C．研究結果

1．抗GPCMV抗体g－1の性状

抗GPCMVマウスモノクローナル抗体g−1は1986年に筒井らによりGPCMV感染細胞をマウスに免疫して作成されたものであり、g－1と同時にb−16、b−29、d−13、e−16などのいくつかのGPCMVに対するクローンが得られている。GPCMV感染組織に上記5つの抗体を用いて免疫組織化学を行ったところ、GPCMVを特異的に検出したのはg−1のみであり、他の抗体ではシグナルが微弱であったり、特異的なシグナルが全く見られないものもあった。感染細胞を用いたウエスタンブロットではg−1抗体は感染後48－72時間で発現する50kDaのタンパクを認識し、このタンパク発現はphosphonoacetic acid（PAA）では阻害されないが、サイクロヘキシミドでは阻害される。こうしたウイルスタンパクのkineticsからg－1抗体はGPCMVの早期タンパクを認識しているものと考えられた。

2．内耳への感染実験

マクロ所見では内耳蝸牛管に点状出血が認められた。組織では前庭階、鼓室階の著明な出血とリンパ球浸潤が見られ、らせん神経節にもリンパ球浸潤、出血が確認され、多くの巨細胞封入体も見られた。抗GPCMVマウスモノクローナル抗体g−1を用いた免疫組織化学ではライスナー膜の前庭階側、前庭階、鼓室階の出血、炎症巣の中に、多くのGPCMV感染細胞を認めた。蝸牛管の中には軽い出血を認めたが、GPCMV感染細胞は認められなかった。らせん神経節では変性、壊死部分に一致してGPCMV感染細胞が認められた。これらの所見を総合すると、GPCMVが直接接種された内耳では外リンパ液領域にGPCMV感染細胞が分布し、コルチ器などの感覚器細胞を含む内リンパ液領域ではGPCMV感染細胞は認められない。また、神経節領域にもGPCMV感染細胞が見られることから、内耳ではGPCMVは外リンパ液と神経組織を介して広がっていくものと推察された。

感染モルモットには内耳以外にも唾液腺、腎臓、膵臓、脈絡叢に免疫組織化学でGPCMVのGPCMV感染細胞が認められた。一方で、対照群のモルモットにはこれらの変化は認められなかった。

3．垂直感染実験

10匹の妊娠モルモット（妊娠5週）にGPCMVを接種した。胎児の内耳には前庭階、鼓室階に出血、炎症が、らせん神経節に神経細胞の脱落と壊死が認められ、これらの領域に免疫組織化学でGPCMV感染細胞が認められた。出血、炎症の程度やGPCMV感染細胞の数は胎児ごとにばらつきがあったが、蝸牛管、コルチ器、卵形嚢、球形嚢、平衡斑、三半規管、内リンパ嚢などの内リンパ液領域にはGPCMV感染細胞は見あたらなかった。また、内耳以外では中耳腔の内壁にGPCMV感染細胞を認めた。こうした所見より、垂直感染モデルの胎児の内耳においても外リンパ液領域および神経組織がGPCMV感染細胞の主な分布であり、内リンパ液領域にはGPCMV感染細胞は見られなかった。

D．考　察

本研究でわれわれはGPCMV感染実験モデルにおけるGPCMV感染細胞の局在を免疫組織化学で明らかにした。内耳におけるGPCMV感染細胞の局在はおもに外リンパ液領域とらせん神経節内であり、感覚器であるコルチ器を含む蝸牛管などの内リンパ液領域にはGPCMV感染細胞は見られなかった。このことはGPCMVが水平感染モデルでも、垂直感染モデルでも、内耳においては外リンパ液、および神経組織を伝わって、広がることを示唆する。

感覚器細胞である内毛細胞、外毛細胞が存在するコルチ器には感染は認められず、周辺の血管には感染による破壊と出血があり、炎症細胞浸潤が著明であったことから、コルチ器周辺には循環障害が起きていることが予想される。また、らせん神経節にはGPCMV感染による明かな組織破壊と神経細胞の脱落、壊死が認められたことから、らせん神経節内で、機能的な神経伝導障害が起こっていることが予想される。これらの知見から、予想されるGPCMV感染による難聴のメカニズムとしては、コルチ器周辺の循環障害とらせん神経節内の神経伝導障害が考えられる。これらの知見はヒトのサイトメガロウイルスによる難聴のメカニズムを考える上で重要な示唆を含むものと思われる。今後、ヒトの臨床サンプルや他の動物を使った実験により、確認していくことが望まれる。

E．結　論

モルモット動物モデルを用いてGPCMV水平感染、垂直感染実験系におけるGPCMV感染細胞の局在を免疫組織化学により明らかにした。GPCMV感染細胞は外リンパ液領域、および、らせん神経節に見られ、蝸牛管、コルチ器などの内リンパ液領域にはGPCMV感染細胞は見られなかったことから、コルチ器周辺の出血等による循環障害、および、神経節の破壊による神経伝導障害が難聴のメカニズムであると考えられた。

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H．知的財産権の出願・登録状況

1．特許取得

なし

2．実用新案登録

なし

3．その他

なし

厚生労働科学研究費補助金（感覚器障害研究事業）
Ⅰ．総合研究報告
Ⅱ．分担研究報告

動物実験モデルにおける聴覚評価、組織評価方法の確立

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研究要旨

実験動物の聴力障害の程度を評価する方法の一つに聴性脳幹反応（ABR）を用いる方法がある。聴性脳幹反応を安定して正確に記録するためには、検査機器の整備と検査方法に習術することが必要である。われわれはモルモット、およびマウスにおけるABR記録の装置を整備しABRの記録を行った。さらに組織学的検討を加えるため、マウスにおける至適観察面を検討した。

A．研究目的

先天性CMV感染による聴覚障害の発症貴女を解明するためには、CMV感染小動物を用いた他覚的聴力検査が必須である。この研究の目的は、聴性脳幹反応（ABR）を安定して正確に記録するシステムを構築することであり、検査機器の整備、検査方法の習熟が必要不可欠である。本年度は最新のABR検査装置、電気シールド防音室を整備し、実際にコントロールとして感染していない小動物のABRの記録を行った。さらに病理組織学的検討を行うため、マウス蝸牛における至適観察面の検討を行った。

B．研究方法

Ⅰ．ABRを計測する際に必要な機器、環境の整備

ABRを計測するためには刺激音発生装置とABR検査装置が必要である。刺激発生装置はpowerlab（ADInstruments社製、オーストラリア）を用い、刺激音はtone burstを用いスピーカーはTucker Davis社製ES1。ABR検査装置はTucker Davis社製（オーストラリア）、sound level meterはOno Sokki LA511、amplifierはGRASS P55を用いた。できるかぎり電磁波などの影響の少ない環境とするために、金網で囲まれた聴力検査室において、動物、アイソレーター準備しアースをとり測定した。

組織学的評価はマウス頭部を脱灰後、マウス上顎骨硬口蓋に対して平行な平面に対し、30度、45度、60度の角度で切り出してパラフィン包埋し、切片を作製した。

Ⅱ．ABR記録法（麻酔、電極の取り付け）

動物に対して、ジエチルエーテルで吸入麻酔後、フェントバルビタール（商品名：ネンブタール）を30mg/kgで腹腔内投与し図1のように電極を刺入し、音刺激（刺激音は20000Hzのtone burst）を与え、ABRを測定した。

（倫理面の配慮）

実験動物の取り扱いに関しては福島県立医科大学動物実験ガイドラインに沿って、愛護的に行った。

C．研究結果

刺激音の周波数は20000Hzで刺激音圧は76.5dbSPLから36.5dBSPLまで変化させ、ABRを測定した。そのうち、図1に76.5dBSPL、66.5dBSPL、51.5dBSPL、におけるABRの波形を示す。音圧の低下とともにⅠからⅢ波の波形が小さくなり、36.5dBの刺激音圧ではⅠからⅢ波は消失していた。

組織学的検討においては45度の角度で切り出した標本が蝸牛軸に対してほぼ平行な角度となり、標準的に評価されているヒト側頭骨病理標本と一致する結果となった。（図2）

D．結　　論

本装置を用いることにより、モルモット、マウスのABRを安定して記録することが可能であった。CMV感染モデルにおける聴力の評価、聴力障害の程度を推察することが可能であると考えられた。本装置を用いることにより、今後はCMV感染をしたモルモット、マウスの感染実験系を用い、ABRを計測し、聴覚障害の評価が可能である。

図1－1　76.5dBSPL

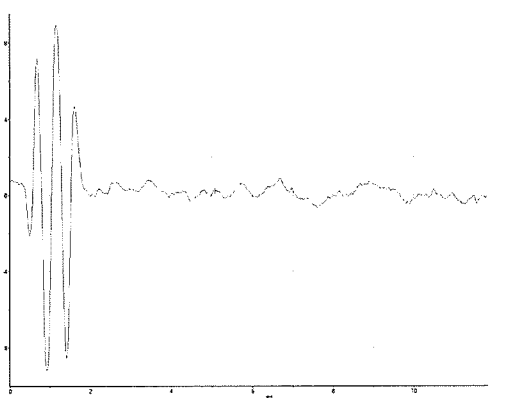


図１－２　66.5dBSPL

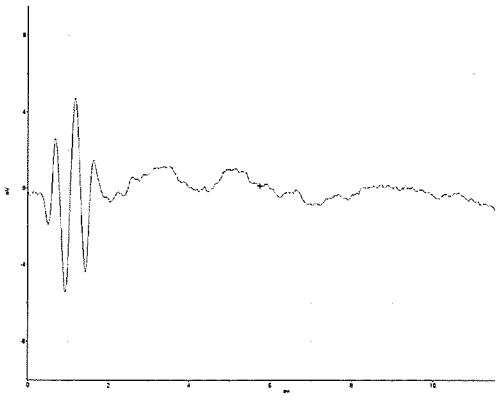
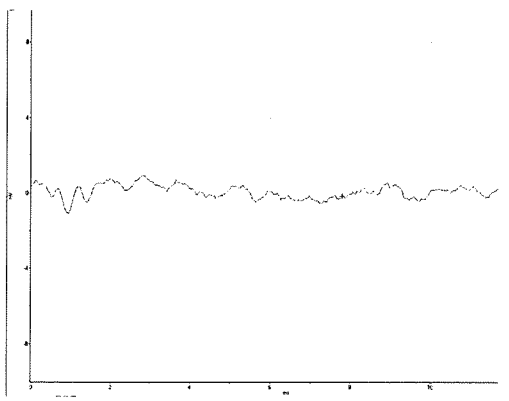


図１－３　51.5dBSPL



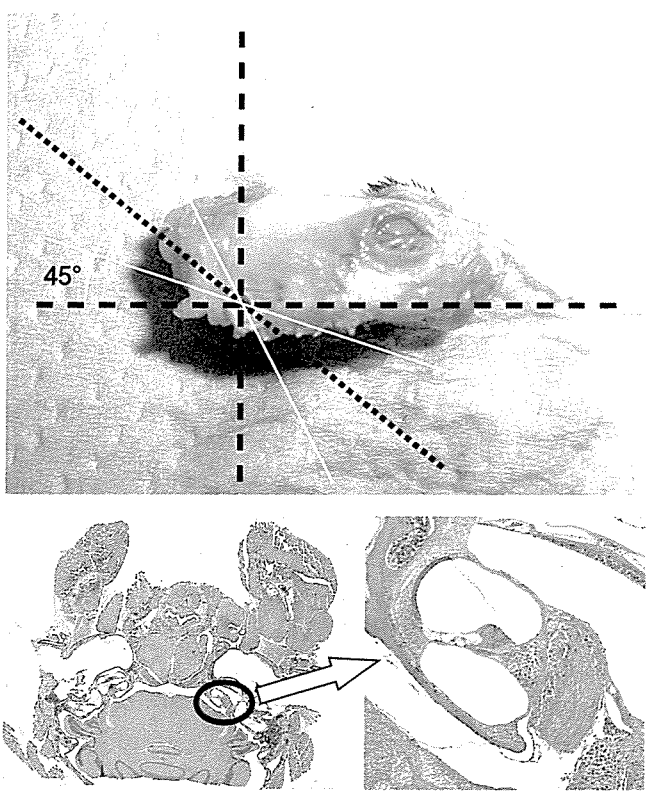


図２　マウス頭部に対する至適観察面の決定。45°が至適観察面であった。

E．まとめ

当科における小動物に対するABR測定の実際について報告した。小動物に対するABR評価のための聴力検査システムを整備した。病理組織学的評価を行うための至適観察面を決定することができた。

F．研究発表

なし

G．知的財産の出願・登録状況

1．特許取得
なし
2．実用新案登録
なし
3．その他
なし

Ⅲ. 研究成果の刊行に関する一覧表

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関連する刊行物

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報道

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Ⅳ. 研究成果の刊行物・別刷

MAJOR ARTICLE

Etiology of Severe Sensorineural Hearing Loss in Children: Independent Impact of Congenital Cytomegalovirus Infection and *GJB2* Mutations

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(See the editorial commentary by Pass, on pages 767–9, and the article by Schleiss et al., on pages 789–98.)

Background. Sensorineural hearing loss (SNHL) is the most common congenital disease. Longitudinal studies of infants with congenital cytomegalovirus (CMV) infection have demonstrated an association between CMV and SNHL. However, because of the lack of suitable neonatally collected specimens, the proportion of CMV-associated SNHL has not been defined, nor has the relationship between CMV and the major genetic causes of SNHL, such as mutations in the *GJB2* gene.

Methods. Sixty-seven children with severe SNHL were analyzed for CMV and human herpesvirus 6 (HHV-6) infections and for *GJB2* mutations. DNA specimens were prepared from dried umbilical cords, which are available for everyone born in Japan. Four children with typical symptomatic infection at birth served as positive control subjects.

Results. Congenital CMV infection and *GJB2* mutations were identified in 15% and 24% of the patients, respectively. HHV-6 was not detected. All children with CMV-associated cases developed SNHL before they were 2 years old. Most children with CMV-associated SNHL had no obvious clinical abnormality at birth, and their viral loads were lower than those of symptomatic children.

Conclusions. Congenital CMV infection is an important cause of severe SNHL, and it has an incidence comparable to that of *GJB2*-associated SNHL.

Sensorineural hearing loss (SNHL) is the most common congenital disease, with an incidence of 1–3 cases/1000 live births, or ~3 times the frequency of Down syndrome [1–4]. Universal hearing screening programs for neonates have been established in several countries and communities on the basis of research findings that early intervention in infants with hearing loss facilitates language development to a level comparable to that of their audiologically normal peers. The Joint Committee on Infant Hearing in the United States issued a statement of principles and guidelines to support the implementation of screening and intervention programs for SNHL in newborns [5].

Hereditary and environmental factors are involved in the etiology of pediatric SNHL (reviewed in [6, 7]). More than 20 genes are associated with autosomal-recessive inheritance, and 20 are associated with autosomal-dominant inheritance. Among them, mutations in the *GJB2* gene, which encodes the connexin 26 protein, represent the leading hereditary cause of hearing loss. Among environmental factors, viral infection is thought to play a major role in congenital SNHL. Successful vaccination for rubella in developed countries has virtually eliminated congenital rubella infection as a cause of SNHL; however, a decrease in the seroprevalence of cytomegalovirus (CMV), which may increase the potential of congenital CMV infection, has

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been observed in these same countries [8]. SNHL is detected at birth in 5%–10% of neonates with evidence of congenital CMV infection. In addition, longitudinal studies of infants with congenital CMV infection clearly have shown that many infants who are asymptomatic at birth and have normal hearing will develop SNHL during early childhood. Thus, universal newborn screening for hearing loss misses a number of children who will ultimately have late-onset SNHL that is linked to congenital CMV infection [9–13].

Although many studies have reported an association between CMV and hearing loss, the precise proportion of SNHL cases caused by congenital CMV infection has never been measured directly. In addition, to our knowledge, no previous study has compared the prevalences of CMV and any genetic mutations, such as in the *GJB2* gene, in a single cohort. Discrimination of congenital infection from postnatal infection requires specimens obtained immediately after birth. Blood-spot samples routinely collected for phenylketonuria assessment have value for this purpose [14]. However, the limited retention period of the materials and issues related to privacy protection [15, 16] have hampered comprehensive and retrospective studies of the infectious etiology of SNHL. Previously, we demonstrated the feasibility of retrospective diagnosis of congenital CMV infection by using dried umbilical-cord specimens [17], which are traditionally provided by Japanese obstetric hospitals to parents as a precious keepsake. In the present study, we took advantage of this tradition to perform DNA-based assessments of the proportions of congenital CMV infection and *GJB2* mutations in children with early- and late-onset SNHL.

SUBJECTS, MATERIALS, AND METHODS

Study subjects. The collection and use of human materials for the present study were approved by the Ethics Committee on Human Subjects of each institute, and informed consent was obtained from parents of all patients. We enrolled 67 children (28 boys and 39 girls) with severe SNHL who were referred to the Fukushima Rehabilitation Center for Disabled Children and the Department of Otolaryngology, Fukushima Medical University, for early speech therapy, an externally worn hearing aid, or surgical intervention, including cochlear implant. The mean ± SE of age of the patients at the time of referral was 68 ± 52 months (range, 0 months–16 years).

As a negative control, dried umbilical-cord specimens from 17 healthy children were used. As a positive control, dried umbilical cords were used from 4 infants (subjects 1, 12, 15, and 16) with symptomatic CMV infection who attended the affiliated hospitals of the Asahikawa Medical Colleges. Their clinical manifestations and laboratory findings were as follows: subject 1, porencephaly, intracranial calcifications, positive CMV culture and polymerase chain reaction (PCR) results, normal auditory brain stem response (ABR), and developmental delay; subject 12, small for gestational age, petechia, seizure, hepatosplenomegaly, thrombocytopenia, intracranial calcifications, positive CMV culture and IgM results, developmental delay, and unilateral SNHL; subject 15, small for gestational age, hepatosplenomegaly, intracranial calcifications, positive CMV IgM and antigenemia results, developmental delay, and unilateral SNHL; and subject 16, petechia, intracranial calcifications, and positive CMV IgM and antigenemia results. Because their hearing levels were evaluated at a facility different from the ones at which we enrolled children with SNHL and because any genetic tests were not allowed for them, the results from control subjects were not integrated into the analyses of the SNHL cohort.

Clinical and audiological evaluations. Birth weight, gestational age, any clinical manifestations, and abnormal laboratory findings were identified from medical records. Interviews with family member(s), audiological tests, computed tomography (CT) imaging, and a mental and physical developmental evaluation were performed, if necessary, when patients with SNHL attended our facilities.

Hearing levels of the patients were measured by at least 1 of the following objective audiological tests: ABR and/or auditory steady-state response using Audera (GSI) or Navigator Pro (Biologic). The patients also underwent at least 1 of the following subjective tests: play audiometry, conditioned orientation reflex audiometry, and/or pure-tone audiometry. These tests were performed by audiological experts and were repeated at least twice to confirm measurements. Hearing levels of the patients were classified into 5 categories on the basis of the severity of the worst ear: profound (>90 dB), severe (71–90 dB), moderate to severe (56–70 dB), moderate (41–55 dB), and mild (20–40 dB). The onset of SNHL was estimated from the medical records and the interview. Cerebral palsy, autism, and other disorders were clinically evaluated by appropriate specialists.

DNA extraction. Dried umbilical cords were cut into 5-mm squares with a disposable scalpel. Genomic DNA was isolated using the QIAamp DNA Mini Kit (Qiagen) in accordance with the manufacturer's directions, except that specimens were incubated in the presence of proteinase K overnight. From 5-mm-square specimens, 5–10 μg of DNA was recovered. DNA specimens were coded to ensure that assay operators were blinded to clinical information.

Analysis of the* GJB2 *gene. A 0.9-kb DNA fragment containing the entire coding region of *GJB2* was obtained by PCR using *Pful* polymerase (Promega). Primer sets 5′-TCTTTTCCAGAGCAAACCGC-3′ and 5′-GGGCAATGCGTTAAACTGGC-3′ or 5′-TCCAGAGCAAACCGCCCAGAGTAG-3′ and 5′-TTGCCTCATCCCTCTCATGCTGTC-3′ were used for PCR amplification. The PCR products were separated on agarose gels and purified by using a DNA extraction kit (QIAEX II; QIAGEN). The purified DNA fragments were sequenced with

the BigDye Terminator Cycle Sequencing Kit (PE Applied Biosystems).

Diagnostic PCR assays. To detect CMV DNA, a 451-bp fragment derived from the conserved region of the CMV glycoprotein H gene was amplified from the DNA specimens by a nested PCR with primer sets as follows: outer primers 5′-TCTAAACAGAATCAGCAACATCTC-3′ and 5′-CCTTGCGTGTCGTCGTATTCTA-3′ and inner primers 5′-CAAGAACTCTACCTCATGGG-3′ and 5′-ATGATGAGGCTCTGGCCTAC-3′. Cycle conditions for the first round were as follows: 1 cycle of 2 min at 95°C and 40 cycles of 20 s at 95°C, 1 min at 53°C, and 40 s at 72°C, followed by 1 cycle of 10 min at 72°C. Conditions for the second round were the same, except for 1 min at 48°C in place of 1 min at 53°C.

Real-time PCR was performed using TaqMan Universal PCR Master Mix (PE Applied Biosystems). Twenty-five microliters of reaction mixture contained 0.2–1.0 μg of umbilical-cord DNA, 1× TaqMan universal master mix, 0.2 mmol/L dNTP, 0.2 μmol/L each primer, and 0.25 μmol/L TaqMan probe. The PCR conditions consisted of 1 cycle of 2 min at 50°C and 10 min at 95°C followed by 50 cycles of 30 s at 95°C and 1 min at 60°C for human albumin and CMV UL83 genes and 1 min at 58°C for human herpesvirus 6 (HHV-6) UL67. The reaction and data analysis were performed using the ABI PRISM 7500 system. For human albumin gene, primers and a probe described elsewhere [18] were used. DNA extracted from a human B cell line was used as a standard for quantification. For CMV, primers 5′-CGCAACCTGGTGCCCATGG-3′ and 5′-CGTTTGGGTTGCGCAGCGGG-3′ [19] and the probe 5′-TTCGGCGAAGATGC-3′ were used. The entire UL83 open reading frame (ORF) of CMV strain AD169 was amplified by PCR and cloned into pcDNA3 (Invitrogen), and dilutions of the resulting plasmid were used as the quantitative standard. For HHV-6, primers and a probe were used as described elsewhere [20], except for 1 nucleotide alteration in the primer TAQ6 (AGGAT→AGAAT). pH6Z-802 [21] containing the HHV-6B U67 gene was used as the quantitative standard. Cutoff values for positive detection of CMV and HHV-6 were defined to be 5 copies/reaction on the basis of detection of standard plasmid DNA.

Determination of CMV UL144 genotypes. A 577-bp DNA fragment containing the entire UL144 ORF was obtained by a nested PCR using *Pful* polymerase. The following primer sets were used: outer primers 5′-TCTCGTATTACAAACCGCGGAGAGGATG-3′ and 5′-ACTCAGACACGGTTCCGTAAAGTGCTTC-3′ and inner primers 5′-TTCCGGTAGGAGGCATGAAG-3′ and 5′-GTGACTTCATCGTACCGTGA-3′. The first-round PCR cycle parameters were 1 cycle of 5 min at 96°C, 40 cycles of 45 s at 94°C, 45 s at 55°C, and 2 min at 72°C, followed by 1 cycle of 10 min at 72°C. The second-round PCR parameters were 1 cycle of 3 min at 94°C, 45 cycles of 45 s at 94°C, 45 s at 55°C, and 80 s at 72°C, followed by 1 cycle of 10 min at 72°C. The PCR products were purified and sequenced as described for the *GJB2* gene. The obtained sequences were analyzed using the ClustalW program (available at: http://www.ddbj.nig.ac.jp/search/clustalw-e.html).

Statistical analysis. Statistical significance was evaluated using the χ^2 test.

RESULTS

Clinical and audiological assessment of SNHL cohort. All cases of SNHL analyzed were nonsyndromic, and there were no familial cases. Sixty-three case patients had bilateral SNHL, and 4 had unilateral SNHL. Cases of bilateral SNHL were categorized into profound ($n = 36$), severe to moderately severe ($n = 19$), and moderate to mild ($n = 8$). All 4 cases of unilateral SNHL were profound or severe.

CT imaging of temporal bones identified 2 cases of Mondini malformation and 1 case of malformation of the external auditory canal and middle ear among 28 patients with SNHL for whom the test was performed. Two patients had Down syndrome, and 1 had a chromosomal abnormality. Of the 67 cases, mental retardation was diagnosed in 31, cerebral palsy in 4, and autism in 3.

Prevalence of congenital infection with CMV and HHV-6. All 4 umbilical-cord DNA specimens from infants with symptomatic CMV infection at birth, but none of 17 healthy infants were CMV positive both by the nested PCR and by real-time PCR, which confirmed the accuracy of PCR using umbilical-cord DNA. Ten (15%) of 67 patients with SNHL were CMV positive by both assays. All CMV-positive cases had unique CMV UL144 gene sequences (data not shown), indicating that the positive PCR results were not due to laboratory contamination. Medical records indicated that 2 of these CMV-positive patients had intrauterine growth retardation (IUGR). One of the patients with IUGR had thrombocytopenia, ventricular dilation, and slight intracranial calcification at birth, although there was no laboratory confirmation of congenital CMV infection. Congenital CMV infection was confirmed by laboratory tests in the other patient. In addition to the patients with IUGR, 1 patient had cerebellar dysplasia, hydrocephalus, ventricular dilation, and intracranial calcification at birth, with laboratory confirmation of CMV involvement both by PCR of blood and urine specimens and by detection of anti-CMV IgM. The remaining 7 CMV-positive patients with SNHL had no clinical manifestations at birth; therefore, no laboratory examination was conducted. HHV-6 DNA was not detected in any of the 88 umbilical-cord specimens analyzed from our collection.

Incidences of the* GJB2 *defect. Connexin 26–inactivating mutations in the *GJB2* gene were present in 16 (24%) of 67

patients with SNHL. Eleven had homozygous mutations, including a 1-bp deletion at nt 235 (235delC) ($n = 10$) and a 16-bp deletion between nt 176 and 191 (176–191del16) ($n = 1$). Five patients had compound heterozygous mutations, including 235delC plus an alteration of G→A at nt 134 (G134A) ($n = 2$), G401A ($n = 1$), or 176–191delC ($n = 1$), and 176–191delC plus a 2-bp deletion at nt 299 and 300 ($n = 1$). Most of these mutations have been reported elsewhere [22]. Nonpathological polymorphic changes, including G79A, G341A, and T608C, were detected in more than one-third of patients. All CMV-positive patients had normal *GJB2* genes.

Possible etiologies other than CMV infection and* GJB2 *defects. Other than CMV infection and *GJB2* defects, there were 5 patients with obvious genetic problems, including Down syndrome ($n = 2$), Mondini malformation ($n = 2$), and a chromosomal abnormality ($n = 1$). The group with unknown etiology included infants with extremely low birth weight (<1600 g) ($n = 4$) and those with a malformation of the external auditory canal and middle ear ($n = 1$), hyperbilirubinemia ($n = 2$), and the use of extracorporeal membrane oxygenation ($n = 2$). No cases were associated with rubella infection or meningitis. The group with unknown etiology may have contained patients with genetic mutations other than in *GJB2,* such as those in *GJB6* and in mtDNA.

Factors related to the etiology of SNHL. SNHL developed before age 2 years in all but 1 of the CMV-positive or *GJB2*-deficient patients with SNHL, whereas more than one-quarter of the patients with unknown etiology developed SNHL after age 2 years (table 1; CMV, $P < .05$). Importantly, at least 5 of the CMV-positive patients lost their hearing capability after age 6 months. Because there was some ambiguity of the onset age because of gaps in records and recollections, to verify the earlier onset of CMV- or *GJB2*-associated SNHL, we examined the ages when the patients were referred to our facilities for thorough audiological examinations and language therapy. Means and SDs of the ages at referral were 39 ± 20, 46 ± 38, and 83 ± 56 months for the CMV-positive, *GJB2*-deficient, and unknown etiology groups, respectively, which indicates that the patients with CMV- and *GJB2*-associated SNHL were referred earlier. The CMV- or *GJB2*-associated cases were characterized by more-severe hearing impairment than that in patients with unknown etiology (table 2; CMV and *GJB2,* $P < .005$).

Comparison of CMV loads. Viral loads of CMV-positive patients were measured and expressed as CMV DNA copy numbers per microgram of cellular DNA. As shown in figure 1, the patients with CMV-associated SNHL had substantially lower viral loads than the 4 positive control subjects—that is, children with typical symptomatic infection at birth. Because of the small number of CMV-positive patients, it was impossible to find any significant relationship between CMV copy numbers and either estimated onset age or degree of hearing impairment.

DISCUSSION

In the present retrospective study of the etiology of SNHL, congenital CMV infection was associated with a substantial number of severe cases of SNHL. One-fifth of cases of SNHL with onset before 2 years of age were ascribed to congenital CMV infection, a proportion similar to that attributable to hereditary mutations in *GJB2.*

Previously, population-based rates of SNHL caused by congenital CMV infection were indirectly estimated from the following 3 components: population-based rates of congenital infection, rates of SNHL in cohort studies of infants with congenital CMV infection, and population-based rates of SNHL. Because the population-based rates of SNHL caused by congenital infection and of overall SNHL were estimated to be 0.2–0.6 and 1–3 cases/1000 live births, respectively, simple algebra yields an estimate that congenital CMV infection may account for 10%–60% of cases of SNHL (reviewed in [23]). Barbi et al. [14] reported that dried blood spots were positive for 9 (10%) of 87 infants with SNHL who had hearing loss at >40 dB. This prevalence may have been an underestimate, because (1) the relatively small amount of DNA that was extracted from the blood spots and (2) testing was conducted only for children whose SNHL was diagnosed at age <2 months. By contrast, we used specimens that afforded greater analytic sensitivity and enrolled patients spanning a wide range of age, from birth to age 16 years. Therefore, our retrospective study is the first comprehensive study that substantiates the estimates based on population-based studies.

A survey conducted by the Japanese government in 2002

Table 1. Etiology of sensorineural hearing loss (SNHL) and estimated age at onset.

Estimated age at onset[a]	Congenital CMV ($n = 10$)	Genetic defects: *GJB2* mutation ($n = 16$)	Genetic defects: Others[b] ($n = 5$)	Unknown ($n = 36$)
<2 years	10	15	5	25
Birth	2	7	2	5
<6 months	3	1	0	7
6–12 months	2	2	3	3
12–24 months	3	2	0	2
Not clear[c]	0	3	0	8
After 2 years	0	1	0	11

NOTE. CMV, cytomegalovirus.

[a] Based on medical records and interviews with family members

[b] Mondini malformation ($n = 2$), Down syndrome ($n = 2$), or chromosomal abnormality ($n = 1$).

[c] Age at onset known to be <2 years but could not be specified.

Table 2. Severity of sensorineural hearing loss (SNHL) and etiology.

Etiology	Bilateral SNHL			Unilateral SNHL, profound and severe (n = 4)
	Profound (n = 36)	Severe and moderately severe (n = 19)	Moderate and mild (n = 8)	
Congenital CMV infection (n = 10)	8 (22)	1 (5)	0	1
Genetic defects				
GJB2 (n = 16)	14 (39)	2 (11)	0	0
Others (n = 5)	4 (11)	0 (0)	0	1
Unknown (n = 36)	10 (28)	16 (84)	8 (100)	2

NOTE. Data are no. (%) of subjects. CMV, cytomegalovirus.

reported that 15,200 children with severe (>70 dB) hearing loss were officially registered as "disabled." Because ~90% of them had SNHL, the incidence of severe SNHL was estimated to be 0.6 cases/1000 children <18 years old. Two-thirds of these children with SNHL had profound hearing loss (>90 dB). In the area with a population of 369,000 children from which our facilities have accepted referred cases, ~180 cases of SNHL have been officially registered. More than one-third of these were referred to us, and most of them were enrolled without any bias in the study. The enrolled cases had a spectrum of severities similar to that in Japan as a whole. Because only cases with hearing loss at >40 dB were referred, our study could not assess cases of mild SNHL. Further study is needed to evaluate congenital CMV infection in such cases.

The use of dried umbilical cords had 2 advantages: (1) Japanese tradition has made dried umbilical cords a specimen that is available for almost every person born in Japan, and (2) these are large specimens that provide much more DNA (5–10 μg) than can be recovered from conventional dried blood spots (~30 ng of DNA from a disc of 3 mm in diameter). The latter factor permitted us to perform a wider variety of tests for the patients with SNHL with a higher sensitivity than would have been possible using dried blood-spot specimens. As a result, we were able to compare the relative contributions of congenital CMV and HHV-6 infections and *GJB2* gene defects on the SNHL disease burden in the same population.

The progressive nature of congenital CMV-associated SNHL has been carefully described [11, 24, 25]. Consistent with prior descriptions, we found that all cases of CMV-associated SNHL occurred before age 2 years, but, in 5 of 10 cases, CMV-related SNHL was not detected before 6 months of age. In another study, congenital CMV infection was detected in only 1 of 20 cases of SNHL identified through newborn hearing screening, although the rate of *GJB2*-related cases (30%) was equivalent to what we observed in the present study (24%) [26]. Collectively, these findings demonstrate that the identification of infants at risk for SNHL will require a combination of universal auditory screening and newborn CMV screening. Screening for congenital CMV infection might lead to new treatment options for CMV-infected infants with antiviral agents such as ganciclovir [27]. Importantly, CMV loads in patients with delayed-onset SNHL tended to be lower than those in positive control subjects (i.e., 4 children with symptomatic infection at birth). Although further study with a bigger sample size is required to confirm this finding, our finding is consistent with that of a previous report that demonstrated higher CMV loads in infants with symptomatic infection [28]. Thus, a sensitive assay for CMV detection is required for newborn CMV screening to identify asymptomatic patients who might develop SNHL later on. From this point of view, blood specimens may not be the best choice of specimens for the screening, because viral loads in blood specimens are >2 logs lower than those in urine specimens [29] (N.I. and S.K., data not shown). Because the use of dried umbilical-cord specimens is not practical for screening,

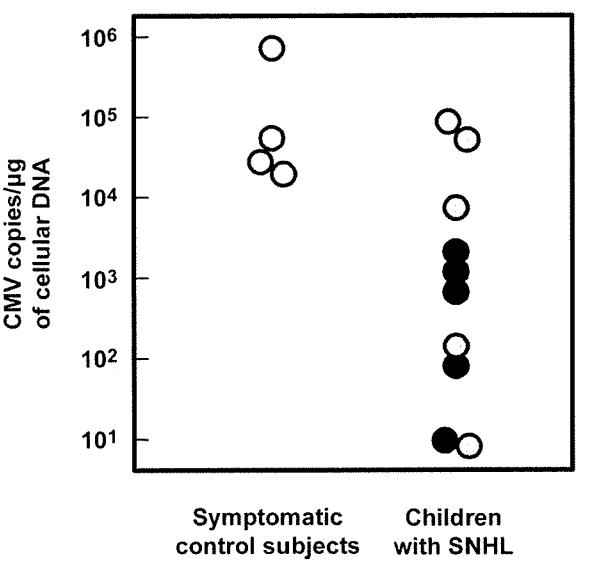

Figure 1. Comparison of cytomegalovirus (CMV) loads. White circles indicate the positive control children with symptomatic CMV infection at birth. Shaded and black circles indicate the CMV-positive children identified in the sensorineural hearing loss (SNHL) cohort with their estimated onset before and after the age of 6 months, respectively.

the development of rapid and convenient methods for the detection of CMV in urine specimens would be essential.

It is noteworthy that congenital CMV infection and *GJB2* mutations appear to be independently associated with SNHL. However, because CMV infection is known to increase the risk of chromosomal abnormality (reviewed in [30]), this does not exclude possible association between CMV infection and mutations in genes other than *GJB2*.

Like CMV, HHV-6 belongs to the betaherpesvirus subfamily, and the viruses have a similar genomic structure. A recent study demonstrated asymptomatic congenital infection with HHV-6 but not HHV-7 at a frequency of 1% [31]. In addition, it is well known that HHV-6 is neurotropic and that it causes neurological diseases [32]. Therefore, it would be interesting to determine whether HHV-6 causes SNHL, as CMV does. However, we found no evidence for congenital HHV-6 infection in any of the patients with SNHL. There are 2 possible explanations: congenital HHV-6 infection rarely causes SNHL and/or there is a lower frequency of congenital HHV-6 infection in our general population that hampered our attempts to identify HHV-6–associated SNHL in our sample size. Because HHV-6 DNA can be detected in maternal blood, our negative data on HHV-6 suggest the specificity of our assay using dried umbilical cords.

In conclusion, our retrospective analysis of the etiology of SNHL demonstrates directly that congenital CMV infection is responsible for a substantial proportion of early-childhood SNHL and that almost half of the infants at risk for the development of late-onset CMV- or *GJB2*-associated SNHL show no clinical and audiological indications at birth. Our results support the results of previous studies that advocated the need for neonatal screening programs for both CMV and the prevalent genetic causes of SNHL.

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Human Herpesvirus 8 Genoprevalence in Populations at Disparate Risks of Kaposi's Sarcoma

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The prevalence of human herpesvirus 8 (HHV-8) in populations at different risks of developing Kaposi's sarcoma (KS) was assessed using a protocol involving immunomagnetic fractionation of CD45+ blood cells prior to detection of the HHV-8 genome by nested PCR. Preliminary studies using blood of eight gay men infected by human immunodeficiency virus-1 (HIV-1) revealed that, for the detection of HHV-8 DNA derived from open reading frame (ORF) 26 of the HHV-8 genome, this protocol provided substantially higher rates (100%) compared to one involving red blood cell (RBC) lysis (0%) and to another requiring double density gradient centrifugation (DDGC) of leukocytes (13%). When the CD45+ fractionation protocol was applied to samples from 103 other HIV-1-infected patients (the vast majority of whom were gay men) and 100 blood donors, the ORF 26 DNA detection rates obtained were 37% and 8%, respectively. When DNA from the variable region 1 of ORF K1 was additionally amplified from samples of the blood donors, a detection rate of 9% was achieved. This rate was highly concordant with the ORF 26 DNA detection rate. Furthermore, the ORF K1 sequences were predominantly unique, assignable to genotypes A1, A4, and C3. When assays for anti-HHV-8 and anti-herpes simplex viruses (HSV) 1 and 2 were applied, significant concordances between HHV-8 DNA detection rates and those relating to anti-HHV-8 and to anti-HSV 1 and 2 were more frequently observed for HIV-1-infected patients than for blood donors. The higher-than-expected HHV-8 genoprevalence rate in blood donors requires further confirmation in view of its implications for post-transfusion HHV-8 transmission. ***J. Med. Virol. 79:52–59, 2007.*** © 2006 Wiley-Liss, Inc.

KEY WORDS: human herpesvirus 8; blood transfusion; human immunodeficiency virus; Kaposi's sarcoma; AIDS

INTRODUCTION

The epidemiology of human herpesvirus 8 (HHV-8) has been much clarified by the availability and wide application of serological tests for estimating the prevalence of infection [Chatlynne and Ablashi, 1999; Martin, 2003]. More accurate estimations of HHV-8 prevalences may be made when seroprevalence data are complemented by data generated from the detection of the HHV-8 genome. When the HHV-8 genome is examined, an improvement in specificity would be expected, since by identifying the genome rather than the antibody a more direct marker of HHV-8 infection is sought. Better sensitivity may also be achieved, as infection can be identified in the early stages before the host mounts a detectable humoral response [Cook et al., 2002].

In peripheral blood, β- and γ-herpesviruses can be present in quantities below the threshold of detection, reflecting viral persistence in the latent state. Innovative approaches have been devised to identify and characterize the viremic state of infected hosts. Thus, for human cytomegalovirus (HCMV), peripheral blood mononuclear cells (PBMCs) of blood donors were

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stimulated allogeneically by blood of other donors to reveal evidence of genome carriage and transcription [Soderberg-Naucler et al., 1997]. Four hundred fifty milliliters of blood from each of the subjects was collected for Epstein-Barr virus to determine the frequency of the viral genome in B lymphocytes of healthy seropositive individuals [Wagner et al., 1992]. For HHV-8, PBMCs of donors were cultured in phytohemagglutinin and then enriched for CD19+ cells enabling subsequent HHV-8 DNA detection [Blackbourn et al., 1997]. An investigation is described of HHV-8 genome detection in blood of two groups of people representing disparate risks of HHV-8 infection and of developing Kaposi's sarcoma (KS)–patients infected by human immunodeficiency virus type 1 (HIV-1) and HIV-negative blood donors.

MATERIALS AND METHODS

Enrichment of Blood Cell Subsets

Between 8 and 10 ml of peripheral blood from study individuals was collected in edetic acid-treated vacutainers. To 1 ml of the sample, immunomagnetic cell separation (ImCS) of the pan-leukocyte CD45+ fraction was carried out using Dynabeads M-450 coated with monoclonal antibodies that specifically bind to the CD45 antigen (Dynal AS, Oslo, Norway). The cells were thereafter rosetted. The same approach was applied to facilitate ImCS of CD31+, CD19+, CD14+, and CD2+ fractions (to isolate, respectively, endothelial cells, B lymphocytes, monocytes, and T lymphocytes) for a subset of samples collected. To another 1 ml of the sample a red blood cell (RBC) lysis protocol was applied using the Amplicor HIV-1 Monitor Lysis Reagent (Roche Diagnostics, Basel, Switzerland). A further 3 ml of the sample was submitted to double density gradient centrifugation (DDGC) [English & Anderson, 1974]. The double gradient was formed by layering 1.5 ml of HISTOPAQUE 1077 over an equal volume of HISTOPAQUE 119, after which blood was layered. After low-speed centrifugation, the plasma fraction was aspirated, followed by the mononuclear/platelet layer, and the layer between the 1077/1119 interphase which represents cells of the granulocyte series. Cells derived following the application of each of these three approaches were reconstituted in 250 μl of nuclease-free water, aliquotted, and stored at −70°C until processed further.

HHV-8 Genome Detection

DNA was extracted from samples using the Geneclean III kit (BIO 101, La Jolla, CA). The presence of DNA in each extract was verified by amplifying a fragment of the β-globin gene as previously described [Saiki et al., 1985]. A nested PCR to amplify a 171-base pair (bp) fragment within the KS330 segment of open reading frame (ORF) 26 of the HHV-8 genome, hereafter referred to as KS330 [Chang et al., 1994], was undertaken using, as outer primers 5′AGCCGAAAGGATTCCACCAT3′ (sense) and 5′TCCGTGTTGTCTACGTCCAG3′ (anti-sense); and as inner primers 5′TTCCACCATTGTGCTCGAAT3′ (sense) and 5′TACGTCCAGACGATATGTGC3′ (anti-sense) [Di Alberti et al., 1997]. The PCR was undertaken in a 25 μl reaction mixture containing nuclease-free water, 1.5 mM $MgCl_2$, 10 nM each of dNTP, 1 unit Taq DNA polymerase (Invitrogen; Paisley, UK), 20 pmol of each pair of primers, and 2.5 μl of the extract. Nested PCR amplification for the 213-bp segment from variable region 1 of ORF K1, hereafter referred to as K1/V1, was done using, as primers 5′CCCTGGAGTGATTTCAACGC3′ (sense) and 5′ACATGCTGACCACAAGTGAC3′ (anti-sense); and as inner primers 5′GAGTGATTTAACGCCTTAC3′ (sense) and 5′TGCTGACCACAAGTGACTGT3′ (anti-sense) [Zong et al., 1999]. This PCR was undertaken in a 50 μl reaction mixture containing nuclease-free water, Buffer 8 (Stratagene, Amsterdam, The Netherland), 3.2 mM $MgCl_2$, 10 nM each of dNTP, 1 unit Taq DNA polymerase, 20 pmol of each pair of primers, and 2 μl of the first-round PCR product. Nested PCR is capable of amplifying, in a reaction tube, as little as 5 zg of HHV-8 subgenomic DNA (equivalent to one copy of the target sequence) [Di Alberti et al., 1997].

The sensitivity of PCR presents problems with DNA contamination, which generates potentially false positive results. In view of this, strict adherence was applied to adopt laboratory practices that minimize contamination, and both positive and negative controls were used for every PCR run [Kwok and Higuchi, 1989]. The positive control was obtained from a diluted BCBL-1 cell line, kindly provided by Dr. E. Cesarman (Cornell University, New York). PCR detection of HHV-8 DNA was repeated at least twice from each sample extract.

Amplification of both regions was carried out under the same PCR thermocycling conditions. Samples were initially heated for 94°C for 5 min, followed by 35 cycles of 94°C for 1 min (denaturation), 60°C for 1 min (annealing), 72°C for 1 min (extension), and finally 5 min at 72°C.

Sequencing

All the purified PCR products underwent sequencing of both strands of the DNA product. Cycle sequencing was applied, using the PRISM™ Ready Reaction DyeDeoxy™ Terminator Cycle Sequencing Kit with AmpliTaq™ FS DNA polymerase (Perkin Elmer, Forest City, CA). Sequence alignments were generated using the CLUSTAL X, BIOEDIT, and PHYLIP programs.

Anti-HHV-8 and Anti-HSV-1 and -2 IgG Assays

Two immunofluorescence assays (IFAs) were applied. The first, IFA^{KS-1}, used a mouse monoclonal antibody-enhanced indirect assay based on the KS-1 cell line [Said et al., 1996] treated with 12-O-tetradecanoyl-phorbol-13-acetate for the expression of lytic antigens, produced by Biotechnologies incorporated for research use. The second, $IFA^{SFV/K8.1}$, is an indirect assay utilizing BHK cells infected with recombinant Semliki Forest virus expressing the HHV-8 ORF K8.1 protein [Inoue et al., 2000]. The anti-HHV-8 detected by the two IFAs are

correspondingly designated anti-HHV-8^{KS-1} and anti-HHV-$8^{SFV/K8.1}$. For both IFAs, only samples that scored 3+ reactivities or greater (within a visual scale of 0–4+) were considered positive. For anti-herpes simplex virus-1 (HSV-1) and anti-HSV-2-testing, enzyme-linked immunoassays (EIAs) based on the blocking of type-specific epitopes by test sera [Slomka et al., 1995] were applied.

Statistics

The strength of associations between pairs of variables was determined using logistic regression and presented as odds ratios (ORs) with 95% confidence intervals (CIs). Differences in proportions of variables in related samples were tested using McNemar's test, while differences in proportions of variables in independent samples were tested using the Chi-squared test with a continuity correction.

STUDY PATIENTS

Samples from a total of 111 HIV-1-seropositive patients who attended the outpatient clinic of the Department of Genitourinary Medicine at Charing Cross Hospital, London in 1996 were assembled. Thirty-three of these patients had participated in a study examining the impact of HIV protease inhibitors on HHV-8 replication [Leao et al., 2000]. Informed consent and ethical committee approval were obtained prior to the study. The group consisted, in total, of 109 males and 2 females, with the date of the initial HIV antibody positive test ranging from 2 months to 12 years prior to testing for HHV-8. Risk factors identified for their HIV infection were: gay men (108 patients), injecting drug use (1 patient) and being of African origin (2 patients).

A hundred blood donors were recruited in 2001 at a blood donation center in North London. Only donors screened free of markers of infection by HIV-1 and -2, hepatitis B virus (HBV), hepatitis C virus (HCV), and *Treponema pallidum* were included.

RESULTS

Evaluation of Approaches to the Fractionation of HHV-8-Carrying Blood Cells

In this phase of the study, blood from the first eight HIV-1-seropositive patients who agreed to participate in the study was examined to compare sample-processing protocols. The patients (Patients a–h) were all gay men (Table I). Peripheral blood samples from three patients (Patients a–c) were split and processed by four different methods to isolate white blood cells: RBC lysis, DDGC to fractionate monocytes, and granulocytes, ImCS to fractionate CD45+ cells, and DDGC followed by ImCS (Fig. 1A). For each of these patients, RBC lysis did not permit any successful PCR. Following DDGC, the monocyte fraction of one of the patients (Patient c) was positive but only after nested PCR (Table IIA). ImCS consistently provided a 100% detection rate, with samples from all three patients being positive after nested PCR. Substantially better detection rates were obtained for samples subjected to DDGC followed by ImCS than for those subjected to DDGC only (Table IIA). In five patients (Patients d–h) whose blood samples were processed by three rather than four methods (Fig 1B), ImCS by contrast to RBC lysis and DDGC resulted in successful PCRs: HHV-8 DNA could be detected by nested PCR in CD45+ cells of all the samples; and for one sample (from Patient f), HHV-8 DNA could be amplifiable by first-round PCR. Peripheral blood samples collected from five other HIV-1-infected gay men with no clinical KS (Table I, Patients i–m) were used to evaluate the sub-cellular distribution of HHV-8 DNA detection following ImCS. This was as follows (in descending order): CD45+ cells (4/5 samples positive); CD31+ cells (1/5); and CD19+, CD14+, and CD2+ cells (0/5) (Table IIB).

HHV-8 Genome Detection in HIV-Infected Patients

An additional 103 anti-HIV-1-seropositive patients (Table III) were investigated. Of these, 100 were gay

TABLE I. Characteristics of HIV-1-Seropositive Patients Who Participated in the Evaluative Phase of the Study

Patient	Age (year)	Presence of KS	CD4 count (cells/μl)	Antiretroviral therapy
a	39	No	420	None
b	49	Yes	24	Zalcitabine, saquinavir, lamivudine
c	26	Yes	96	Stavudine, lamivudine
d	54	No	140	Zidovudine, zalcitabine, saquinavir
e	29	No	255	None
f	54	No	40	Zidovidine, lamivudine, indinavir
g	25	No	477	None
h	41	Yes	437	Zidovudine, zalcitabine, saquinavir
i	58	No	72	Zidovudine, zalcitabine, indinavir
j	39	No	240	Zidovudine, zalcitabine, indinavir
k	37	No	192	None
l	39	No	353	None
m	30	No	448	None

KS, Kaposi's Sarcoma.