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Mini-review

# Human herpesvirus 6 (HHV-6)

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## 1. Introduction

Human herpesvirus 6 (HHV-6) was discovered in 1986 in the laboratory of Robert Gallo, at the National Cancer Institute in Bethesda (Salahuddin et al., 1986). The first viral isolates were derived from cultured leukocytes of immunocompromised patients with various forms of lymphoproliferative disorders. At that time, five human herpesviruses were known: herpes simplex virus type 1 and 2 (HSV- or HHV-1 and 2), varicella-zoster virus (VZV or HHV-3), Epstein–Barr virus (EBV or HHV-4) and human cytomegalovirus (hCMV or HHV-5). The discovery of HHV-6, which shortly thereafter was characterized as the first T-lymphotropic human herpesvirus (Lusso et al., 1988), stimulated new interest in the field. Four years later, using culture conditions similar to those established for the isolation of HHV-6, a second human herpesvirus with a predominant T-lymphocyte tropism, HHV-7, was identified (Frenkel et al., 1990b; Berneman et al., 1992). More recently, the human Herpesviridae family has acquired an additional member, designated HHV-8 or, provisionally, Kaposi's sarcoma herpesvirus (KSHV), for its suggested association with this neoplasia (Chang et al., 1994). Preliminary evidence suggests that, among blood cells, HHV-8 preferentially infects B-lymphocytes (Ambroziak et al., 1995).

HHV-6 and HHV-7 share close genetic, biological and immunological relatedness and differ from all the other known human herpesviruses for the combination of two peculiar features: their primary T-cell tropism (Lusso et al., 1988; Takahashi et al., 1989; Frenkel et al., 1990b; Berneman et al., 1992) and their inability to directly induce cellular transformation in vitro (Salahuddin et al., 1986; Frenkel et al., 1990b). These unique characteristics may eventually lead to the classification of HHV-6 and HHV-7 into a novel subfamily among the Herpesviridae. At present, the two agents have been classified as β-herpesviruses on the basis of their genetic homology with hCMV, in spite of the fact that their lymphotropism is a typical feature of the γ-herpesviruses.

This review is focused on the biological and medical importance of HHV-6. The history of this

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virus is peculiar and has been animated by heated controversies. Salahuddin et al. obtained the first viral isolates while attempting to identify a causative agent of the B-cell lymphomas that frequently occur in patients with acquired immunodeficiency syndrome (AIDS). After the discovery of HHV-6, however, no conclusive evidence for its involvement in lymphoproliferative disorders has been provided and, for a long period, HHV-6 has remained 'a virus in search of a disease'. There have been controversies about the pathological role of HHV-6, related at least in part to the confusion about the appropriate methods for the clinical diagnosis of HHV-6 infection. Even today, there is still some resistance to accept an etiologic role of HHV-6. The cellular tropism of HHV-6 has also been a controversial issue. Initially, HHV-6 was thought to infect preferentially B-lymphoid cells; hence, the original designation of human B-lymphotropic virus (HBLV). With the improvement of the virus propagation systems in vitro, it was soon realized that the primary target cell of HHV-6 is the T-lymphocyte (Lusso et al., 1988), whereas B-cells can be infected only when immortalized by EBV (Ablashi et al., 1988; Cuomo et al., 1995). Another long-debated question has been the classification of the different viral isolates: with the characterization of an increasing number of viral strains collected throughout the world, it has become evident that heterogeneity exists and that the different isolates segregate into two well-defined subgroups, referred to as HHV-6 A and B (Ablashi et al., 1991; Aubin et al., 1991; Schirmer et al., 1991), leading some authors to even propose their classification as different viruses. Last but not least, there is still uncertainty about the efficacy of antiviral compounds against HHV-6. Along with all the controversies, however, HHV-6 has always generated a remarkable interest, undoubtedly stimulated by the recognition of its unique interactions with the cells of the immune system and, more recently, by the accumulating evidence for its role as an important human pathogen, particularly in immunocompromised individuals. It was this continuous interest that led to dramatic progress, in a relatively short period of time, in our knowledge of the genetic, biological and epidemiological features of HHV-6.

## 2. Structure and genetic characteristics

On a morphological basis, HHV-6 is a typical herpesvirus, indistinguishable from the other members of the family (Biberfeld et al., 1987). The diameter of the mature particle is 200 nm. Its structure includes an internal core formed by the capsid, consisting of 162 capsomers arranged according to the typical icosadeltahedral symmetry, which enclose a double-stranded DNA genome of approximately 160 000 base pairs (bp). The nucleocapsid is surrounded by an amorphous tegument and, externally, by a lipid-containing envelope with spikes projecting from its surface (Fig. 1). HHV-6 replicates by a rolling circle mechanism, with the formation of head-to-tail concatamers in infected nuclei (Martin et al., 1991b). A lytic-phase origin of DNA replication has been identified upstream from the major DNA-binding-protein gene (Dewhurst et al., 1993a, 1994).

The genome of HHV-6 is large and complex, containing more than 100 genes. The complete nucleotide sequence of strain U1102 (Downing et al., 1987), which is closely related to the GS prototype (Salahuddin et al., 1986), has been recently obtained (Gompels et al., 1995). The overall content of G + C is 43%, one of the lowest among the Herpesviridae. HHV-6 shares with HHV-7 the highest degree of nucleotide sequence homology, while both HHV-6 and HHV-7 are more distantly related to hCMV (Efstathiou et al., 1988; Frenkel et al., 1990b; Lawrence et al., 1990; Neipel et al., 1991; Berneman et al., 1992; Gompels et al., 1995). The genome of HHV-6, HHV-7

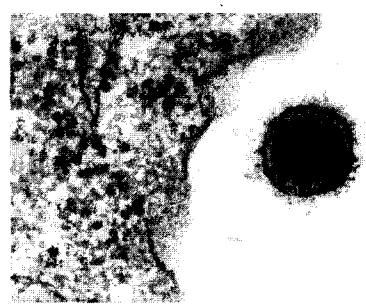

Fig. 1. Electron micrograph of a mature HHV-6 virion.

and hCMV are roughly colinear. More than 65% of the HHV-6 genes have a recognized counterpart in hCMV, as determined either by sequence similarity or by positional homology (Gompels et al., 1995). The genome of HHV-6 consists of a single long unique (UL) element of approximately 140 000 bp, rich in A + T, flanked by two identical direct repeat (DR) segments, rich in G + C, of approximately 8000 bp each. The direct repeats, which contain essential elements for the viral DNA packaging and replication, as well as, possibly, for the maintenance of the latency state (Lindquester and Pellet, 1991; Martin et al., 1991b; Thomson et al., 1994a; Gompels and Macaulay, 1995), include a tandem repetitive sequence (GGGTTA) that is also present in the genome of the Marek's disease virus (Kishi et al., 1988) and in the human telomers. Restriction endonuclease polymorphism has been documented among different HHV-6 isolates (Josephs et al., 1988a; Jarrett et al., 1989; Kikuta et al., 1989); as discussed below, two major viral subspecies (A and B) have been defined. The overall genetic divergence between the two variants is approximately 5%, ranging from 2 to 25% according to the loci analysed (Aubin et al., 1991; Gompels et al., 1993, 1995).

The genome of HHV-6, like that of other herpesviruses, has coding regions on both DNA strands and contains only limited non-coding segments. Only minimal splicing occurs after transcription. The analysis of the complete nucleotide sequence of HHV-6 (Gompels et al., 1995) has led to the identification of five major families of genes (i.e. hCMV US22 homologues, DR1/6, U4/5, G-protein coupled receptor-like and immunoglobulin-like families). Several genes of HHV-6 have been characterized, mostly based on genetic homology or colinearity with other human herpesviruses, particularly with hCMV. A conserved HHV-6 homologue has been found for all seven genes believed to be necessary and sufficient for the replication of HSV-1, i.e. the transactivator U27, the DNA polymerase (U38), a single-stranded DNA-binding protein (U4l), the helicase/primase complex (U43, U74, U77) and the origin binding protein (U73). Additional genes which are critical for the replication of hCMV also have homologues in HHV-6 (Gompels et al., 1995). The major HHV-6 genes hitherto characterized include putative immediate early (IE) genes (Martin et al., 1991a; Nicholas and Martin, 1994; Chou and Marousek, 1994; Schiewe et al., 1994; Yamamoto et al., 1994) which are now classified into two distinct regions, IE-A and IE-B (Gompels et al., 1995), a candidate IE enhancer and genes homologous to the US22 family of hCMV (Thomson and Honess, 1992; Efstathiou et al., 1992), the DNA polymerase gene (Teo et al., 1991), the uracyl-DNA glycosylase gene (Sato et al., 1994), and genes coding for a nuclear phosphoprotein (Chang and Balachandran, 1991), a major immunogenic 100-kD structural protein (Neipel et al., 1992; Pellett et al., 1993), the major capsid protein (Littler et al., 1990), a transport/capsid assembly protein (Jones and Teo, 1993), the large tegument protein and glycoprotein H (Josephs et al., 1991), glycoprotein B (Chou and Marousek, 1992), glycoprotein M (Lawrence et al., 1995) and a gp82–gpl05 glycoprotein complex (Pfeiffer et al., 1993, 1995). Interestingly, HHV-6 possesses a unique gene with a striking homology to the *rep* gene of adeno-associated virus type 2 (AAV-2), a defective parvovirus dependent on adenovirus for its replication (Thomson et al., 1991). Although the possible role of this gene in the virus life cycle is presently unknown, its presence in the HHV-6 genome is intriguing because the Rep protein of AAV-2 has a transregulatory activity and inhibits cellular transformation by papillomaviruses. The *rep*-like gene product of HHV-6 is functional, as it was recently shown to mediate AAV-2 DNA replication (Thomson et al., 1994a), to block cellular tranformation by H-*ras* (Araujo et al., 1995) and to either increase (Thomson et al., 1994b) or suppress (Araujo et al., 1995) transcription driven by the long terminal repeat (LTR) of HIV-1. Another gene that is unique to HHV-6, U83, presents structural features typical of the C–C chemokine subfamily. It is noteworthy that HHV-6, like hCMV, lacks a thymidine kinase, but possesses a gene (U69) homologous to the ganciclovir kinase of hCMV (Gompels et al., 1995).

Strictly connected with the study of the HHV-6 genome has been the attempt to identify viral gene

products. Using specific polyclonal or monoclonal antisera, Balachandran et al. (1989) identified more than 30 immunoreactive polypeptides in HHV-6-infected cells, ranging from 31 to 180 kD. None of these proteins was present in uninfected homologous cells. In a subsequent study, more than 20 infection-specific polypeptides were identified using human sera containing antibodies to HHV-6 (Balachandran et al., 1991). Similarly, Yamamoto et al. (1990) detected more than 20 different polypeptides in purified HHV-6 virions, including a major immunogenic protein of 101 Kd. Eizuru and Minamishima (1992) reported evidence for IE proteins, detectable as early as 3 h after infection in the nucleus of infected cells. Bapat et al. (1989) and Williams et al. (1989) demonstrated a unique DNA polymerase activity present in HHV-6-infected cells. Agulnick et al. (1993) identified a DNA-binding protein (p41) homologous to the hCMV UL44 gene product which serves as a polymerase-associated stimulatory factor. A DNase activity, but no thymidine kinase, uracil-DNA glycosylase or deoxyuridine triphosphate nucleotide hydrolase were detected in infected cells (Williams et al., 1989). Antisera generated against expressed portions of the gene encoding the major viral capsid protein were found to recognize a 135-Kd protein present in infected cells (Littler et al., 1990). Antibodies directed against glycoprotein B, glycoprotein H and the gp82–gp105 complex were shown to neutralize the viral infectivity (Okuno et al., 1990; Foà-Tomasi et al., 1991; Chang and Balachandran, 1991; Ellinger et al., 1993; Liu et al., 1993a,b).

Table 1
Biological features of the two major HHV-6 subspecies

| | A (GS-like) | B (Z29-like) |
|---|---|---|
| Cellular tropism | | |
| Primary cells | | |
| CD4+ T-cells | + | + |
| CD8+ T-cells | + | +/− |
| NK-cells | + | − |
| $\gamma\delta$ T-cells | + | − |
| Cell lines | | |
| HSB-2 | + | − |
| Molt-3 | +/− | + |
| Sup-T1 | + | + |
| Jurkat | + | + |
| EBV+LCL | + | − |
| Cytopathic effect | + | + |
| Cell immortalization in vitro | − | − |
| Downregulation of CD3 | + | + |
| Upregulation of CD4 (CD4+$^{low}$ cells) | + | + |
| Induction of CD4 (CD4− cells) | + | (?) |
| HIV-LTR transactivation | + | + |

## 3. Viral subgroups or 'variants'

As mentioned earlier, two major subgroups (A and B) of HHV-6 have been identified (Ablashi et al., 1991; Aubin et al., 1991; Schirmer et al., 1991). The two subgroups, also referred to as 'variants', differ genetically (restriction endonuclease pattern), immunologically (monoclonal antibody reactivity) and biologically (cellular tropism) (Table 1). Evidence, albeit mostly anecdotal, suggests that HHV-6 A and B have a different epidemiological distribution in the human population (B more prevalent than A) and, possibly, different disease associations. The most rapid and efficient methods currently available for subgroup identification are based either on the reactivity of specific monoclonal antibodies with productively infected cells (in vitro or in vivo), or on the restriction patterns of specific regions of the viral genome. After amplification by the polymerase chain reaction (PCR) or by virus isolation, a consistent pattern is observed for each of the two subgroups upon digestion with specific restriction enzymes (e.g., *Bgl*II, *Hind*III, *Taq*1). Endonuclease restriction analysis has permitted to

define at least one further subdivision within subgroup B (B1 and B2) (Di Luca et al., 1992).

The prototype of HHV-6 A is strain GS, originally isolated from a young black male with acute T-cell lymphoblastic leukemia (Salahuddin et al., 1986). The prevalence of HHV-6 A in the general population and the time of primary infection are still undefined. Albeit less frequently detected and isolated than subgroup B, HHV-6 A has been thoroughly characterized biologically. To date, no definitive link of this subgroup with human disease has been established, but the fact that A-type isolates were derived mostly from immunocompromised patients has led many investigators to suggest a possible role in immunodeficiency conditions, including AIDS. This concept is strengthened by the broad 'immunotropism' demonstrated by these isolates in vitro (see below).

HHV-6 subgroup B is prototyped by strain Z29 which was first isolated at the Centers for Disease Control (CDC) in Atlanta from a patient with AIDS (Lopez et al., 1988). It is responsible for most of the cases of primary infection documented in early childhood in the United States (Dewhurst et al., 1993b) and has been etiologically associated with exanthema subitum (Yamanishi et al., 1988), a usually benign febrile disorder, also referred to as roseola infantum or sixth disease. The prevalence of type-B isolates in the general population in Western countries is almost universal. The main biological features of the two major viral subgroups will be analyzed in detail in the next section.

## 4. Biological features

Similar to other herpesviruses, HHV-6 is believed to establish latent infection in vivo and thereby to persist in the host indefinitely after primary infection. No well-characterized in vitro models of latent infection are currently available, but persistent non-productive infection has been documented in cultured macrophages (Kondo et al., 1991). Although circulating monocytes and epithelial cells of the bronchial and salivary glands have been suggested as possible reservoirs (Kondo et al., 1991; Krueger et al., 1990), there is still uncertainty regarding the exact sites of viral persistence and latency in vivo.

In vitro, HHV-6 behaves as a cytopathic virus. Exposure of primary human mononuclear cells, previously activated in vitro, to HHV-6 results in the appearance, after a period of 3–7 days, of peculiar cytomorphological changes. The cells enlarge, lose their structured, blastic shape and eventually become evenly rounded and refractile. These large cells survive in culture for 3–4 days, while increasing amounts of infectious virus particles are released into the culture supernatant. Unlike EBV and the monkey T-lymphotropic viruses (e.g., *Herpesvirus saimiri* and *Herpesvirus ateles*), HHV-6 does not directly cause immortalization of its target cells, at least in vitro. HHV-6 is a broadly 'immunotropic' herpesvirus that may affect, directly or indirectly, both the cellular and humoral arms of the immune system. There is now consensus that HHV-6 has a primary tropism for CD4+ T-lymphocytes, both in vitro (Lusso et al., 1988) and in vivo (Takahashi et al., 1989). When activated mononuclear cells of different tissue origin (e.g., peripheral blood, umbilical cord blood, thymus, lymph node, tonsil, bone marrow) are exposed to HHV-6, the vast majority of the infected cells display the phenotype of activated CD4+ T cells (CD2+ CD4+ CD5+ CD7+ CD26+ CD38+ CD71+). Although both major viral subgroups (A and B) infect preferentially CD4+ T-cells, some important differences exist in their cellular tropism. For example, they differ in the capacity to infect established human T-cell lines and B-lymphoblastoid cell lines (LCL) (Table 1). Moreover, HHV-6 A has the unique ability to infect productively and cytopathically several types of cytotoxic effector cells, such as CD8+ T-lymphocytes (Lusso et al., 1991a,b), natural killer (NK) cells (Lusso et al., 1993) and $\gamma\delta$ T-lymphocytes (Lusso et al., 1995). Because these cells are involved in the mechanisms of antiviral defense in vivo, HHV-6 A may exploit this strategy to counteract the protective immune surveillance of the host and thereby establish persistent infection. In contrast, HHV-6 B seems to be unable to attack efficiently cytotoxic effector cells (Lusso, P., unpublished).

Besides T- and NK-lymphocytes, HHV-6 can infect other types of immune cells, like B-lymphoid cells and mononuclear phagocytes, as

well as non-immune cells, like fibroblasts and cells of neural, muscular and epithelial origin (Fig. 2). Nevertheless, the full lytic viral cycle appears to be efficiently completed only in lymphoid cells. The biological basis for this restriction is presently unknown. It has been shown that B-lymphocytes become susceptible to HHV-6 infection only after their immortalization with EBV (Ablashi et al., 1988; Cuomo et al., 1995), suggesting that EBV infection might induce the expression of a membrane receptor for HHV-6. Other types of interaction between HHV-6 and EBV have also been reported (Flamand et al., 1993). Cells of the mononuclear phagocytic system, such as terminally differentiated macrophages, can be infected and killed by HHV-6, but the infection is typically non-productive and the cytopathic effect (CPE) is likely to be indirect (Kondo et al., 1991; Carrigan, 1992).

Little information is currently available on the surface membrane receptor(s) of HHV-6. The fact that activation signals are necessary to induce susceptibility to HHV-6 infection in T-cells (Lusso et al., 1989b; Frenkel et al., 1990a) indicates that at least one component of the receptor complex is a T-cell activation antigen. The differences in cellular tropism between A- and B-type strains suggest that the composition of the receptor complex may be different for the two viral subgroups. Evidence has been reported that the receptor for HHV-6 is not the CD4 glycoprotein (Lusso et al., 1989b) which serves as the major receptor for HIV (Dalgleish et al., 1984; Klatzmann et al., 1984) and for HHV-7 (Lusso et al., 1994b); in

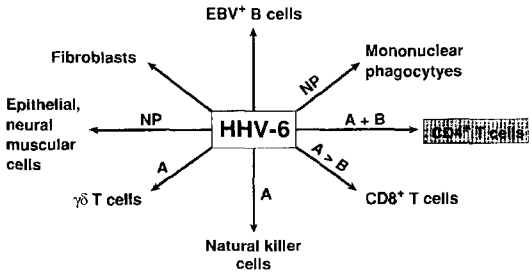


Fig. 2. Cellular tropism of HHV-6. A and B denote the two major HHV-6 subgroups. NP = non-productive.

fact, HHV-7 and HIV-1 compete for CD4 occupancy and reciprocally interfere both in CD4+ T-cells (Lusso et al., 1994b) and in mononuclear phagocytes (Crowley et al., 1996).

Diverse interactions have been demonstrated between HHV-6 and its host cells. Evidence is emerging that HHV-6 infection induces either activation or suppression of the expression of cellular genes. It has been reported that terminally infected T-cells fail to express either CD3 or the T-cell receptor $\alpha\beta$ heterodimer on their surface membrane (Lusso et al., 1988, 1991b). This phenomenon, which occurs with both A and B strains (P. Lusso et al., unpublished), is related to the ability of HHV-6 to transcriptionally downregulate, by yet unknown mechanisms, the expression of CD3 (Lusso et al., 1991b). Because the CD3/TCR complex plays a critical role in the processes of T-cell activation, downregulation of CD3 may have an immunosuppressive effect. Another unusual phenotypic feature documented in HHV-6-infected T-lymphocytes is that a variable proportion of them coexpresses the glycoproteins CD4 and CD8 on their surface membrane, a feature typical of the early T-cell development within the thymus (Lusso et al., 1988). This phenomenon is related to the unique ability of HHV-6 to transcriptionally activate the expression of CD4 in cells that physiologically do not express it, such as mature CD8+ T-cells, NK-cells and $\gamma\delta$ T-cells. Activation of CD4 is mediated by immediate early gene products of HHV-6 (Lusso et al., 1991a) and seems to involve activation of the CD4 promoter (L. Flamand et al., in preparation). Due to the inefficient growth of subgroup B-isolates in CD4− cells, such as cytotoxic effectors, de novo induction of CD4 has been documented exclusively with HHV-6 A. Nonetheless, increased levels of CD4 expression were observed after infection with different HHV-6 B strains in Jurkat, a CD4+$^{low}$ neoplastic T-cell line (P. Lusso et al., unpublished). Among the other virus–host interactions hitherto documented, HHV-6 infection has been shown to induce the release of inflammatory cytokines, such as interferon-$\alpha$(Kikuta et al., 1990), tumor necrosis factor (TNF)-$\alpha$ and interleukin (IL)-1$\beta$ (Flamand et al., 1991), as well as the expression of the G-protein-

Table 2
Susceptibility of animal species to HHV-6 infection

| Species | Strain tested | Susceptibility | |
|---|---|---|---|
| | | In vitro | In vivo |
| Chimpanzee (*Pan troglodytes*) | A/B | +/+ | nt/nt |
| Pig-tailed macaque (*Macaca nemestrina*) | A/B | +/+ | +/nt |
| African green monkey (*Cercopythecus aethiops*) | A/B | −/+ | −/+ |
| Rhesus macaque (*Macaca mulatta*) | A/B | −/+ | −/nt |
| Cynomolgus macaque (*Macaca fascicularis*) | B | + | + |
| Marmoset (*Callithrix jacchus*) | A | − | − |
| Tamarin (*Sanguinis labiatus*) | A | − | − |
| Cat (*Felix catus*) | A | − | nt |
| Rabbit (*Oryctolagus cuniculi*) | A | − | − |
| Guinea pig (*Cavia porcellus*) | A | − | − |
| Mouse (*Mus musculus*) | A | − | − |
| Duck (*Anas platyrhyncos*) | A | − | − |
| Chicken (*Gallus domesticus*) | A | − | − |

nt = not tested

coupled receptor EBI 1 (Hasegawa et al., 1994). Interestingly, HHV-6 also contains other genes with homology to G-protein-coupled receptors, which include the major chemokine receptors, as well as a unique gene with a striking structural homology to C–C chemokines (Gompels et al., 1995). It can be speculated that the virus may exploit molecular mimicry strategies to generate decoys for the immune system and thereby evade the immunologic control of the host.

## 5. Animal models of HHV-6 infection

Several animal species have been tested, both in vitro and in vivo, for their susceptibility to infection by HHV-6 (Table 2). The prototype of subgroup A, strain GS, has a very restricted species host-range, as it was shown to replicate in vitro in activated T-cells from only two nonhuman primate species: the chimpanzee (*Pan troglodytes*) (Lusso et al., 1990) and the pig-tailed macaque (*Macaca nemestrina*) (Lusso et al., 1994a). The latter species has also been successfully infected with HHV-$6_{Gs}$ in vivo (P. Lusso, R.W. Crowley and R.C. Gallo, in preparation), whereas no signs of infection were observed in a series of other animals tested by in vivo inoculation (Table 2). In contrast, both cynomolgus macaques (*Macaca fascicularis*) and African green monkeys (*Cercophytecus aethiops*) were successfully infected with a subgroup-B strain (HST), both in vitro and in vivo (Yalcin et al., 1992). Thus, not only the cellular tropism, but also the species tropism of the two major HHV-6 variants appears to be different.

## 6. Transmission, diagnosis and epidemiology

Infection by HHV-6 B is ubiquitous in the human population in different geographic areas and is acquired almost universally within the first 2 years of life (reviewed in Lusso and Gallo, 1995a). The infection is believed to be transmitted predominantly via oropharyngeal secretions, as also suggested by the frequent detection of the virus, by different techniques, in saliva or salivary glands (Pietroboni et al., 1988; Gopal et al., 1990; Harnett et al., 1990; Fox et al., 1990a; Cone et al., 1993b). In contrast, HHV-6 DNA sequences were not found in breast milk (Dunne and Jevon, 1993), suggesting that breast feeding is not an important route of transmission. In one case, intrauterine transmission of HHV-6 has been suggested (Aubin et al., 1992). At present, no conclu-

Table 3
Specificity of different tests for the diagnosis of HHV-6 infection

| | Primary infection | Latency | Reactivation/reinfection |
|---|---|---|---|
| IgG serology | + [a] | + | + |
| IgM serology | + [a] | − | +/− |
| Virus isolation | + | +/− | +/− |
| Qualitative DNA-PCR (cells) | + | + | + |
| Quantitative DNA-PCR (cells) | + | + | + |
| Cell-free DNA-PCR (serum or other body fluids) | + | − | + |
| RNA-PCR (cells) | + | − | + |
| Immunohistochemistry | + | − | + |
| In situ hybridization (DNA) | + | + | + |
| In situ hybridization (RNA) | + | − | + |

[a] Positive during the convalescence period, after a seronegative window of variable length.

sive information regarding the epidemiology and route of transmission of HHV-6 A is available.

The question of the diagnosis of HHV-6 infection is complex and has been a source of considerable confusion in the field. To understand the reasons for this complexity, one has to consider the characteristic life cycle of HHV-6 in the host. The virus enters the body during primary infection which, at least for HHV-6 B, occurs almost universally in early childhood. The virus is probably never cleared from the organism and persists in selected anatomical sites (including blood leukocytes), most likely in a latent state, for the entire lifetime. Subsequently, either reactivation or exogenous reinfection may occur, particularly in concomitance with episodes of immune disregulation or deficiency. After such episodes, latency may be established again. In light of these considerations, it is obvious that diagnostic methods which cannot discriminate between latent (clinically silent) and active (clinically relevant) infection, such as serum IgG antibody testing or DNA-PCR on blood cells, may be inappropriate or even yield misleading results for the clinical diagnosis of HHV-6 infection (Table 3). In particular, serological IgG antibody tests have several important shortcomings: a) the generalized seropositivity in the adult population (Saxinger et al., 1988; Briggs et al., 1988), which is due to the ubiquitous distribution of the infection, at least by HHV-6 B; b) the inability to discriminate between active and pregressed infection; c) the persistence of maternal IgG antibodies during the first 6 months of life; d) the frequent fluctuations of the IgG titers, even in the absence of appreciable clinical manifestations; e) the immunologic cross-reactivity of HHV-6 with other herpesviruses (mostly hCMV and HHV-7) (Irving et al., 1988; Larcher et al., 1988; Chou and Scott, 1990; Adler et al., 1993; Berneman et al., 1992); f) the inability of the current tests to identify the two major viral subgroups (A and B). In spite of these shortcomings, IgG antibody serology has been widely used, especially in the early days of HHV-6 research, allowing important, yet unwarranted, conclusions to be drawn. A particularly unfortunate example is an extensive seroepidemiological survey on a cohort of HIV-infected patients, the results of which were interpreted as evidence that HHV-6 plays no significant role in the course of HIV infection (Spira et al., 1990). Although conclusive evidence on this issue is still wanting, subsequent studies have contradicted these conclusions (see below). In this respect, it is noteworthy that the lack of correlation between the IgG antibody titers and the actual levels of HHV-6 replication in vivo may be further accentuated in HIV-infected patients, because of the progressive decline of the CD4+ T-cell helper function during the course of the disease.

A summary of the specificities of the different diagnostic tests currently available for HHV-6 infection is given in Table 3. Unlike IgG anti-HHV-6, specific IgM antibodies may provide a

reliable marker of active infection (Fox et al., 1990b; Suga et al., 1992; Secchiero et al., 1995a). The main limitations of this test are its relatively low sensitivity and the possible cross-reactivity with other human herpesviruses. To date, no HHV-6 antigen capture test with sufficient sensitivity to be used for clinical diagnotic purposes has been developed. Virus isolation methods, though more frequently positive in patients with active virus replication in vivo, do not distinguish, in principle, between active and latent infection, as they usually involve a cellular activation step in vitro. Even less accurate is the merely qualitative PCR analysis on DNA extracted from blood cells or tissues. In fact, DNA-PCR has almost the same lack of specificity as IgG antibody testing, with the additional complication of the wide variations in the sensitivity of the tests in different laboratories. For example, in five studies performed in western countries, the proportion of HHV-6 DNA-positive cases in peripheral blood leukocytes of unselected healthy individuals ranged from 5 to 95% (Cone et al., 1993b; Cuende et al., 1994; Di Luca et al., 1994; Rajcani et al., 1994; Wilborn et al., 1994). Even semi-quantitative or quantitative methods, like semi-quantitative PCR with an external standard (Cone et al., 1993a), quantitative PCR with an internal standard (Secchiero et al., 1995b), DNA in situ hybridization and Southern blot hybridization, do not permit diagnosis of active infection (reviewed in Carrigan, 1995); nevertheless, they provide, with different levels of accuracy, an estimate of the viral load in vivo, which often correlates with the degree of virus replication. In particular, quantitative methods may be useful to establish an etiologic link when analyzing tissues involved by inflammatory or neoplastic disorders, in which the occasional presence of infiltrating infected cells may be totally irrelevant to pathogenesis.

All the above considerations emphasize the fact that the clinical diagnosis of HHV-6 infection must rely on the use of direct markers of virus replication in vivo. In this respect, the most accurate and practical test currently available is the detection of HHV-6 DNA by PCR in serum or plasma (Secchiero et al., 1995a). By this method, HHV-6 replication has been documented both in immunocompetent children, early in the course of primary infection (exanthema subitum), and in different immunocompromised patients. A good correlation was observed between serum PCR and serum IgM antibody reactivity (Secchiero et al., 1995a). Other suitable methods for the demonstration of active HHV-6 infection in circulating cells or tissues are reverse transcriptase (RT) PCR, immunohistochemistry (Carrigan et al., 1991; Knox and Carrigan, 1994) and in situ hybridization on RNA, the latter two offering the additional advantage of an exact identification of the lineage of the infected cells. In conclusion, the diagnosis of HHV-6 infection is a delicate process that requires the use of markers of active virus replication in vivo and, preferably, the assessment of multiple parameters simultaneously. Once again, it is important to underline that clinical studies based exclusively on markers with limited specificity, or lacking a critical evaluation of the diagnostic potential of the techniques used, may lead to confusing and sometimes erroneous conclusions on the pathogenic role of HHV-6.

## 7. Clinical manifestations of HHV-6 infection

During the last 8 years, there have been numerous claims of possible associations of HHV-6 with human diseases. Many of these claims, however, have not been adequately substantiated and some have been contradicted by subsequent studies. In several instances, as discussed above, these inconsistencies were related to the inappropriate choice and/or interpretation of the diagnostic techniques.

As first reported in Japan by Yamanishi et al. (1988), primary infection with HHV-6 B is associated with exanthema subitum. The disease occurs in infants and is manifested by high-grade fever, typically lasting for 3 days, followed by the appearance of a skin eruption (roseola). However, primary HHV-6 infection is often manifested by an acute febrile illness without the typical rash (Suga et al., 1989; Pruksananonda et al., 1992). Although the febrile disease is usually self-limited and does not require specific therapeutic interventions, HHV-6 has been reported as a major cause of visits to hospital emergency departments

(Breese-Hall et al., 1994), in a high proportion of cases because of high fever and/or neurologic complications, such as meningitis and meningoencephalitis, with or without seizures (Ishiguro et al., 1990; Huang et al., 1991; Asano et al., 1992; Yanagihara et al., 1995). The neurologic manifestations seem to be related to the direct invasion of the central nervous system by the virus (Kondo et al., 1993). Other abnormalities that may be associated to exanthema subitum are granulocytopenia and liver dysfunctions (Takikawa et al., 1992). It is at present unclear whether all the cases of exanthema subitum are due to HHV-6: in fact, it has been suggested that HHV-7 infection may also be linked to a similar disease (Tanaka et al., 1994). Other disorders which have been sporadically associated with HHV-6 in children are fulminant hepatitis (Asano et al., 1990), idiopathic thrombocytopenic purpura (Yoshikawa et al., 1993; Kitamura et al., 1994), Rosai-Dorfman disease (Levine et al., 1992), hemophagocytic syndrome (Huang et al., 1990) and multiple organ failure (Prezioso et al., 1992).

In immunocompetent children and adults, HHV-6 infection has been associated with several pathological entities, albeit in some instances only by anecdotal evidence. They include EBV-negative infectious mononucleosis (Steeper et al., 1990; Akashi et al., 1993), persistent lymphadenopathy (Niederman et al., 1988), fulminant hepatitis (Sobue et al., 1991), autoimmune disorders (Krueger et al., 1991) and chronic fatigue syndrome (Buchwald et al., 1992). Recently, HHV-6 infection has been documented in the brain tissue of more than 70% of patients with multiple sclerosis (MS). An apparently MS-specific expression of viral antigens has been detected by immunohistochemistry in the nucleus of oligodendrocytes around the plaques that constitute the hallmark of the disease (Challoner et al., 1995). However, HHV-6 DNA and antigen expression were also detected in a similar percentage of non-MS brains, although oligodendrocytes were not specifically involved. Though suggestive as these data may be, it is difficult, at this stage, to draw any conclusions on the role of HHV-6 in MS.

Like other human herpesviruses, HHV-6 has been suggested as a possible etiologic factor in neoplastic disorders. It has been linked to Hodgkin's disease (HD) by IgG serology and PCR (Clark et al., 1990; Torelli et al., 1993; Di Luca et al., 1994), to non-Hodgkin's lymphoma (NHL) by Southern blot and PCR (Jarrett et al., 1988; Josephs et al., 1988b; Fillet et al., 1995), to angioimmunoblastic lymphadenopathy with dysproteinemia (AILD) by PCR (Luppi et al., 1993a), to Langerhans cell-hystiocytosis by PCR (Leahy et al., 1993), to KS by PCR (Bovenzi et al., 1993), to oral carcinoma by immunohistochemistry and in situ hybridization (Yadav et al., 1994) and to cervical carcinoma by PCR, Southern blot and in situ hybdridization (Chen et al., 1994b). Unfortunately, most of these studies have been conducted without adequate controls and the analysis has been limited to non-quantitative DNA-PCR. Without a precise identification of the cell type involved by HHV-6 infection (i.e. neoplastic or infiltrating cells) and in the absence of a precise quantitative evaluation, it is impossible to understand the exact significance of these claims. That HHV-6 may have a tumorigenic potential is nevertheless indicated by in vitro studies on the transforming potential of isolated HHV-6 genes transfected into murine fibroblasts (Razzaque, 1990; Thompson et al., 1994) or human keratinocytes (Razzaque et al., 1993). Moreover, it has been documented that HHV-6 can integrate into the host cell's chromosomes (Luppi et al., 1993b) and can accelerate the tumorigenesis induced by human papillomavirus-immortalized human cervical cells heterotransplanted into immunodeficient mice (Chen et al., 1994a). Further investigation is important to elucidate the relationship of HHV-6 with human cancer.

An increasing body of evidence suggests that HHV-6 may act as an opportunistic agent in patients with immune deficiencies, particularly those who received bone-marrow or organ transplantation and HIV-infected individuals (reviewed in Lusso and Gallo, 1995a). The clinical manifestations that have been associated with HHV-6 infection in immunocompromised hosts include interstitial pneumonitis (Carrigan et al., 1991; Cone et al., 1993a; Knox and Carrigan, 1994),

encephalitis (Drobyski et al., 1994; Knox et al., 1995), retinitis (Qavi et al., 1992; Reux et al., 1992) and bone-marrow graft failure (Drobyski et al., 1993; Carrigan and Knox, 1994). Consistent with the latter observation, in vitro studies have demonstrated a suppressive activity of HHV-6 on the maturation and growth of normal bone-marrow precursors committed toward different lineages (Knox and Carrigan, 1992). The evidence suggesting a possible etiopathogenic role of HHV-6 in these conditions is based on the direct detection of viral antigen expression or viral DNA in diseased tissues and on the temporal association between signs of disease and serum IgM antibody positivity or virus isolation. In a longitudinal study performed by serum PCR and IgM antibody testing in three bone-marrow transplanted patients, repeated HHV-6 reactivation/reinfection episodes were documented in concomitance with fever and respiratory or neurologic signs (Secchiero et al., 1995a). Most of the viral isolates obtained from circulating leukocytes or bone marrow of immunosuppressed patients were found to belong to subgroup B (Carrigan et al., 1991; Drobyski et al., 1994), whereas the viruses detected in serum from post-transplant and AIDS patients were mostly of the A-type (Secchiero et al., 1995a). Thus, coinfection by HHV-6 A and B may be common in immunocompromised hosts. Despite the increasing number of clinical reports, the evidence thus far accumulated is still insufficient to prove definitively the role of HHV-6 as a bona fide opportunistic agent. Carefully controlled, longitudinal studies will be essential to address this important question. Moreover, important information may also derive from the implementation, in patients with life-threatening infections of suspected HHV-6 etiology, of effective therapeutic measures directly targeted at this virus.

## 8. HHV-6 in AIDS

It is commonly accepted that herpesviruses can become reactivated in the advanced stages of HIV infection and may cause severe systemic or organ-specific disorders. As mentioned above, accumulating evidence suggests that HHV-6 may also act as an opportunistic pathogen in patients with AIDS. Nevertheless, the role of HHV-6 in AIDS may be more complex and important for the pathogenesis of the immune deficiency itself. Indeed, because of its peculiar biological characteristics, particularly its primary CD4+ T-cell tropism, HHV-6 has been suggested to act as a cofactor in the progression of the disease. A similar role has been proposed for hCMV which, however, lacks the ability to directly infect CD4+ T-cells. The first suggestion that HHV-6 could play a role in AIDS dates back to 1988, after the demonstration of its preferential tropism and cytopathicity for CD4+ T-lymphocytes (Lusso et al., 1988), the T-cell subset that is directly targeted by HIV and selectively depleted in AIDS. Subsequently, a series of intriguing experimental observations on the interactions between HHV-6 and HIV have corroborated this hypothesis. Similar to other DNA viruses, HHV-6 (both A and B) is a potent transactivator of the HIV-LTR (Lusso et al., 1989a; Ensoli et al., 1989; Horvat et al., 1989), a phenomenon that is likely to have biological significance because HHV-6, unlike other DNA viruses, can productively coinfect individual CD4+ T-cells together with HIV (Lusso et al., 1989a) and thus a direct interaction between the two viruses may take place within the same cell. In turn, the Tat transactivator of HIV-1 was recently shown to enhance the replication of HHV-6 (Sieczkowski et al., 1995). These observations may help to explain the accelerated HIV-1 expression and CPE observed in primary mononuclear cell cultures coinfected with HHV-6 and HIV-1 (Lusso et al., 1989a). Although some authors have described a suppressive, rather than enhancing, effect of HHV-6 on HIV-1 replication (Carrigan et al., 1990; Levy et al., 1990), there is consensus that coinfection leads to accelerated cell death. The discrepancies between different studies are possibly related to the different multiplicity of infection (m.o.i.) used for HHV-6 infection. As mentioned above, HHV-6 is also a powerful inducer of inflammatory cytokines, such as TNF-$\alpha$ and IL-1$\beta$ (Flamand et al., 1991) which are known to enhance the replication of HIV in vitro. However, the most striking and unique type of interac-

tion between HHV-6 and HIV occurs at the receptor level. As discussed above, infection with HHV-6 has a positive regulatory effect on the expression of the major HIV receptor, CD4. Not only is CD4 upregulated in cells that already express it at low levels (e.g. Jurkat), but even induced de novo by transcriptional activation mechanisms, in cells that physiologically do not express it. Evidence has been provided that the novel, HHV-6-induced CD4 receptor is functional, as it can mediate productive HIV infection in previously refractory cells, such as mature CD8+ T-cells (Lusso et al., 1991a), NK-cells (Lusso et al., 1993) and $\gamma\delta$ T-cells (Lusso et al., 1995). By this mechanism, HHV-6 may significantly expand the range of cells susceptible to HIV. Positive interactions have also been documented between HHV-6 and SIV, in coinfected T-lymphocyte cultures from pig-tailed macaques (Lusso et al., 1994b). Altogether, the above observations indicate that HHV-6 is an excellent candidate to play a cofactorial role in the course of HIV infection: it is a broadly 'immunotropic' herpesvirus that, when actively replicating in vivo, has the potential to cause direct damage to the immune system and, by direct or indirect mechanisms, to dramatically boost the replication and the spread of HIV in coinfected individuals.

Although the possible role of HHV-6 as a cofactor in AIDS still remains hypothetical, some clinical evidence is consistent with this concept. Besides the frequent isolation of HHV-6, particularly subgroup A, from HIV-infected individuals (reviewed in Lusso and Gallo, 1995b), there have been reports of high rates of detection of HHV-6 by PCR both in circulating leukocytes and in lymph nodes of HIV-infected patients (Buchbinder et al., 1988; Dolcetti et al., 1994). A correlation was observed between the HHV-6 viral load in vivo and the number of circulating CD4+ T-cells, with a decline of the HHV-6 copy number in blood cells in parallel with the depletion of CD4+ T-cells (Fairfax et al., 1994). Although this phenomenon may be interpreted as a mere consequence of the depletion of the main target cells for HHV-6 infection, it demonstrates a link between this virus and the disappearance of CD4+ T-cells. Active replication of HHV-6 was documented by serum PCR in patients with symptomatic HIV infection, but not in asymptomatic HIV-seropositive individuals (Secchiero et al., 1995a). Moreover, two studies performed on necropsy specimens, one conducted by PCR (Corbellino et al., 1993) and the other by immunohistochemistry (Knox and Carrigan, 1994), demonstrated that HHV-6 infection is active and disseminated in terminal AIDS patients. Unfortunately, it is impossible, based on necropsy studies, to know the time of acquisition (or reactivation) of HHV-6 and thereby to distinguish between a primary (pathogenetic) and a secondary (epiphenomenal) event.

Three major approaches may shed light on the role played by HHV-6 in AIDS. Firstly, controlled longitudinal studies of HHV-6 replication in vivo during the course of HIV infection will help to establish if a temporal association exists between active HHV-6 infection and the progression of the immunodeficiency. A second important approach is the experimental coinfection of susceptible animal models (e.g. *M. nemestrina*) with HHV-6 and HIV (or SIV). Finally, as discussed in detail in the next section, critical information on the relative role played by HHV-6 in AIDS could come from the implementation of therapeutic trials in vivo, employing selective anti-HHV-6 compounds in combination with antiretroviral drugs (e.g. zidovudine [AZT]).

## 9. Efficacy of antiviral agents against HHV-6

The progressive unraveling of the pathogenic role of HHV-6 in humans emphasizes the need for the rapid identification of effective therapeutic measures against this agent. The efficacy of several antiviral agents against HHV-6 has been evaluated in cell culture systems in vitro (Table 4). By contrast, apart from sporadic attempts, no systematic trials have been performed on well-documented HHV-6 infection in vivo. Most authors concur that acyclovir exerts no significant inhibitory effect on the replication of HHV-6 in vitro. In contrast, the efficacy of ganciclovir is still debated: a very narrow therapeutic window has been reported by some authors (Streicher et al.,

Table 4
Susceptibility of HHV-6 to antiviral agents

| Compound | Viral subspecies | Selectivity index $ID_{50}/TD_{50}$ | Reference |
|---|---|---|---|
| Acyclovir | A | <5 | Streicher et al. (1988) |
| | B | <5 | Kikuta et al. (1989) |
| | B | <5 | Russler et al. (1989) |
| | B | <5 | Di Luca et al. (1990) |
| | B | <10 | Åkesson-Johansson et al. (1990) |
| | A, B | <10 | Agut et al. (1991) |
| | A | <5 | Reymen et al. (1995) |
| Ganciclovir | A | <5 | Streicher et al. (1988) |
| | B | >50 | Russler et al. (1989) |
| | B | >10 | Burns and Sandford (1990) |
| | A | 8 | Åkesson-Johansson et al. (1990) |
| | A, B | >50 | Agut et al. (1991) |
| H2G | A | 47 | Åkesson-Johansson et al. (1990) |
| CyA, CyG | A | >40 | P. Lusso et al. (unpublished) |
| PMEDAP | A | 18 | Reymen et al. (1995) |
| PFA | A | >20 | Streicher et al. (1988) |
| | A | 31 | Åkesson-Johansson et al. (1990) |
| | A, B | >100 | Agut et al. (1991) |
| | A, B | >100 | P. Lusso et al. (unpublished) |
| | A | 125 | Reymen et al. (1995) |

1988; Åkesson-Johansson et al., 1990), but not by others (Russler et al., 1989; Burns and Sandford, 1990; Agut et al., 1991). Whether these discrepancies reflect differences in the viral strains used (e.g. A- versus B-variants, laboratory passaged versus field isolates) or in the experimental conditions is still unclear. In this respect, it is noteworthy that HHV-6, like hCMV, lacks a thymidine kinase, although both viruses encode a different kinase (Gompels et al., 1995). Mutations of this kinase have been shown to confer resistance to ganciclovir in the hCMV model (Littler et al., 1992).

The compounds with the best therapeutic window against HHV-6 include some novel nucleoside analogues, such as H2G (Åkesson-Johansson et al., 1990) and cyclobut-A and -G (CyA, CyG) (P. Lusso et al., unpublished data), selected acyclic nucleoside phosphonates, such as PMEDAP (Reymen et al., 1995), and the pyrophosphate analogues phosphonoformic acid (PFA or foscarnet) (Streicher et al., 1988) and phosphonoacetic acid (Di Luca et al., 1990). In particular, foscarnet is already available for clinical use and is of proven efficacy against drug-resistant HSV and hCMV infections in patients with AIDS (Öberg, 1989). Interestingly, foscarnet is also one of the few agents which has been shown to increase the survival time in patients with AIDS (Studies of Ocular Complications of AIDS Research Group, 1992). It is tempting to speculate that the beneficial effects of this compound in AIDS patients may be due, at least in part, to direct inhibition of the replication of HHV-6 or another foscarnet-sensitive cofactor. However, this hypothesis is not currently supported by any direct experimental or clinical evidence and it has to be considered that foscarnet does not have a selective activity against herpesviruses, but also exerts a direct antiretroviral effect (Öberg, 1989). The concept that herpesviruses may act as cofactors in AIDS is corroborated by the generally favorable, albeit still debated, results of several clinical trials employing AZT in combination with acyclovir (reviewed in Griffiths, 1995). In this respect, it must be considered that, although acyclovir seems to be poorly effective against HHV-6

in vitro, marked discrepancies have been documented between studies of acyclovir efficacy performed in cell culture systems and in vivo (Griffiths, 1995). Ideally, future trials in HIV-infected patients should employ compounds with selective activity against HHV-6 in combination with selective antiretroviral agents. This approach, paralleled by careful monitoring of markers of HHV-6 replication in vivo, will be extremely valuable for the definition of the relative role played by HHV-6 in AIDS.

Besides chemotherapy, cytokines should also be considered as potential therapeutic agents against HHV-6 infection. For example, it has been reported that IFN-$\alpha$ (Kikuta et al., 1990) and IL-2 (Roffman and Frenkel, 1990) exert inhibitory effects on infection by HHV-6 in cell culture systems. At present, it is unclear whether their antiviral action is direct or indirect (i.e. mediated by the functional activation of antiviral effector cells). The use of immunomodulating agents may favorably combine virus-suppressing effects with a positive action on the immune system.

## 10. Conclusions

In less than a decade since the discovery of HHV-6, our knowledge of this unique 'immunotropic' agent has grown rapidly. After some years of uncertainty, sensitive and specific methods for the clinical diagnosis of HHV-6 infection have been developed and HHV-6 is emerging as an important new human pathogen. Efforts should therefore be devoted to identifying and testing in controlled clinical trials effective therapeutic measures for the control of HHV-6 infection.

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