., 1995; ^^Cesarman et al., 1995.

4.3.2. Oral granulomatous disorders

HHV-8 DNA could be amplified from 17 out of 17 biopsies from OFG patients, and none of 21 non-OFG oral biopsies. PCR-SSCP and DNA sequencing demonstrated that HHV-8 sequences of 8 of the 17 positive tissue samples were unique. A common HHV-8 DNA sequence was identified in 10 samples. Figure 4.3 illustrates sequence polymorphism, as revealed by an PCR-SSCP assay, of HHV-8 DNA amplified from all the OFG study samples. Mycobacterial sequences were found in 2 out of 17 biopsies.

From each of the OFG tissues, amino acid sequences predicted from the inter-primer segment of the amplified ORF26 fragment (171 bp in length, representing 57 codons) were aligned and compared with sequences previously obtained and with sequences reported by other laboratories from various geographical regions. All except one segregated with sequences that had previously been characterised, with the largest group bearing a peptide sequence identical to the BC-1 cell line and a wide range of clinical specimens from North America, Europe and Australia (Figure 4.3). Two sequences were in common to the BC-2 cell line and specimens derived from a predominantly European setting. None of the sequences from the OFG samples clustered with those from Africa.

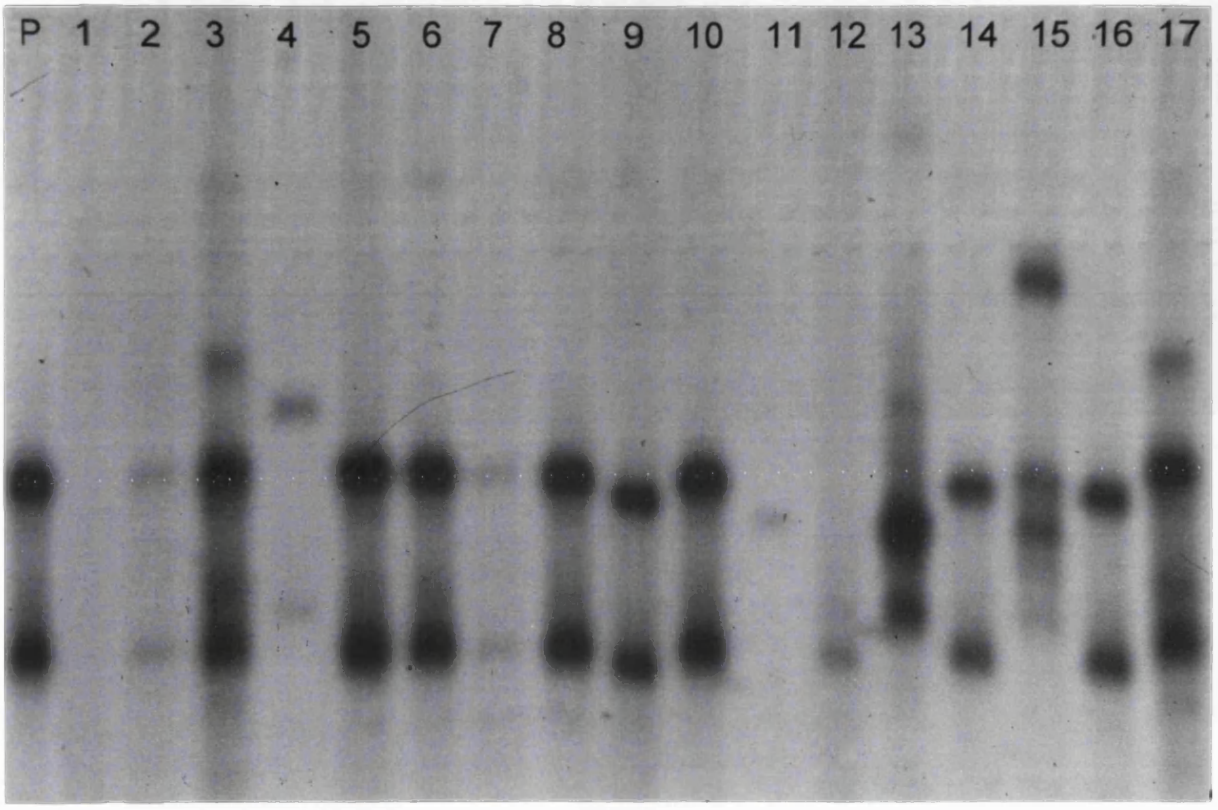


Figure 4-3 PCR-SSCP profile of HHV-8 ORF26 amplified from tissues taken from patients with oral granulomatous disorders.

Autoradiographic banding patterns produced from a representative nondenaturing gel in which was electrophoresed single-stranded PCR products of HHV-8 ORF26 sequences. Specimen codes are as indicated from 1 to 17. Lane P: DNA from BC-1 cells.

Different banding patterns predict differences in nucleotide sequences.

4.4. Discussion

The finding of a very high detection rate of HHV-8 DNA in sarcoid tissues, but not in non-sarcoid tissues (including those with intense inflammatory infiltration) and in OFG but not in normal tissues points to HHV-8 as agent linked to the pathogenesis of these disorders. The high proportion of sarcoid tissues that were positive for HHV-8 DNA after the first round of PCR amplification suggests that they harbour a relative abundance HHV-8 genomes.

Of the granulomatous diseases, sarcoidosis is the one whose epidemiology has been most closely studied. It is a common disease (the world-wide prevalence is estimated to be 10 to 50 per 100,000) (Bresnitz et al., 1983). That HHV-8, whose markers of infection are observed only in certain high-risk groups or particular populations (Whitby et al., 1995; Moore et al., 1996; Kedes et al., 1996; Gao et al., 1996), should be associated with so widespread a disease, therefore seems incongruous. However, these markers relate to viral DNA amplified from peripheral blood cells (Whitby et al., 1995; Moore et al., 1996) and HHV-8-specific antibody detected by first-generation serological tests (Kedes et al., 1996; Gao et al., 1996). When viral sequences are sought in tissues and body fluids other than the blood (Bigoni et al., 1996; Monini et al., 1996; Staskus et al., 1997) and serological testing using more sensitive techniques are conducted (Lennette et al., 1996), HHV-8 infection in populations not at risk for KS has been found to be more prevalent than initially thought.

The very high frequency of HHV-8 DNA detection in the granuloma-containing tissues examined here is unlikely to be due to PCR cross-contamination,

because of the variety of DNA sequences recovered. Ten new HHV-8 variants, all from sarcoid tissues, were discovered in the course of this study. Despite the diversity, the segregation of most sequences to groups B and C accord with the observation in chapter 3 that HHV-8 variants are geographically restricted. The current study indicates that yet another group, E, albeit a rarer one, may also be of European provenance. Further evidence of the geographical restriction of HHV-8 has been independently provided by another laboratory (Zong et al., 1997).

An intriguing finding in this study is the coexistence of HHV-8 variants in individual hosts. As the mutation rate of herpesviruses is slow (Smith et al., 1987), it is unlikely that such multiplicity can be due to evolution from a single strain first transmitted to each host. Multiple infection episodes occurring in the past is a more probable situation. It follows that infection by one HHV-8 variant does not confer immunity to infection by another.

Mycobacterial 16S rRNA sequences were not frequent in the granuloma-containing tissues in this study, except in lymph node tissues. Curiously, the overall frequency of mycobacterial sequences in skin samples was high, but accords with a previous PCR study that reported amplification of sequences of atypical mycobacteria from control as well as sarcoid tissues (Bocart et al., 1992). Nevertheless, the range and extent of atypical mycobacterial sequences recovered in this study are wider. The failure to identify any known species reflects the abundance in nature of species that are commensals and saprophytes.

The complete absence of DNA from *M. tuberculosis* in the lymph node and other study samples here shows that this bacterium cannot be associated with

sarcoidosis in these patients. The lack of data is in agreement with some reports (Gerdes et al., 1992; Ghossein et al., 1994; Richter et al., 1996) but contrasts with others (Saboor et al., 1992; Fidler et al., 1993; Mitchell et al., 1992). It is important to note that other observations do implicate *M. tuberculosis* as a causative agent. In an extension of the mouse footpad experiments, *M. tuberculosis* could be cultured from the viscera of the mice even though the human tissues that served as sources of the inocula were negative in culture (Mitchell et al., 1983). More recently, cell-wall deficient forms, also known as L-forms or protoplasts, of mycobacteria resembling *M. tuberculosis* have been reported to be isolated from the blood of patients with sarcoidosis but not controls (Almenoff et al., 1996). However, another recent study reported from sarcoid skin specimens the long-term culture of, and amplification of sequences specific to, the *M. avium* complex and *M. paratuberculosis*, or both, but not *M. tuberculosis* (El-Zaatari et al., 1996). These findings are difficult to reconcile. An alternative possibility, that coinfection with mycobacteria and HHV-8 may be associated with granulomas, is also not supported by this study, since there was no correlation between HHV-8 and mycobacteria detection in the granulomatous samples. The granuloma response need not, however, be viewed as a consequence of stimulation by antigens presented exclusively by one specific microbe. Rather, disparate exogenous agents can produce similar, if not identical factors that become granulomagenic in genetically predisposed hosts. There is evidence for susceptibility to sarcoidosis as being HLA-associated (Martinetti et al., 1995). The histologic hallmark of sarcoidosis and granulomatous disorders is the presence of

granulomas, which are inflammatory foci largely formed by alpha/beta T-lymphocytes and variably by fibroblasts aggregating around epithelioid or multinucleated cells of macrophage origin.

Since of all the human herpesviruses HHV-8 is the only one that possesses a wide variety of genes potentially expressing homologues of inflammatory and proinflammatory cytokines as vIL-6 (ORF K2), vMIP1α (ORF K4), vMIP1β (ORF K6), vIRF (ORF K9) (Russo et al., 1996; Nicholas et al., 1997; Cesarman et al., 1996), it is conceivable that this virus can induce the granulomas in sarcoidosis and other granulomatous conditions. A possible mechanism is its selective infection of monocytes or macrophages, which, having taken residence in tissue sites then expresses viral products that either act as cytokines or induce cellular production of cytokines, e.g. IL-1, followed by recruitment of CD4+ cells of the TH-1 subset. (Kawakami et al., 1995). Also, increased expression of IL-1β has been reported in non-perforating variant of Crohn's disease (Gilberts et al., 1996).

Circulating levels of IL-6 may also be elevated in patients with sarcoidosis and Crohn's disease but not in these with ulcerative colitis or in control population (Gilberts et al., 1994). This cytokine is known to be involved in the proliferation of Kaposi's sarcoma cells and in the development of KS. IL-6 promotes growth and facilitates maturation of B cells causing immunoglobulin secretion; in fact resting B cells do not express the IL-6 receptor (IL-6R) but are induced to express it following activation. In view of my findings, it is now necessary to determine if the IL-6 that may be responsible for this observation is of viral rather than cellular origin.

Protein homologous for IL-8R is encoded by HHV-8 ORF74. IL-8R has as its closest viral homologue the HVS *ECRF3* gene product, which encodes a functional IL-8 receptor (Ahuja et al., 1993). IL-8, which is a potent cytokine for recruitment and activation of neutrophils and a major contributing factor in angiogenesis, is located in mucosal cells of Crohn's disease tissues (Mazzucchelli et al., 1994). Again, it is now necessary to determine the extent to which increased expression of vIL-8R plays in the induction of granulomas.

The HHV-8 proteins similar to MIP chemokines and IRF may also contribute to the development of granuloma formation. Macrophage T-cell activation is hypothesised to be critical in the initiation of inflammation and granuloma formation. MIPs 1α and 1β have been shown to be chemoattractant for monocyte and promotes accumulation of CD4+ T cells and elevated levels of macrophage-derived cytokines, such as IL-6, TGF-β, and TNF-α, have been previously demonstrated in granulomas (Seitzer et al., 1997; Homolka et al., 1993; Minshall et al., 1997). Whether the MIPs studied here are of viral or host origin would now require examination in the light of my observations described in this chapter.

Thus the recent data showing HHV-8 proteins bearing homologies with and sharing functional characteristics of cytokines (Russo et al., 1996; Moore et al., 1996; Neipel et al., 1997) may provide insights into the potential for this virus to induce granulomas. Furthermore, they may explain why corticosteroids (Gibson et al., 1996), in attenuating the effects of these effector molecules, can retard the progression of sarcoidosis and granulomatous disorders. The tropism and state of

activation of HHV-8 activation in granuloma tissues, and the role of antiherpesvirals in suppressing both viral and disease activities merit attention.

5. HHV-8 GENOMIC VARIATION

5.1. Inter-host genomic variation in herpesviruses

5.1.1. General comments: herpesviruses

Over 100 different herpesviruses have been characterised and classified. The herpesviral genome ranges between 80 and 150 million daltons in molecular weight with a G+C base composition that ranges from 32 to 74 moles percent. The virion has an icosadeltahedral capsid composed of 160 capsomers and an envelope with glycoprotein spikes on its surface. Eight herpesviruses have been isolated so far from humans and they are classified in three different sub-families: *alphaherpesvirinae, betaherpesvirinae, and gammaherpesvirinae.*

5.1.1.1. Alphaherpesvirinae

The members of this sub-family are classified on the basis of a variable host range, relatively short reproductive cycle, rapid spread in culture, efficient destruction of infected cells, and capacity to establish latent infections. The sub-family accommodates the genera *Simplexvirus* and *Varicellovirus.*

5.1.1.2.Betaherpesvinae

This sub-family has a long reproductive cycle and the infection progresses slowly in culture. Infected cells normally become enlarged (cytomegalia), and the virus is able to maintain itself in a latent form in secretory glands, lymphoreticular

cells, kidney and other tissues. The genus *Cytomegalovirus* (CMV) is in this sub-family.

5.1.1.3. Gammaherpesvirinae

In-vitro all members of this sub-family replicate in lymphoblastoid cells and some cause lytic infection in certain epithelioid and fibroblastic cells. Virus latency is frequently demonstrated in lymphoid tissue. This sub-family includes two genera of viruses infecting humans, *Lymphocryptovirus* and *Radhinovirus*.

5.2. Variation in Herpes simplex viruses

The herpes simplex virus (HSV) genome is about 150 kb in length. The two genotypes, HSV-1 and HSV-2 are closely related: their genetic organisation, which is largely colinear, is similar but they differ in restriction endonuclease (RE) cleavage sites and in the size of ORFs (Roizman and Sears, 1990). Coding sequences of homologous genes show 70-80% nucleotide sequence identity, with non-coding sequences showing greater divergence. During persistent productive infection, viruses may change their biological properties as a result of genetic alterations, making possible the selection of virus variants in carrier cultures (Mannini-Palenzona et al., 1985; Furukawa et al., 1984). Genetic variability of HSV is frequently assessed by analysis of RE cleavage patterns. These analyses have provided information on the wide extent of intratypic variation among HSV strains.

5.2.1. HSV-1

HSV-1 has the capacity to persist in a latent state in the human host and is genetically stable even when recovered from recurrent infections. (Klauck et al., 1995). For most of the HSV-1 variants, biological differences, replication and tropism for particular cell lines have been demonstrated, and it is known that some genomic regions of HSV, such the L and S terminal fragments and middle S fragment, are heterogeneous and prone to variation (Tognon et al., 1983). HSV genome alterations were observed by Klauck and colleagues (1995), after hybridization and cloning of a 4.3kbp *Bam*HI-fragment to the *Bam*HI pattern of all re-isolated of the series, in the *Bam*HI-J fragment in the series of HSV-1 (F-Raji) variants 1-IV. It was also noted, using the same techniques, that a 290-320 bp sequence deletion occurred in 13 out of 16 isolates in the BamHI-B fragment and a 360 bp insertion in the *Bam*HI-F fragment (HSV-1 F-BJAB) (Klauck et al., 1995).

A quantitative analysis of the diversity of HSV-1 genomes, on a world-wide level, was carried out by Sakaoda et al. (1994), by examining 225 RE sites. Two hundred forty two HSV-1 strains originating from six different countries (Japan, Korea, China, Sweden, USA and Kenya) which clustered in 186 distinct HSV-1 genotypes were tested. Genotypes originating from East Asia's countries were phylogenetically related and differed from the ones from Sweden and USA, which clustered together (Sakaoda et al., 1994).

5.2.2. HSV-2

HSV-2 has a world-wide distribution and is endemic in all human population groups. The prevalence of infection and the timing of primary infection of HSV-2 differs from the one of HSV-1, reflecting the differences in the major modes of transmission of the two viruses. HSV-2 transmission is less efficient than that observed for HSV-1. The primary route is intimate contact during sexual activity. By using 16 variable markers selected from 97 RE sites mapped after digesting by 5 enzymes, inter-host HSV-2 variation has been observed from 307 epidemiologically unrelated strains of HSV-2 from four areas of the world (Korea, Japan, Sweden and USA) and 68 different genotypes could be distinguished. Significant differences among the genotypes relating with the country of isolation were noted (Sakaoda et al., 1995): genotypes 30, 26 and 3 were more predominant in Japan, Sweden and USA, respectively. Furthermore, genotypes 1 and 6 were significantly different, in frequency of detection, between Japan and Sweden and between Japan and USA, respectively (Sakaoda et al., 1995).

5.3. Variation in varicella-zoster virus

The genome of varicella-zoster virus (VZV) is 125 kb long and is composed of two covalently joined segments, designated L (long segment) and S (short segment). It can also be subdivided into four distinct domains based on sequence redundancy. The VZV genome exists as two predominant isomeric forms

result from an inversion of the S segment. These two isomers, designated P (prototype) and Is (inverted S), are present in equimolar amounts and represent 90-95% of packaged VZV DNA. The remaining 5-10% consists of two more isomeric forms, from the region L, and from S and L linked together (Ostrove, 1991).

Five repeats elements, R1 to R5, have been mapped on the VZV genome with the number of repeats varying between strains (Davison et al., 1986). The use of R1 and R3 to discriminate between strains is inadequate, due to their instabilities, therefore elements R2, R4 and R5, and, furthermore, a PstI-site-less region, are preferentially used to identify different strains of the VZV genome (Takada et al., 1995). Takayama et al. (1996), using RFLP analysis of long PCR products identified 12 patterns among 40 isolates on the basis of the presence or absence of RE cleavage sites. The combination of these patterns with the R5 copy number allows the classification of the isolates into 17 groups. Similarly, Thawarnantha et al., (1995), were able to discriminate 16 different strains in 17 isolates of VZV obtained from Thai individuals with varicella or zoster infections.

5.4. Variation in Epstein-Barr Virus

EBV is associated with infectious mononucleosis, nasopharyngeal carcinoma (NPC), and with lymphoproliferative disorders in the immunosuppressed. The two EBV subtypes circulating in most of the human population have been previously classified as EBV type A and type B, or more

recently, EBV-1 and EBV-2, consistent with the HSV-1 and HSV-2 nomenclature. The EBV-1 and EBV-2 genomes are nearly identical except for the genes that encode nuclear proteins (EBNAs). The EBNA-1, EBNA-2, EBNA 3A, EBNA 3B and EBNA 3C genes of EBV-1 differ in their predicted primary amino acid sequences compared with those of the corresponding EBV-2 proteins, varying from 20% to 50%. Apart from these genes, the genomes appear to have few differences beyond those relating to individual EBV strains. In fact type 1 and type 2 EBV strains have extensive homologous genomes, and the RE sites in most part of their genomes of type 1 and type 2 EBV strains are very similar. Thus, EBV-1 and EBV-2 are more closely related to each other than HSV-1 and HSV-2 (Gratama and Ernberg, 1995).

Studies of the BNLF1 gene have revealed several polymorphisms with relevance for the EBV epidemiology, especially a point mutation leading to a loss of the *Xho*I restriction site in the first exon; infection with variants with this mutation are associated with an increased risk for NPC (Hu et al., 1991). Lung et al. (1990) also grouped EBV genome into other two further types, C and c, and F and f, based on the *Bam*HI restriction site between the *Bam* HI W1 and I1 fragments. Although the biological relevance of these polymorphisms is not well studied, they might provide useful markers for epidemiological studies of EBV infection. For example, it has been noted that the predominant EBV genotype in southern Chinese NPC tumours is Cf, while a minority of such tumours carry the CF genotype; this latter genotype is common among healthy carriers from south

China (Lung et al., 1990). By contrast, Caucasian NPC tumours carry either DF or CF genotypes (Lung and Chang, 1992)

5.5. Variation in cytomegalovirus

CMV is ubiquitous. It causes significant morbidity and mortality in immunocompromised patients, although infection in immunocompetent individuals is generally asymptomatic. However, not all immunocompromised patients with CMV infection develop CMV-related diseases. It is possible that genetic variation of functional important genes may influence the virulence of CMV strains. Two important components of the CMV genome are the glycoproteins B and H (gB, gH). These are targets for virus neutralising antibody and are involved in both cell-to-cell spread of CMV and virus entry (Rasmussen et al., 1985, 1991).

Certain regions of the gB gene are highly variable between different virus strains. Based on the nucleotide sequences in this variable region, Chou et al. (1992) classified CMV into four gB and two gH genotypes. gB genotypes are more frequently isolated in HIV-positive patients and in allograft transplant recipients. The four different gB genotypes can also be distinguished using RE mapping. Using this approach Rasmussen and colleagues (1997) studied the distribution of genotypes in different study populations: gB1 and gB2 were found to be occurring more frequently in both leukocytes and urine samples in HIV-infected persons and in allograft recipients, while gB4 occurred more often in semen samples than in leukocytes of HIV-infected patients; moreover, the same gB

genotype was detected consistently in some patients in more than one body site. However, Vogelberg et al. (1996) and Chou and Dennison (1991) could not find any correlation between infection with the gB type and the clinical outcome of CMV infection in renal transplant recipients.

5.6. Variation in human herpesvirus-6

HHV-6 consists of two closely related yet distinct viruses, classified as HHV-6 variants A and B. HHV-6A and HHV-6B are members of the betaherpesvirinae subfamily, in the Roseolavirus genus, along with HHV-7. This classification is based on the relatively high level of sequence conservation and general genetic colinearity between these viruses and CMV (Braun et al., 1997).

Serologic assays cannot presently differentiate prior infection with one virus from the other. In vitro, HHV-6 replicates most efficiently in activated primary T cells, but in vivo the host tissue range of HHV-6 is very broad and includes lymph nodes, lymphocytes, macrophages and monocytes, kidney tubules, endothelial cells, salivary glands and CNS tissues; furthermore, viral gene products have been localized to neurons and oligodendrocytes (Challoner et al., 1995; Cone et al., 1993; Fox et al., 1990; Kondo et al., 1991; Levine et al., 1992). The effect on host cells differs between the two HHV-6 variants. HHV-6A does not shut off cell protein synthesis and infection of mononuclear cells, and leads to production of IFN-α, IL-1β and TNF-α (Balachandran et al., 1989). By contrast, HHV-6B shuts

off host cell DNA synthesis within 65 hours of primary infection; it also stimulates protein synthesis (Di Luca et al., 1990).

Epidemiologically over 95% of people older than 2 years old of age are seropositive for either or both viruses (Braun et al., 1997) The two variants differ between them with respect to epidemiology, growth properties, reactivity to monoclonal antibodies and nucleotide sequences. Nonetheless, they are closely related with some genes showing greater than 95% homology. Variant B is nearly always associated with exanthem subitum (ES). Two variants of type A (GS and U1102), simultaneously with the B variant (Z29), have been found in an ill child with ES (Dewhurst et al., 1993). Of HHV-6 strains detected in BMT recipients, 88% were B variants, 7% were A variants and 5% were mixtures of HHV-6A and HHV-6B (Drobyski et al., 1993, Gao et al., 1996, Kadakia et al., 1996). Di Luca et al. (1996) found that there were significant variant-specific sequence differences in the frequencies of HHV-6 DNA PBCs of patients with various category of diseases; in descending order, these were: ES patients (100%), healthy donors (30%), chronic fatigue syndrome patients (44%), asymptomatic HIV-infected patients with a median CD4+ count of 754/ml (11%), AIDS patients with a median CD4+ count of 69/ml (5%) and patients with the lymphadenopathy syndrome (8%). This data set confirms the findings of Fairfax et al. (1994) who found that the prevalence of HHV-6 decreased with the decreasing number of CD4 T-cells, and that variant B was more prevalent than variant A both in HIV-infected and HIV-seronegative individuals. On the other hand, in the second part the study, which examined lymph nodes tissues, different results were observed: there was a

striking difference between HIV-negative patients with reactive lymphadenitis (20%) and HIV-positive-lymphadenopathy syndrome (LAS) lymph nodes (67%), while in lymph nodes biopsied from patients affected by Hodgkin lymphomas in HIV-positive and -negative patients, HHV-6 DNA was detectable with the same frequency (30%).

Di Luca et al. (1996) showed, using RE analysis, significant differences in the HHV-6 variants A and B distribution in human tissues, suggesting differences in tissue tropism: HHV-6A was more frequently detected in skin and primary fibroblast cultures while HHV-6B was more frequent in the lymphoid system.

5.7. Variation in human herpesvirus-7

HHV-7 is another recently isolated betaherpesvirus. It is also prevalent in the human population, with primary infection usually occurring in early childhood causing fever and a non-specific rash. First isolated from healthy blood donor, it has since been detected in, or isolated from the blood and saliva of many other individuals (Berneman et al., 1992; Fenkel et al., 1990; Wyatt et al., 1992). HHV-7 is a CD4+ T lymphotropic virus with a cytopatic effect characterised by marked enlargement of infected cells (Asano et al., 1995, Clarck et al., 1995). No genetic variation of HHV-7 has been reported in the literature so far.

5.8. Variation in human herpesvirus-8

As discussed in chapter 1, KS330Bam was one of two fragment isolated from lesional tissue of an AIDS patient with KS (Chang and Moore, 1994). The ORF from which KS330Bam originates is also termed ORF26, in emphasis of the close relationship between HVS and HHV-8 (Moore et al., 1996; Russo et al., 1996). Early studies alluded to sequence variability in ORF26 of HHV8 (Huang et al., 1995; Buonaguro et al., 1996) and BCBL cell lines (Cesarman et al., 1995b). Recently, Zong et al. (1997) reported that, in a 500-bp ORF26 segment that encompasses $KS330_{233}$, most of nucleotide substitutions occurred at a restricted number of sites, with the sequences falling into three groupings. In this chapter, the finding of a study of variant HHV8 sequences found in >250 tissues samples are reported. I show that the hot-spot mutations in ORF26 allow at least eight genetic groupings of HHV8 to be made.

5.9. Materials and Methods.

To derive $KS330_{233}$ sequences from study tissues, a 210-bp DNA segment of $KS330_{233}$ was amplified by nested PCR from PEFF pathological tissues, as described in chapter 2. Tissues were taken from HIV-infected and presumably non-HIV-infected patients with KS and various non-KS diseases. A number of geographical regions was represented. Inter-primer sequences of the amplicons (171 bp long) were assessed from both strands of the PCR product. DNA extracts that yielded unique sequences were re-evaluated by amplification from a fresh aliquot of the extract, to exclude PCR-induced

mutation as a cause of the base changes observed. In addition, $KS330_{233}$ sequences from 21 previous reports (Moore and Chang., 1995; Rady et al., 1995; De Lellis et al; 1995; Cesarman et al., 1995; Huang et al., 1995; Barozzi et al., 1996; Buonaguro et al., 1996; Chuck et al., 1996; Collandre et al., 1996; Gyulai et al., 1996; Humphrey et al., 1996; Inagi et al., 1996; Kikuta et al., 1997; Koizumi et al., 1996; Luppi et al., 1996; Marchioli et al., 1996; Monini et al., 1996; O'Neill et al., 1996; Elgelbrecht et al., 1997; Memar et al., 1997; Zong et al., 1997) that contain information on mutations in the 171-bp segment were combined with my data set. In total, 156 sequences from my specimens and 128 reported from other laboratories were evaluated.

5.10. Results and Discussion

The large majority of point mutations were observed to cluster in five codons: 134, 141, 152, 167 and 169 (Table 5.1) (Codon and nucleotide positions are numbered according to Chang et al. (1994)). Of these, base changes in codons 134, 141 and 167 are missense mutations. At codon 134, a C to T transition at the second position (nt 1033) was the most frequent, leading to a proline-leucine switch. The next most frequent mutation in this codon was a C to A transversion at the adjacent nucleotide position 1032, which, depending on the base occupying position 1033, codes for proline, leucine, isoleucine or threonine. In codon 141, mutation was observed only at the third codon position (nucleotide 1055), and was allelic, leading to a proline-leucine change. In codon 167, the mutation always occurred at the second codon position (nt 1132), and was allelic, leading to an aspartate-glycine switch. The silent base changes in

codons 152 and 169 were also allelic, involving C to T, and A to C changes in nucleotides 1086 and 1139, respectively.

The variant sequences could be segregated to eight main groups, A through H, based on mutations in codons 134, 167 and 169 (Table 5.1). Hence, in addition to groups A to E identified in chapter 4, three more groups (F,G and H) were identified. It is noted that in codon 169, an A to C change at position 1139 almost always correlated with an A to G change in codon 167, at position 1132. Furthermore, subgrouping could be done. This was based on nucleotide changes in positions 141 and 152, and point mutations occurring elsewhere. Assignment of the subgroups was also based on the frequency at which each sequence appeared (thus the most frequently occurring sequence in each group was assigned the number 1, the next most frequent was assigned 2, and so on). Minor and unique sequences were assigned subgroup numbers according to the order of their entry into my data base.

To determine if the grouping system could be phylogenetically based, all the variant sequences were analysed using the Clustal algorithm in the MEGALIGN program of the LASERGENE System. The dendrogram in Figure 5.1 shows that the majority of the sequences clustered according to the grouping system proposed. Clustering of the following sequences were observed: sequences in groups A and H; all the B sequences except B6; group C sequences and those of D1, D2, E3 and variants 1 and 2 of the miscellaneous group; and all the F sequences except F3.

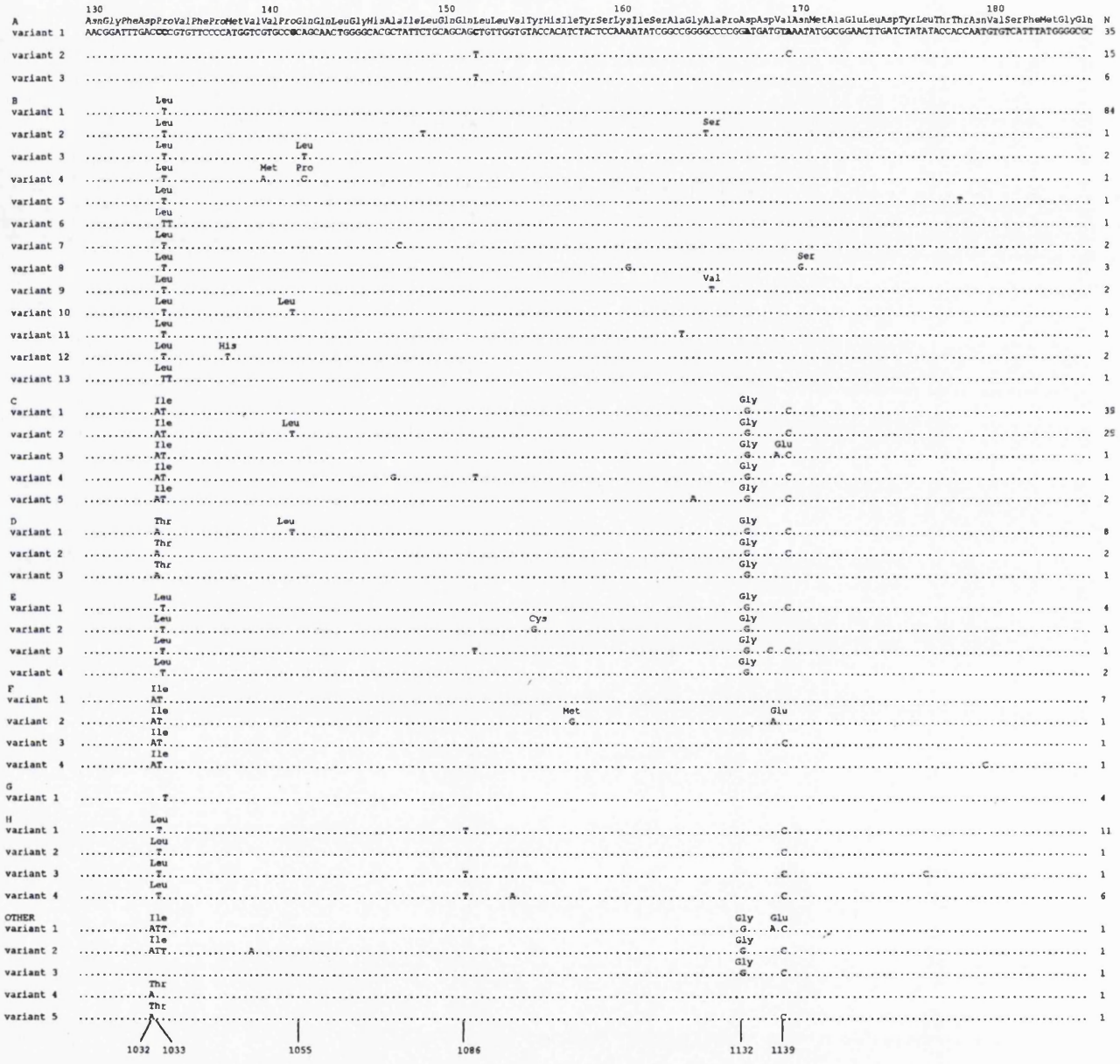

130
140
150
160
170
180
A
AsnGlyPheAspProValPheProMetValValProGlnGlnLeuGlyHisAlaIleLeuGlnGlnLeuLeuValTyrHisIleTyrSerLysIleSerAlaGlyAlaProAspValAsnMetAlaGluLeuAspTyrLeuThrThrAsnValSerPheMetGlyGln
N
variant 1
AACGGATTTGACCCCGTGTTCCCCATGGTCGTGCCGCAGCAACTGGGGCACGCTATTCTGCAGCAGCTGTTGGTGTACCACATCTACTCCAAAATATCGGCCGGGGCCCCGGGATGATGTAAATATGGCGGAACTTGATCTATATACCACCAATGTGTCATTTATGGGGCGC
35
variant 2
15
variant 3
6
B
variant 1
84
variant 2
1
variant 3
2
variant 4
1
variant 5
1
variant 6
1
variant 7
2
variant 8
3
variant 9
2
variant 10
1
variant 11
1
variant 12
2
variant 13
1
C
variant 1
35
variant 2
25
variant 3
1
variant 4
1
variant 5
2
D
variant 1
8
variant 2
2
variant 3
1
E
variant 1
4
variant 2
1
variant 3
1
variant 4
2
F
variant 1
7
variant 2
1
variant 3
1
variant 4
1
G
variant 1
4
H
variant 1
11
variant 2
1
variant 3
1
variant 4
6
OTHER
variant 1
1
variant 2
1
variant 3
1
variant 4
1
variant 5
1
1032
1033
1055
1086
1132
1139

Table 5-1 Alignment of variant HHV-8 ORF26 sequences

Variant sequences in the middle segment of ORF 26 of HHV 8 (171 bps) grouped according to nucleotide and cognate amino acid motifs. The total number (N) of variants in each category is indicated on the right. Italicised numbers refer to nucleotide positions at which hot-spot mutations occur, un-italicised numerals to codon positions. Base and amino acid sequences of variant A1 are shown in full. Dots alongside non-A1 sequences signify base residues matching those of A1 aligned above. Identity of amino acids resulting from missense mutations in non-A1 sequences is indicated.

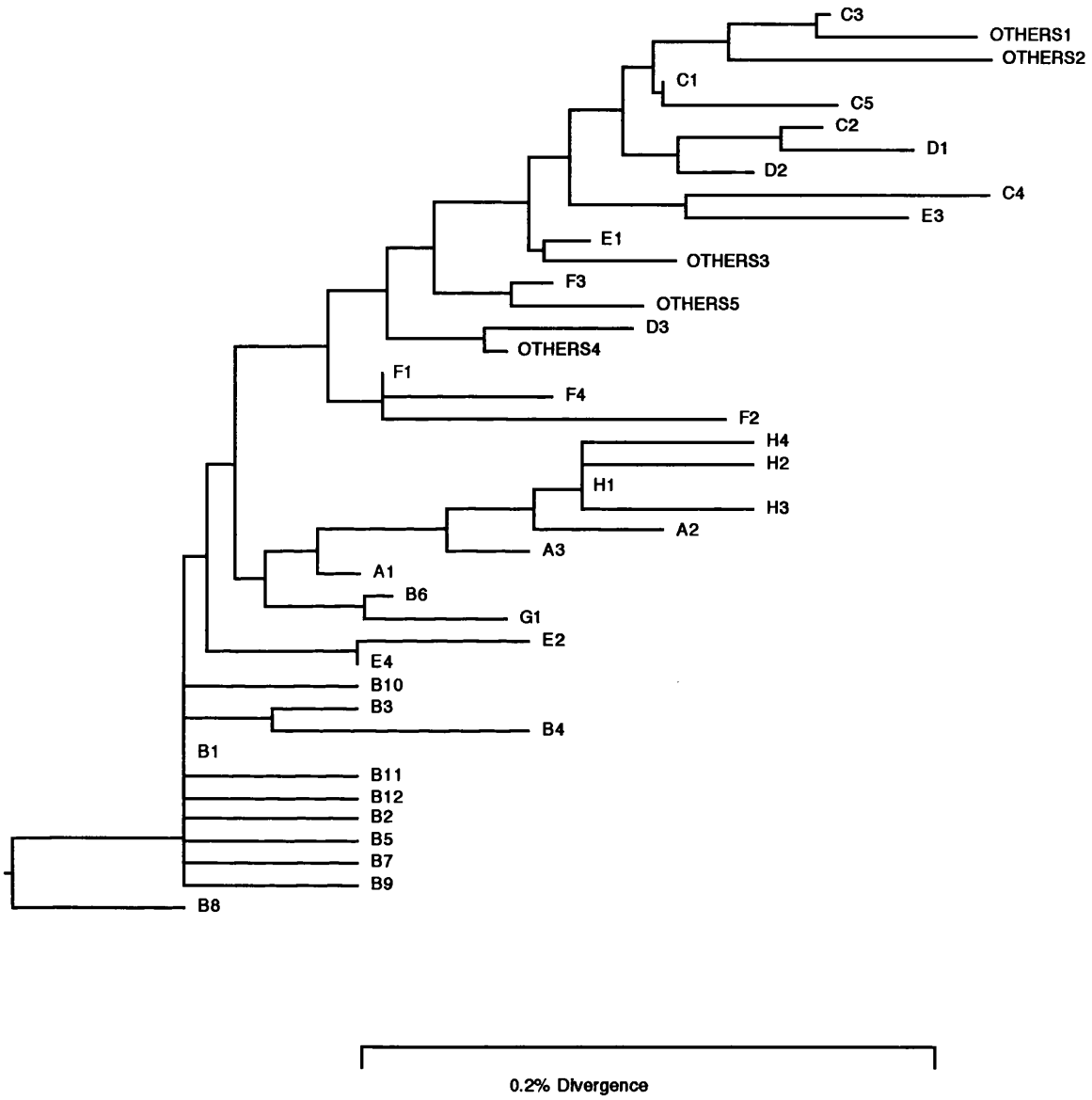


Figure 5-1 Dendrogram showing possible phylogenetic relationship of variant HHV-8 ORF26 sequences.

Variant	North America	West Europe	East Europe	Israel	Central Africa	South Africa	East Asia	Australia
A1	0/27[d,i,o]	0/1[t]			0/5[r,i]		0/2[l]	
A2	0/7[g,i,p]	0/6[q]				0/2[g]		
A3	0/6[i,p]							
B1	6/12[p,r,t,u]	43/0	0/1[t]	4/0			3/14[k,l,n]	1/0
B2				1/0				
B3		2/0						
B4		1/0						
B5			0/1[q]					
B6		1/0						
B7								2/0
B8	0/3[p]							
B9				2/0				
B10						0/1[g]		
B11		1/0						
B12	1/0	1/0						
C1		30/0		1/0	0/2[u]		6/0	
C2	5/4[n,r,t]	12/5[a,e]		2/0			1/0	
C3		1/0						
C4		1/0						
C5	1/0	1/0						
D1	0/8[i,p]							
D2	0/2[i,p]							
D3	0/1[i]							
E1		2/0			0/2[u]			
E2						0/1[g]		
E3		1/0						
E4		1/0					0/1[l]	
F1		3/0					4/0	
F2								1/0
F3		1/0						
F4		1/0						
G1	1/0							3/0
H1		5/5[n]			0/1[u]			
H2		1/0						
H3		1/0						
H4	0/6[u]							
Others								
1		1/0						
2		1/0						
3						0/1[g]		
4	0/1[i]							
5	0/1[i]							
Total	14/78	112/17	0/2	10/0	0/10	0/5	14/17	6/0

Table 5-2 Distribution of HHV-8 variants according to geography.

Distribution of variant ORF26 sequences of HHV-8 according to geography. In each cell, numbers in bold (to left of oblique) refer to sequences I characterised, those not in bold (to right of oblique) to sequences published by other laboratories. Superscript letters relate to papers listed below:

(a) Barozzi et al., 1996; (b) Buonaguro et al.,1996; (c) Cesarman et al., 1995; (d) Chuck et al., 1996; (e) Collandre et al., 1995; (f) De Lellis et al.,1995; (g) Engelbrecht et al., 1997; (h) Gyulai et al., 1996; (i) Huang et al., 1995; (j) Humphrey et al., 1996; (k) Inagi et al., 1996; (l) Kikuta et al., 1997; (m) Koizumi et al., 1996; (n) Luppi et al., 1996; (o) Marchioli et al., 1996; (p) Memar et al., 1997; (q) Monini et al., 1996; (r) Moore et al., 1995; (s) O'Neill et al., 1996; (t) Rady et al., 1995; (u) Zong et al., 1997.

Table 5.2 shows how the sequences are distributed geographically. In North America, A1 and the group D sequences prevail, while B1 and C2 occur as minor populations. In West Europe, groups B, C and H sequences are predominant, the most common variants being B1, C1, C2 and H1 (in decreasing order of frequency). The C2 sequence is significant in being absent from North America, although it is found frequently in West Europe, and to a lesser extent, elsewhere. The pattern for East Asia reflects West Europe, in that B1 is dominant, followed by C1. Of the few sequences so far recovered from tissues of patients in Israel, Central Africa and Australia, the dominant sequence appears to be B1, A2 and G1, respectively. For Central Africa in particular, it is notable that the more frequently recovered sequences in this series, A1, B1 and C2, are absent, although C1 is found. Overall, the current set of data provides evidence of geographical restriction for the more frequently recovered sequences. The evidence for restriction of the minor sequences is less conclusive, but is suggestive. The sequences that appear to be particularly prevalent in specific regions (group D sequences in North America, and the A2 and G1 sequences are in Central Africa and Australia, respectively, alludes to their origin from indigenous viruses.

The above observations permit preliminary insights to be made into the global epidemiology of HHV-8. There appears to be one transmission route that principally involves North America-Central Africa cross-traffic of HHV-8 carriers; this is evident from the high rate of recovery of the prototypic A1 sequence (Chang et al., 1994) in the two continents. There is another transmission route that, on a global scale, is more inclusive, but nevertheless does not involve Africa. This is evident from the pattern of distribution of the B1 sequence, which, being dominant in West Europe, is found

frequently in North America, East Asia and (to a lesser extent) Israel, but is yet to be recovered from African patients .

The epidemiology of HHV-8 bearing the C1 and C2 sequences is more difficult to discern, because they appear almost equally frequently and are closely related genetically; further studies to define their true genotypic relationship (v.i.) are necessary before their pattern of transmission can be ascertained. Nevertheless, the complete absence of the C1 sequence from North America is notable and may be of epidemiological significance.

As Table 5.3 shows, there is a wide scatter of the minor sequences across the range of pathologies examined. Inferences of correlation with diseases can therefore only be made for the more frequently occurring sequences. In making these inferences, the confounding effect of geographical restriction of the sequences has to be taken into consideration. Thus, the frequent identification of the A1 sequence from AIDS-related, classical and African KS lesions reflects more its frequent occurrence in tissues of North American and Central African patients (Table 5.2) rather than its association with KS of the three epidemiological forms. Similarly, the frequent representation of the C2 sequence in AIDS-related KS reflects its high prevalence in tissues of North American and West European patients.

The C1 sequence is unique in that was heavily represented in a single disease category: sarcoidosis. Thus, the C1 sequence was carried by 20/38 (52%) of sarcoid tissues (Figure 5.5). As these tissues were all taken from Italian patients, and there were 26 C1 sequences recovered from tissues of Italian patients altogether, the over-

representation of C1 (20/26, 77%) in sarcoid tissues may reflect a specific link between sarcoidosis and HHV8 of the C1 group.

Some sequences were identified in both diseased and healthy tissues. The A2 sequence isolated from North American and South African patients were all derived from KS, while that recovered from West Europe originated from prostate tissue and sperm of Italian men (Monini et al., 1996). The B1 sequence was the most common sequence recovered from AIDS-related and classical KS tissues from North American and West European patients, oral lesions in HIV-infected British patients and labial biopsies of British patients with OFG. However, the B1 sequence was also observed in peripheral blood of Japanese children with febrile illnesses (Kikuta et al., 1997), suggesting that it may not always be carried in diseased tissues. Thus the virus bearing this sequence, like the one bearing the A2 sequence, may be both pathogenic and non-pathogenic.

The three HHV-8 genetic groups of Zong et al. (1997) (referred to as A, B and C) may have a genotypic basis, as evidence was provided that most of the polymorphic substitutions in the 500-bp segment of ORF26 co-segregated with base changes at specific positions in two other domains in the HHV-8 genome, ORF75 and UPS75. Our study, which was confined to examining a shorter segment of the HHV-8 genome, but covered a much wider range of sequences, confirms that groups B, C and H (corresponding respectively to groups A, B and C of Zong et al. (1997)) possess distinct nucleotide motifs, and that these motifs permit segregation to be done.

Variant	KS			non-KS						
					HIV+ve				HIV-ve	
	AIDS	endemic	classical	iatrogenic	oral ulcers	other oral lesions	sarcoidosis	OFG	lymph-adenitis	others
A1	0/21[i,l,p]	0/5[d,f]	0/9[d,f,i]							
A2	0/4[p]	0/3[i]	0/1[g]	0/1[g]						0/6[q]
A3	0/4[f,p]	0/1[i]	0/1[i]							
B1	15/10[k,l,r,t]	0/1[u]	3/6[n,r,t]	4/0	5/0	7/0	7/0	10/0	6/0	0/10[l,r,u]
B2				1/0						
B3			1/0		1/0					
B4							1/0			
B5										0/1[g]
B6							1/0			
B7	2/0									
B8										0/3[p]
B9				2/0						
B10				0/1[g]						
B11					1/0					
B12			1/0		1/0					
C1	1/2[u]		2/0	1/0	3/0		20/0	4/0	5/0	1/0
C2	15/4[e,r,t]		2/1[r]	2/0				1/0	1/3[n]	0/1[a]
C3							1/0			
C4							1/0			
C5	1/0				1/0					
D1	0/8[i,p]									
D2	0/1[p]	0/1[i]								
D3		0/1[i]								
E1		0/2[u]					2/0			
E2			0/1[g]							
E3					1/0					
E4	1/0									0/1[l]
F1							2/0		4/0	
F2	1/0									
F3							1/0			
F4								1/0		
G1	1/0				1/0	1/0				1/0
H1	3/1[u]		1/0					1/0	0/1[n]	0/4[n]
H2									1/0	
H3						1/0				
H4	0/6[u]									
others										
1							1/0			
2							1/0			
3		0/1[g]								
4		0/1[r]								
5	0/1[i]									

Table 5-3 Distribution of variant ORF 26 sequences of HHV-8 according to disease categories.

Superscript letters relate to papers listed below:

(a) Barozzi et al., 1996; (b) Buonaguro et al.,1996; (c) Cesarman et al., 1995; (d) Chuck et al., 1996; (e) Collandre et al., 1995; (f) De Lellis et al.,1995; (g) Engelbrecht et al., 1997; (h) Gyulai et al., 1996; (i) Huang et al., 1995; (j) Humphrey et al., 1996; (k) Inagi et al., 1996; (l) Kikuta et al., 1997; (m) Koizumi et al., 1996; (n) Luppi et al., 1996; (o) Marchioli et al., 1996; (p) Memar et al., 1997; (q) Monini et al., 1996; (r) Moore et al., 1995; (s) O'Neill et al., 1996; (t) Rady et al., 1995; (u) Zong et al., 1997.

Thus, in the middle segment of ORF26, the $C^{1032}/T^{1033}/A^{1132}/A^{1139}$ motif is peculiar to group B, $A^{1032}/T^{1033}/G^{1132}/C^{1139}$ to group C and $C^{1032}/T^{1033}/A^{1132}/C^{1139}$ to group H. Zong et al. (1997) had suggested that the nucleotide residues at these positions might be "diagnostic" of three formal genotypes of HHV-8. Whether the motifs that we found to be characteristic of five more groups, namely $C^{1032}/C^{1033}/A^{1139}/A^{1139}$ for group A, $A^{1032}/C^{1033}/G^{1139}$ for group D, $C^{1032}/T^{1033}/G^{1032}$ for group E, $A^{1032}/T^{1033}/A^{1132}$ for group F and $C^{1032}/C^{1033}/T^{1034}/A^{1132}/A^{1139}$ for group G, are similarly diagnostic of other genotypes requires further examination of longer stretches of the HHV-8 genome recoverable from the study specimens. It is moreover likely that the additional base changes occurring at positions 1055 and 1086 may co-segregate with changes in non-neutral sites of codons 134, 152, 167 and 169, and with those elsewhere in the genome (Zong et al., 1997).

From its homology with the herpes saimiri ORF26 protein, the HHV-8 ORF26 protein is likely to be a minor capsid protein (Chang et al., 1995; Russo et al., 1996). Sera from KS patients failed to immunoprecipitate recombinant proteins expressed from this ORF (O'Neill et al., 1997), indicating that the native ORF26 protein is either not expressed in the host or does not elicit a specific humoral response. Consequently, antibody-mediated selective pressure does not explain why hot-spot mutations are regularly found in the ORF26 protein. However, the selective effect of the cytotoxic T cell response might more account for the mutability of this segment. Peptide fragments from the segment could possibly encompass HLA-restricted cytotoxic T-lymphocyte (CTL) epitopes. Over time, polymorphisms in the peptides would have selected for viral mutants that circulate in host populations that do not carry HLA-restricting alleles. Indeed, in the EBV model, mutations in CTL epitopes of the EBNA 4 protein have been reported to result in the loss

of recognition by HLA alleles that prevail in certain human populations (de Campos-Lima et al., 1994). HHV8 variants bearing "CTL-epitope-loss-mutants" similar to those in EBNA 4 may thus persist in a geographically confined population. In this respect, the amino acid polymorphism in codon 134, and to a lesser extent in codon 167, may be significant: the amino acid residues encoded from these codons could be constituents of viral epitopes that anchor to HLA molecules.

In this study a large number of sequences derived from $KS330_{233}$, the most commonly amplified DNA segment of the HHV-8 genome was analysed. Point mutations were observed to preferentially occur in codons 134, 141, 152, 167 and 169 of the ORF26 protein. Mutations in codon 134 involved all three codon positions; the vast majority were missense, leading to four possible amino acid changes. Mutations in codons 152 and 167 (missense) and in 141 and 169 (neutral) were all allelic, involving one specific position in each codon. At least eight genetic groupings, A through H, could be made based on permutations in nucleotides occupying codons 134, 167 and 169. Moreover, the mutations in codons 141 and 152, and those occurring elsewhere, permitted subgroups to be defined. The grouping system was further determined to be phylogenetically based. Different patterns in the global distribution of the more frequently recovered sequences were evident: the prototypic A1 sequence was recovered almost exclusively from North America and Central Africa, while the B1 sequence, being very common in North America, West Europe and East Asia, was absent from Africa. No groups or variants could be regarded as disease-specific; however, the C1 sequence was frequently recovered from tissues of Italian patients with sarcoidosis. Comparative analysis of larger stretches of the HHV8 genome of major variants is necessary to determine whether the eight groups

identified here reflect distinct genotypes. Such an evaluation should allow the epidemiology and pathogenesis of this newly discovered virus to be assessed with greater certainty.

5.10.1. Intra-host genomic variation in herpesviruses

Intra-host variability of DNA viruses is a relatively unexplored field of study in virology. Very few reports have been published in literature in the past regarding genome diversity of herpesviruses within the same host and these are summarised here. With regards to HSV-1, intra-host DNA polymorphism was first observed by Alam et al. (1985) in a 7-year-old patient affected by recurrent ulcerations of the skin and internal organs. Single HSV isolates were recovered from mesenteric lymph nodes, skin, brain, lung and liver taken over three years. Digestion of the biopsies with Nal I from skin, mesenteric lymph nodes and lungs showed a common RFLP pattern which was however different from that from the brain and from the liver. However, the degree of heterogeneity between these three isolates was small, suggesting that they originated from a single infecting strain (Alam et al., 1989).

Bongarts and colleagues (1996) found evidence of mixed infection by different strains of CMV of an HIV-positive patient. Viral DNA was derived from blood, urine and aqueous humour, and gB genotypes identified by PCR and RLFP analysis. A mixture of gB types was found in 15 out of 99 patients; in these 15, different gB types were found in the same patient in 6 patients and multiple sites of the same patient in 9 patients.

Boghart et al. (1996) also searched for CMV DNA sequences in blood, urine and aqueous humour samples of renal transplant recipients and found sequences belonging to different gB genotypes in multiple sites of a single patient in 6 out of 15 tested and in multiple sites in 9 out 15 tested. Vogelberg et al. (1996), found in 8 out

of 39 renal and bone marrow transplant recipients the presence of CMV variants with different gB glycoproteins.

Rasmussen and her colleagues (1997) monitored 5 HIV-infected patients and immunocompromised allograft recipients at 2-month intervals for over several months to assess changes in CMV genotypes over time. Three of the 5 consistently yielded the gB2 genotype in both blood and urine during the monitoring period. One patient began the study period with type gB1 in urine and blood, but after 4 months of observation, while type 1 remained the sole genotype found in urine, type 3 began to be detected in the blood, followed at 6 months by type 2. The last observations at 7 and 8 months revealed complex mixtures of all 4 gB genotypes. However, one patient over a 14-month period consistently showed the presence of types gB2 and gB3.

A comparison of gB types of multiple CMV isolates from different sites in bone marrow transplant recipient was undertaken by Fries at al. (1994). In fifteen out of 17 patients who died of CMV pneumonia, the gB groups of the early and late isolates did not change. One patient with fatal CMV disease originally presented with gB type 4 and later gB type 3 CMV infection; a second patient with an early gB type 1 isolate from blood presented with gB type 3 in his gut and lung isolates; finally, in one patient with non-fatal CMV infection the gB genotype of two isolates were different.

Intra-host variability has been observed for human herpesvirus 6 in patients with lymphoproliferative disorders (Becker et al., 1989), using an hybridisation

approach with the pZVH-14 DNA probe; two different bands of hybridisation corresponding to DNA lengths of 5 kb and 23 kb were isolated from the same patients instead of a single band at 10 kb.

As intra-host variability of HHV-8 has never been examined, I extended my work on inter-host variability to look into variability at the intra-host and intra-lesional levels.

5.11. Source of clones

Cloning of the ORF26 of the HHV-8 genome was performed on several samples with different histopathological diagnosies and from different patients. The cloning procedure was carried out following the procedure previously discussed in chapter 2. The samples tested were biopsies taken from patients affected by sarcoidosis (Figure 5.5), reactive lymphadenitis (Figure 5.4), granulomatous lymphadenitis (Figure 5.4), Mediterranean KS (Figure 5.3), non-Hodgkin's lymphoma (Figure 5.4), Kikuchi's histiocytic necrotizing lymphadenitis (Figure 5.2), and, as controls, the BCP-1 cell line and plasmid pHHV-8.

A note here about Kikuchi's disease is appropriate. It is a lymphadenitis characterised by focal reticulum cell hyperplasia, with nuclear debris and phagocytes. The lymph nodes involved are massively infiltrated by plasmacytoid T-cells, macrophages and small lymphocytes. Necrotic changes in the centre of the lesion are distinctive, with the degree of necrosis varying from the appearance of small pyknotic cells to an extensive necrosis affecting the entire lymph node (Kikuchi, 1972).

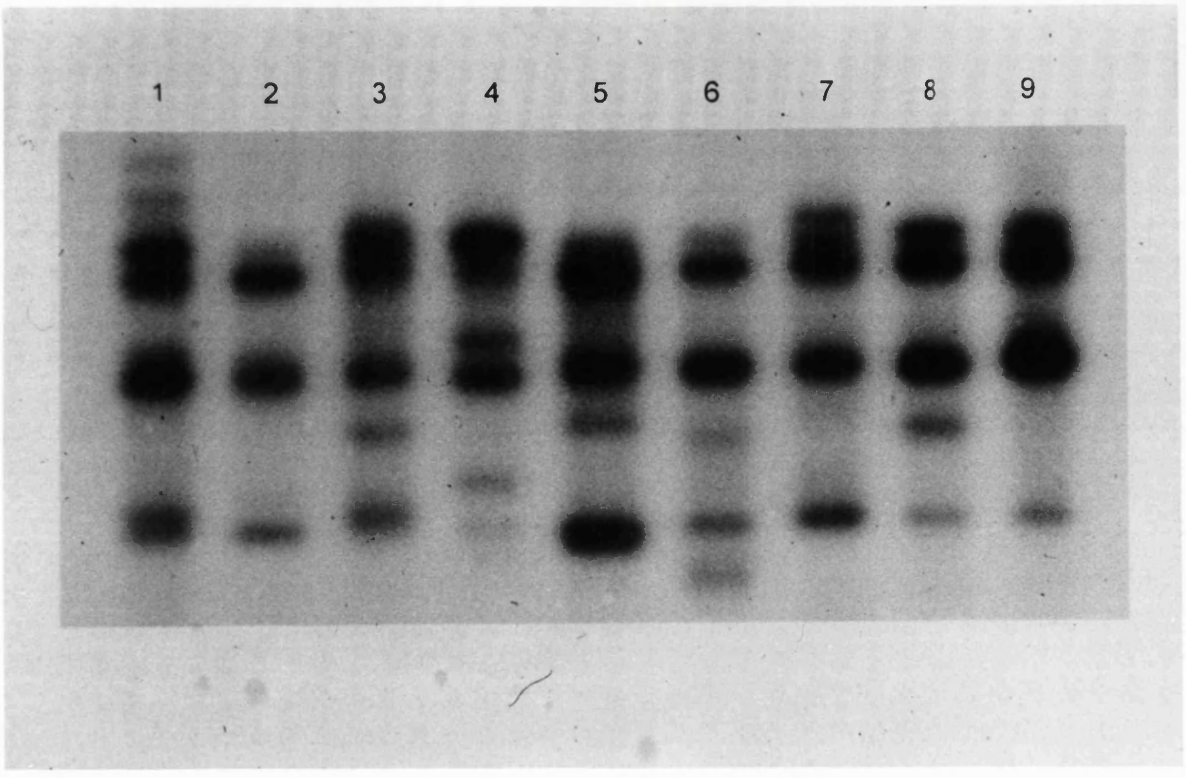


Figure 5-2 SSCP profile of HHV-8 ORF26 amplicons derived from lymph nodes of patients with Kikuchi's disease.

Different banding patterns predict differences in nucleotide sequences.

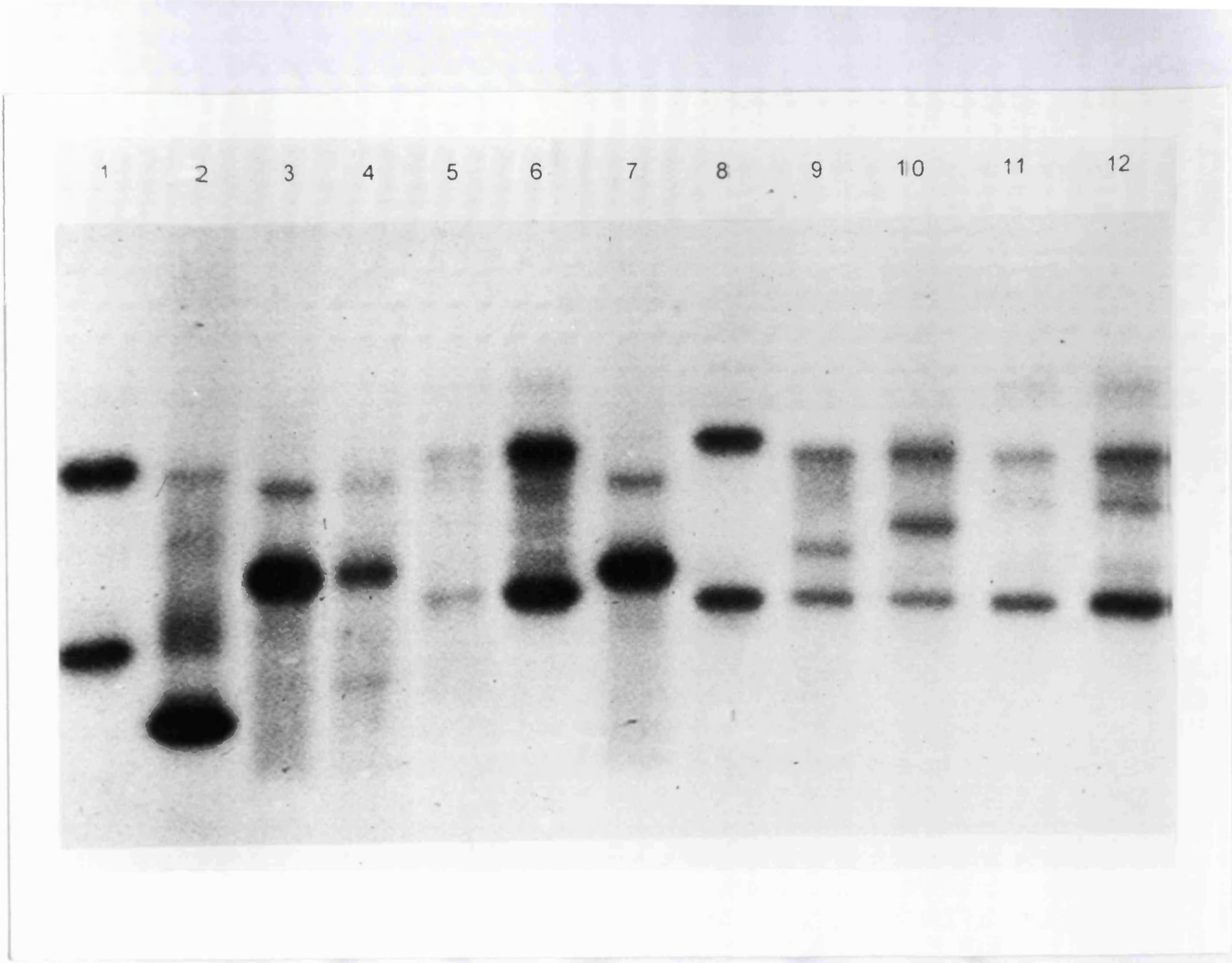


Figure 5-3 SSCP profile of HHV-8 ORF26 amplicons derived from skin biopsies of patients with Mediterranean KS.

Different banding patterns predict differences in nucleotide sequences.

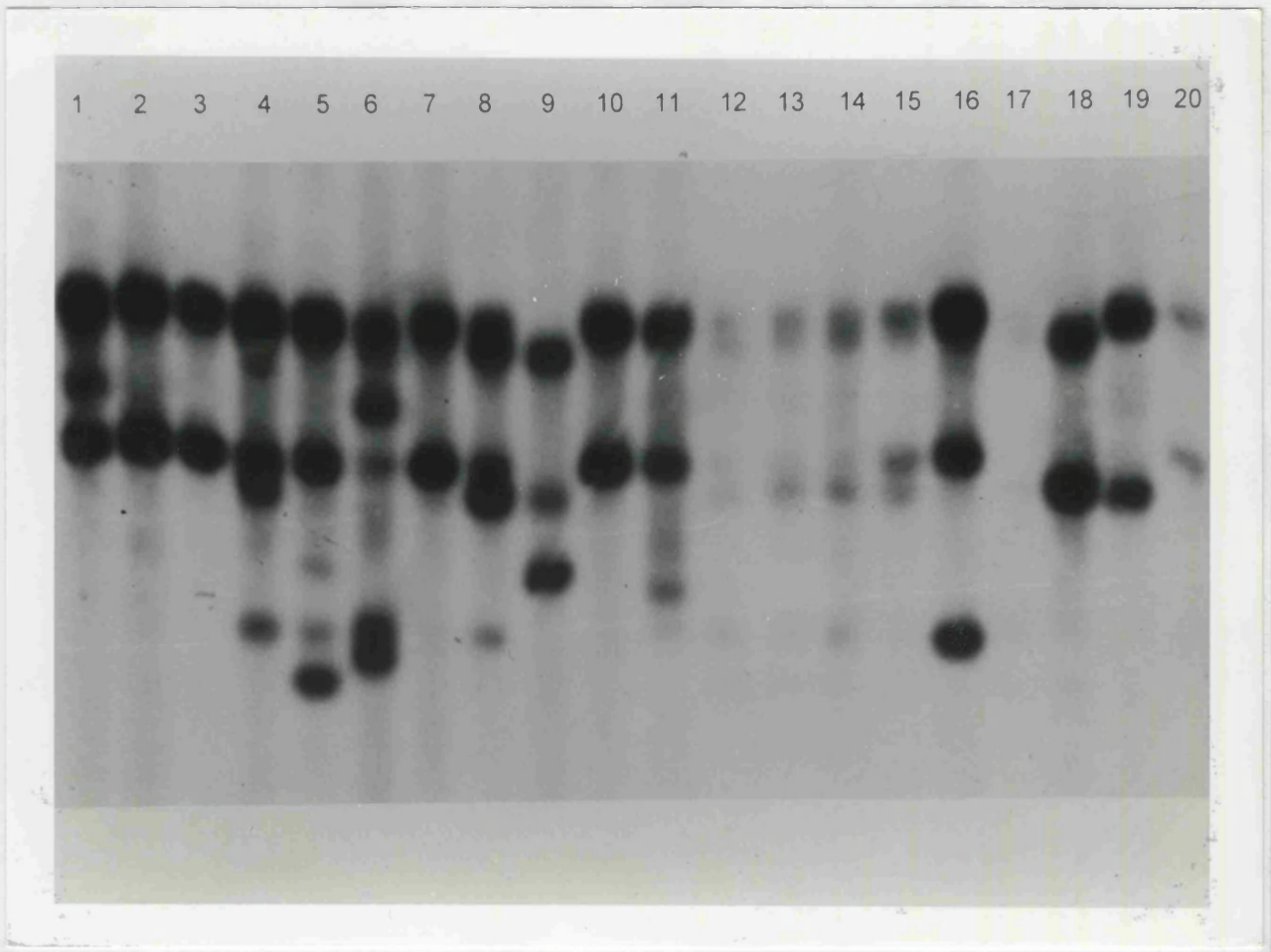


Figure 5-4 SSCP profile of HHV-8 ORF 26 amplicons derived from tissue biopsies of patients with various lymphadenitides and with B-cell lymphoma.

Lanes 1-7: reactive lymphadenitis; lanes 8-13: granulomatous lymphadenitis; lanes 14-18: B-cell lymphomas; lane 19: BC-1 cell line; lane 20: HIV+ KS.

Different banding patterns predict differences in nucleotide sequences.

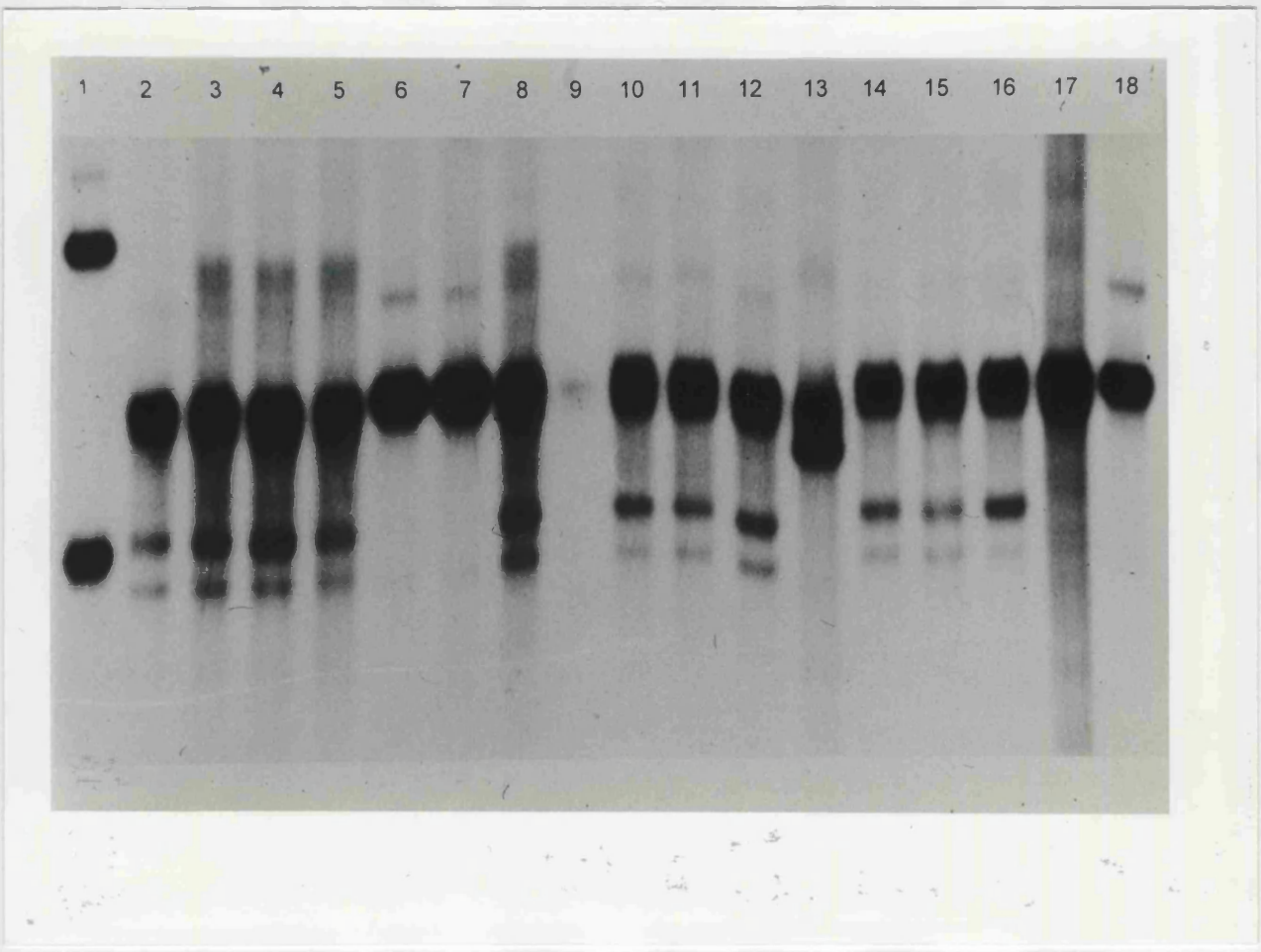


Figure 5-5 SSCP profile of HHV-8 ORF26 amplicons derived from biopsies of patients with sarcoidosis (G1, H1, A2, D2, D4, H2, J1, A3, A4, L1, M1, E3, D5, D3, N1, O1); lanes 17 and 18 are positive control BCP-1 and BC-1 cell lines.

Different banding patterns predict differences in nucleotide sequences.

5.12. Intra-host ORF 26 sequence variation: SSCP analysis

Clones recovered from skin, lymph nodes and oral mucosa with histopathological evidence of sarcoidosis showed different SSCP banding patterns between different biopsies and within the same lesion. Figure 5.6 shows a banding pattern derived from a single skin lesion, and Figures 5.7, 5.8, and 5.9 depict the profiles of biopsies from lymph nodes. In these, a wide variation of sequences is evident, reflecting the presence of multiple infections in a given tissue taken from a given patient. The profiles in Figures 5.9 and 5.10 show the presence of a predominant variant, each of which yield a distinct banding pattern, in the background of unique variants that yield banding patterns different from each other and from the dominant variant. Sequencing from the clones confirmed the differences suggested by the SSCP band patterns (Figures 5.6 to 5.9). Lymph node preparations from patients with Kikuchi's disease also showed a wide diversity of SSCP patterns (Figures 5.11-14). Overall, these results suggest the presence of multiple HHV-8 infection of lymphoid tissues of patients with sarcoidosis and Kikuchi's disease.

By contrast, SSCP profiles from lymph nodes taken from patients with reactive and granulomatous lymphadenitis (Figures 5.15-17) revealed a more restricted pattern. A further contrast to the multiclonal and oligoclonal patterns is the monoclonal pattern seen in neoplastic tissues. In Figure 5.18, SSCP bands from clones from a biopsy of B-cell lymphoma are shown, and in Figures 5.19 and 5.20 patterns from clones of Mediterranean KS tissues are shown. Figures 5.21 show uniform patterns from clones derived from the BCP-1 cell line.

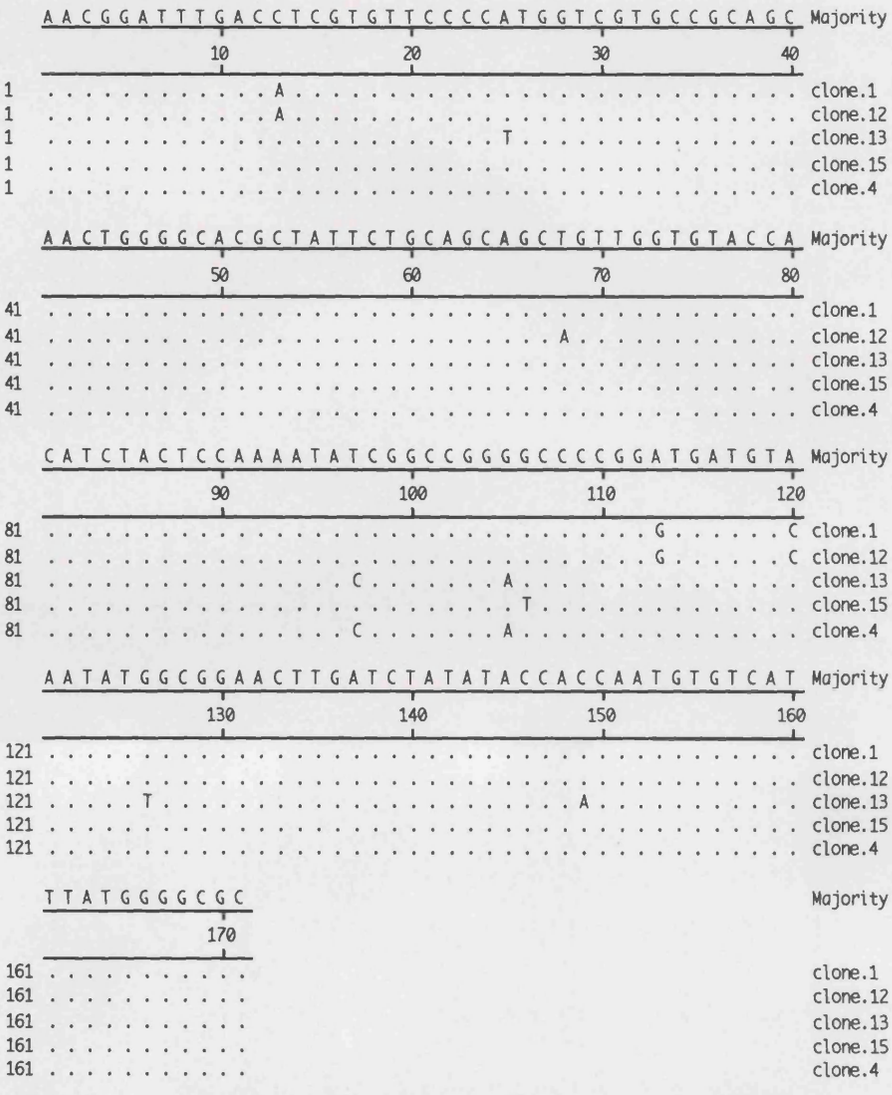
5.13. Intra-host ORF 26 variation in sarcoid tissues: DNA analysis

There is genomic variation in sarcoid tissues of the skin and lymph nodes but not in oral mucosa tissues (Figures 5.6-9). In a sarcoid skin biopsy it was possible to observe the sequences of two main variants, one belonging to group C1 and the other deriving from group C1 but with a point mutation (A^{1087}). In addition, there were four unique variants with point mutations, affecting: positions 1032, 1044, 1116, 1124, 1132, 1139 and 1168 for clone 11.9, positions 1032, 1044, 1116, 1124, 1132 and 1139 for clone 11.13, positions 1032, 1116, 1124, 1132 and 1139 for clone 11.4, and positions 1032, 1125, 1132 and 1139 for clone 11.5 (Figure 5.6). All these four mutants could all be classified as variants of group B.

Sequence analysis showed that 12 out of 19 clones from a lymph node biopsy of a patients with sarcoidosis were identical, with the following changes: T^{1033}, C^{1116} and A^{1124}; this variant then falls into group B. Four of the 19 clones showed the following changes: A^{1032}, C^{1116} and G^{1132}; they fall into group D. One clone possessed A^{1032}, C^{1116}, G^{1132} and T^{1108} and two clones possessed G^{1021}, T^{1086}, A^{1032}, C^{1116} and G^{1132}; all these three were group D variants as well (Figure 5.7).

Sequencing from nine clones from a lymph node biopsy from another patient with sarcoidosis showed the presence of a single group B1 variant with no mutations at any position (Figure 5.8). Figure 5.9 shows as well sequence data from clones derived from a sarcoid lymph node. Two variants could be distinguished in the six clones. The first one were A^{1032}, T^{1033}, G^{1132} and C^{1139}, and therefore belong to variant C1; the other were at T^{1033}, C^{1116}, A^{1124}, which allows it to be classified as a group B variant.

Figure 5.10 shows the sequence data of 14 clones derived from an oral biopsy of a sarcoid patient. In this case a major variant was present with a T^{1032} mutation, classifying it as group B1. One single sequence showed T^{1034}, C^{1116} and A^{1124}, which is the motif of the group B variants.



Decoration 'Decoration #1': Hide (as '.') residues that match the Consensus exactly.

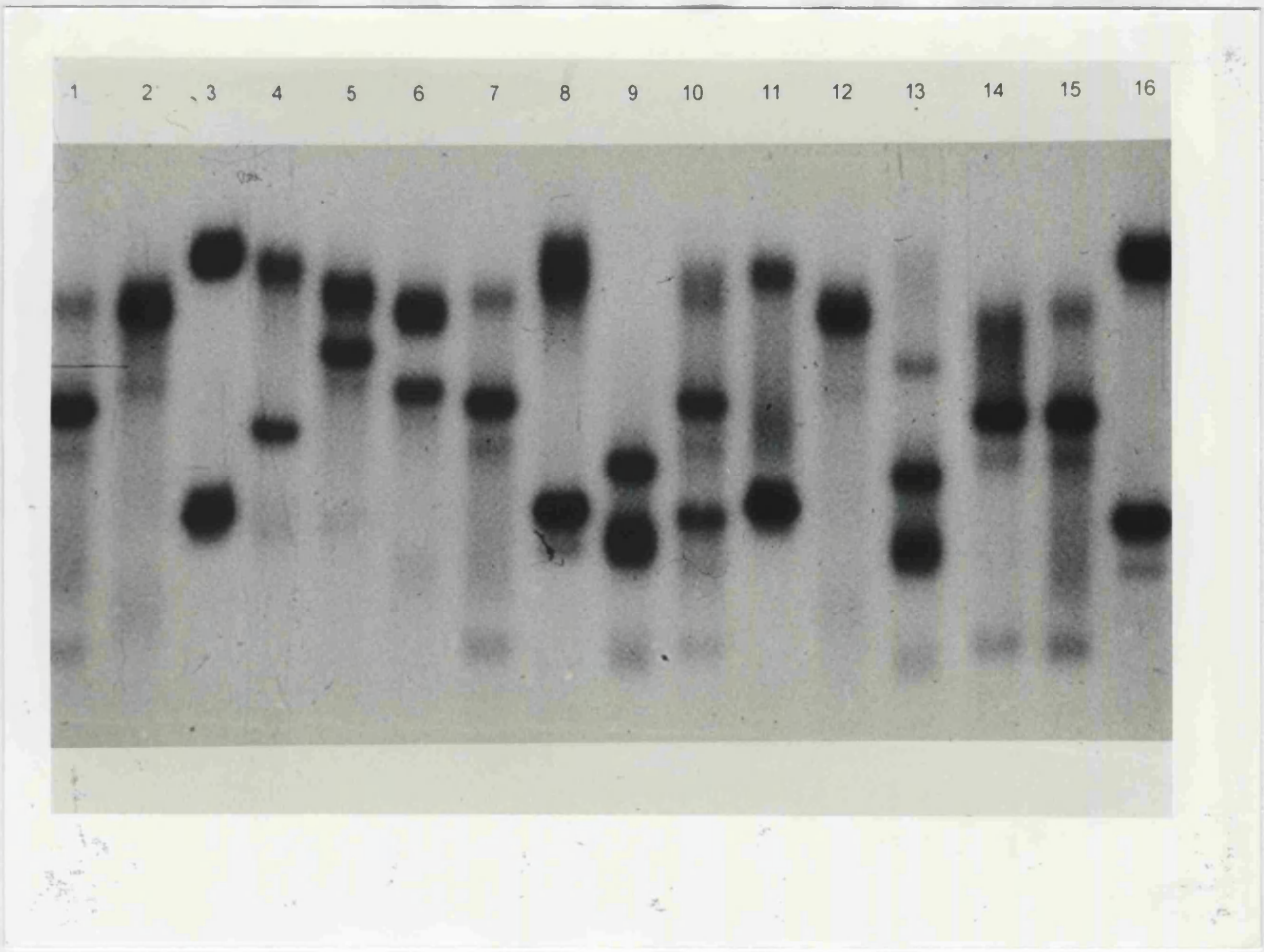
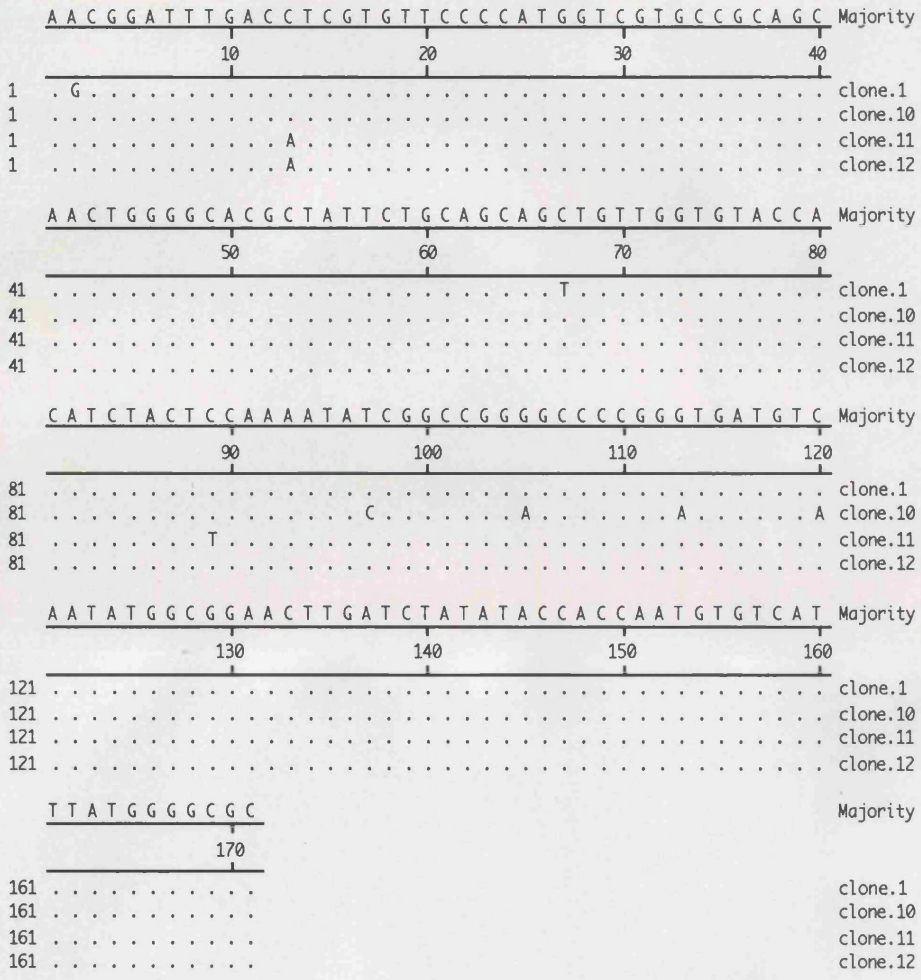


Figure 5-6 SSCP profile (above) and sequence alignment (left) of clones selected from amplicons derived from skin tissue of a patient (G1) with sarcoidosis.

Clone numbers indicated at left correspond to lane numbers above.

(Specimen number in this figure and in Figure 5.7 to 5.10 correspond to specimen numbers in Table 4.1).

Different banding patterns predict differences in nucleotide sequences.



Decoration 'Decoration #1': Hide (as '.') residues that match the Consensus exactly.

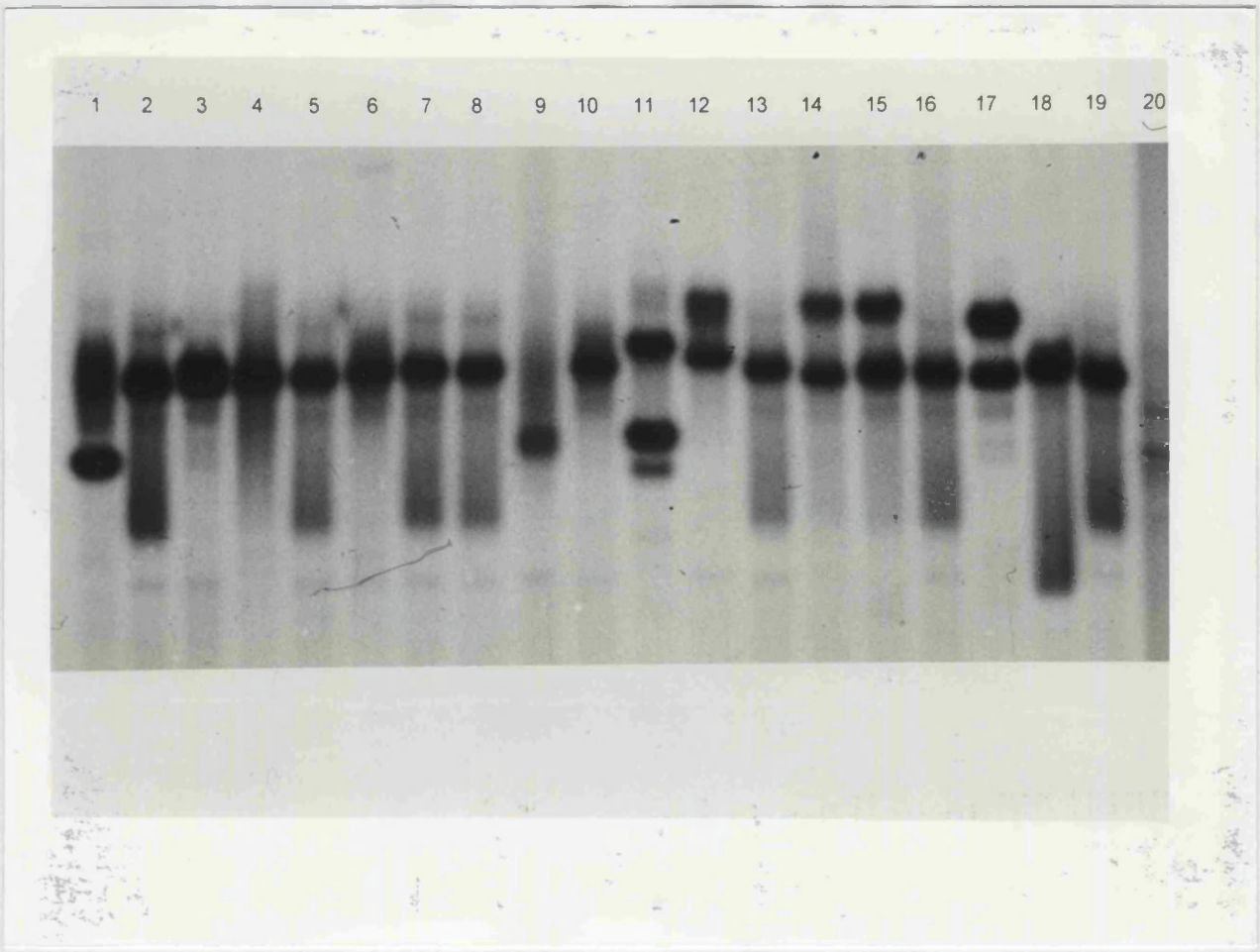


Figure 5-7 SSCP profile (above) and sequence alignment (left) of clones selected from amplicons derived from lymph node tissue of a patient (H1) with sarcoidosis.

Clone numbers indicated at left correspond to lane numbers above.

Different banding patterns predict differences in nucleotide sequences.

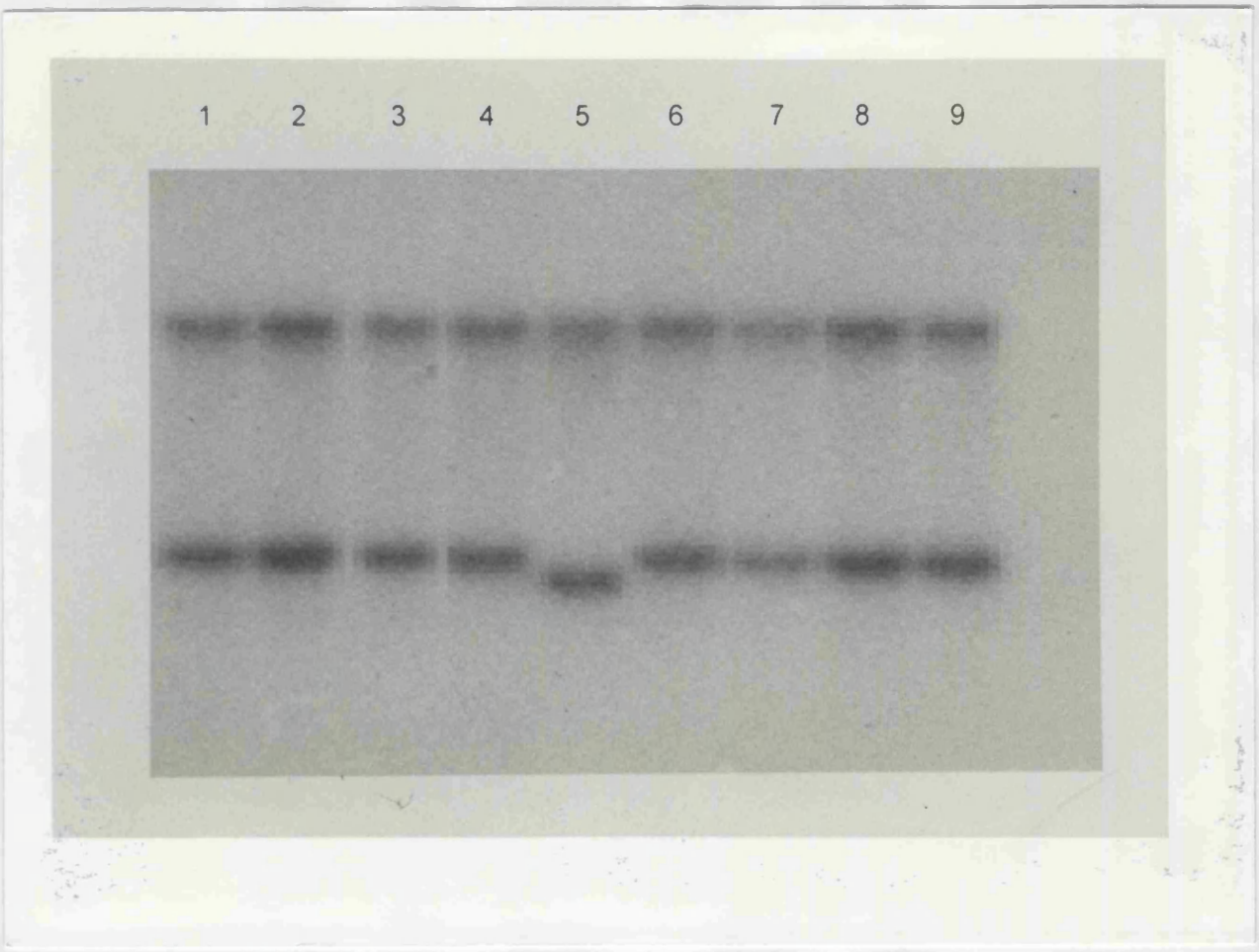
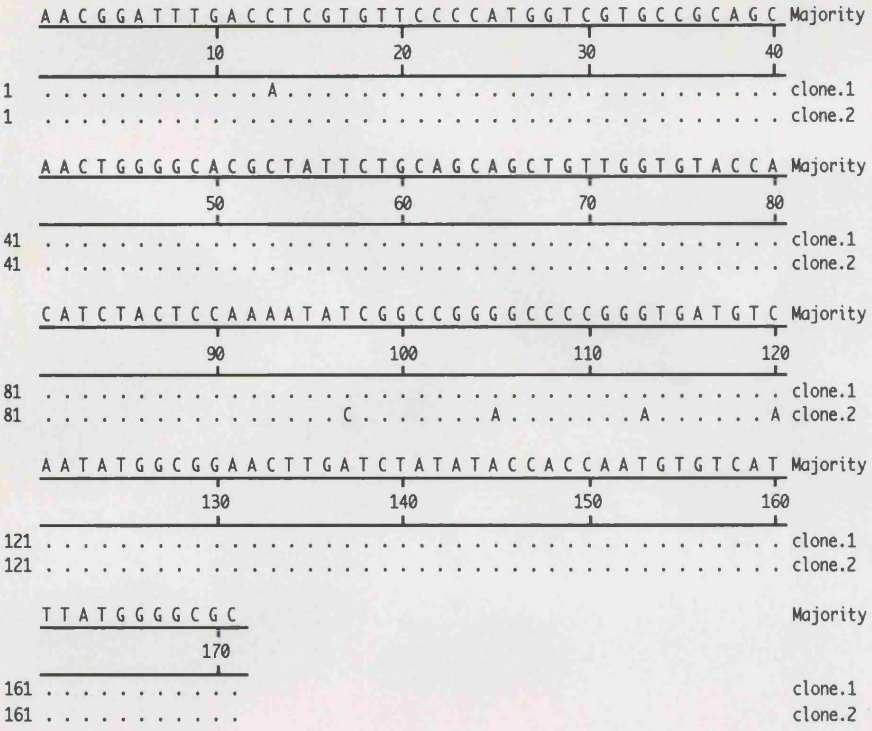


Figure 5-8 SSCP profile of clones (above) selected from amplicons derived from lymph node tissue of a patient (A2) with sarcoidosis.



Decoration 'Decoration #1': Hide (as '.') residues that match the Consensus exactly.

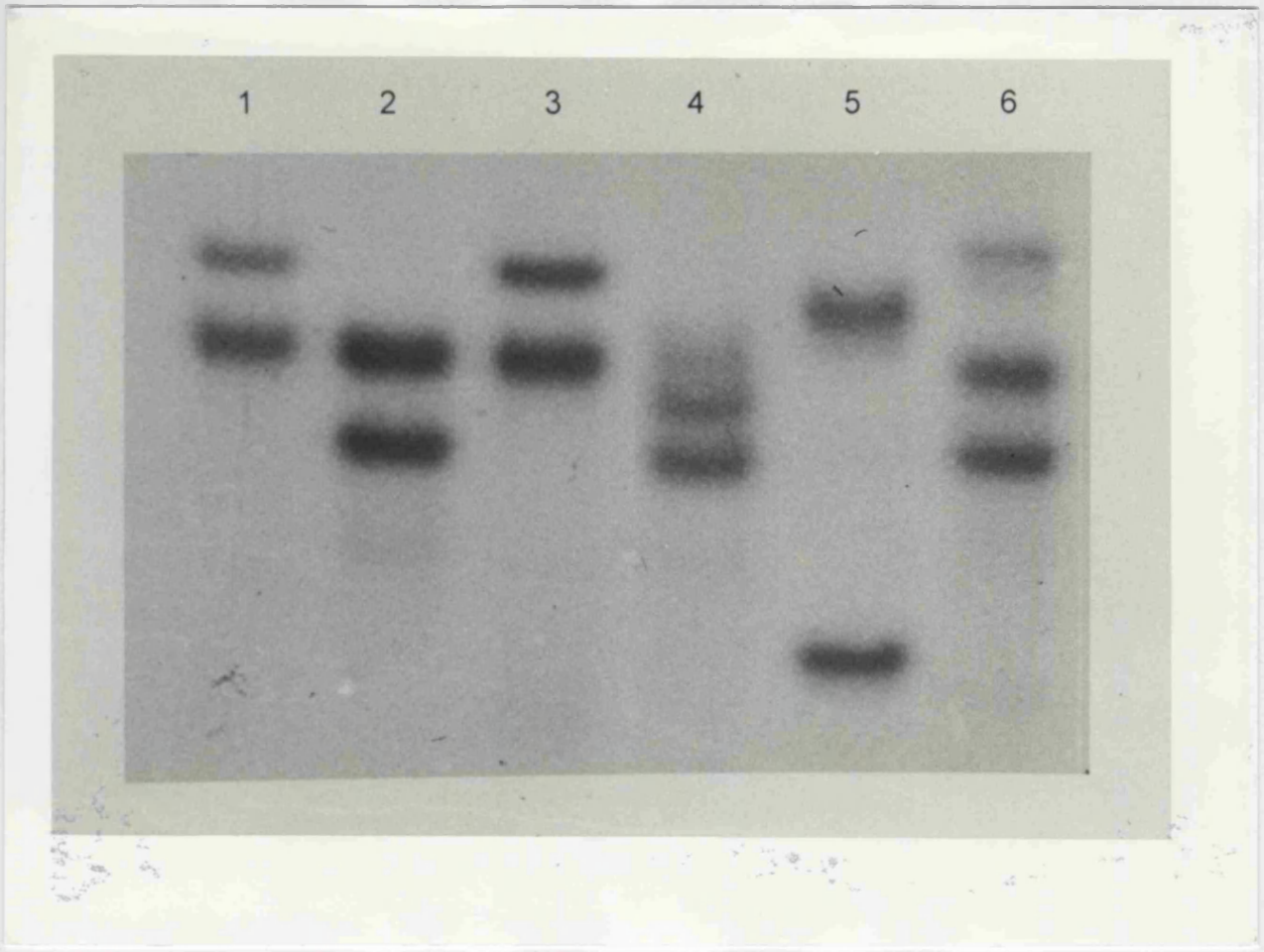
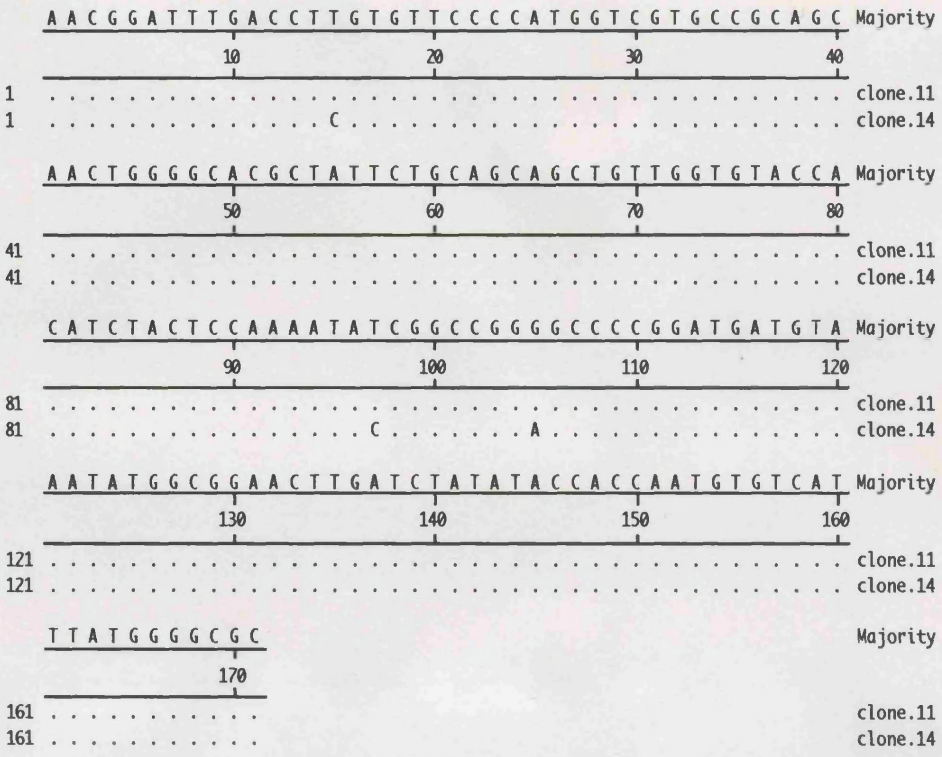


Figure 5-9 SSCP profile (above) and sequence alignment (left) of clones selected from amplicons derived from lymph node tissue of a patient (D2) with sarcoidosis.

Clone numbers indicated at left correspond to lane numbers above.

Different banding patterns predict differences in nucleotide sequences.



Decoration 'Decoration #1': Hide (as '.') residues that match the Consensus exactly.

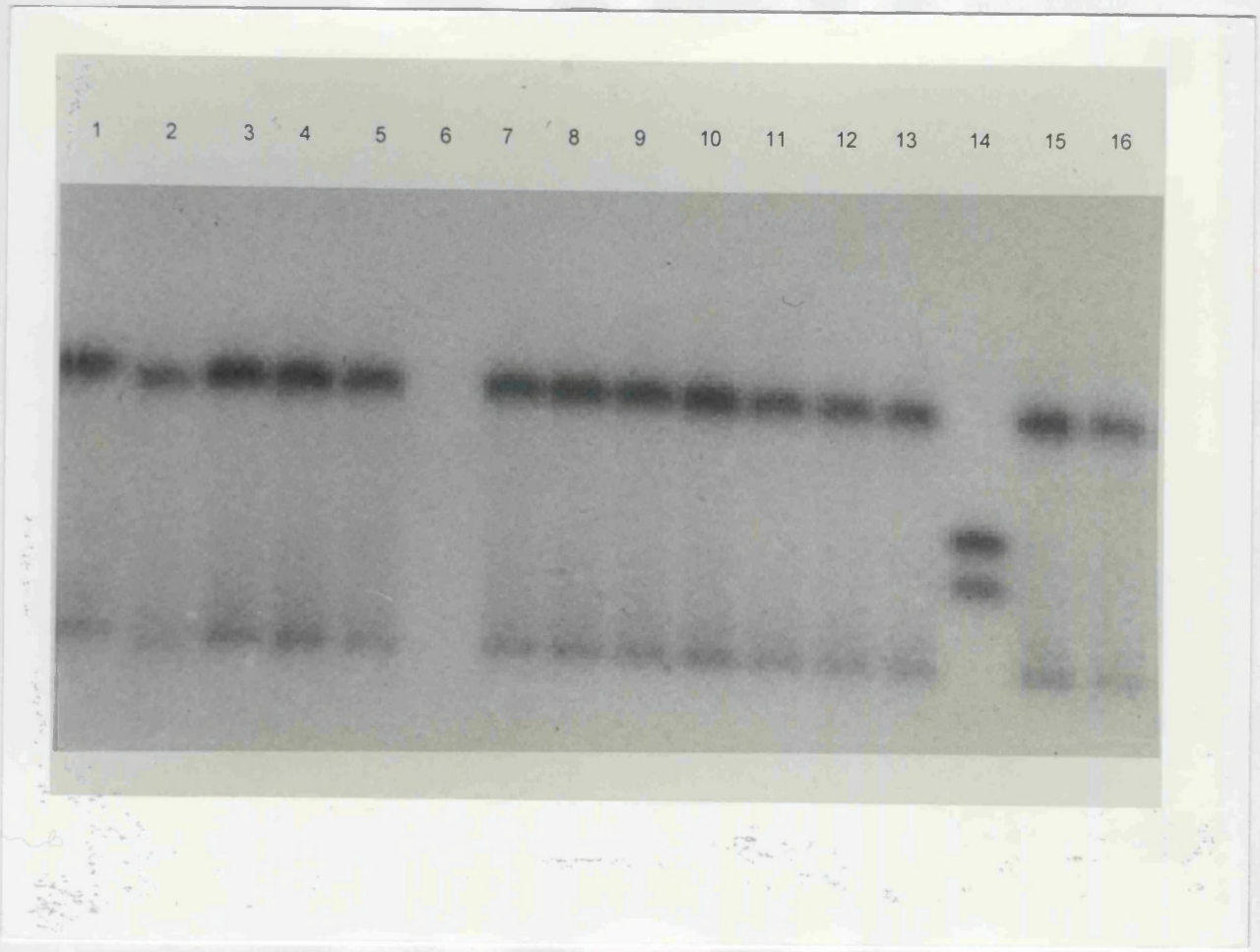


Figure 5-10 SSCP profile (above) and sequence alignment (left) of clones isolated from amplicons derived from oral mucosa tissue of a patient (Q1) with sarcoidosis. Clone numbers indicated at left correspond to lane numbers above.

Different banding patterns predict differences in nucleotide sequences.

5.14. Intra-host ORF 26 variation in Kikuchi's disease: DNA sequence analysis

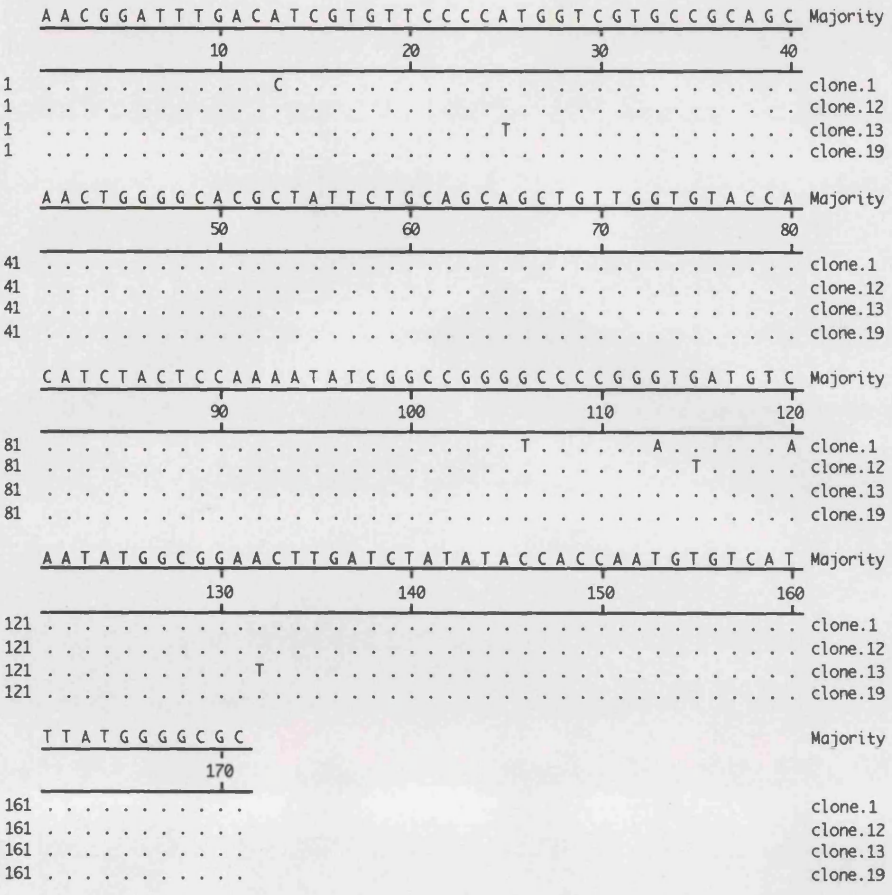
Four PCR amplicons obtained from different Kikuchi's disease lymph node biopsies were cloned and sequenced. Figure 5.11 shows sequence data from 20 different clones from the same biopsy of patient 6. Twelve out of the clones showed the same banding pattern, possessing A^{1032}, T^{1033}, G^{1132} and C^{1139}, and so could be classified as group C1. Clone 6.1, 6.2 and 6.17 showed C^{1032}, T^{1033} and T^{1125} changes and fall into group B. Clone 6.12 is regarded as variant of group C, with the following point mutations: A^{1032}, T^{1033}, G^{1132}, C^{1139} and T^{1134}. Changes in clone 6.13 were at A^{1032}, T^{1033}, T^{1055}, G^{1132}, C^{1139} and T^{1151}, which marked the clone as a group C variant. Changes in clone 6.11 were at A^{1032}, T^{1033}, G^{1132} and C^{1139}, correlating with group C1. Clones 6.3 and 6.4 possessed T^{1033} mutation, placing it in group B1.

Figure 5.12 shows the sequence data derived from 19 clones from the lymph node of patient 7. Eleven of the 19 clones were identical, with changes at positions 1032, 1033, 1132 and 1139, and they thus fall into group C1. Three clones showed C^{1033}, C^{1116} and A^{1124}, and could be classified as group B variants. One clone showed the characteristic changes of group C1 with an additional mutation at T^{1044}. The last clone sequenced showed changes at 1032 and 1125, classifying it as a group B variant.

Twelve clones were sequenced from another Kikuchi's disease lymph node biopsy (of patient 5) and their sequences are shown in Figure 5.13. Five clones bore the C^{1116} and A^{1124} mutations, and were classified as group B variant. Three clustered

together and showed mutations at positions 1021, 1086, 1132 and 1139; they fall into group E. Two clones showed a change at T^{1121}, and they fall into group B. Clone 5.3 showed mutations at positions 1032 and 1033 and is thus a B1 variant. Clone 5.7 showed changes at positions 1032, 1033, 1132 and 1139, possessing the motifs of C1.

Figure 5.14 shows sequence data from 20 clones from the biopsy of patient 8. Nine out of 20 clones were identical and showed mutations characteristic of variant C1. Four clones showed changes at position 1033; they therefore belong to B1. Clone 8.16 was classified as variant E1 because of the changes at positions 1033, 1132 and 1139. Clones 8.10 and 8.6 appear to be C1 variants, with a single additional change at 1079 and 1151, respectively. Clones 8.13 and 8.18 showed a C2 pattern with a change at A^{1144} and A^{1172}, respectively. Clone 8.12 revealed the following changes: A^{1032}, T^{1033}, G^{1044}, G^{1132}, C^{1139} and G^{1151}, thus this variant falls into group C. Clone 8.20 showed point mutations at 1032, 1033, 1189, 1106, 1132 and 1139, and was therefore considered as a variant of group C.



Decoration 'Decoration #1': Hide (as '.') residues that match the Consensus exactly.

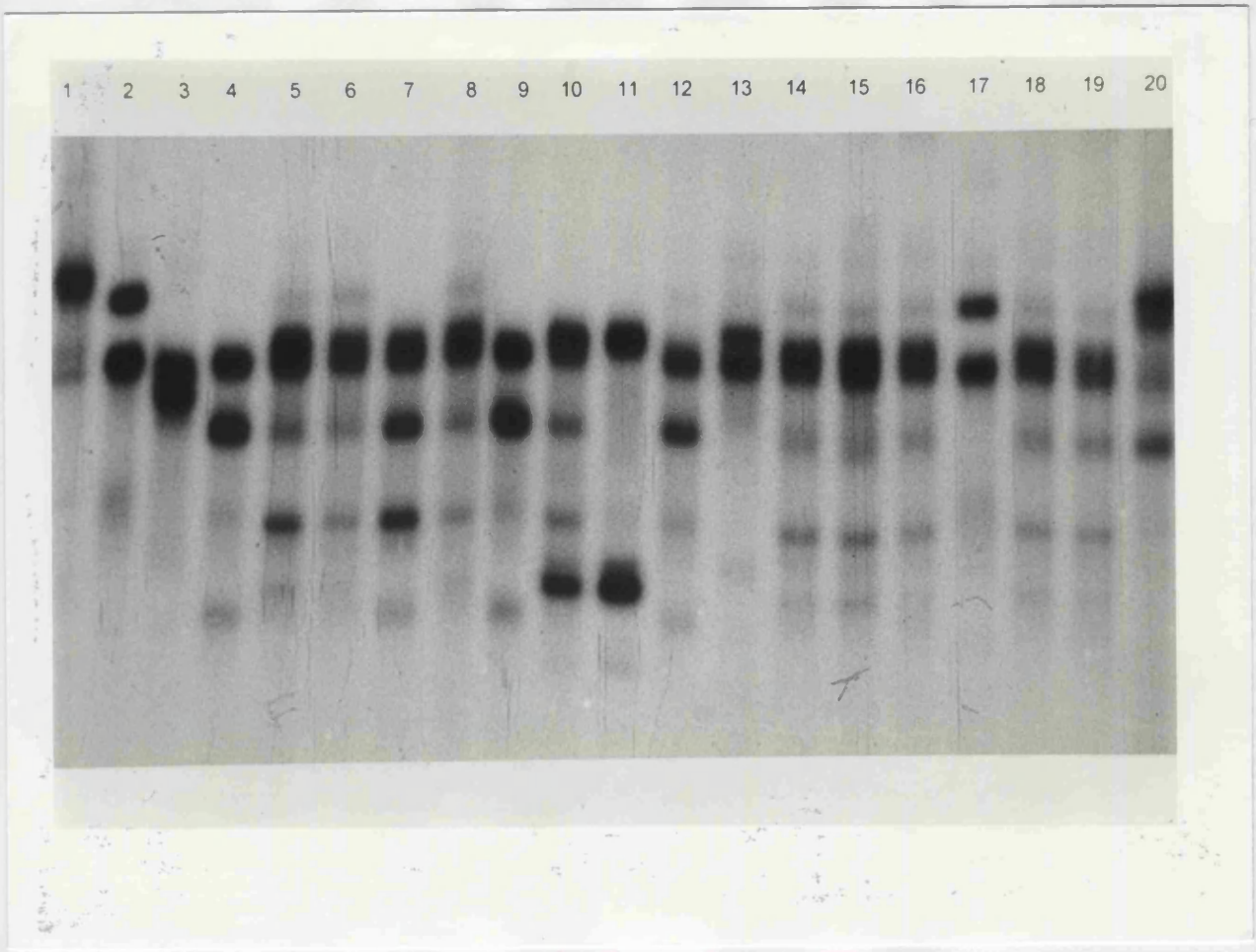
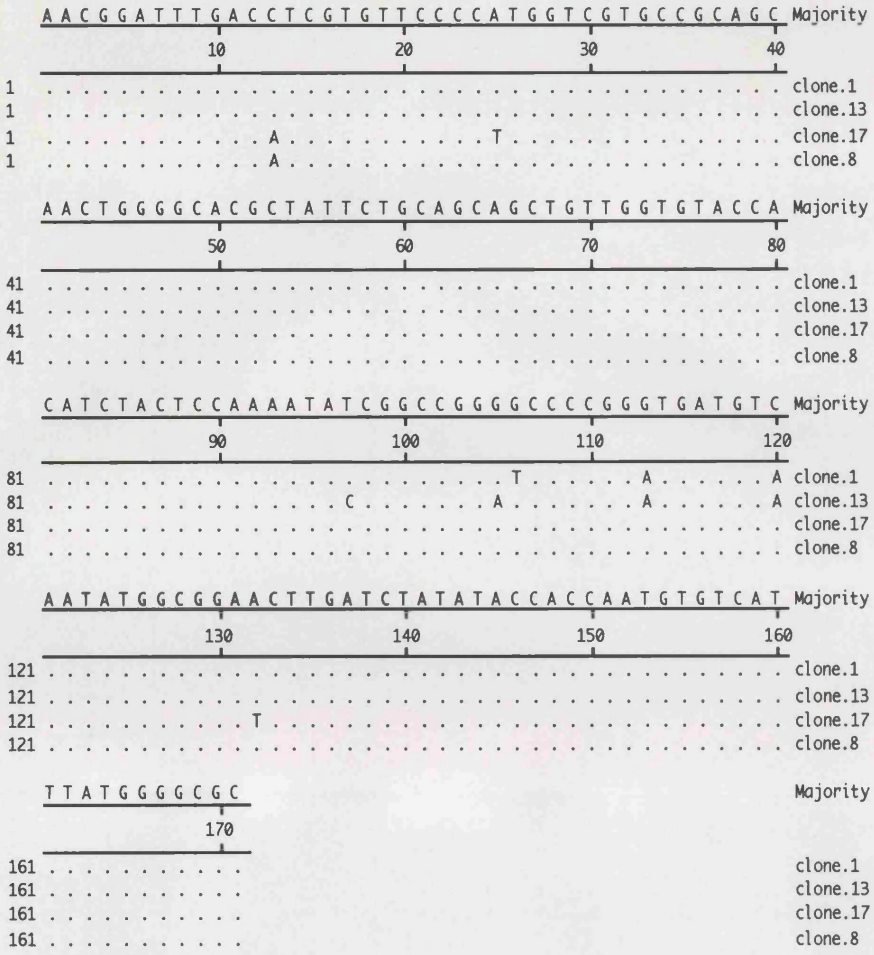


Figure 5-11 SSCP profile (above) and sequence alignment (left) of clones selected from amplicons derived from lymph node tissue of a patient with Kikuchi's disease (patient 6 of Figure 5.2).

Clone numbers indicated at left correspond to lane numbers above.

Different banding patterns predict differences in nucleotide sequences.



Decoration 'Decoration #1': Hide (as '.') residues that match the Consensus exactly.

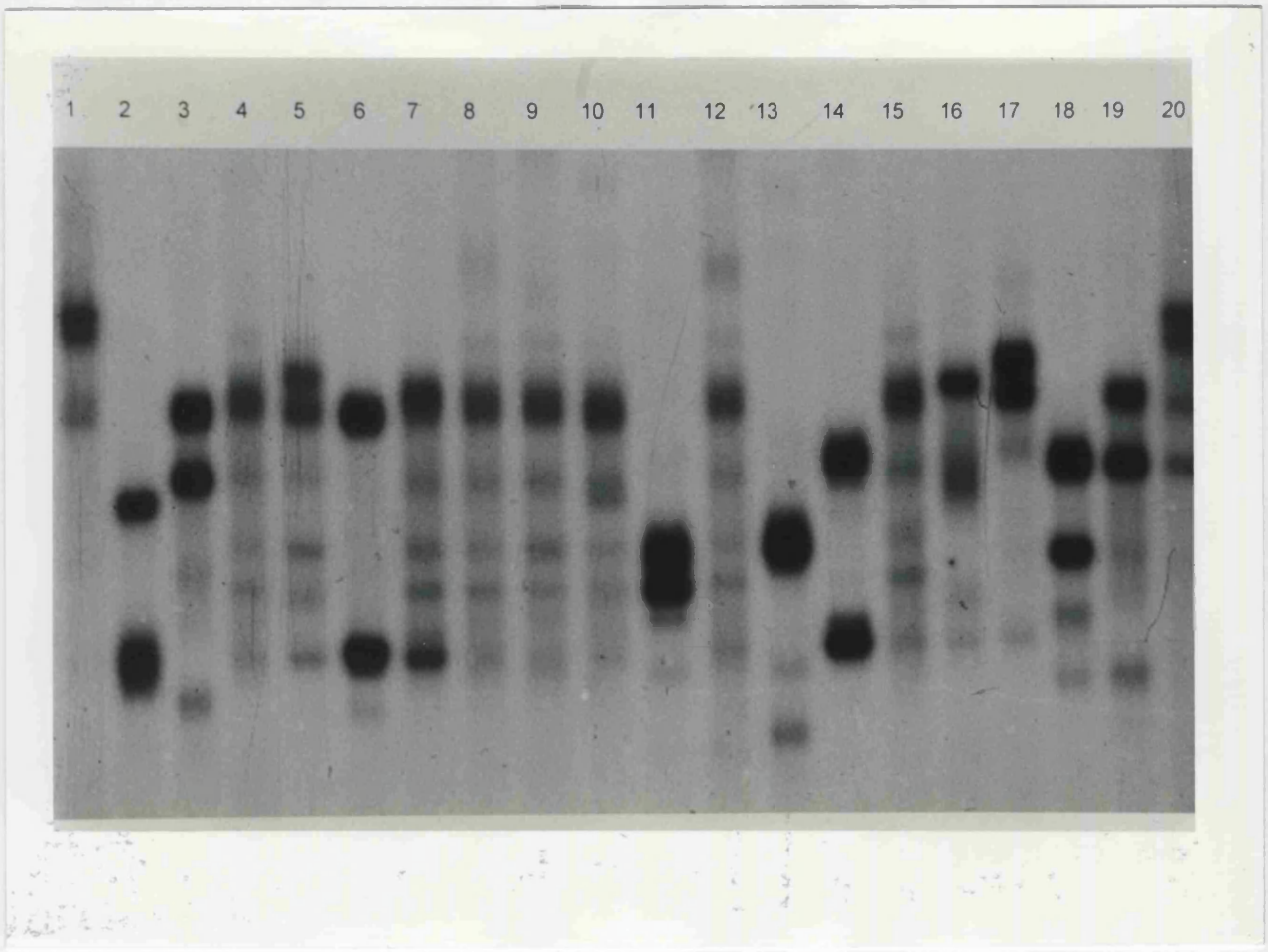
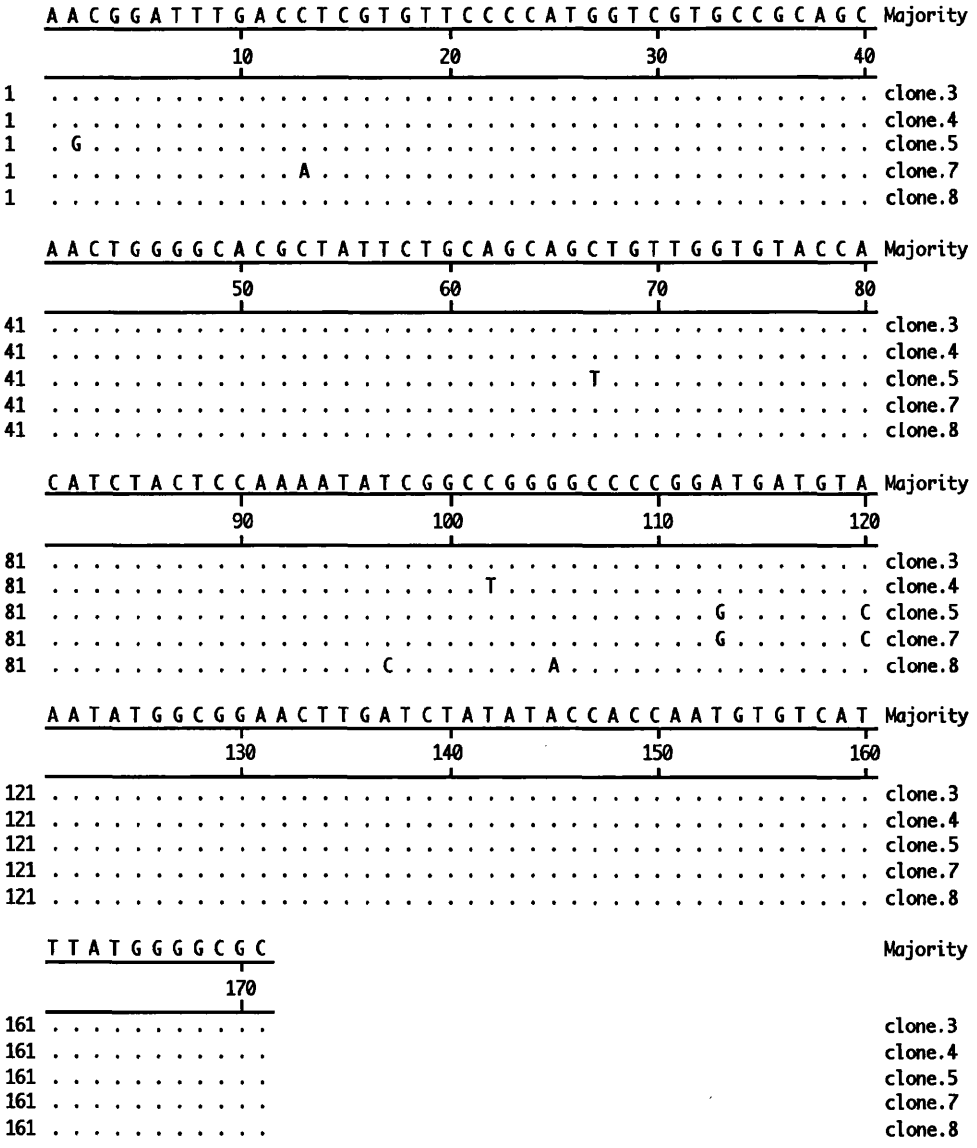


Figure 5-12 SSCP profile (above) and sequence alignment (left) of clones selected from amplicons derived from lymph node tissue of a patient with Kikuchi's disease (patient 7 of Figure 5.2)

Clone numbers indicated at left correspond to lane numbers above.

Different banding patterns predict differences in nucleotide sequences.



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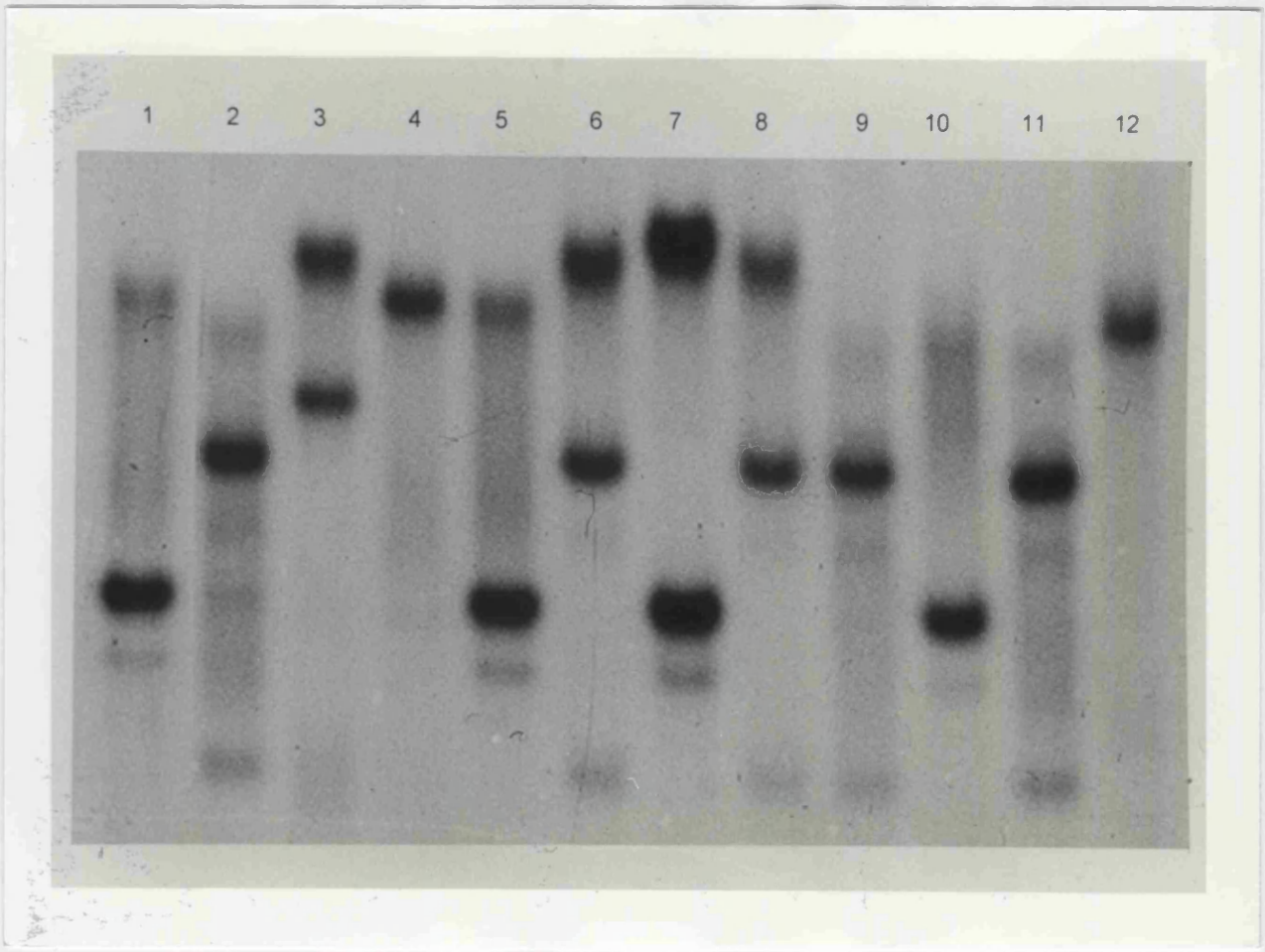
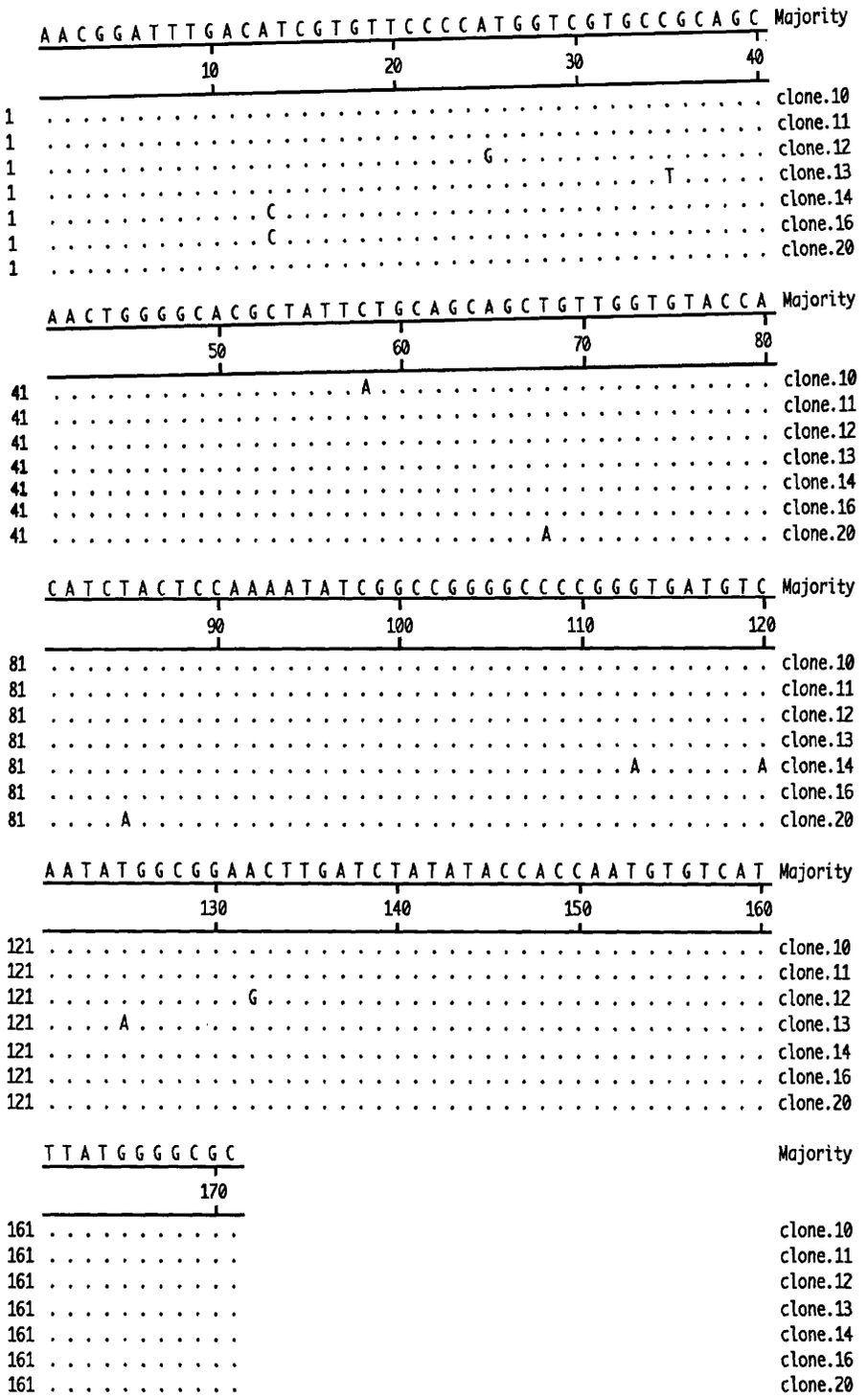


Figure 5-13 SSCP profile (above) and sequence alignment (left) of clones selected from amplicons derived from lymph node tissue of a patient with Kikuchi's disease (patient 5 of Figure 5.2).

Clone numbers indicated at left correspond to lane numbers above.

Different banding patterns predict differences in nucleotide sequences.



Decoration 'Decoration #1': Hide (as '.') residues that match the Consensus exactly.

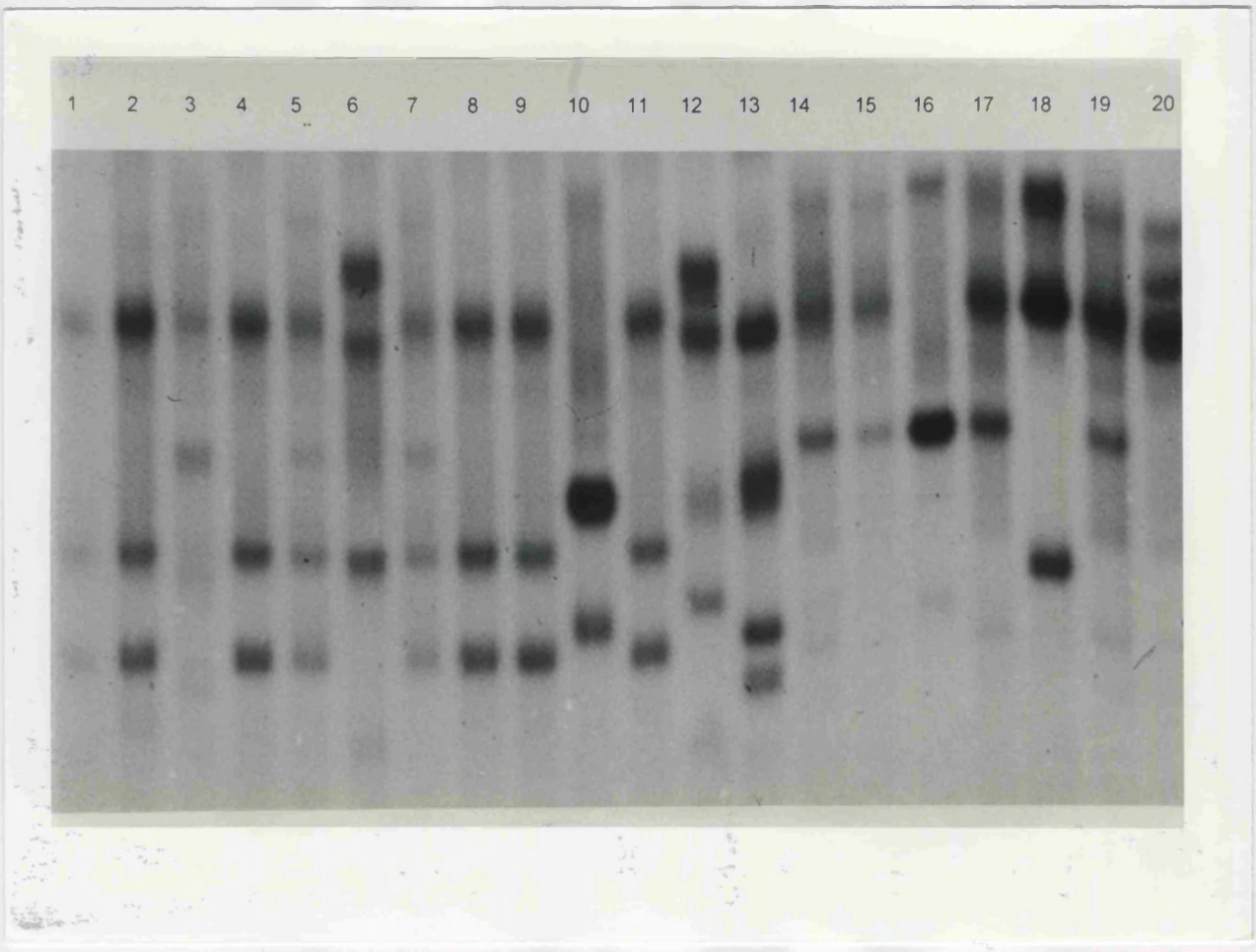


Figure 5-14 SSCP profile (above) and sequence alignment (left) of clones selected from amplicons derived from lymph node tissue of a patient with Kikuchi's disease (patient 8 of Figure 5.2).

Clone numbers indicated at left correspond to lane numbers above.

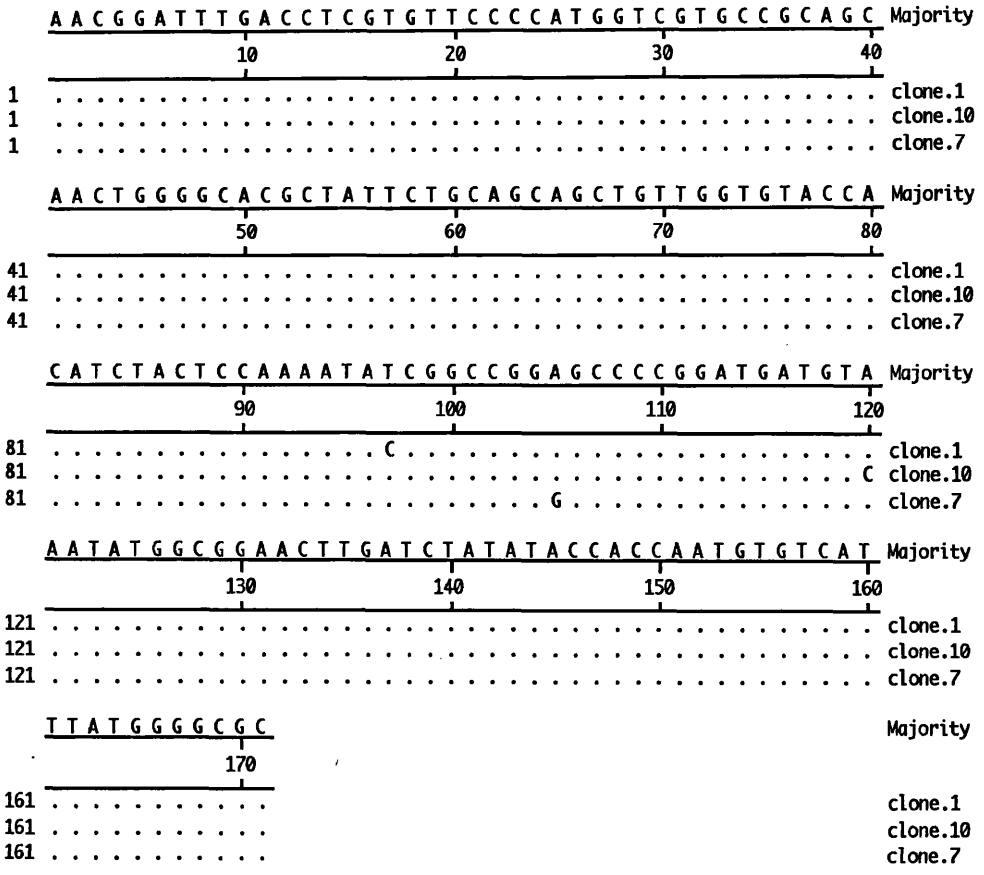
Different banding patterns predict differences in nucleotide sequences.

5.15. Intra-host ORF 26 variation in other forms of lymphadenitis: DNA analysis

Results from cloning of a biopsy of a patient with reactive lymphadenitis showed the presence of three different banding patterns (Figure 5.15). Nine out 18 clones showed mutations at position 1032, classifying them as B1 variants. Five clones showed the following changes: T^{1033}, A^{1124}, C^{1139}; these changes are characteristic of group H2. Four clones appeared to be a variant of group B, with point mutation at positions 1116 and 1124 .

Figure 5.16 shows an SSCP banding pattern from a biopsy of a patient with granulomatous lymphadenitis. Nine out of 13 clones showed an identical sequence with a change at position 1033 and they are thus in group B1. Clone 7.13 is also related to group B, but with a mutation at position 1052. Clone 7.10 showed changes at positions A^{1032}, T^{1033}, G^{1059}, C^{1116}, A^{1124}, G^{1132}, C^{1139} and G^{1164}; this variant falls into group C. Clone 7.3 showed changes at position 1116 and 1124, and can therefore be classified as variant of group B. Clone 7.6 appears to be variant C1.

Figure 5.17 shows 20 clones from a biopsy of another patient with granulomatous lymphadenitis. Sixteen out of 20 clones showed the same mutation at position 1033 and are therefore related to group B1. Clone 13.6 showed changes at position 1033, 1055, 1168, so classifying it as a variant of group B. Clones 13.4 and 13.11 showed mutation at T^{1033}, T^{1086}, G^{1132} and C^{1139}; these variants therefore fall into group B. Finally, clone 13.20 appears to be a variant of group C, with mutations at positions 1032, 1033, 1132, 1139, 1151 and 1153.



Decoration 'Decoration #1': Hide (as '.') residues that match the Consensus exactly.

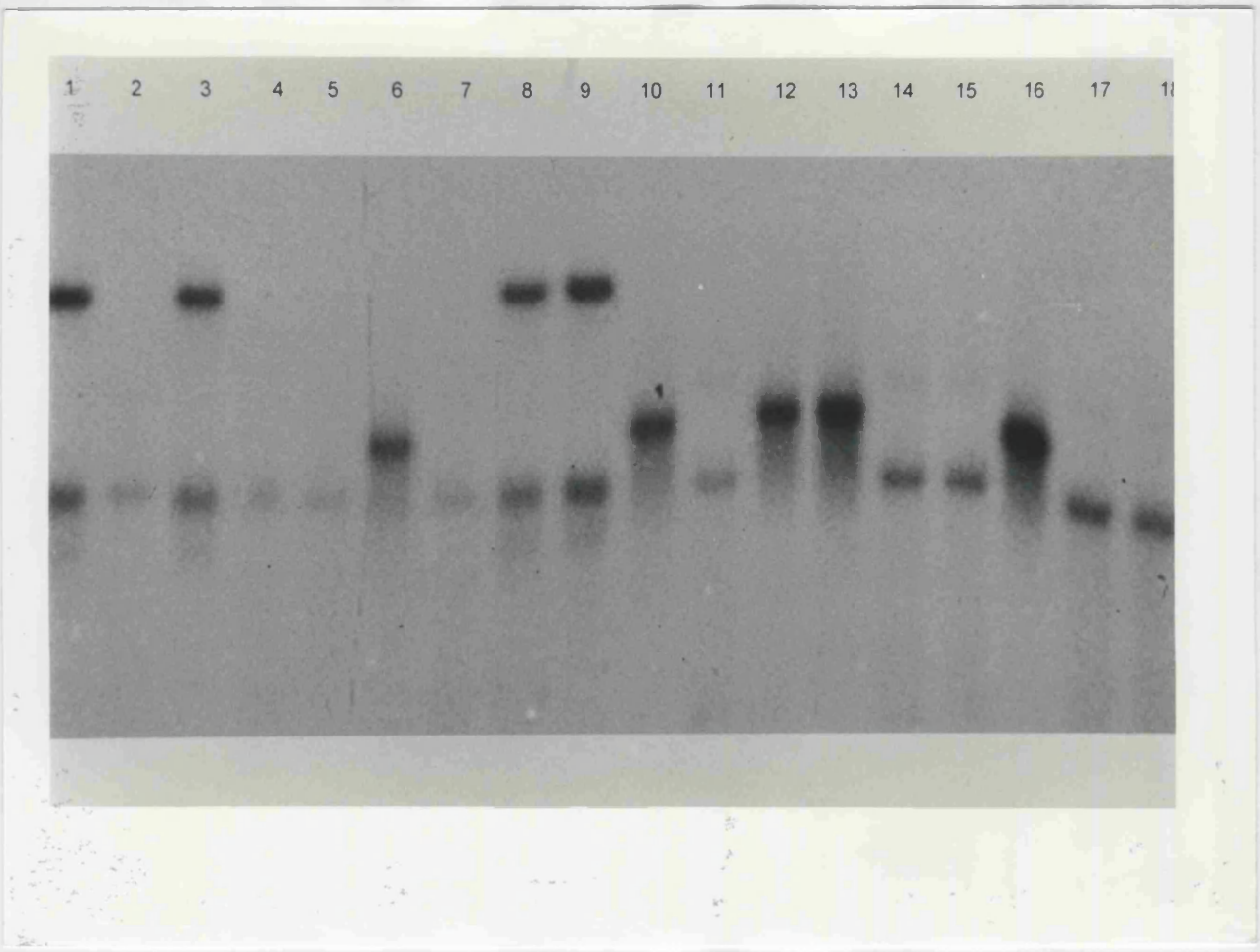
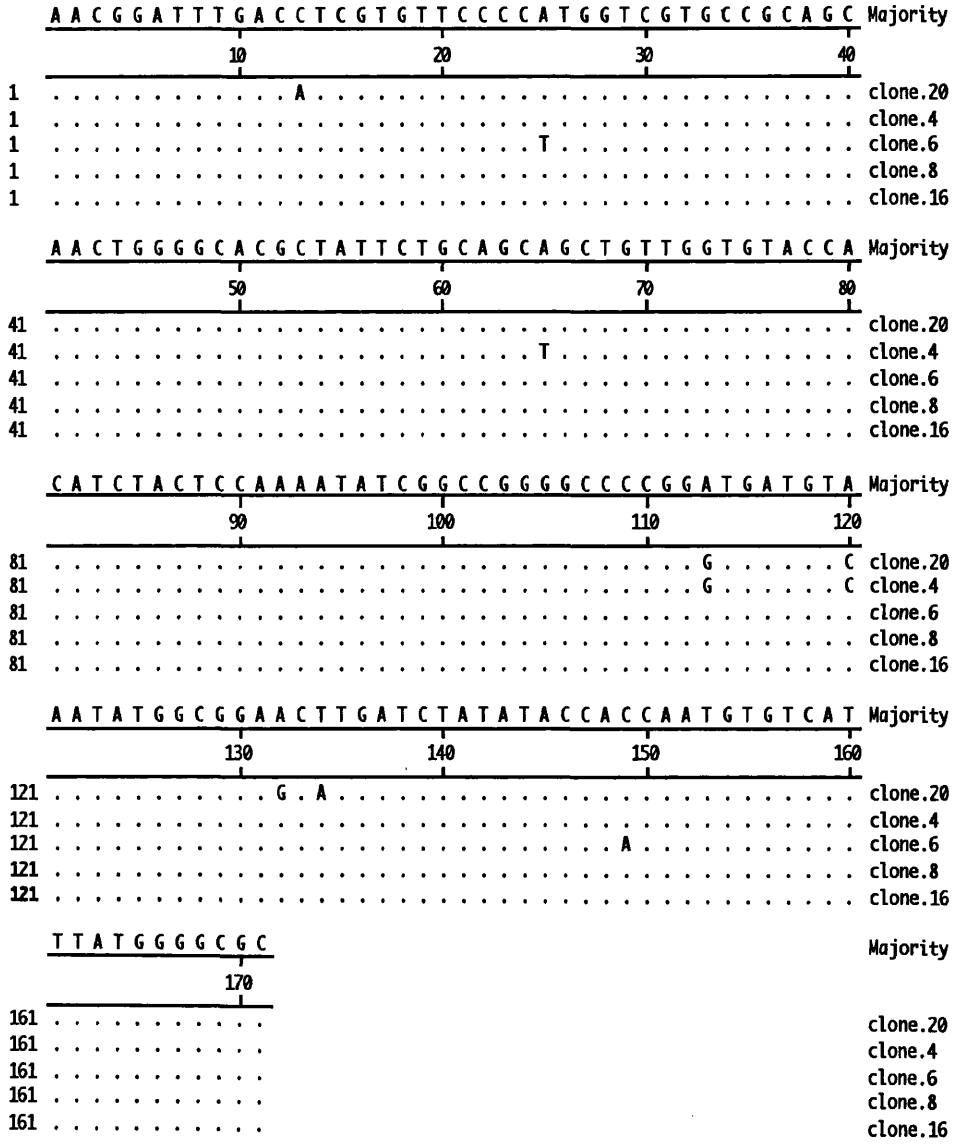


Figure 5-15 SSCP profile (above) and sequence alignment (left) of clones selected from amplicons derived from lymph node tissue of a patient with reactive lymphadenitis (patient 1 of figure 5.4).

Clone numbers indicated at left correspond to lane numbers above.

Different banding patterns predict differences in nucleotide sequences.



Decoration 'Decoration #1': Hide (as '.') residues that match the Consensus exactly.

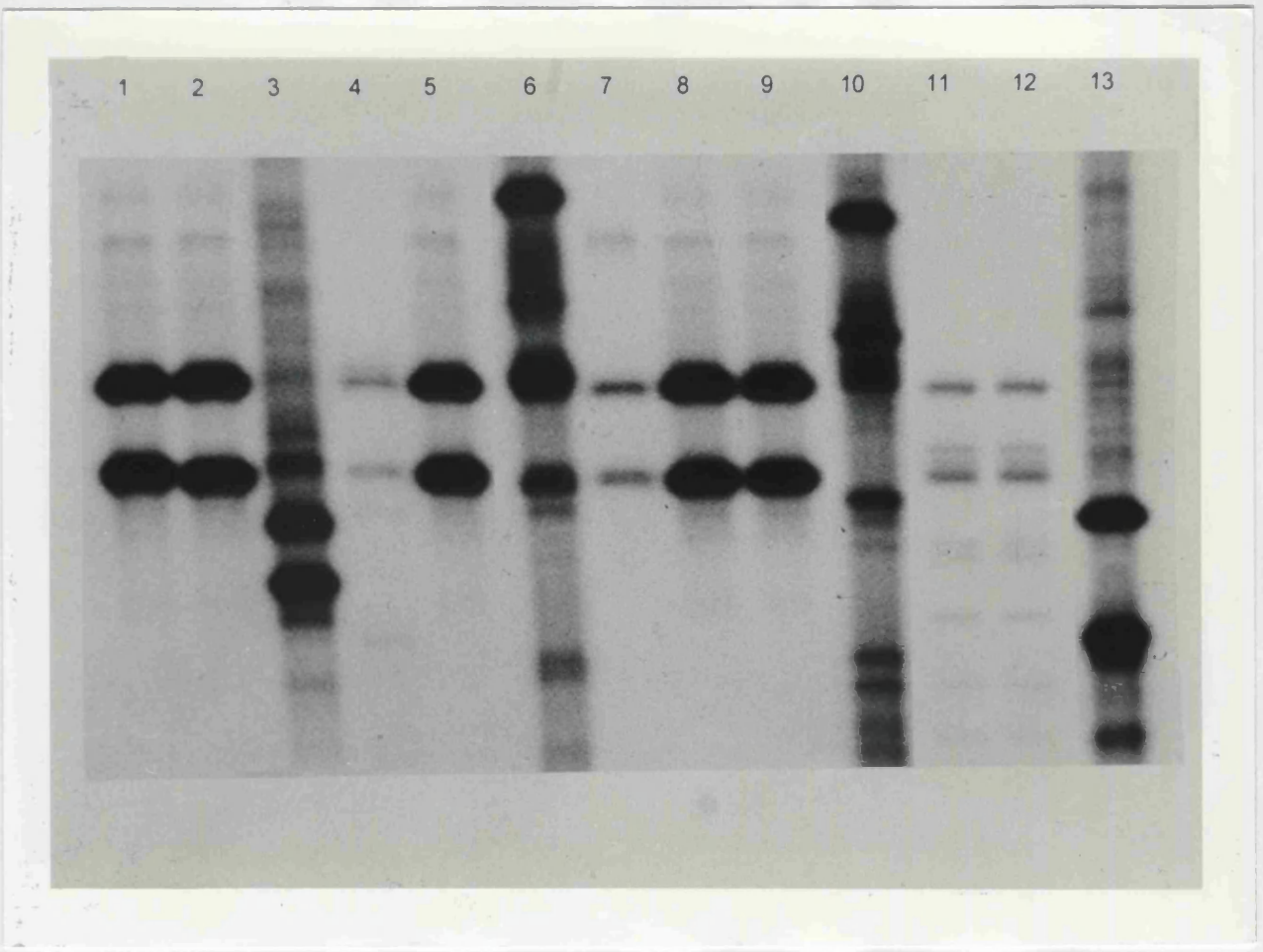
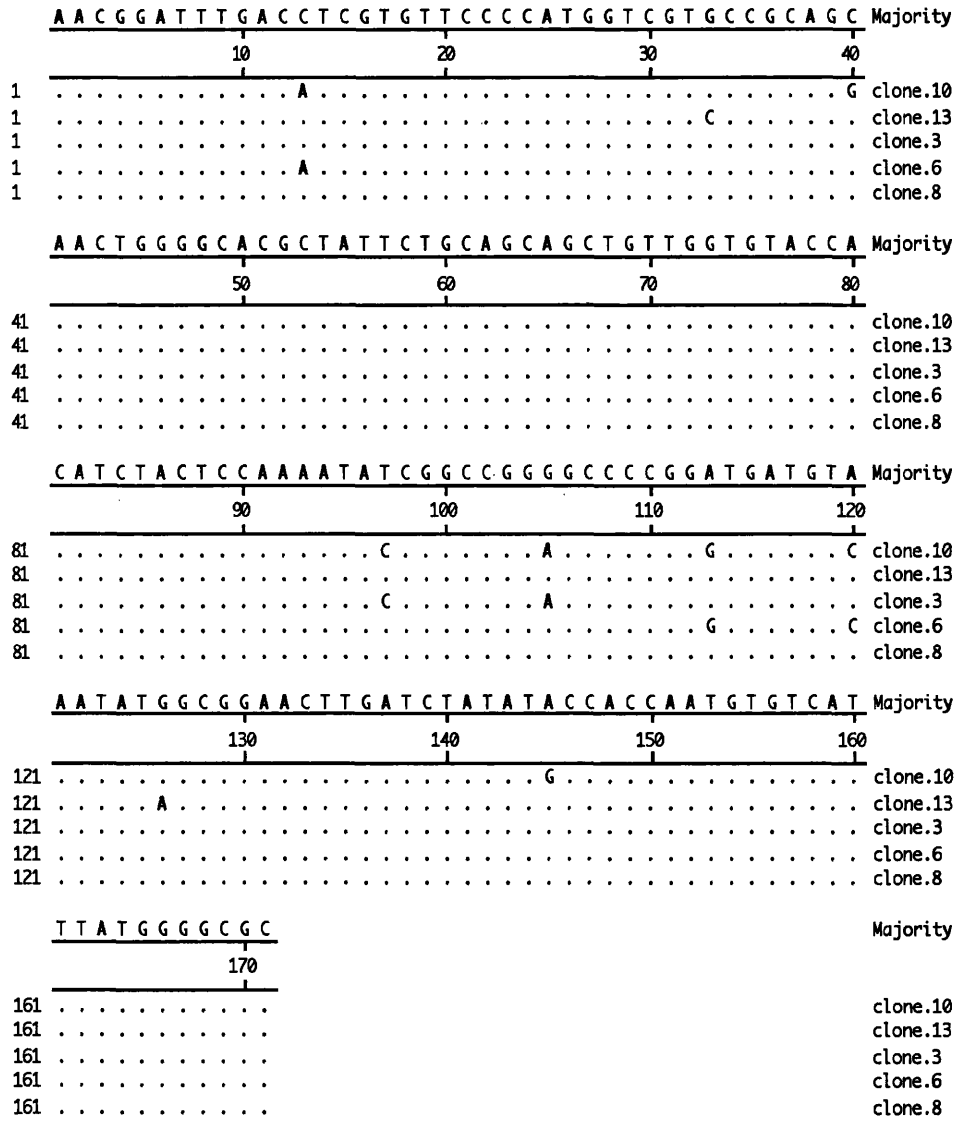


Figure 5-16 SSCP profile (above) and sequence alignment (left) of clones selected from amplicons derived from lymph node tissue of a patient with granulomatous lymphadenitis (patient 8 of figure 5.4).

Clon numbers indicated at left correspond to lane numbers above.

Different banding patterns predict differences in nucleotide sequences.



Decoration 'Decoration #1': Hide (as '.') residues that match the Consensus exactly.

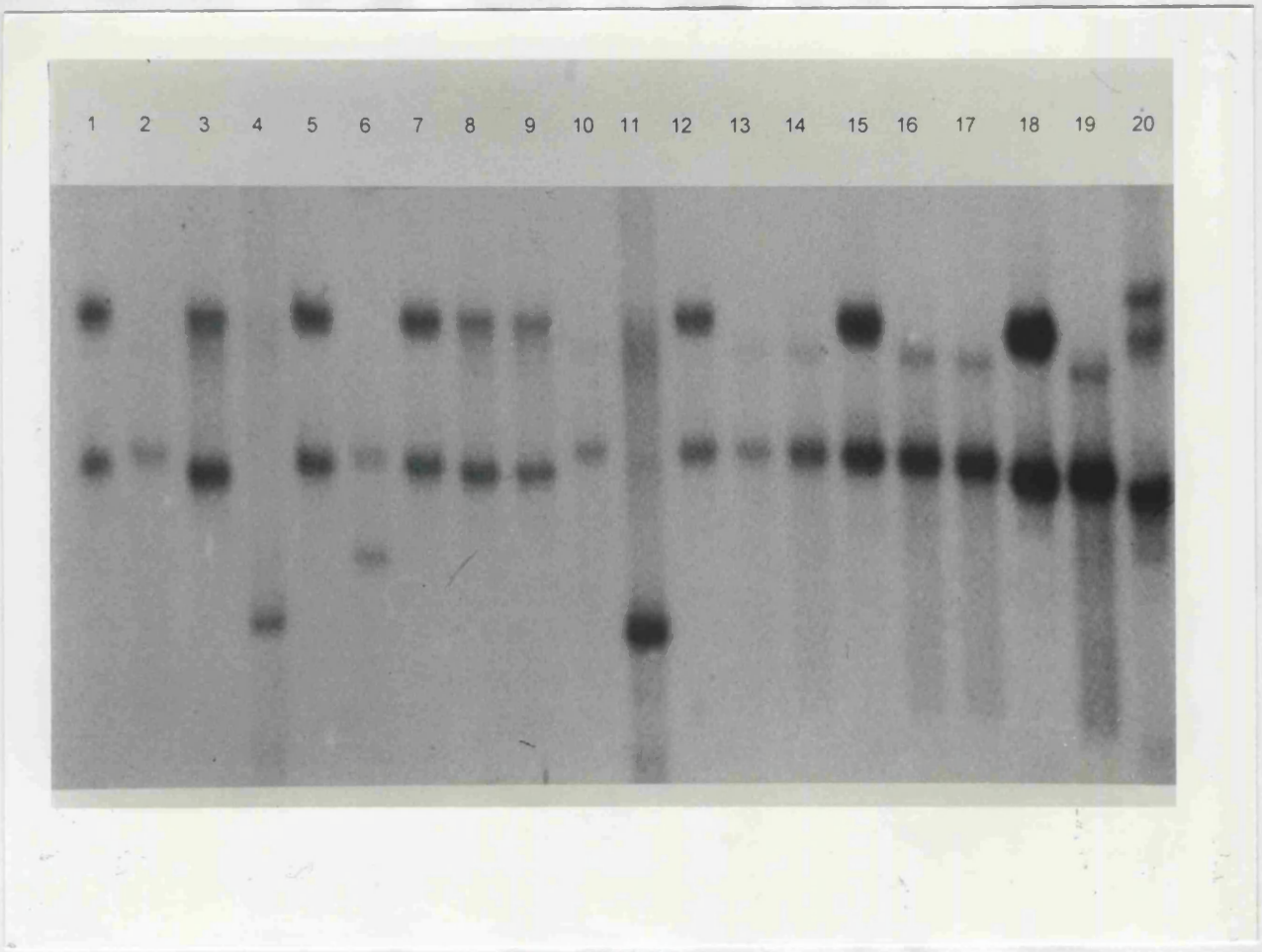


Figure 5-17 SSCP profile (above) and sequence alignment (left) of clones selected from amplicons derived from lymph node tissue of a patient with granulomatous lymphadenitis (patient 9 of figure 5.4).

Clone numbers indicated at left correspond to lane numbers above.

Different banding patterns predict differences in nucleotide sequences.

5.16. Intra-host ORF 26 variation in B-cell lymphoma: DNA analysis

Clones form a B-cell lymphoma were sequenced. 16 clones share a common C1 sequence, with mutations at positions 1032, 1033, 1132 and 1139 (Figure 5.18).

5.17. Intra-host ORF 26 variation in Mediterranean KS: DNA analysis

The nineteen clones derived from a Mediterranean KS biopsy showed three distinct sequences (Figure 5.19). The first sequence present in 12 clones showed mutations at positions 1032, 1033, 1132 and 1139, which correspond to changes of variant C1. Three clones bore the same sequence with the following changes: C^{1116} and A^{1124}; they are therefore related to group B. Four other clones showed the following changes: 1033, 1124 and 1139; they could be classified as variant of group H (Figure 5.19).

Clones from another Mediterranean KS biopsy specimen were sequenced and all possessed the characteristics of C1 variant with changes at positions 1032, 1033, 1132 and 1139 (Figure 5.20).

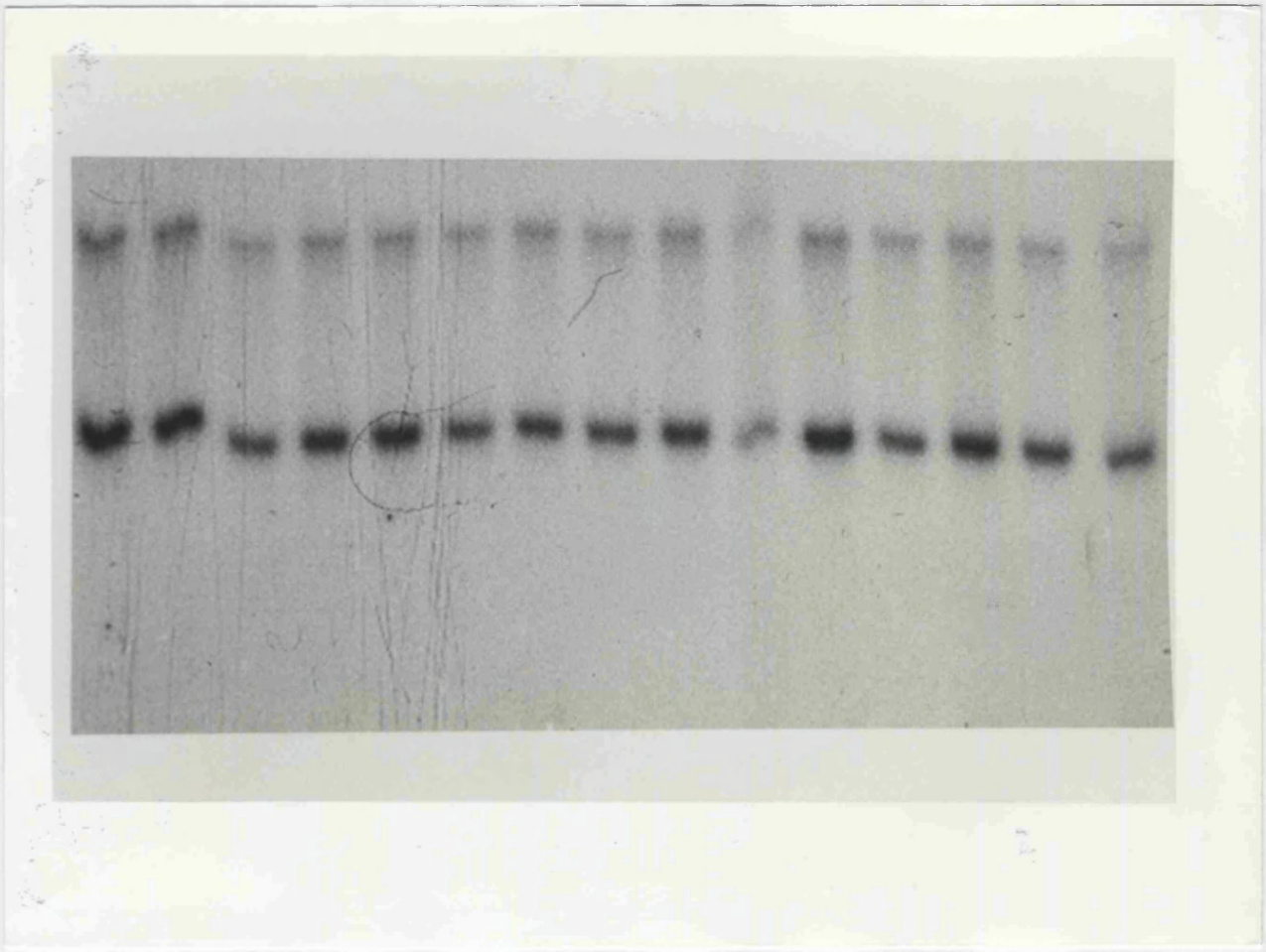
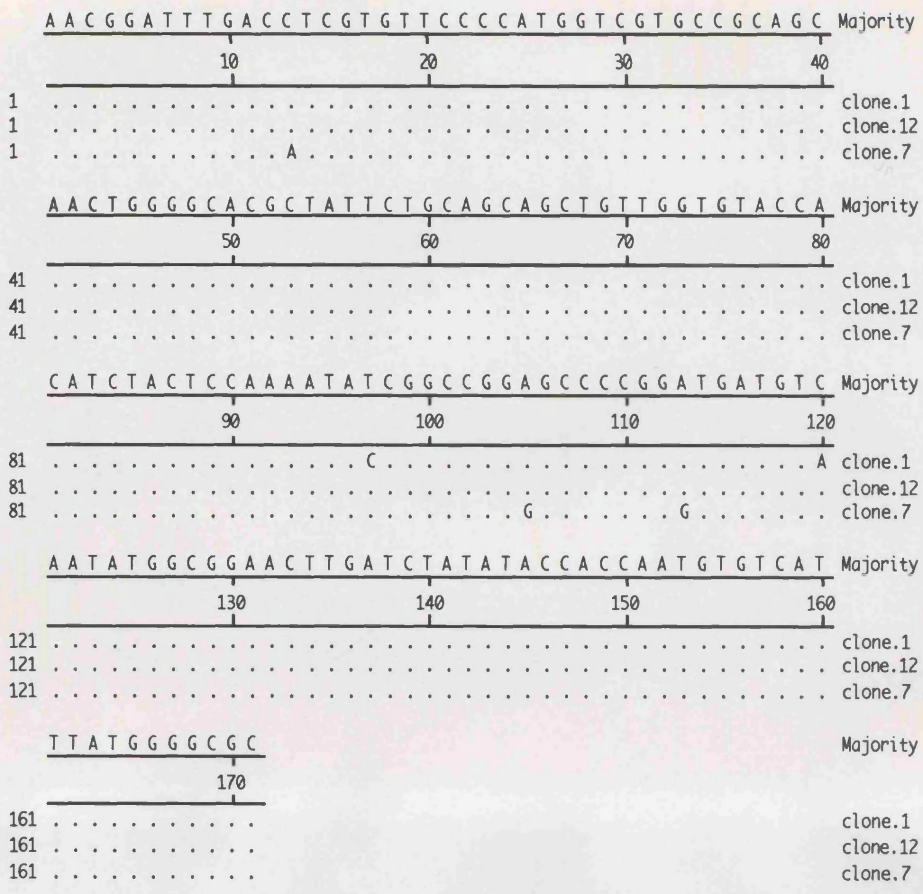


Figure 5-18 SSCP profile of clones isolated from amplicons derived from lymph node tissue of a patient with B-cell lymphoma (patient 18 of figure 5.4).



Decoration 'Decoration #1': Hide (as '.') residues that match the Consensus exactly.

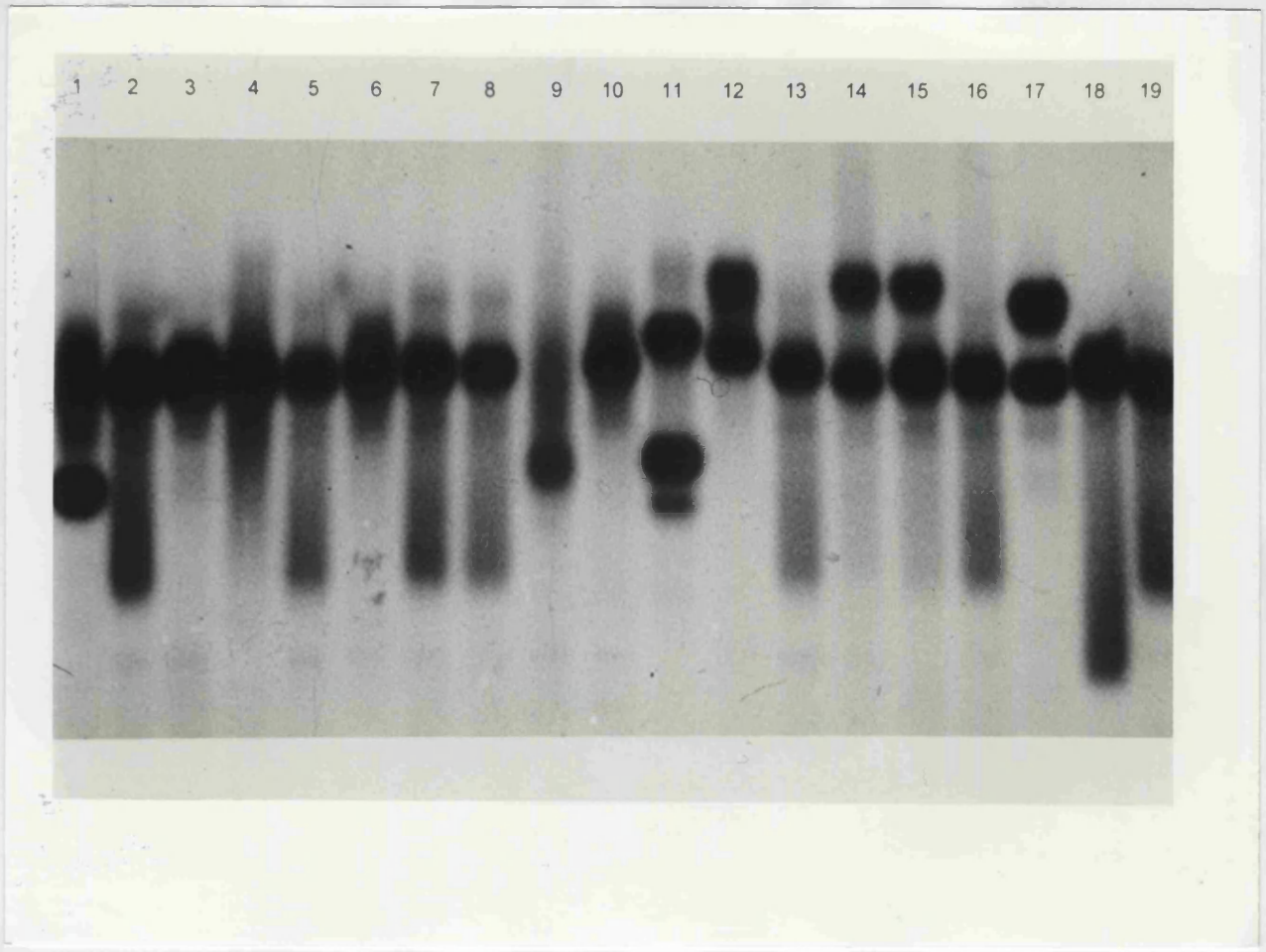


Figure 5-19 SSCP profile and sequence alignment of clones isolated from amplicons derived from skin tissue biopsy of patient with Mediterranean KS (patient 5 of figure 5.3).

Clone numbers indicated at left correspond to lane numbers above.

Different banding patterns predict differences in nucleotide sequences.

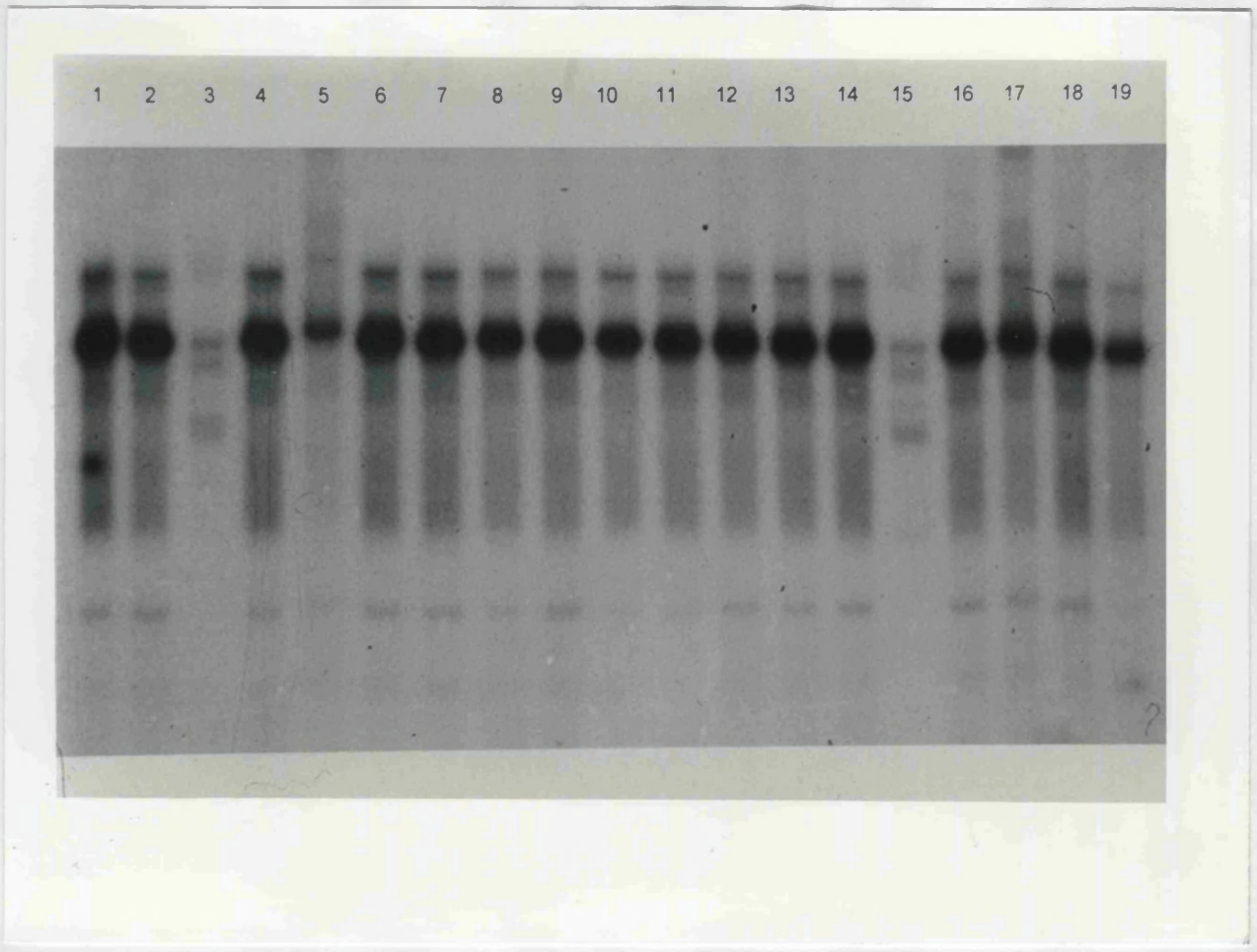


Figure 5-20 SSCP profile of clones isolated from amplicons derived from skin tissue of patient with Mediterranean KS (patient 6 of figure 5.3).

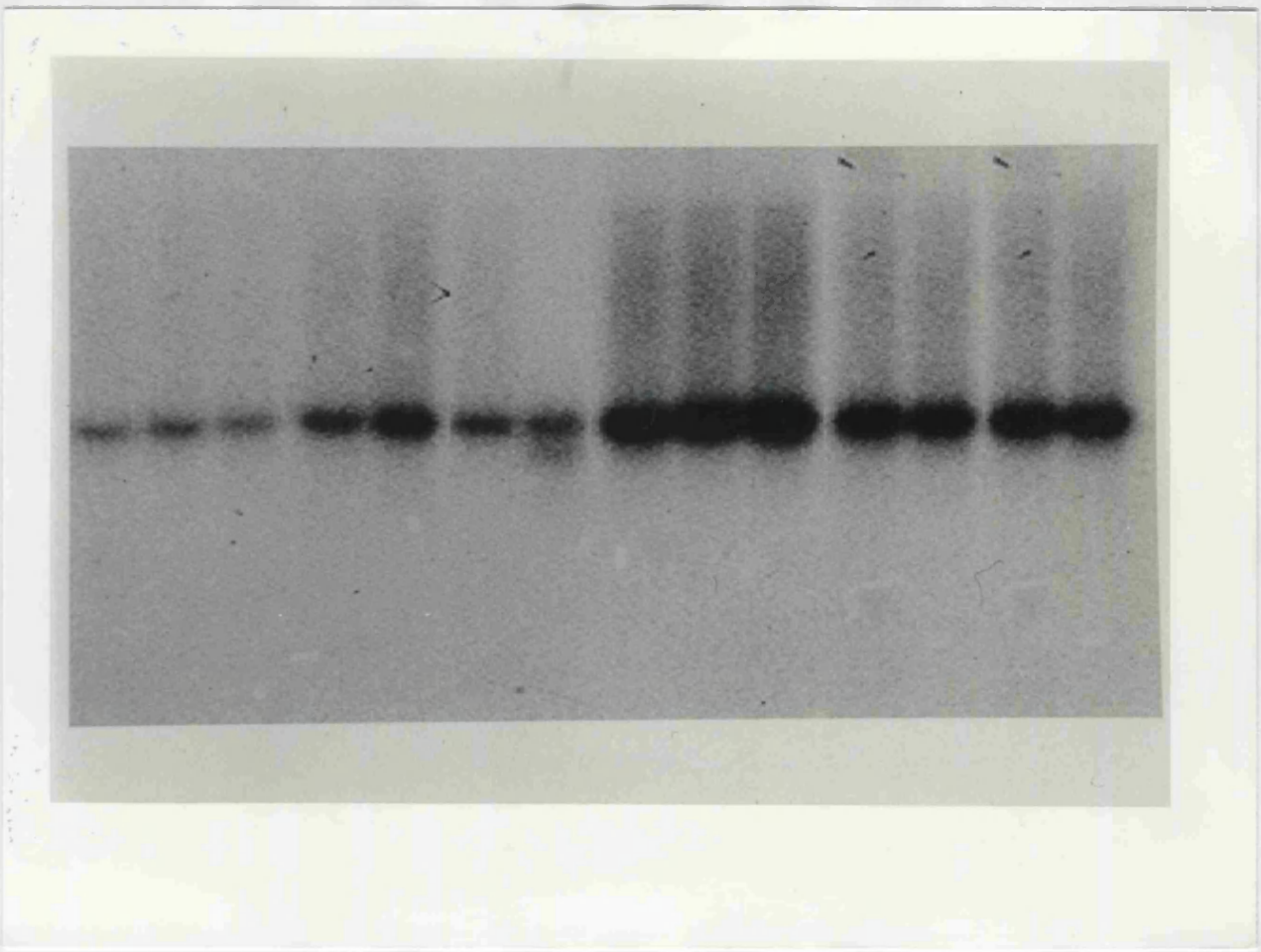


Figure 5-21 SSCP profile of clones isolated from amplicons derived from the BCP-1 cell line.

5.18. Discussion

In this study, I have been able to demonstrate the presence of intra-patient variability of HHV-8 genome using different tissue samples originating from different diseases. This variability can arise in three ways: 1. as artefacts of the PCR process; 2. existence of HHV-8 quasispecies; 3. co-infection of multiple HHV-8 variants.

One difficulty with interpreting the significance of the wide sequence diversity presented here is that all information was derived from PCR amplification of the viral genome. For clones of a PCR product 150 bp in length, the expected frequency of mutations is equivalent to 0.1-1 artefactual substitutions (rate of 0.3-3 x 10^{-3}/nt), approximately two-thirds of which may be expected to be non-synonymous (Smith et al., 1997). However, sporadic artefacts can be distinguished from genuine segregating polymorphisms by sequence analysis of variants, as substitutions that are present in more than one clone are likely to represent segregating polymorphisms actually present in the virus population.

Results from this study showed that clones from some specimens can produce a wide range of sequences. The apparent random distribution of the point mutations seen in the clones suggests that some of the changes could result from *Taq*-polymerase-induced errors. However, it seems unlikely that artefactual misincorporation would have accounted for the majority of the changes. This is because the rate of mutation is less than what is expected if misincorporation did occur. For example, eight out fifteen clones from a biopsy of skin sarcoid (Figure 5.6) are of the same sequence, while three others (11.2, 11.6 and 11.12) clustered

together, and only four others showed point mutations; the mean mutation frequency of these clones, in aggregate, was 1.5 x 10^{-3}/nt Similarly, clones from a lymph node sarcoid (Figure 5.7) showed a frequency rate of 9.2 x 10^{-4}/nt, and in lymph node tissues from patients with Kikuchi's disease (Figures 5.11, 5.12, 5.14) were noted the presence of point mutation in single clones with mutant frequency rates of 5.8 x 10^{-4} /nt, 6.1 x10^{-4}/nt and 1.7 x 10^{-3}/nt. Furthermore, the rate of mutation frequency in clones derived from a granulomatous lymphadenitis biopsy (Figure 5.17) was 5.8 x 10^{-4}/nt, while a reactive lymphadenitis tissue showed the presence of three variants and absence of random base substitutions (Figure 5.15), giving a mutation frequency of 1.7 x 10^{-2}/nt. Most of these rates are therefore lower then expected.

That artefactual misincorporation occurs infrequently is supported by the finding of the complete sequence homogeneity from pHHV-8, the BCP-1 line and a biopsy from a B-cell lymphoma tissue. Thus while PCR-induced mutations may account for sequence heterogeneity in the non-lymphoma tissue studied here, it is unlikely that it played a substantial role in producing the heterogeneity. The explanation for sequence heterogeneity must lie elsewhere.

The existence of HHV-8 as quasispecies might offer this explanation. The quasispecies concept was first introduced by Eigen (1971) to apply information theory to molecular evolution. The concept was introduced to describe replicons in the early stage of the evolution of life; it has since been extended to describe a population of viruses that share a common origin but eventually displaying different genomic sequences following evolution from the founder strain. Virus populations therefore need not be viewed to be homogeneous but to consist of "quasispecies", variants that

may differ from the population average at one or more positions (Eigen, 1996). This concept principally applies mainly to rapidly evolving RNA viruses, e.g. HIV and hepatitis C virus. However, it could also apply to DNA viruses, whose rate of genomic mutation may be similar to RNA viruses ($>10^{-4}$/nt). This is suggested by studies showing that the frequency of variants resistant to neutralization by monoclonal antibodies or bearing defined nucleotide substitutions are similar between RNA and DNA viruses (Smith et al., 1987).

The third possible cause of variability of HHV-8 is infection by multiple strains of the virus. Multiplicity can arise from different ways: different infection episodes, each by a different variant; single infection episode, by different variants transmitted during that episode; and the reactivation of different variants that hitherto have remained latent in the host following past infection. Prospective studies of clones in tissues taken from the host at different times in the natural history of the diseases examined here will much clarify which processes have been operative in the production of intra-host HHV-8 diversity.

5.19. Conclusions

The studies described in this chapter have revealed the heterogeneity of HHV-8. At the inter-host level, this heterogeneity intimates the existence of multiple groups of HHV-8. In this respect, HHV-8 is more similar to human papillomavirus that to the other herpesviruses. At the intra-host level, the existence of HHV-8 heterogeneity points either to multiple infection episodes or to HHV-8 genomic instability. The findings of this chapter therefore have implications for the epidemiology, immunology,

and evolutionary genetics of this fascinating virus. They pave the way to studying HHV-8/mankind co-evolution, the nature of host defences against HHV-8, and the mechanisms that underlie the high mutability of HHV-8

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7. APPENDIX 1: METHODS FOR AUTOMATED SEQUENCING

7.1. Estimation of amount of ds DNA required for cycle sequencing

The optimal conditions for cycle sequencing are a 4:1 molar ratio of primer to purified DNA i.e. 3.2 pmol of primer are used per sequencing reaction. Therefore, 0.8 pmol of DNA is required.

The relationship between size of DNA, picomoles and mass is:

$$\frac{2 \times 10^6}{(660 \times \text{number of bp})} = \text{pmol ends}/\mu g$$

[2×10^6 adjusts for 2 ends and μg; 660 is the mean molecular weight of a bp]

In practice:

$$\frac{0.8 \times (660 \times \text{number of bp})}{1000} = \text{ng of DNA required for sequencing}$$

7.2. Precipitation of sequenced PCR products

After cycling the sequencing reaction is placed in 50 μl of 95% ethanol containing 2 μl of 3 M sodium acetate (pH 4.5). This is then kept at -20°C for a minimum of 10 mins. The reaction vessels are then spun in a microfuge for 20 mins at maximum speed, following which the supernatant are removed and the pellets washed with 250 μl of 75% ethanol, then respun for a further 5 mins. The supernatant is aspirated and the pellets dried (these pellets are stable for 3 months if kept in the dark at -20°C).

7.3. Computer analysis of sequencing data

After each sequencing run, launch the programme "Analysis" (Applied Biosystems) to display the gel image. Grey tracker lanes indicate where each sample is and the sample files are generated from these positions. Each sample file can be viewed in one of four ways: (1) file information, which is details about the run; (2) raw data, which are the data prior to analysis by the "Analysis" programme; (3) analysed data; and (4) sequence data, i.e., data found in the standard IUPAC 5 letter code (A,G,C,T,N).

7.3.1. Base calling to reanalyse data

This enables the raw data to be reanalysed, and is often necessary because the computer will have assigned first and last bases incorrectly which may result in the contig assembly programme being unable to recognise overlapping sequences.

Launch the programme "Analysis", pull down the File menu and select Open. When the dialogue box appears it is possible to select the sample file requiring reannalysis. The window menu is used to select Raw Data, Analysed Data and Controller. The custom tool on the controller is used to locate the beginning of the data, normally located after the dye terminator peaks at the start of the raw data (these are produced from the unincorporated dye terminators). Record the X-axis number for the first base, found in the lower left hand corner of the Raw Data window. Similarly record the number of the last base. As small PCR products are used, discrete raw data are produced and it is easy to locate the end point. Pull the Analysis menu down, select Call Bases and enter the start and end co-

ordinates into the appropriate boxes. This process produces a new sample file that is written over the existing file. However, the raw data remains unaltered. Using the tools on the controller it is possible to look at the reanalysed Analysed Data. Check the chromatogram to ensure that the correct bases have been called, and where ambiguous data are. The base in question can be changed to an "N" in hope that it can be resolved from the sequencing of the other strand. The sequence data are presented in two formats: the sample file and ASCII text files.

7.3.2. **Using SeqEd version 1.03**

This programme allows contigs to be assembled from the ABI sequence data. Open SeqEd (Applied Biosystems) and pull the File menu down to select New Layout. To import the ABI sequences, pull the Sequences menu down and choose Import Sequence. The sequences of interest are then imported. Any anti-sense sequences can be made to read in the sense direction by highlighting the file name on the left hand side of the layout, then pulling down the Sequences menu and selecting reverse complement sequence.

The sequences can now be aligned by highlighting the sequences of interest, pulling down the Align menu, then choosing overlap, comparative or multiple. Once the alignment is complete, the sequences can be compared for mismatches; this is done by using the Sequences menu, selecting create shadows and then compare two sequences; stared areas indicate mismatches. These areas are highlighted and viewed once again using the Sequences menu and then Display Chromatograms. Inspect the chromatograms closely. Those bases that are indeterminate are labelled as "N" for unknown. Repeat this process

until all stared sites have been examined. A unanimity sequence is created by highlighting the names of all the sequences used, using the Sequences menu pick create shadow and then unanimity sequence. Freeze this unanimity sequence by using freeze shadow from the Sequences menu. This can now be exported in the form of a text file by using export sequence, and also from the Sequences menu. The unanimity sequence can also be used to determine the amino acid sequence. By selecting create shadow and translate codons to amino acids from the sequence menu it is possible to get the DNA sequence translated into "universal" code in all three reading frames with either three letter or single letter amino acid code. This layout can now be saved for future reference.

7.3.3. **Using EDITSeq of Lasergene Navigator**

This programme enables sequence data to be converted into a format easily recognisable to the *Lasergene Navigator* suite of programmes.

Open the *Lasergene Navigator* and select the Sequence Editing and Analysis option. Enter sequence data in one of two ways, by importing text files created by SeqEd or by manually typing in the sequence. If the data is to be manually entered, the computer can be used to proof read the data vocally. This is done by using the Digitizer menu and choosing Macintosh Voice. To import data, pull the File menu down and select Open, the data has to be in the DNAstar format. If, however, the data is in another format use the File menu to select Import. This can then be translated into an amino acids by highlighting the DNA sequence and selecting Translate DNA from the Goodies menu. Export these sequences as documents

containing sequence data with comments. Save these files for use in other programmes in the *Lasergene Navigator* suite.

7.3.4. **Using MegAlign of Lasergene Navigator**

This programme enables the degree of similarity between different sequences to be analysed by comparing nucleotides and/or amino acids.

To use this programme select Multiple Sequence Alignment from the *Lasergene Navigator*. Using the File menu and selecting New enables sequences to be entered into the worktable for alignment. Alternatively a pre-existing alignment can be opened and altered by choosing Open from the File menu. To enter sequences, pull the File menu down and select Enter Sequences. When the dialogue box appears, enter in sequences and/or other alignment files by highlighting their names and selecting >>Add>>. Once the desired sequences have been entered, select Done. Add both DNA and amino acid sequences at the same time with the DNA sequences being automatically being translated into amino acids from base one. If only a selected part of the sequence is required, it is possible to select this part from within MegAlign; this is known as subranging, alternatively alter the EditSeq file before entry into the programme. To set the subrange, open the Options menu followed by Set Sequence Limits and By Coordinates. A dialogue box will appear; enter positions of the first and last base required.

Alignment of multiple sequences is performed either By Clustal Method or By Jotun Hein Method from the Align menu. Clustal is generally preferred. However, Jotun Hein is used when the sequences are derived from a common source. The alignment can be viewed in four different formats: the Alignment Report, Sequence Distances, Residue Substitution and Phylogenetic Tree.

7.3.5. **Using Protean of Lasergene Navigator**

This programme is used to evaluate at the antigenic profiles of amino acid sequences. To use this programme, select Protein Analysis from the *Lasergene Navigator*. By using the File menu, import in New an Editseq protein file, following which an Assay Document will appear.

7.3.6. Using Map Draw of Lasergene Navigator

This programme was used to seek RE sites within DNA sequences. To use this programme, select Restriction Analysis and Mapping from the *Lasergene Navigator*. By using the File menu, import in the New an Editseq DNA file and scan the DNA for RE sites. To visualise the RE sites, open the Map menu and choose site and sequence or linear minimap was chosen.

8. APPENDIX 2: PUBLICATIONS AND PRESENTATIONS RELATED TO THE THESIS.

8.1. Publications

Di Alberti L, Teo CG, Porter SR, Zawkreska J, Scully C. Kaposi's Sarcoma HerpesVirus in oral Kaposi's sarcoma (1996). *European Journal of Cancer-Oral Oncology* 32B(1):68.

Di Alberti L, Ngui SL , Porter SR, Scully C, Speight PM, Zawkreska J, Williams J, Artese L, Piattelli A, Teo CG (1997). Presence of herpesvirus 8 (HHV8) in the oral mucosa of HIV positive patients. *Journal of Infectious Diseases* 175:703-707.

Di Alberti L, Piattelli A, Artese L, Favia G, Patel S, Saunders N, Porter SR, Scully CM, Ngui SL, Teo CG (1997). Human herpesvirus 8 variants in sarcoid tissues. *Lancet* 350:1655-61.

Di Alberti L, Porter SR, Speight PM, Scully C, Zawkreska J, Williams J, Artese L, Piattelli A, Ngui SL, Teo CG (1997). Detection of human herpesvirus 8 DNA in the oral ulcer tissues of HIV-infected individuals. *Oral Diseases* 3 (Suppl 1):S133-S134.

Porter SR, **Di Alberti L**, Kumar N (1998). Human herpesvirus 8 (HHV-8). *European Journal of Cancer-Oral Oncology* (In press).

Di Alberti L, A. Trattner, E. Hodak, A. Piattelli, L. Artese, G. Favia, G. Campisi, V. Margiotta, P. A. Reichart, J. Epstein, G. Ficarra, P. Reade, I. Sng, P. M. Speight, S. R. Porter, C. M. Scully ,C. G. Teo. Hot-spot mutations in open reading frame 26 of the human herpesvirus-8 define at least eight genetic groups. (Paper submitted).

Di Alberti L, Piattelli A, Artese L, Favia G, Speight P.M., Porter SR, Scully CM, Teo CG. Intra-host and intra-lesional variability of HHV-8 ORF26. (Paper in preparation).

8.2. Oral and poster presentations

Di Alberti L. Herpesvirus 8: a new virus. Principles and new associated diseases. Chieti Dental School, Chieti. March 26 1996.

Di Alberti L. New pathological entities associated with KSHV (Kaposi sarcoma herpes virus) (Nuove entità patologiche associate con il KSHV) 3° Congresso Nazionale del "Collegio dei Docenti di Odontoiatria". Dalla ricerca all clinica. Rome. March 27-30 1996.

Di Alberti L. HHV8: a new virus. Genitourinary Medicine Department. Charing Cross Hospital, London. 15 May 1996.

Di Alberti L. KSHV: Preliminary research results and review of the literature. Eastman Dental Institute Research Seminars 1996. Eastman Dental Institute, London, Uk 22 May 1996.

Di Alberti L, Porter SR, Scully C, Speight P, Zakrzewska J, Williams I, Artese L, Piattelli A, Ngui SL Teo CG. KSHV in oral tissues of AIDS patients without Kaposi's sarcoma. Third International Workshop on the Oral Manifestations of HIV Infection. The Royal College of Physicians, London. 29 May - 1 June 1996.

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Human herpesvirus 8 variants in sarcoid tissues

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Summary

Background The cause of sarcoidosis is unknown, although mycobacteria have been implicated. We examined sarcoid tissues for human herpesvirus 8 (HHV-8) in addition to mycobacterial genomic sequences.

Methods Biopsy samples from 17 patients with sarcoidosis were studied (eight transbronchial, 27 lymph node, two skin, and two oral mucosa). We used tissues (n=137) from 96 patients without sarcoidosis as negative controls. A nested PCR was applied to amplify a segment of open reading frame (ORF) 26 of the HHV-8 genome, and a heminested PCR was used to amplify a segment of ORF 25 of HHV-8 and of the 16 S rRNA gene of mycobacteria. Differences in base sequences of the amplified fragments were resolved with single-strand conformation polymorphism and dideoxy sequencing.

Findings HHV-8 ORF 26 DNA was detected in significantly higher proportions of sarcoid than of non-sarcoid tissue samples from lung (8/8 *vs* 0/54; p<0·0001), lymph nodes (26/27 *vs* 6/29; p<0·0001), skin (2/2 *vs* 0/17; p=0·006), and oral tissues (2/2 *vs* 1/13; p=0·029). 31 (82%) of the 38 ORF 26 DNA-positive sarcoid specimens were also positive for ORF 25 DNA. For mycobacteria-like 16 *S* rRNA DNA, the proportion positive was significantly higher in sarcoid than non-sarcoid tissues for lymph node samples (11/27 *vs* 2/29; p=0·003) but not for other tissues (lung 3/8 *vs* 22/54; skin 2/2 *vs* 15/17; and oral tissues 1/2 *vs* 0/13). Overall, the prevalence of HHV-8 ORF 26 sequences was higher in sarcoid tissues than in non-sarcoid tissues (p<0·0001). When patients whose tissues were included in a masked phase of the study were treated as units of analysis, eight of eight sarcoidosis patients were positive for HHV-8 ORF 26 DNA, compared with three of 56 control patients (p<0·0001); for mycobacteria-like sequences, three of eight sarcoidosis patients were positive, compared with four of 56 controls (p=0·0464). The HHV-8 ORF 26 sequences, ten of which were unique, could be segregated into four groups according to peptide motifs. In seven of nine patients from whom biopsy samples were taken from various sites, different sequences were recovered. The mycobacterial sequences amplified from sarcoid tissues were also varied, but none was homologous to those of known species.

Interpretation Variant HHV-8 DNA sequences are found in a wide range of sarcoid but not non-sarcoid tissues. Mycobacteria-like 16 *S* rRNA sequences are more frequently present in sarcoid lymph nodes and not in other tissue types, but do not indicate infection by a particular mycobacterial species.

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Introduction

Sarcoidosis has been described as a systemic disorder characterised by the presence in multiple tissues of non-caseating epithelioid-cell granulomas, which may spontaneously resolve or convert to hyaline connective tissue.[1] In the active phase of the disease, activated macrophages aggregate at several tissue sites and coalesce as giant and epitheloid cells.[2] Also accumulating at these sites are CD4 cells, which show restriction in their use of T-cell-receptor genes,[3] indicating stimulation by a limited range of antigens. Clinical expression of sarcoidosis is variable and can be protean, but the disease frequently presents on chest radiograph as bilateral hilar lymphadenopathy with or without pulmonary mottling.[2]

The aetiology of sarcoidosis is uncertain. Many agents have been implicated as possibly providing the antigens that elicit the sarcoid granulomatous response.[2] In particular, the role of *Mycobacterium tuberculosis* and other mycobacteria is the subject of many studies and much debate.[4–7] Granuloma formation has been shown to follow infection by herpesviruses such as varicella zoster virus and herpes simplex virus type 1, and, in some cases, sarcoid-type granulomas are seen.[8–10] Specific associations between granulomatous diseases and herpesviruses, however, are very rare. In this study, we looked for the presence of a newly discovered herpesvirus, human herpesvirus 8 (HHV-8; also known as Kaposi's sarcoma-associated herpes virus),[11,12] in sarcoid tissues.

Methods

Tissue samples

We studied paraffin-embedded formalin-fixed tissues obtained by transbronchial, lymph-node, and skin biopsies that were done to confirm sarcoidosis in 15 patients attending the University Hospital of Chieti, Italy. In addition, we examined two oral-biopsy specimens, one from each of two patients with oral ulceration presenting to the University Hospital of Bari, Italy. All 17 patients had chest changes, detected by radiography, suggestive of intrathoracic sarcoidosis, were Kveim-test-positive, and had not been on corticosteroid therapy. Specimens were included for study if they contained multiple granulomas that were predominantly non-necrotising and were negative for acid-fast bacilli and fungi after staining with Ziehl-Neelsen, periodic acid/Schiff, and Gomori's methenamine silver. As negative controls, paraffin-embedded formalin-fixed tissues of patients with disorders other than sarcoidosis from the lung, lymph node, skin, and oral mucosa were used. Paraffin-embedded formalin-fixed skin samples from patients with classic (Mediterranean) Kaposi's sarcoma and postnasal space samples from patients with nasopharyngeal carcinoma were used as controls for the DNA amplification of HHV-8 and Epstein-Barr virus,

Patient	Patients characteristics			Histological characteristics					
	Age (years)	Sex	Chest staging by radiography	Specimen code	Site of biopsy	Necrosis*	Fibrosis*	Asteroid cells	Schaumann bodies
A	44	F	I	A1	Scalene lymph node	0	1	No	Yes
				A2	Scalene lymph node	0	1	No	No
				A3	Mediastinal lymph node	0	2	Yes	No
				A4	Mediastinal lymph node	0	1	Yes	Yes
B	56	F	II	B1	Scalene lymph node	0	0	No	No
				B2	Mediastinal lymph node	0	0	No	Yes
				B3	Lung	1	2	No	No
				B4	Lung	0	0	Yes	No
C	43	M	III	C1	Mediastinal lymph node	0	3	Yes	No
D	48	F	III	D1	Mediastinal lymph node	0	3	No	No
				D2	Retroperitoneal lymph node	2	2	Yes	No
				D3	Mediastinal lymph node	0	1	Yes	No
				D4	Mediastinal lymph node	0	3	Yes	Yes
				D5	Lung	0	3	No	Yes
				D6	Lung	0	3	Yes	No
E	75	F	II	E1	Mediastinal lymph node	1	1	Yes	Yes
				E2	Scalene lymph node	1	1	Yes	Yes
				E3	Mediastinal lymph node	1	1	Yes	Yes
F	63	F	I	F1	Mediastinal lymph node	1	1	No	Yes
G	65	F	I	G1	Skin	0	0	No	No
H	49	F	II	H1	Mediastinal lymph node	1	1	No	Yes
				H2	Mediastinal lymph node	1	1	Yes	Yes
				H3	Scalene lymph node	0	2	No	Yes
I	86	F	I	I1	Mediastinal lymph node	1	0	Yes	Yes
J	60	M	II	J1	Mediastinal lymph node	0	2	No	No
L	58	F	I	L1	Mediastinal lymph node	0	1	No	No
				L2	Scalene lymph node	0	1	Yes	No
M	63	F	III	M1	Lung	1	3	No	No
				M2	Lung	1	0	No	No
N	45	F	I	N1	Skin	0	0	No	No
O	71	F	II	O1	Mediastinal lymph node	1	1	No	No
				O2	Mediastinal lymph node	0	1	Yes	No
				O3	Mediastinal lymph node	1	2	No	Yes
				O4	Scalene lymph node	0	1	No	No
P	30	F	III	P1	Lung	0	2	Yes	No
				P2	Lung	1	3	Yes	Yes
				P3	Mediastinal lymph node	1	3	No	No
Q	36	M	III	Q1	Inside of cheek	0	1	No	No
R	44	F	II	R1	Inside of cheek	0	1	No	No

*On microscopy (×2·5 magnification): 0=absent; 1=1–25%; 2=26–50%; 3=>50%.

Table 1: **Characteristics of study patients and tissue specimens**

respectively. Sputum samples that stained positive for acid-fast bacilli referred from patients with suspected tuberculosis were included as controls for detection of mycobacterial DNA.

Study structure

The study had three phases. First, HHV-8 DNA was sought from tissue sections sent to the PHLS Central Public Health Laboratory, London, UK, from 37 biopsy samples taken from the 15 sarcoidosis patients from Chieti, Italy. When 36 specimens were found positive, another set of sections from the same 37 tissue blocks and 40 control tissues were sent from Chieti. A technical-staff member in Chieti coded this set of specimens without revealing the code (until after testing) to investigators in Chieti or London. The two specimens from oral-sarcoidosis patients from Bari, Italy, were also despatched. Sections were examined for HHV-8, mycobacterial, and Epstein-Barr virus DNA during this second phase of the study. Nucleotide sequences of HHV-8 and mycobacterial amplicons were also characterised during this phase. In the third phase, sections from ten sarcoid tissues from six Chieti patients (a subset of the 37 tissues originally examined) were batched with a further 61 tissues from 43 patients without sarcoidosis, coded in Chieti and sent to the Eastman Dental Institute, London, UK. The code was kept by investigators at the institute (to be broken after testing) and the specimens sent to the Central Public Health Laboratory for examination for the presence of HHV-8, mycobacterial, and Epstein-Barr virus DNA. Also despatched to this laboratory were coded sections from the two oral-sarcoid tissues from Bari, together with 13 oral tissues each from 13 patients not known to have sarcoidosis. This third phase is hereafter referred to as the masked phase. In all, for each pathogen, sarcoid tissues were tested at least four times and non-sarcoid tissues twice.

DNA extraction

30 μm sections were cut from each tissue block and deparaffinised in xylene. We extracted DNA using the Geneclene Kit II (BIO 101, Vista, CA, USA) and eluted it in 60 μL sterile water. For extraction of DNA from sputum containing acid-fast bacilli from patients subsequently confirmed by sputum culture to have been infected with *M tuberculosis*, phenol-chloroform was used. We measured the concentration of DNA recovered by spectrophotometry. About 100 ng DNA from each extract was loaded in each PCR reaction.

PCR amplification

We used a nested PCR approach to amplify HHV-8 DNA. First-round PCR was done to amplify the $KS330_{233}$ HHV-8 DNA fragment.[11] When amplification was completed, 1 μL PCR solution was added to 24 μL second-round PCR mix, which contained the same set of reagents as first-round PCR except that the primers used were KSin1 (5'TTCCACCATTGTGCTCGAAT3') and KSin2 (5'TACGTCCAGACGATATGTGC3'); this mixture underwent 35 cycles under the same conditions as the first amplification. Second-round amplification yields a product that is 210 bp in length (encompassing positions 366–576 of open reading frame [ORF] 26 of the HHV-8 genome). The sensitivity limit of nested PCR, as assessed by amplification of serial dilutions of the plasmid pHHV-8, which contains an insert of the $KS330_{233}$ fragment of HHV-8 derived from the BC-1 cell line,[12] was equivalent to unit copy of the plasmid.

Sarcoid-tissue extracts from which DNA from ORF 26 of HHV-8 was found were subjected to a heminested PCR procedure for the amplification of a 212-bp segment within ORF 25. The sequences of the first-round PCR primers were 5'CATGGATACATTGTCAGGACCTC3' (sense) and

Type of lesion	Total tested	Number of samples positive		
		HHV-8 DNA	Mycobacterial DNA	EBV DNA
Sarcoid tissues				
Pulmonary	8	8	3	0
Lymphoid	27	26	11	0
Dermal	2	2	2	0
Oral	2	2	1	0
Non-sarcoid tissues				
Pulmonary	54	0	22	??
Lung cancer	22	0	18	2
Non-granulomatous alveolitis	17	0	2	3
Granulomatous alveolitis	2	0	0	1
Emphysema	6	0	0	0
Atelectasia	1	0	0	0
Normal mucosa	6	0	2	6
Lymphoid	29	6	1	0
Non-specific lymphadenitis	17	5	1	0
B-cell lymphoma	12	1	0	0
Dermal				
Basal-cell carcinoma	17	0	15	2
Oral	22	1	0	0
Squamous-cell carcinoma	9	0	0	0
Control tissues	13	1	0	0
Other control tissues				
Nasopharyngeal carcinoma	3	0	1	3
Mediterranean Kaposi's sarcoma	16	10	1	0
Tuberculosis sputum samples	6	0	6	0

Table 2: **Frequency of HHV-8, mycobacterial, and Epstein-Barr virus (EBV) DNA sequences in sarcoid and non-sarcoid tissues**

5'GTCACATCTGACGTTGCCT3' (antisense), and 35 cycles were applied: the cycle conditions were 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min. Second-round PCR was then carried out, with a sense primer replaced by one of sequence 5'GGCAACCTGCGAGATAATTCC3'; cycle conditions were the same as for the first round, except that the annealing temperature was 60°C. This heminested procedure was about ten to 100 times less efficient in the amplification of HHV-8 DNA than the procedure described above for ORF 26.

To amplify mycobacterial DNA, we used a heminested PCR procedure with primers that anneal specifically to sequences in the 16 *S* rRNA gene common to 38 species of mycobacteria,and which flank sequences that are species-specific.[13] The procedure yields products whose lengths vary from 565 to 590 bp, depending on the mycobacterial species. First-round PCR was carried out in 50 µL volumes containing primers P1 (5'GCGTCGTTAACACATGCAA3') and P2 (5'CGCTCACAGTTAAGCCGT3'). Amplification was done over 35 cycles, each consisting of 93°C for 30 s, 55°C for 30 s, and 72°C for 60 s. Second-round amplification was carried out as in the first round, except that primer P1-2 (5'CTGGCTCAGGACGAACGCT3') was used instead of P2. The sensitivity of the heminested PCR procedure, as estimated by the amplification of serial dilutions of *M tuberculosis* (strain H37Rv) chromosomal DNA was equivalent to the DNA content of three bacilli.

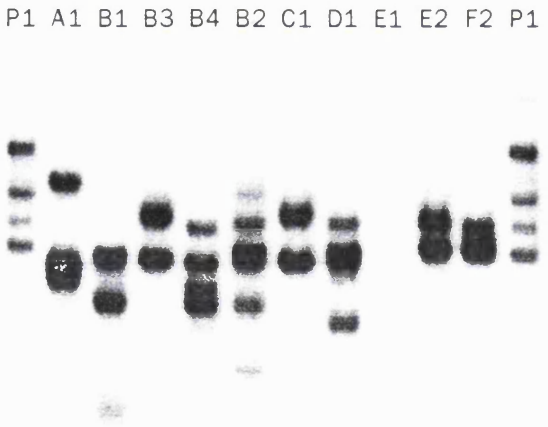

Figure 1: **Autoradiographic banding patterns from a representative non-denaturing gel showing single-stranded PCR products of HHV-8 sequences**
Specimen codes as indicated. Pl=plasmid (pHHV-8).

As an additional control, we used a nested PCR procedure to amplify a stretch of DNA within the *Bam HI* K fragment of the Epstein-Barr virus genome, which is 470 bp in length (from coordinates 109311 to 109780). The outer primers were K01 (5'TGATGGAGCAGGCGCAAAAAAG3') and K02 (5'GAAACCAGGGAGGCAAATCTACT3'); the inner primers were KI1 (5'CGCAAAAAAGGAGGGTGGTTT3') and KI2 (5'CATCGTCAAAGCTGCACACAG3'). The first PCR round consisted of 35 cycles, each of 94°C for 60 s, 68°C for 40 s, and 72°C for 60 s; the second round involved 35 cycles, each of 94°C for 60 s, 60°C for 40s, and 72°C for 60 s.

We used sterile water as a negative control in each PCR reaction. As positive controls for HHV-8, mycobacterial, and Epstein-Barr virus DNA amplification, appropriate dilutions of pHHV8, chromosomal DNA from the H37Rv strain of *M tuberculosis*, and DNA extracted from the C15 tumour, respectively, were used in each reaction. Extracts also underwent PCR to amplify a 110 bp segment of the β-globin gene.

Single-strand conformation polymorphism assay

HHV-8 and mycobacterial amplicons were processed in the phosphorus-32-based single-strand confirmation polymorphism assay,[14] to scan for sequence differences. Tissue extracts in which DNA sequences were amplified and observed in the assay to produce different band patterns were reamplified and analysed in a second gel run, to exclude PCR-induced mutations as a cause of any differences.

DNA sequencing

HHV-8 PCR products that yield differential banding profiles in the single-strand confirmation polymorphism assay and representative HHV-8 amplificates that yielded identical band patterns were subjected to DNA sequencing (Taq DyeDeoxy Terminator Cycle Sequencing Kit and ABI 373A DNA Sequencer; Perkin Elmer, Forest City, CA, USA). Both strands of each product were sequenced routinely. Mycobacterial amplicons were similarly sequenced, and the sequences of the V2 hypervariable region[15] were compared with those lodged with the rRNA database of the University of Illinois, USA.

Further analyses of HHV-8 sequences

HHV-8 sequences (171 bp in length, not including the inner primer sequences) were aligned against HHV-8 sequences reported previously, and analysed for relatedness with the Clustal algorithm in the Megalign program (version V.2.60) of the Lasergene System (DNAstar, Madison, USA). We also categorised the HHV-8 sequences into genomic groups with a system we had previously proposed based on nucleotide and cognate aminoacid motifs present within the 171 bp segment.[16]

Statistical analysis

One approach was to use each specimen as the unit of analysis, because of the wide sequence diversity of HHV-8 ORF 26 and mycobacterial 16 *S* rRNA amplicons discovered in the tissue samples. For this analysis, all observations from the latter two phases of the study were combined. The other approach was to use each patient as the unit of analysis. A patient was classified as positive for HHV-8 or mycobacteria if at least one tissue derived from the patient amplified positively for HHV-8 ORF 26 or mycobacteria 16 *S* rRNA sequences. For this latter approach, only observations from the masked phase of the study were analysed. The data were analysed by χ^2 or Fisher's exact test (two-tailed).

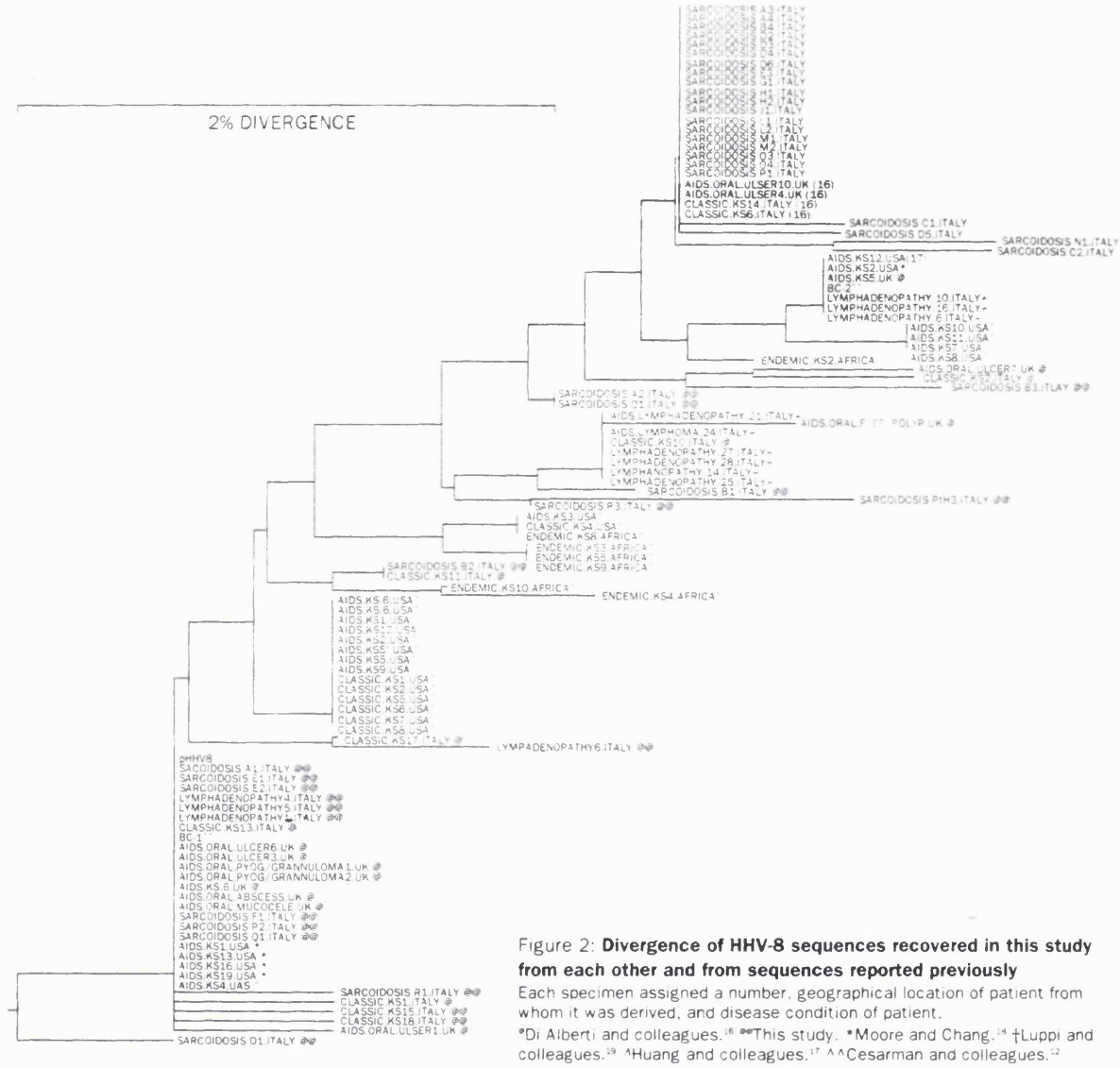

Figure 2: **Divergence of HHV-8 sequences recovered in this study from each other and from sequences reported previously**
Each specimen assigned a number, geographical location of patient from whom it was derived, and disease condition of patient.
#Di Alberti and colleagues.[16] ##This study. *Moore and Chang.[18] †Luppi and colleagues.[19] ^Huang and colleagues.[17] ^^Cesarman and colleagues.[12]

Results

The majority of tissues from patients with sarcoidosis-like changes showed no or negligible necrosis, and the extent of fibrosis was variable (table 1). 17 (44%) and 15 (38%) of the 39 specimens contained asteroid and Schaumann bodies, respectively.

Of the 38 specimens found positive for ORF 26 DNA, 31 (82%) were also positive for ORF 25 DNA (table 2). The 12 specimens from eight patients that were found positive by nested PCR for ORF 26 in the second phase also tested positive in the third phase (masked). From each of the body sites sampled, the frequency of HHV-8 ORF 26 sequences was higher in sarcoid than in non-sarcoid tissues (p values for comparison between pulmonary, lymphoid, dermal, and oral tissues <0·0001, <0·0001, 0·006, and 0·029, respectively). The frequency of mycobacterial sequences was significantly higher in sarcoid than in non-sarcoid lymph-node tissues (p=0·003), whereas the differences between sarcoid and non-sarcoid tissues from other sites were not significant. Overall, HHV-8 ORF 26 sequences were found in a significantly higher proportion of sarcoid tissues than of non-sarcoid tissues (38/39 *vs* six of 113; p<0·0001). The difference between the two tissue groups in proportion with mycobacterial DNA (17 of 38 *vs* 39 of 113) was not significant.

When patients whose tissues were studied in the masked phase were included as units of analysis, all of eight sarcoidosis patients compared with three of 56 controls were positive for HHV-8 ORF 26 DNA (p<0·0001). By contrast, three of eight sarcoidosis patients compared with four of 56 controls were positive for mycobacteria-like sequences (p=0·0364).

Epstein-Barr virus DNA was amplified in no sarcoid tissues, in eight non-sarcoid (six pulmonary, two dermal), tissues, and in all three nasopharnygeal carcinoma tissues.

The 38 HHV-8 ORF 26 sequences amplified from sarcoid tissues were diverse. This is exemplified by the appearance on autoradiography in a single-strand conformation polymorphism assay of ten of the amplified sequences (figure 1); none of the samples (except the plasmid controls) gave rise to identical banding patterns, thus reflecting DNA sequences unique to each. The extent of diversity is shown by the dendrogram (figure 2), which compares the 38 sarcoid tissue-derived HHV-8 sequences with 71 others reported previously.[11,12,17–19] Most

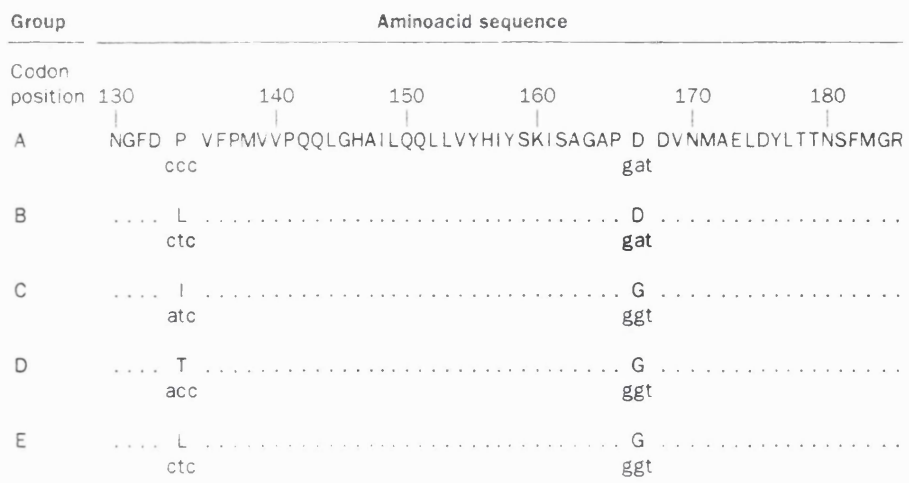

Figure 3: **Molecular basis of grouping of HHV-8 sequences**

Peptide sequence, from codon position 130–186, predicted from nucleotide sequence of 170 bp HHV-8 DNA segment amplified from study tissues. Single letter aminoacid code used. Aminoacid residues that allow classification of variants into other groups shown in alignment with group-A sequence. Dots signify residues identical to group A.

of the sequences (n=28) were identical to those previously characterised; they had base changes at certain preferred positions, which allow them to be assigned to four groups according to predicted changes in the aminoacid sequence (figure 3; table 3). In ten sequences (amplified from B1, B3, C1, D1, D5, H3, N1, O2, P3, and R1), nucleotide substitutions occurring at locations other than the group-specifying positions were also observed (table 3).

In seven of nine patients from whom several tissue sites were biopsied (A, B, D, E, H, O, and P), different HHV-8 ORF 26 sequences could be recovered from each sample (figures 1 and 2). For patient B, HHV-8 sequences amplified from the scalene and mediastinal lymph nodes and from the two transbronchial tissues were disparate (figure 1). For patient E, the sequence amplified from the scalene node differed from that from the mediastinal node. Figure 2 is a fuller representation of the multiplicity of sequences recovered from tissues of all seven patients.

Various mycobacteria-like 16 *S* rRNA sequences could be identified in 14 of the 18 amplicons derived from sarcoid tissues. Several bore unique sequences in the V2 hypervariable region. For this study, a mycobacterial sequence that differed from the sequence of the most closely related species by no more than two nucleotides in the V2 region was classified as related to that species; sequences that diverged by more than two bases were deemed to belong to uncharacterised mycobacterial species (table 3).

Discussion

The sarcoid granulomatous response has been thought to result from an immune action mounted to sequestrate foreign agents.[2] Our finding of a very high detection rate of HHV-8 ORF 26 DNA in sarcoid tissues, but not in non-sarcoid tissues (including those with intense inflammatory infiltration), points to a close association between HHV-8 and sarcoidosis. Whether this association is causal or not requires further study. Nevertheless, our study has not formally excluded a general association between HHV-8 and granuloma formation, since the number of tissues and patients with granulomatous conditions other than sarcoidosis was small.

Sarcoidosis is prevalent worldwide at a rate of ten–50 per 100 000.[20] That HHV-8, the infection markers of which are observed only in certain high-risk groups or in particular populations,[21–23] should be associated with the disease thus seems incongruous. However, these markers relate to viral DNA amplification from peripheral blood cells,[21] and to antibodies that recognise a limited range of HHV-8 antigens (which are principally latency-associated).[22,23] Evidence since 1996 has shown that HHV-8 infection in populations not at risk of Kaposi's sarcoma may be more prevalent than initially thought; this evidence comes from studies that used better techniques to seek viral sequences in blood,[24] tested tissues[25–27] and body fluids[28] other than blood, or tested for antibodies to lytic viral antigens.[29,30]

We used nested or heminested PCR to look for HHV-8, mycobacterial, and Epstein-Barr virus DNA sequences. These methods were preferred to non-nested procedures to maximise the likelihood that pathogen-specific amplicons could be obtained from archived tissues. In such material, DNA tends to be fragmented and to become cross-linked by formalin to itself and to surrounding proteins.[16] Our inability to amplify HHV-8 ORF 26 sequences from all the Kaposi's-sarcoma tissue controls in this study, which contrasts with the higher frequency reported in other studies with frozen or fresh biopsy tissues,[11,17,18] probably reflects the poor integrity of target DNA.

The very high frequency of HHV-8 DNA in the archived sarcoid tissues we examined is striking. This high frequency is unlikely to result from PCR cross-contamination, for two reasons. First, a large proportion (82%) of specimens that were positive for HHV-8 ORF 26 DNA were also positive for ORF 25 DNA, which also raises the possibility that the entire HHV-8 genome may be carried by sarcoid tissues. Second, various ORF 26 DNA sequences were recovered. Ten new HHV-8 variants, all from sarcoid tissues, were discovered during this study.

Despite the diversity of the ORF 26 sequences, most could be classified into two groups, designated B and C, based on nucleotide substitutions at preferred mutational

Specimen code	HHV-8 group	Location of base substituting in unique HHV-8 sequences	Mycobacterial species
A1	B	–	Unidentified
A2	E	–	ND
A3	C	–	ND
A4	C	–	Relative to *M cookii*
B1	A	Codon 132 (c→t, first position); codon 167 (g→a, second position)	Unidentified*
B2	A	–	ND
B3	C	Codon 146 (c→g, second position); codon 152 (c→t, second position)	Unidentified
B4	C	–	Unidentified*
C1	C	Codon 169 (g→a, first position)	Unidentified*
D1	B	Codon 140 (g→a, first position); codon 175 (t→c, third position)	Related to *M fortuitum*
D2	C	–	ND
D3	C	–	ND
D4	C	–	Unidentified
D5	C	Codon 181 (t→c, second position)	ND
D6	C	–	ND
E1	B	–	Unidentified*
E2	B	–	ND
E3	C	–	ND
F1	B	–	ND
G1	C	–	Unidentified
H1	C	–	ND
H2	C	–	ND
H3	C	Codon 157 (c→g, third position); codon 168 (t→a, third position)	ND
I1			Related to *M cookii*
J1	C	–	Unidentified*
L1	C	–	ND
L2	C	–	ND
M1	C	–	Unidentified*
M2	C	–	ND
N1	C	Codon 134 (c→t, third position)	Unidentified
O1	E	–	ND
O2	C	Codon 134 (c→t, third position); codon 168 (t→a, third position)	ND
O3	C	–	ND
O4	C	–	Unidentified*
P1	C	–	ND
P2	B	–	ND
P3	A	Codon 134 (c→t, first position)	Related to *M fortuitum*
Q1	B	–	ND
R1	B	Codon 134 (c→t, third position)	Unidentified

ND=none detected. Dashes denote no changes other than in group-specifying positions (figure 3). Lower-case single letters refer to nucleotides. Reference KSHV nucleotide sequence is that of prototype.[20] *Species with identical V2 sequence; mycobacterial species amplified from all other positive specimens have unique V2 sequences.

Table 3: **Assignment of HHV-8 sequences into groups and mycobacterial DNA sequences into species, and identification of base substitutions in unique HHV-8 sequences**

sites.[16] We have shown previously that groups B and C are common to Italian and UK patients, in contrast to groups A and D, which are shared by individuals in North America and Africa.[16] This study also reveals the presence of a group we had not identified previously—group E—which may also be of European origin. Another laboratory[31] provided independent data that substantiate the presence of preferred mutational sites in ORF 26 of the HHV-8 genome. The finding in sarcoid tissues of mutational hot-spots in ORF 26 on the one hand, and the reproducible identification of unique HHV-8 sequences on the other, suggest that the multiplicity of HHV-8 sequences recovered from these tissues is unlikely to be an artefact.

The finding that in some patients with sarcoidosis different body sites harbour different HHV-8 sequences is intriguing, because it reflects the coexistence of HHV-8 variants in individual hosts. Such multiplicity is unlikely to result from evolution from a single strain first transmitted to each host, because the mutation rate in herpesviruses is slow. A more probable explanation is multiple infection episodes, which suggests further that infection by one HHV-8 variant does not confer immunity to infection by another. To examine more closely the phenomenon of HHV-8 diversity within the host by examination of other hypervariable subgenomic regions would be worthwhile.[32]

We did not find that mycobacterial 16 *S* rRNA sequences were frequent in sarcoid tissues, except in lymph-node tissues. Curiously, the overall frequency of mycobacterial sequences in skin samples as a whole was high, and accords with a PCR study,[32] that reported amplification of sequences of atypical mycobacteria from control as well as from sarcoid tissues. Nevertheless, the range and extent of atypical mycobacterial sequences recovered in our study are wider. Our inability to identify any known species in this study reflects the abundance in nature of species that are commensals and saphrophytes.

The total absence of DNA from *M tuberculosis* in the lymph-node sample and in other samples here indicates that this bacterium cannot be associated with sarcoidosis in our patients. Our findings are consistent with some reports[5,33,34] but conflict with others.[5] Other observations, importantly, do implicate *M tuberculosis* as a causative agent; in particular, forms with cell-wall deficiency (also known as L-forms or protoplasts) of mycobacteria resembling *M tuberculosis* were isolated from the blood of patients with sarcoidosis but not from that of controls.[5] However, other investigators who analysed sarcoid skin specimens reported the long-term culture of—and amplification of sequences specific to—the *M avium* complex and *M paratuberculosis*, or both; *M tuberculosis* could not be cultured.[6] These findings are difficult to reconcile. Another possibility, that coinfection with mycobacteria and HHV-8 may be associated with sarcoidosis, is not supported by our study either, since there was no correlaton between HHV-8 and mycobacteria detection in the sarcoid samples.

Reports of HHV-8 proteins bearing homologies with, and sharing functional characteristics of, cytokines[35] provide insights into the potential for this virus to induce granulomas, and may explain why corticosteroids, in attenuating the influence of these effector molecules, can slow the progression of sarcoidosis. The tropism and state of HHV-8 activation in sarcoid tissues, and the role of antiherpesvirus drugs in suppressing both viral and disease activities, require further investigation.

Contributors

L D Alberti, A Piatteli, N Saunders, S R Porter, C M Scully, and C-G Teo designed the study. L D Alberti, S-L Ngui, and S R Porter carried out laboratory work. A Piatteli, L Artese, and G Favia were responsible for clinical and histopathological investigation. L D Alberti, A Piatteli, S R Porter, and C-G Teo wrote the paper.

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Presence of Human Herpesvirus 8 Variants in the Oral Tissues of Human Immunodeficiency Virus–Infected Persons

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A 210-bp DNA segment specific to the human herpesvirus 8 (HHV-8) genome was amplified by nested polymerase chain reaction from 10 of 14 archived oral biopsy samples of HIV-positive patients in London who had no evidence of oral Kaposi's sarcoma (KS). Various oral sites were represented. Oral tissues from 20 general dental patients not known to be HIV-infected were negative. When DNA sequences of these products were compared with sequences derived from 5 oral KS tissues of AIDS patients in London and 10 skin biopsies of Italian patients with Mediterranean KS (total number of positive tissues = 25), 11 were found to be unique. DNA and predicted peptide motifs of these sequences were also different from those in 28 of 36 HHV-8–positive lesions previously reported from American and African patients. HHV-8 is tropic for the oral mucosa of HIV-infected persons, and HHV-8 variants, though diverse, may be geographically restricted.

Unique DNA sequences homologous to a capsid protein gene of γ-herpesviruses have been identified in cutaneous Kaposi's sarcoma (KS) lesions [1–3], blood of human immunodeficiency virus (HIV)–infected patients with KS [4], effusions of patients with AIDS-related body cavity–based lymphoma [5], semen [6], prostatic and urogenital tissue [6], and lymph nodes [7]. The putative virus from which these sequences derive has been assigned to the *Rhabdinovirus* genus [8]. The virus has been described as KS-associated herpesvirus or, more formally, human herpesvirus 8 (HHV-8). Here, we investigate the presence of HHV-8 DNA sequences in oral tissues of HIV-infected people in London and compare these with sequences identified in cutaneous KS lesions of Italian patients and those reported from HHV-8–infected tissues of patients in the United States and Africa.

Materials and Methods

Specimens. We examined formalin-fixed, paraffin-embedded tissues of 25 incisional oral biopsy samples taken from 23 patients with HIV infection who attended the oral medicine clinic of a genitourinary medicine department in central London. Of these, 11 had histologic features consistent with KS, 10 nonspecific ulceration, and 4 other diseases. Various sites of the oral mucosa were represented. Also studied were paraffin blocks of 16 skin biopsy samples from Italian patients with classic (Mediterranean) KS and 20 diagnostic biopsy samples from patients with various oral diseases attending general dental clinics (including 9 with oral squamous cell carcinoma). The HIV antibody status of the patients in the latter 2 groups was unknown.

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Specimen preparation. Multiple sections, totaling 30–50 μm in thickness, were cut from each tissue block using disposable microtome blades that were changed between samples. The sections were deparaffinized, and DNA was extracted using Geneclean (Bio 101, Vista, CA).

HHV-8 DNA amplification. About 100 ng of DNA from each extract was processed for HHV-8 DNA amplification. Conditions and primers used to amplify a 233-bp fragment (the $KS330_{233}$ fragment), which is located in open-reading frame (ORF) 26 of the HHV-8 genome [8], were as described previously [1]. A nested polymerase chain reaction (PCR) was applied to an aliquot of the first-round amplificate to generate 210-bp products. The DNA sequence of the sense inner primer was 5′-TTCCACCATTGTGCTCCAAT (from positions 366–385 of ORF 26) that of the antisense inner primer was 5′-TACGTCCAGACGATATGTGC (positions 557–576). Cycling conditions were identical to those of first-round PCR. The nested PCR procedure is capable of amplifying, in a reaction tube, as little as 5 zg of the $KS330_{233}$ fragment ligated to a plasmid vector (data not shown); this is equivalent to 1 copy of the target sequence. The presence of DNA in each tissue extract was verified by amplifying a 110-bp fragment of the β-globin gene. As positive control, we used either extracts of KS tissue from an Italian patient or DNA from the BCBL-1 cell line (provided by E. Cesarman, Cornell University, Ithaca, NY). PCR detection of HHV-8 DNA was repeated at least once from each sample extract.

Characterization of HHV-8 DNA polymorphism. Amplified HHV-8 DNA was processed for single-strand conformation polymorphism (SSCP) analysis as previously described [9]. DNA sequencing of HHV-8 PCR products, without prior cloning, was performed using the Taq DyeDeoxy Terminator Cycle Sequencing Kit and the ABI 373A DNA Sequencer (Perkin Elmer, Forest City, CA). Routinely, two opposing strands from each product were sequenced.

Amplification of DNA from other human herpesviruses. Extracts from oral tissues subsequently found to be HHV-8–positive were subjected to nested PCR procedures to amplify DNA specific to Epstein-Barr virus (EBV), cytomegalovirus (CMV), and HHV-7. For EBV, the primer sequences were 5′-TGATGGAGGCAGGCGCAAAAAAG-3′ and 5′-GAAACCAGGGAGGCAAATCTACT-3′(outer primers) and 5′-CGCAAAAAAGGAGGGTGGTTT-3′ and 5′-CATCGTCAAAGCTGCACACAG-3′ (inner primers); these flank a 470-bp segment in the *Bam*HI K region of the genome (coordinates 109311–109780). For CMV, the primer sequences were 5′-GGAAACGTGTCCGTCTTCGA-3′ and 5′-GAAACGCGCGCGGCAATCGG-3′ (outer primers) and 5′-GTGTTCCTGGCAAGGCATCAA-3′ and 5′-CGTTGATCCACACACCAGGC-3′ (inner primers), which flank a 245-bp segment in the glycoprotein B–coding region, corresponding to positions 1285–1529. For HHV-7, primer sequences were 5′-TATCCCAGCTGTTTTCATATAGTAAC-3′ and 5′-GCCTTGCGGTAGCACTAGATTTTTTG-3′ (outer primers) and 5′-CAGAAATGATAGACAGATGTTGG-3′ and 5′-TAGATTTTTTGAAAAAGATTTAATAAC-3′ (inner primers), which flank a 124-bp region within the genome.

Heminested PCR was used to amplify a 176-bp segment of the HHV-6 genome in the *Bam*HI S region, which is within the large tegument protein–coding region; the sequences of the primers were 5′-GATCCGACGCCTACAAACAC-3′ (sense first- and second-round primer), 5′-TACCGACATCCTTGACATATTAC (antisense first-round primer), and 5′-GGCTGATTAGGATTAATAGGAGA-3′ (antisense second-round primer). Specificity controls used were DNA extracted from Namalwa cells (European Collection of Animal Cell Cultures [ECACC] catalog no. 87060801) for EBV and SUP-T1 cells (gift of R. Jarret, University of Glasgow) for HHV-7, and plasmids sp64/gB for CMV (gift of J. Sinclair, University of Cambridge) and HD9 for HHV-6 (ECACC catalog no. P91013142).

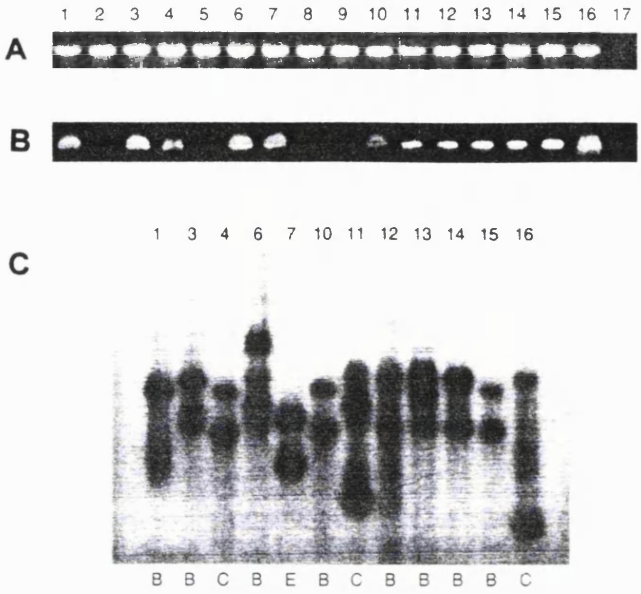

Figure 1. HHV-8 DNA in non KS oral tissues of HIV carriers. **A**, Ethidium bromide–stained bands of electrophoresed 110-bp DNA amplified from human β-globin gene. **B**, Ethidium bromide–stained bands of electrophoresed 210-bp DNA amplified from ORF 26 of HHV-8 genome. **C**, ^{32}P-autoradiographic bands of single-stranded DNA derived from 210-bp HHV-8 DNA polymerase chain reaction products after electrophoresis through nondenaturing gel. Letters below are HHV-8 group assignments (assignment to group E of the unique variant in lane 7 is tentative). Lanes 1–14 are non KS oral tissues; lanes 1–10: ulcer tissues (1 and 2, tongue; 3, angle of mouth; 4 and 5, palate; 6, inner cheek; 7, buccal floor; 8, tongue; 9, buccal sulcus; 10, palate). Lane 11: keratosis (tongue). Lane 12: mucocele (lip). Lane 13: abscess (gingiva). Lane 14: pyogenic granuloma (gingiva). Lane 15: palatal KS (AIDS-related). Lane 16: cutaneous KS (Mediterranean). Lane 17: water.

Results

HHV-8 DNA could be amplified from 5 of 11 KS and 11 of 14 non-KS oral tissues from HIV-positive patients, 10 of 16 classic KS tissues, and none of 20 oral samples taken from general dental patients. SSCP analyses and DNA sequencing demonstrated that HHV-8 sequences of 11 of the 25 positive tissue samples were unique. A common HHV-8 DNA sequence was identified in 3 KS oral samples, 5 non-KS oral samples, and 1 cutaneous KS sample. Another sequence was common to 5 KS tissues (2 oral and 3 cutaneous). Figure 1 illustrates

Variant group	Molecular basis for grouping	Distribution of variants: UK/Italy	USA/Africa	Total
	130 140 150 160 170 180			
A	NGFD P VFPMVVPQQLGHAILQQLLVYHIYSKISAGAP D DVNMAELDYLTTNVSFMGQ	0	20	20
	ccc gat			
B	.... L ..... D	16	6	22
	ctc gat			
C	.... I ..... G	6	2	8
	atc ggt			
D	.... T ..... G	0	7	7
	acc ggt			
others		3	1	4
	(Total)	25	36	61

Figure 2. Molecular basis of HHV-8 grouping and geographic distribution of HHV-8 variants. Predicted group A peptide sequence, codon positions 130–186, is shown in full, using single-letter code. Amino acid residues whose codons are sites of 95% of mutations are in bold. Residues that allow classification of variants into groups B, C, and D are shown in alignment with group A sequence; dots signify residues that are identical to group A. Codons that specify group-specific amino acid changes are depicted as lower-case letters below corresponding amino acid residues. Not shown are codons in which mutations are silent and amino acid changes in group of variants that cannot be classified as A, B, C, or D.

sequence polymorphism of HHV-8 DNA amplified from all of the oral non-KS tissue samples and from representative KS samples. EBV, CMV, and HHV-6 and -7 DNA were not amplified from any of the oral tissue samples.

From each of the 26 HHV-8–positive tissues, the sequence of the ORF 26 segment encompassed by nested primers (170 bp in length, representing 57 codons) was aligned and compared with the others and with 36 sequences reported from previous studies involving patients from the United States and Africa [1–3, 5]. Genetic variation within the segment was not, in most instances, random. Using the prototype virus sequence [1] as the basis for comparison, there were altogether 109 base changes among the 61 sequences examined. Of these changes, 102 (95%) were confined to six positions in 5 codons: 134, 141, 152, 167, and 169 (numbered according to [1]). In codons 134 and 167, nucleotide substitutions occurred at positions that lead to amino acid changes (figure 2). Substitutions in codons 141 (G→T at the third base), 152 (C→T at the first base), and 169 (A→C at the third base) are silent. Based on amino acid changes predicted from substitutions in codons 134 and 167, all sequences, including those that were unique and possessed mutations in other codons, could be segregated into 4 main groups, labeled A–D, and a miscellaneous group (figure 2).

Figure 2 also shows the geographic distribution of variants. Group A and D variants were exclusive to tissues of patients from the United States and Africa. Group B and C variants were also identified in American and African tissues (group B: 6/36 [17%]; group C: 2/36 [6%]) but were less prevalent than in tissues of London and Italian patients (group B: 16/25 [64%]; group C: 6/25 [24%]). Of the 10 oral non-KS tissues from the London patients, 70% belonged to group B.

Of the 109 base substitutions, the 7 that did not involve the 5 codons aforementioned were located at codons 130 (A→G at the second base), 138 (A→T at the first base), 142 (A→T at the second base), 146 (G→T at the third base), 163 (C→T at the third base), 164 (G→A at the third base), and 168 (T→C at the third base). Only the substitutions at codons 138 and 142 are predicted to lead to amino acid changes. Tissue extracts whose PCR products yielded these base changes were reamplified and resequenced. The changes remained the same, discounting the possibility that Taq polymerase misincorporation had led to the detection of unique sequences.

Discussion

This study identifies the oral mucosa as another site of HHV-8 infection in people who are HIV-infected. In designing this study, we were aware of factors that could lead to false-negative results after PCR amplification for HHV-8 DNA from fixed archived tissues: fragmentation of target DNA [10] and formaldehyde-mediated cross-linking of nucleic acid polymers to each other and to proteins [11]. We therefore chose to use a nested approach in searching for HHV-8 DNA (to amplify the population of target DNA that had escaped reaction with formalin) and to adopt $KS330_{233}$ primers as outer primers (to keep the segment of amplifiable DNA short, thereby increasing the chance of detecting nonfragmented target DNA). Nevertheless, 6 of 11 oral KS and 4 of 15 cutaneous KS tissues were negative for HHV-8 DNA in our nested PCR assay. This is likely to reflect the combined effects of nucleic acid polymer degradation [10] and formalin [11] on the specimens. We note that

most other PCR assays for HHV-8 DNA in KS tissues [1–3] have used snap-frozen or fresh biopsy specimens.

The overall rate of positive results in our study is, notwithstanding, high. It is unlikely to be due to carryover PCR contamination, because an array of unique sequences was found. We are thus confident that the results obtained from the KS samples and, in particular, the oral non-KS tissues arose from HHV-8 DNA that was already present in tissue samples before the study.

That HHV-8 may be present in oral non-KS, albeit pathologic, tissues of people who are HIV-infected points to the oral mucosa of such persons as a site of HHV-8 infection. The pleiotropism of HHV-8 in the mouth of HIV-positive persons is similar to that of CMV [12] and contrasts with EBV, which preferentially replicates in the tongue [13]. However, unlike CMV and EBV, it is unclear if the oral mucosa can support active HHV-8 replication. Comprehensive PCR studies of saliva, and of viral RNA and protein expression in tissues, will be necessary to establish if saliva is a vehicle of HHV-8 transmission.

HHV-8 DNA was detected exclusively in oral tissues of people with HIV infection but not in those who were presumed to be uninfected. As this study has not defined which cell types in the oral compartment were infected, the possibility that the detection of the viral genome in the oral tissues is due to the presence of HHV-8–infected mononuclear cells [4] cannot be excluded. However, <10% of HIV-positive patients without KS are positive for HHV-8 DNA in their peripheral blood mononuclear cells [4]; this contrasts with the 70% positivity rate in the oral non-KS tissues examined here. It is, hence, unlikely that the high positivity rate found in the oral tissues was entirely due to infiltration by HHV-8–infected blood cells. In situ hybridization studies are in progress to define precisely which cells in the oral mucosa harbor HHV-8 DNA.

The presence of HHV-8 in oral tissues may be related to the immunosuppressive state that follows HIV infection. That immunosuppression per se may heighten the activity of HHV-8 is suggested by the relatively quick appearance of KS lesions in patients who undergo immunosuppressive therapy and their resolution after discontinuation of therapy [14]. However, as HIV-1 proviral DNA can be located in oral epithelial cells [15], HIV may play a more direct role, for example, by heterologous (HIV–HHV-8) transactivation, in promoting HHV-8 infection in the oral compartment. To confirm this, an examination of the state of activation of HIV-1 in oral epithelial cells and the precise localization of HHV-8 to the oral epithelium will be needed.

The absence of EBV DNA in the oral tissues examined was surprising. The small sample size and the consequent susceptibility to sampling errors are probable contributory factors. Furthermore, none of the samples showed histologic changes suggestive of oral hairy leukoplakia. The absence of DNA from the other herpesviruses known to be shed into saliva is less remarkable, since they are not considered to persist to any significant extent in the oral mucosa of people who are HIV-infected. In contrast, the finding that HHV-8 DNA is found in the majority of this small set of tissues does underscore the prominence of HHV-8, relative to the other herpesviruses, in oral tissues of HIV-positive patients.

We found diversity in the HHV-8 DNA sequences derived from our study samples: 11 of 25 sequences were different from each other. Polymorphism in this region of the viral genome has also been observed in a study of American and African KS patients [3], and another study [5] has demonstrated the hypervariability of the $KS330_{233}$ fragment compared to other regions in the 965-bp ORF. As the genome of DNA viruses is relatively immutable, the high degree of polymorphism in this middle segment of ORF 26 is likely to confer some selective advantage to the virus. The putative peptide encoded from this region might be an immunologic target.

Despite the polymorphism of the HHV-8 DNA ORF 26 fragment, the presence of nucleotide and peptide motifs in it has permitted HHV-8 variants to be grouped. Although the 170-bp stretch of DNA examined is too short and the number of base differences too few to allow a formal typing system, it is intriguing to observe that certain motifs correlate with the locale in which the hosts seek health care, suggesting that HHV-8 variants circulate in geographically restricted areas. Sequence analysis of this short segment of ORF 26 may allow the molecular epidemiology of HHV-8 to be studied in archived tissues.

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