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Characterization of cytomegalovirus isolates from patients with AIDS by DNA restriction analysis

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SUMMARY

Thirty-seven isolates of cytomegalovirus (CMV) were obtained from a group of 20 promiscuous homosexual men, either suffering from the acquired immunodeficiency syndrome (AIDS) at the time of CMV isolation, or who developed AIDS subsequently. The isolates of CMV were characterized by the method of DNA restriction analysis. All epidemiologically unrelated strains of CMV exhibited different fragment migration patterns and no one strain appeared to be associated with AIDS or any particular disease pattern in these patients.

Sequential isolates of CMV were obtained from nine patients in the study group either from different sites at the same time or from the same site on different dates. In the case of seven of the men, viruses with minor differences in restriction profile were obtained, possibly representing sub-populations of an endogenous strain of CMV. In two of the patients, reinfection with different strains was apparent. We conclude that reinfections with CMV in AIDS patients can occur, but the isolation of strains exhibiting major differences in genome structure seen by restriction enzyme analysis was uncommon.

INTRODUCTION

The sexual transmission of human cytomegalovirus (CMV) has been well described (Chretien, McGinniss & Muller, 1977; Handsfield *et al.* 1985) with virus present in both cervical secretions (Jordan *et al.* 1973) and semen (Lang & Kummer, 1972; Lang & Kummer, 1975). The prevalence of infection with CMV is particularly high in promiscuous homosexual men (Drew *et al.* 1981; Mintz *et al.* 1983; Ross *et al.* 1987) and in one study (Drew *et al.* 1981) antibody was demonstrated in the sera of 94% of homosexual men attending a clinic for sexually transmitted diseases. Although the majority of infections are subclinical, CMV has the ability to cause persistent or latent infections (Jordan, 1983) and virus reactivation can result in serious complications in immunocompromised

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hosts (Meyers, Fleurnoy & Thomas, 1982; Betts, 1982). Not surprisingly, CMV is frequently isolated from homosexual men with the acquired immunodeficiency syndrome (AIDS). In these patients it causes life and sight threatening disease (Macher *et al.* 1983; Quinnan *et al.* 1984; Moskowitz *et al.* 1985; Niedt & Schinella, 1985) although treatment with ganciclovir has shown clinical benefits (reviewed by Tyms, Taylor & Parkin, 1988). There is some belief that CMV is more than an opportunist in this group of patients, possibly having a role in the aetiology of AIDS and being associated with Kaposi's sarcoma (Giraldo & Beth, 1986; Drew, 1984; Drew *et al.* 1982). It is known that CMV infection alone can be immunosuppressive (Rinaldo *et al.* 1980; Carney *et al.* 1981) and it is considered possible that this perpetuates the immunodeficient state in AIDS (Greenberg *et al.* 1984; Drew *et al.* 1985). Furthermore, it has been suggested that reinfection with different strains of CMV occurs in AIDS patients (Drew *et al.* 1984; Spector, Hirata & Neuman, 1984) and the cumulative immunosuppressive effect may have predisposed individuals to infection with the human immune deficiency virus (HIV). Of course predisposition to CMV infection by the immunosuppressive effect of HIV is an alternative possibility.

Only limited information has been published on the nature of CMV infection in AIDS patients. Restriction endonuclease digestion of viral DNA provides a powerful tool for determining the molecular relatedness of isolates of CMV (Kilpatrick *et al.* 1976; Huang *et al.* 1976; Huang *et al.* 1980; Tyms, 1983). Epidemiologically unrelated strains show marked heterogeneity in genome structure with between 80%–90% sequence homology which results in distinct DNA fragment migration patterns (Huang *et al.* 1976). In contrast, epidemiologically related viruses, such as those isolated sequentially from recurrently infected individuals (Huang *et al.* 1980*b*), from mother and baby pairs (Huang *et al.* 1980*a*; Peckham *et al.* 1986) or sexual partners (Handsfield *et al.* 1985) have identical DNA profiles.

We have used restriction enzyme analysis to investigate the DNA profiles of 26 isolates of CMV obtained from 9 homosexual men together with single isolates from 11 individuals in the same study group. All patients either suffered from AIDS at the time of virus isolation or developed the disease subsequently. The aims of the study were (a) to establish if one particular virus type or group of CMV isolates were closely related to AIDS or involved in a particular pattern of disease; (b) to investigate strain heterogeneity within the individual patient.

METHODS

Viruses and cells

Clinical specimens were obtained from homosexual men who either had AIDS or persistent generalized lymphadenopathy (PGL) or were asymptomatic but considered to be in a risk group for AIDS. All patients in the study eventually developed AIDS. Viruses were isolated and cultured in human embryo fibroblast (HEF) cells which were grown in Eagles minimal essential medium (MEM) supplemented with 5% fetal calf serum (FCS), 9 mM sodium bicarbonate and 7 mM-HEPES buffer. In maintenance medium (MM) the supplements were adjusted to 2% FCS, 13 mM sodium bicarbonate and 14 mM-HEPES. Virus

isolates were identified initially as CMV by the use of monoclonal antibodies supplied by Bioscot, Edinburgh, and were passaged from two to five times to increase the infectivity titres to give a multiplicity of infection of 0·1 plaque forming units or greater per cell. The prototype strains of CMV, AD169 and Towne, were from our own laboratory stocks and the simian-like strain of CMV, Colburn (Nigida *et al.* 1975) was obtained from Dr Wade Gibson, John's Hopkins Institute, Baltimore, USA. These prototype strains were used for comparisons of restriction cleavage patterns and unequivocal identification of the clinical isolates as human CMV.

DNA restriction analysis

Confluent HEF cell monolayers (approximately 10^6 cells) were infected with the isolates of CMV and the cytopathic effect allowed to progress until all cells were infected. The MM was then replaced with a phosphate-free maintenance medium containing 15 μCi/ml ^{32}P orthophosphate (Amersham) and incubated for 3 days. Radiolabelled cells were then harvested into 0·1 × standard saline citrate (SSC), lysed with sarcosyl on ice and digested with proteinase k for 2 h at 48 °C. The method used for extraction of the DNA has been described previously (Tyms *et al.* 1984). Briefly, samples were treated with phenol and amyl alcohol/chloroform and the DNA precipitated in ice-cold ethanol. The DNA was redissolved in 5 mM-Tris/EDTA (pH 7·6) before being subjected to digestion with the restriction enzymes EcoR1, BamH1 or Sma1 (Amersham) according to the manufacturers' recommendations. The DNA fragments were separated by agarose gel electrophoresis (2v/cm overnight) and the restriction profile visualized by autoradiography after drying.

Immunofluorescence

HEF cells were grown on 13 mm diameter coverslips, infected with clinical isolates of CMV or prototype viruses and fixed 4–5 days after infection in cold acetone for 10 min before air-drying. Fixed monolayers were treated with filtered rabbit serum to block Fc-receptors and exposed to monoclonal antibody HCMV 19 which recognizes the 66-69KD matrix protein (Helena Hart, BioScot, Edinburgh – unpublished). Immune reactions were visualized using fluorescein isothiocyanate (FITC) anti-mouse conjugate, counterstained with 0·2% Evan's blue and examined with a Leitz epifluorescence microscope and UV illumination.

RESULTS

Thirty-seven isolates of CMV obtained from 20 homosexual men were identified initially as CMV by immunofluorescence with CMV-specific monoclonal antibodies. Details of viruses and nine patients from whom CMV was isolated more than once are summarized in Table 1. In addition, single isolates of CMV were recovered from the other 11 patients all of whom either had AIDS at the time of virus isolation or who developed AIDS subsequently.

By restriction enzyme analysis the 37 viruses identified with the human prototype strains of CMV, AD169 and Towne, by the extensive co-migration of DNA fragments, and not with the simian-like strain, Colburn, irrespective of

Table 1. *Details of nine patients and sequential CMV isolates*

Patient/virus		Site	Date	Clinical details	No differences Sma1
J	1	T/S	03.01.86	CMV isolated	—
	2	Urine	26.02.86	at time of illness	3
	3	T/S	25.01.86	with pneumonitis	2
	4	Saliva	26.02.86	and retinitis	1
B	1	Urine	04.09.84	CMV isolated	—
	2	Urine	25.09.84	at time of illness	4
	3	Urine	05.10.84	with retinitis. K.S.	2
	4	T/S	07.11.84		1
M	1	Urine	09.11.84	First two CMVs	—
	2	Urine	14.11.84	isolated at onset	1
	3	Bronc.	11.06.85	of AIDS. Later	4
	4	Urine	11.06.85	developed pneumonitis. K.S.	2
Br	1	Urine	31.07.84	CMV isolated	—
	2	Semen	31.07.84	when asymptomatic.	3
	3	Semen	16.08.84	Developed pneumonitis and retinitis 1986.	2 1
C	1	Blood	11.04.86	CMV isolated	—
	2	T/S	11.04.86	at time of illness	2
	3	Urine	22.04.86	with retinitis and colitis	0
Par	1	Urine	28.11.85	CMV isolated	—
	2	Semen	28.11.85	when PGL. No CMV associated disease	2
X	1	Semen	11.03.86	CMV isolated	—
	2	Semen	28.04.86	at time of illness with pneumonitis. K.S.	0
P	1	Urine	14.11.84	CMV isolated at	—
	2	Urine	20.11.84	onset of AIDS. No CMV associated disease	8
L	1	Urine	16.08.84	CMV isolated at	—
	2	T/S	30.10.84	onset of AIDS. Later developed retinitis and encephalitis	8

Bronc, Bronchoscopy; T/S, Throat swab; K.S., Kaposi's sarcoma; PGL, Persistent generalized lymphadenopathy.

which restriction enzyme was used. Comparison of the restriction profiles of the DNA from the CMV isolates showed greater than 5 and up to 16 fragment size differences with either Sma1, EcoR1 or BamH1. Sma1 digests distinguished in particular the simian-like strain, Colburn, from the human isolates. These differences are illustrated in Fig. 1 which shows restriction profiles of viral DNA of isolates from different patients, digested with EcoR1 (Fig. 1*a*) or Sma1 (Fig. 1*b*). On the basis of previous studies of DNA restriction analysis with random clinical isolates (Kilpatrick, Huang & Pagano, 1976; Huang *et al.* 1976; Huang *et al.* 1980*b*; Tyms, 1983) all viruses from different patients were classified as epidemiologically unrelated.

The DNA profiles of multiple virus isolates obtained from nine individual

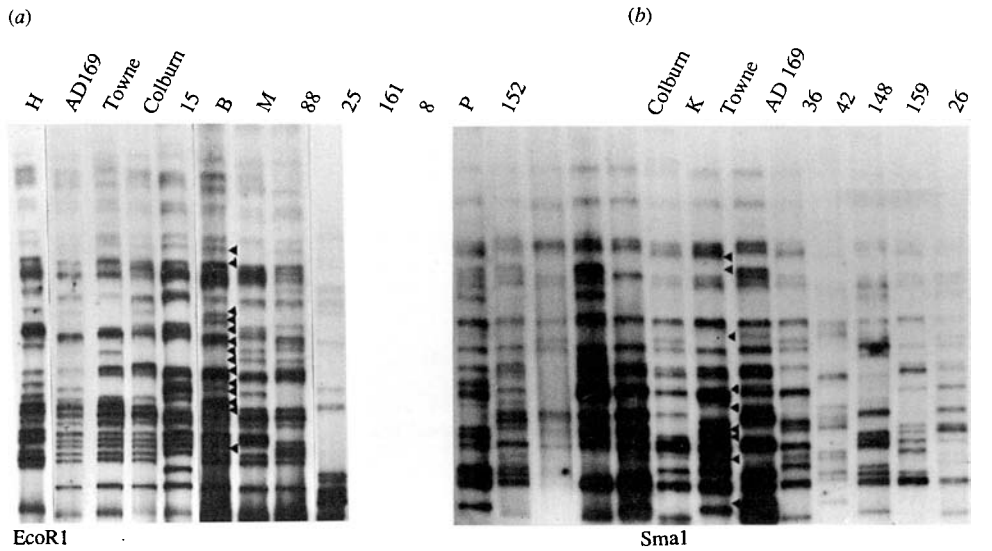

Fig. 1. Comparison of the restriction profiles for a range of clinical isolates of CMV (H; 15; B; M; 88; 25; 161; 8; P; 152; K; 36; 42; 148; 159; 126), prototype strains of human CMV (AD169; Towne) and simian-like CMV (Colburn) after digestion of viral DNA with (a) Eco R1 and (b) Sma-1. The marked bands indicate the 9 fragment size differences between isolates B and M, and the 14 differences between isolates Towne and AD169.

patients (Table 1) were analysed. The first virus isolated was used as the reference strain for calculating the number of restriction site differences. In the case of seven patients there were only minor differences in the DNA restriction profiles of sequential virus isolates. Thus, as shown in Table 1, viruses from patients M, J, B, Br, C, Par and X had a maximum of four restriction site differences when DNA was digested with SmaI. Complete identity or only minor differences were observed also after digestion of viral DNA with EcoR1 or BamH1 (results not shown). It was considered likely that viruses obtained at different times or different sites from these seven patients were variants of a single endogeneous virus strain and did not represent reinfections.

The minor differences in restriction profiles are illustrated by the results obtained for three patients from each of whom four viruses were recovered. Patient J was a 37-year-old male who had a history of syphilis and hepatitis B infection. AIDS was diagnosed in March 1985 and he subsequently suffered CMV pneumonitis and retinitis until his death in March 1986. Analysis of viral DNA from four CMV isolates (J1, throat swab 03.01.86; J2, urine 26.02.86; J3, throat swab 25.01.86 and J4, saliva 26.02.86) recovered during a 2-month period showed minor variations in genome structure, represented by no more than three restriction site differences between any of the four viruses irrespective of the enzyme used (Fig. 2).

Patient B was a 45-year-old male homosexual prostitute with more than 500 partners per year who was diagnosed as having AIDS in May 1984 along with Kaposi's sarcoma. Two months later, he suffered a severe attack of CMV retinitis

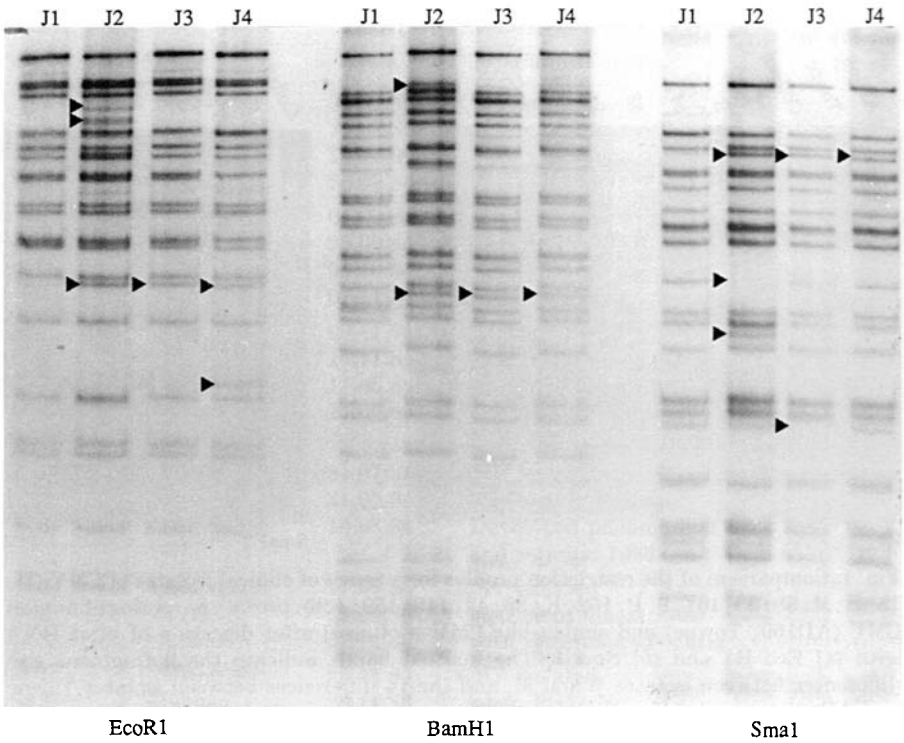

Fig. 2. Restriction profiles of viral DNA from sequential isolates of CMV from patient J (J1; J2; J3 and J4) digested with EcoR1, BamH1 or Sma1. The marked bands indicate the minor variations seen in fragment migration patterns, using the first CMV isolate as the reference strain.

and eventually died in September 1985. The restriction enzyme profiles of four isolates of CMV recovered from this patient during a 2-month period (B1, urine 04.09.84; B2, urine 25.09.84; B3, urine 5.10.84 and B4, throat swab 07.11.84) are shown in Fig. 3. There were only minor variations in genome structure which for viruses from an individual considered to be at high risk for reinfection was surprising.

Patient M was a 40-year-old male who had a history of syphilis. AIDS was diagnosed in November 1984, at which time he suffered from *Pneumocystis carinii* pneumonia (PCP), Kaposi's sarcoma and was shedding CMV in urine. He developed CMV pneumonitis in June 1985 and died shortly after. The restriction enzyme profiles of four isolates of CMV recovered from this patient during a 7-month period (M1, urine 09.11.84; M2, urine 14.11.84; M3, bronchoscopy 11.06.85; M4, urine 11.06.85) are shown in Fig. 4. Five or less fragment size differences were shown when viral DNA's were digested with EcoR1, BamH1 or Sma1, even though the isolates were 7 months apart.

Isolates of CMV from all three patients showed the characteristic 6–16 fragment size differences when compared to epidemiologically unrelated isolates or with DNA from prototype strains. This is illustrated in Fig. 1*a* by the nine differences

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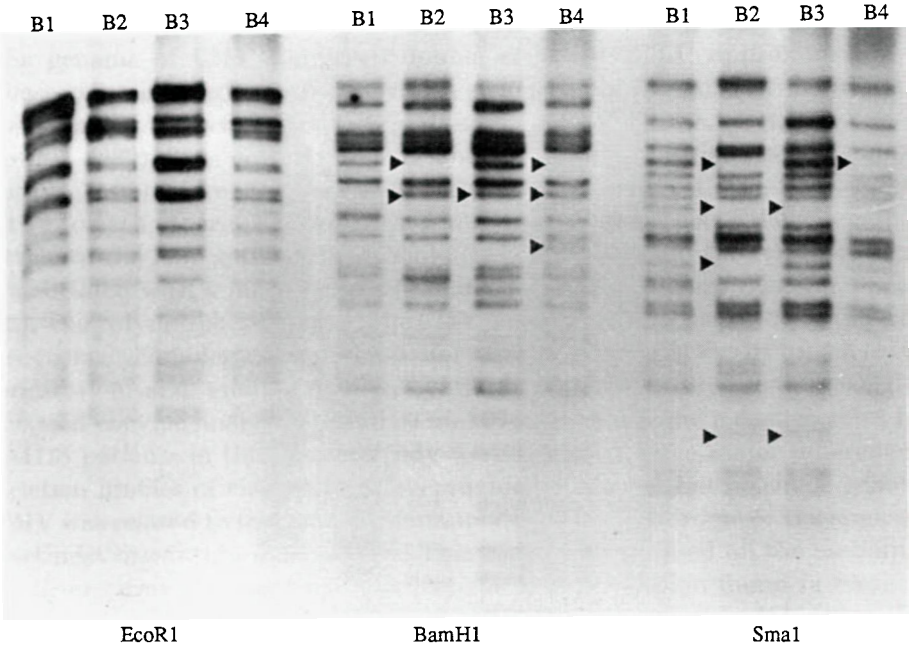

Fig. 3. Restriction profiles of viral DNA from sequential isolates of CMV from patient B (B1; B2; B3 and B4) digested with EcoR1, BamH1 or Sma1. The marked bands again indicate the minor changes in the profiles.

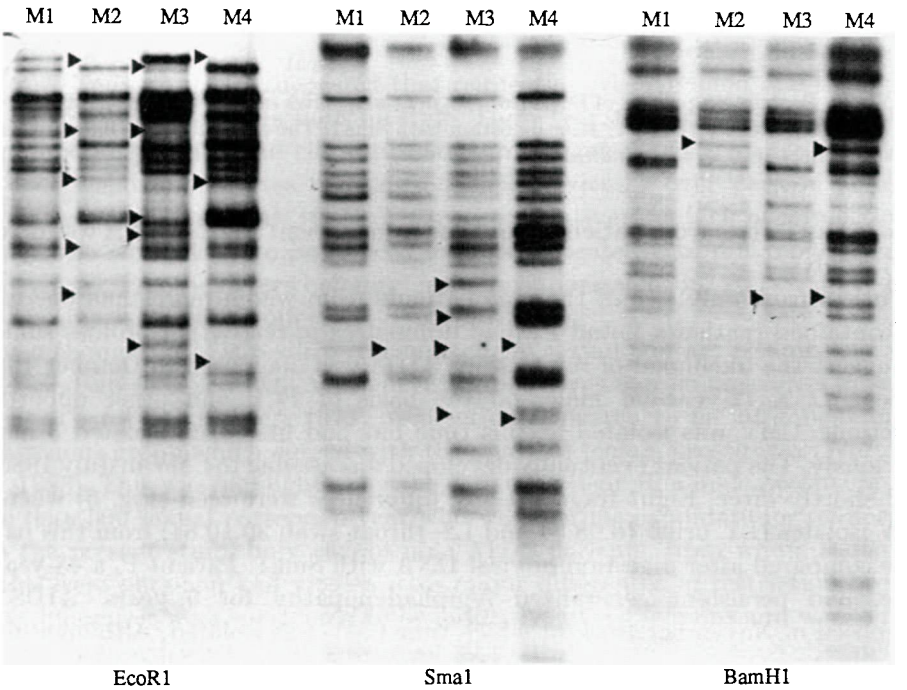

Fig. 4. Restriction profiles of DNA from sequential isolates of CMV from patient M (M1; M2; M3 and M4) digested with EcoR1, BamH1 or Sma1. The marked bands indicate regions of variation in fragment migration patterns.

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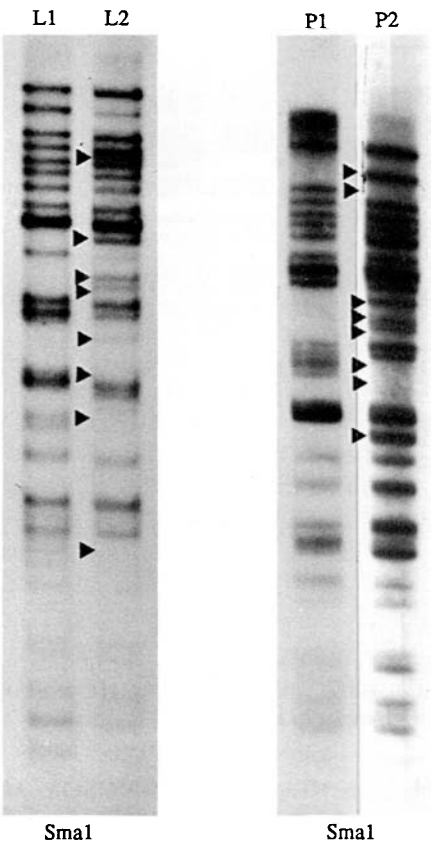

Fig. 5. Restriction profiles of DNA from sequential isolates of CMV from patient L (L1 ; L2) and patient P (P1 ; P2) after digestion with SmaI. The marked bands indicate the differences seen in the profiles.

between an isolate from patient B and one from patient M after DNA was digested with EcoR1.

Viruses from only two of the nine patients from whom more than one isolate was obtained (patients L and P), had differences in restriction profiles sufficient to suggest the likelihood of reinfection. AIDS was diagnosed in October 1984 in patient L, a 42-year-old male, on the basis of PCP and other opportunist infections. CMV was isolated at this time but had first been isolated 3 months previously. The patient eventually developed disease due to CMV in July 1985 and died shortly after. Eight fragment size differences were seen (Fig. 5) when two CMV isolates (L1, urine 16.08.84 and L2, throat swab 30.10.84) from this patient were compared after digestion of viral DNA with SmaI. Patient P, a 45-year-old male, had persistent generalized lymphadenopathy for 5 years. AIDS was diagnosed in November 1984, at which time CMV was isolated. Although disease associated with this virus was not evident, the patient died shortly after. Comparison of the DNA restriction profiles of two viruses isolated from this patient only 2 weeks apart (P1, urine 14.11.84 and P2, urine 20.11.84) showed eight fragment size differences (Fig. 5).

DISCUSSION

The genome of CMV comprises double stranded DNA, approximately 240 kilobase pairs in length with an overall structure similar to that described for herpes simplex viruses (Kilpatrick & Huang, 1977; Westrate, Geelen & Van Der Noordaa, 1980). This consists of long and short unique regions both bound by inverted, terminal repeat sequences. The configuration of the two segments relative to each other appears to result in four isomeric forms of the genome. The junction of the long and short segments (L–S junction) and the terminal regions are associated with a high degree of heterogeneity (Tamashiro, Hock & Spector, 1982). The polymorphic nature of the CMV genome reflects the 10–20% difference in sequence homology established for a range of CMVs by nucleic acid hybridization and enables genetic variants to be readily distinguished by restriction enzyme analysis (Huang *et al.* 1976). By the same means isolates from 20 AIDS patients in the present study were shown to have major differences in restriction profiles of viral DNA. This provided evidence that no single genotype of CMV was related to the acquired immunodeficiency syndrome or the associated opportunist diseases in our patients. This conclusion is based on the assumption that viruses grown in cell culture reflect the true population found *in vivo*.

Genome analysis of sequential isolates of CMV from individual AIDS patients was important in order to establish the incidence of reinfection and any relationship this may have to clinical outcome. It is possible that the suppressive effect by CMV infection on the cell-mediated immune response (Rinaldo *et al.* 1980; Carney *et al.* 1981) may be exacerbated in circumstances where AIDS patients suffer reinfection with different strains of CMV. Previous reports on the incidence of CMV in homosexual men with AIDS (Drew *et al.* 1984; Spector, Hirata & Neuman, 1984) suggested that reinfection was common and this would be consistent with a promiscuous lifestyle. These two studies were limited in both the number of patients and clinical isolates investigated and contrasted with the study of female prostitutes who shed identical viruses over periods of up to 16 months (Wertheim *et al.* 1985). This was indicative of a lack of reinfection in women who were likely to have been exposed repeatedly to different strains of CMV. However, the overwhelming immunosuppression in AIDS may be a predisposing factor for reinfection.

Even though CMV is readily transmitted by sexual contact (Handsfield *et al.* 1985), and up to seven $\log_{10}$ units of infectious virus can be measured in semen samples (Lang & Kummer, 1972), reinfection appeared to be infrequent in our promiscuous group which agreed with the study of female prostitutes (Wertheim *et al.* 1985). This was highlighted by the case of patient B, a male prostitute with more than 500 sexual partners a year who was a prime candidate for reinfection.

In the present study only two of nine AIDS patients, from whom sequential isolates were obtained had viruses with major differences in restriction profiles. These differences were considered to be indicative of reinfections and were similar to those observed when viruses from all 20 patients were compared. Reinfection with CMV has been documented in seropositive patients undergoing renal transplantation but the source of virus could be directly associated with the allograft (Grundy, Super & Griffiths, 1986; Chou, 1986). It seems likely that the

immune response is capable of suppressing reinfection in immune competent individuals but when CMV-infected organs are transplanted or in cases of severe immunosuppression caused by HIV, reinfection is possible. Also, most viruses isolated in the present study were over a relatively short period of time (< 7 months) and, of course, longer term surveillance may reveal a greater incidence of reinfection.

In the majority of our patients only minor differences in genome structure were seen in the multiple isolates obtained. The ability to differentiate between related and unrelated viruses arose by virtue of the comparative nature of our study and the larger number of patients and viruses which were examined. In studies of the molecular epidemiology of CMV in women and their infants (Huang *et al.* 1980*a*; Huang *et al.* 1980*b*), viruses recovered from individual mothers or mother:baby pairs were identical or showed only minor genomic variation. As in our studies, these related viruses although sometimes showing minor variation, were markedly different from those recovered from unrelated persons. These differences in genome structure were consistent with the variations observed after prolonged culture of CMV or after sub-cloning experiments (Huang *et al.* 1980*b*). Interestingly, investigation of what appeared to be homogeneous stocks of herpes simplex virus recovered from cadaveric ganglionic tissue, also revealed different populations of virus after sub-cloning. This reflected the heterogeneity of the *in vivo* virus population present in the latent state (Lewis *et al.* 1984).

It was apparent from our studies of sequential isolates of CMV from patients with AIDS, that viruses showing minor differences in restriction profiles represented variants of a single infecting agent. It is possible that the immunodeficient state may allow viruses, usually present as inapparent sub-populations, to thrive and this may have significance with respect to CMV as an important opportunist in AIDS.

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