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A.J. Zuckerman

Royal Free Hospital School of Medicine
University of London
London, U.K.

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Human Herpesvirus-6

Epidemiology, molecular biology and clinical pathology

Editors

Dharam V. Ablashi

National Cancer Institute, NIH,
Bethesda, MD, U.S.A.

Gerhard R.F. Krueger

University of Cologne, Germany
and
University of Texas, HSC at Houston,
Houston, TX, U.S.A.

and

S. Zaki Salahuddin

University of Southern California,
Los Angeles, CA, U.S.A.

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Editors' foreword

Human herpesvirus-6 (HHV-6), previously called human B lymphotropic virus (HBLV), was discovered during the search for a possible new virus which might be a causative agent for lymphomas associated with acquired immunodeficiency syndrome (AIDS). During the systematic cell culture of peripheral blood mononuclear cells from HIV-1-infected or AIDS patients in Dr. Robert C. Gallo's laboratory (Laboratory of Tumor Cell Biology, National Cancer Institute, Bethesda, MD, U.S.A.), in 1985, frequently a herpesvirus was observed. In 1986, HBLV was reported as a new member of the human herpesvirus family. The previous new human herpesvirus, Epstein–Barr virus (EBV), was reported in 1964. Since the initial isolation of HHV-6, other investigators throughout the world have observed in their cell cultures of mononuclear cells large refractile mono- or binucleated cells which are termed 'juicy' cells. Such cells have been observed in cell cultures from healthy donors as well as from patients with autoimmune diseases, *exanthem subitum*, AIDS, organ transplants, atypical polyclonal lymphoproliferation, and chronic fatigue syndrome (CFS); all have been shown to carry HHV-6. A high prevalence of HHV-6 IgG antibody (>80%) in healthy individuals by seroepidemiology, and infection in the first year of life have been reported. HHV-6 association with HIV-1 infection in AIDS appears to be rather incidental or secondary, like the reactivation of other ubiquitous human herpesviruses, e.g., EBV, cytomegalovirus (CMV), and herpes simplex virus (HSV). Ultrastructural and molecular studies identified HBLV as a member of the human herpesvirus family with a preference for T cells, thus prompting its renaming as HHV-6 in 1987. Since HHV-6 was isolated from HIV-1-infected individuals, interest in the interaction of HHV-6 with HIV-1 has continued because of HHV-6's ability to infect and kill $CD4^+$ cells, the finding of both HIV-1 and HHV-6 virions in the same $CD4^+$ cell and the ability of HHV-6 to induce the CD4 molecule, which is a receptor for HIV-1. In the last 6 years a large volume of data has accumulated worldwide, including isolation of HHV-6 from saliva and other tissues using different propagation systems, and biologic and molecular characterization of new HHV-6 isolates. Because of the availability of new and more sensitive techniques in biology, immunology and molecular virology, great progress has been made in a short time in the study of HHV-6 and its pathogenic role in human diseases. The success in characterization of HHV-6 and its implication in human diseases are largely due to the development of specific reagents such as monoclonal antibodies

and specific molecular probes. A large body of scientific literature has appeared in journals and review articles supporting the fact that HHV-6 is an important pathogen; it is now known to be the primary etiological agent of roseola in young children and some cases of non-EBV and non-CMV infectious mononucleosis. The primary infection of HHV-6 in other acute illnesses in young children, i.e., fulminant hepatitis, febrile seizures, gastroenteritis, and the finding of HHV-6 DNA in malignant lymphomas (Hodgkin's disease, other B cell lymphomas) suggests its association with other human diseases as well. Characterization of HHV-6 isolates obtained from various diseases worldwide led to classification of HHV-6 into 2 groups, group A and group B. Group A isolates are similar to the prototype isolate, GS from National Cancer Institute, Bethesda, Maryland, and group B are similar to the isolate Z-29 from CDC, Atlanta, Georgia.

In 1990, the seventh member of the human herpesvirus (HHV-7) family was discovered in Dr. Niza Frenkel's Laboratory of Molecular Genetics, National Institute of Allergy and Infectious Diseases, Bethesda, MD, U.S.A. from healthy individuals. HHV-6 and HHV-7 share more biological, immunological and molecular homology than they do with CMV, EBV, HSV and VZV. In 1991, in Dr. R.C. Gallo's laboratory, another herpesvirus was observed in the peripheral blood mononuclear cell cultures of a patient with CFS. Later, in collaborations with Dr. Frenkel, this new herpesvirus isolate was determined to be HHV-7. To our knowledge, these are the only 2 reports of isolation of HHV-7. Like other herpesviruses, the HHV-7 is ubiquitous in the general population in the U.S.A. It appears to infect young children later in life than HHV-6. The association of HHV-7 with specific disease is yet to be investigated.

It is the purpose of this book to provide an up-to-date but concise overview of what is currently known about HHV-6. We have attempted to cover as many relevant areas as possible to provide the reader a comprehensive account of HHV-6. The chapters were contributed by the leading researchers working on HHV-6. Since we are in the midst of investigating biological aspects of HHV-6 and its association with diseases, some data or their interpretations by individual authors may appear contradictory. Moreover, some findings may yet be premature and need further evaluation and confirmation. Every author has enjoyed the freedom to provide first hand information which he or she regards to be important; the editors have not interfered with authors' choices of material. We feel that sometimes initial, so-called 'false' concepts have been the basis for critical research leading to important breakthroughs.

The task of this book is to provide, collectively, the most current knowledge of HHV-6 which will serve as the basis for future investigations as well as a reference for epidemiologists, pathologists, clinicians, scientists entering the field of herpesvirus research, medical and microbiology students.

The present monograph is organized essentially into 2 parts: (1) general virology, including studies in molecular virology, ultrastructure and viral biology; and (2) clinical pathology, summarizing the current knowledge on clinical implications of HHV-6 infection and therapeutic considerations, including an outline of potentially effective virostatis or antiviral agents. We gratefully recognize the endeavors

of all the authors who realize the importance of HHV-6 and have contributed to this volume despite their limited time. It was also a great pleasure working with Elsevier Science Publishers. Without their understanding of 'mobile deadlines', this work would probably not have been accomplished.

Bethesda, Maryland — Dharam V. Ablashi
Cologne, FRG and Houston, Texas — Gerhard R.F. Krueger
Los Angeles, California — S. Zaki Salahuddin

Preface by Ronald Glaser

In 1986, human herpesvirus-6 (HHV-6) was isolated from peripheral blood mononuclear cells obtained from an HIV-1-infected AIDS patient. The last new human herpesvirus that had been discovered was the Epstein–Barr virus in 1964. Thus it was very surprising that after all those years in which very sophisticated cell culturing and virological studies had been performed in laboratories throughout the world, that another human herpesvirus was discovered. Indeed many investigators were unwilling to believe initially that this was a new herpesvirus and thought that perhaps it was a contaminate from fetal calf serum. This, however, was shown to not be the case and HHV-6 was confirmed to be a new human herpesvirus and as outlined in the chapters of this book, the virus has now been associated with a broad range of illnesses. What is even a more surprising fact is that another new human herpesvirus, HHV-7, has been identified, but little is known about this virus as of yet. As outlined in the editor's foreword, many findings outlined in these reviews may be premature and additional studies will be necessary to evaluate and confirm these, in some cases, preliminary observations. However, since 5 years have passed since the initial isolation of HHV-6 it is time that a treatise be provided to the clinician/scientist to bring everyone up to date on what is at least presently known about the virus and what is known regarding the pathophysiology and clinical manifestations of the diseases with which the virus has been associated. This book will clearly provide this important information and may be used as a reference for the scientific 'paper trail' that has been generated on HHV-6.

Department of Medical Microbiology
and Immunology
The Ohio State University
Columbus, OH, U.S.A.

Ronald Glaser, Ph.D.
Professor and Chairman

Preface by G.R. Pearson

The recent identification of a new human herpesvirus designated HHV-6 was a remarkable development considering the stability of the herpesvirus family over the centuries. The fact that the virus was initially isolated from patients with immunodeficiency and lymphoproliferative disorders heightened the medical interest in this new infectious entity. Over the past 5 years, progress has been surprisingly rapid in characterizing the epidemiology and molecular biology of the different isolates of HHV-6 and in unveiling its association with human disease. It is remarkable that so much new knowledge has come forth on this virus over such a relatively short period of time. This inaugural comprehensive review prepared by experts on HHV-6 is therefore timely. Since the information presented in this book will serve as the baseline for future studies on this virus, this book will be an important reference in the years to come and therefore will be a valuable addition to the herpesvirus library.

Department of Microbiology
Georgetown University Medical Center
Washington, DC, U.S.A.

G.R. Pearson, Ph.D.
Professor and Chairman

List of contributors

D.V. Ablashi	Laboratory of Cellular and Molecular Biology, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892, U.S.A.
K. Balachandra	Department of Virology, Research Institute for Microbial Diseases, Osaka University, Japan.
N. Balachandran	Department of Microbiology, Molecular Genetics and Immunology, The University of Kansas Medical Center, Kansas City, KS 66103, U.S.A.
Z.N. Berneman	Laboratory of Tumor Cell Biology, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892, U.S.A.
G. Bertram	Ear, Nose and Throat Clinic, Municipal Hospital Dortmund, University Witten-Herdecke, W-4600 Dortmund, Germany.
B. Borisch-Chappuis	Institut für Pathologie, Universität Würzburg, W-8700 Würzburg, Germany.
N. Bourgeois	Department of Pathology, University of Antwerp, 2520 Edegem, Belgium.
D.R. Carrigan	Department of Pathology and the Bone Marrow Transplant Program, Medical College of Wisconsin, Milwaukee, WI 53226, U.S.A.
D. A. Clark	Leukaemia Research Fund Virus Centre, Department of Veterinary Pathology, University of Glasgow, Bearsden, Glasgow G61 1QH, U.K.

L.S. De Clerck	Department of Immunology, Allergy and Rheumatology, University of Antwerp, 2610 Antwerp, Belgium.
K. Ellinger	Institut für Klinische und Molekulare Virologie, Universität Erlangen-Nürnberg, W-8520 Erlangen, Germany.
B. Fleckenstein	Institut für Klinische und Molekulare Virologie, Universität Erlangen-Nürnberg, W-8520 Erlangen, Germany.
S. Gupta	Division of Basic and Clinical Immunology, University of California, Medical Sciences 1, C264A, Irvine, CA 92717, U.S.A.
M.T. Green	Department of Ophthalmology, Baylor College of Medicine, Houston, TX 77030, U.S.A.
C.A. Horwitz	Department of Pathology, Abbott Northwestern Hospital, and University of Minnesota Medical School, Minneapolis, MN 55407, U.S.A.
C.L. Hung	Cell Science Institute, Pharmacia Diagnostic, Inc., Silver Spring, MD 20904, U.S.A.
R. Jarrett	Leukaemia Research Fund Virus Centre, Department of Veterinary Pathology, University of Glasgow, Bearsden, Glasgow G61 1QH, U.K.
S.F. Josephs	Laboratory of Tumor Cell Biology, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892, U.S.A.
M.H. Kaplan	Department of Medicine, Division of Infectious Diseases, North Shore University Hospital/Cornell University Medical College, Manhasset, NY 11030, U.S.A.
M. Kikuchi	First Department of Pathology, Fukuoka University School of Medicine, Nanakuma 7-45-1, Jyonan-ku, Fukuoka 814-01, Japan.
H. Kikuta	Department of Pediatrics, Hokkaido University School of Medicine, Sapporo 060, Japan.

A.L. Komaroff	Division of General Medicine and Primary Care, Brigham and Women's Hospital, Harvard Medical School, Boston, MA 02115, U.S.A.
K. Kondo	Department of Virology, Research Institute for Microbial Diseases, Osaka University, Japan.
T. Kondo	Department of Virology, Research Institute for Microbial Diseases, Osaka University, Japan.
B. Kramarsky	Pharmacia Diagnostics Inc., Columbia, MD 21046-1297, U.S.A.
G.R.F. Krueger	Immunopathology Laboratory, Institute of Pathology, University of Cologne, 5000 Germany 41, Germany, and Department of Pathology and Laboratory Medicine, University of Texas Medical Center, Medical Science Building, Houston, TX 77030, U.S.A.
P.H. Levine	Viral Epidemiology Section, Environmental Epidemiology Branch, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892, U.S.A.
J. Luka	Department of Pathology and Microbiology, University of Nebraska Medical Center, Omaha, NE 68105, U.S.A.
P. Lusso	Laboratory of Tumor Cell Biology, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892, U.S.A.
Y. Minamishima	Department of Microbiology, Miyazaki Medical College, Miyazaki, Japan.
K. Müller-Hermelink	Institut für Pathologie, Universität Würzburg, W-8700 Würzburg, Germany.
T. Mukai	Department of Virology, Research Institute for Microbial Diseases, Osaka University, Japan.
F. Neipel	Institut für Klinische und Molekulare Virologie, Universität Erlangen-Nürnberg, W-8520 Erlangen, Germany.
T. Okuno	Department of Virology, Research Institute for Microbial Diseases, Osaka University, Japan.

H.B. Qavi	Division of Molecular Virology, Baylor College of Medicine, Houston, TX 77030, U.S.A.
S.Z. Salahuddin	Department of Medicine, University of Southern California, School of Medicine, Los Angeles, CA 90033, U.S.A.
I.E. Salit	Department of Microbiology and Division of Infectious Diseases, University of Toronto, Toronto General Hospital, Toronto, Ontario M5S 1A8, Canada.
Ch. Sander	Institute of Pathology, University of Cologne, 5000 Cologne 41, Germany, and Laboratory of Pathology, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892, U.S.A.
G.K. SeGall	Department of Pathology, Baylor College of Medicine, Houston, TX 77030, U.S.A.
S. Seidl	Institut für Pathologie, Universität Würzburg, W-8700 Würzburg, Germany.
T.A. Steeper	Department of Pathology, Abbott Northwestern Hospital, and University of Minnesota Medical School, Minneapolis, MN 55407, U.S.A.
W.J. Stevens.	Department of Immunology, Allergy and Rheumatology, University of Antwerp, 2610 Antwerp, Belgium.
Y. Sumiyoshi	First Department of Pathology, Fukuoka University School of Medicine, Nanakuma 7-45-1, Jyonan-ku, Fukuoka 814-01, Japan.
K. Takahashi	Department of Virology, Research Institute for Microbial Diseases, Osaka University, Japan.
M.V. Williams	Department of Medical Microbiology and Immunology, The Ohio State University and Comprehensive Cancer Center, Columbus, OH 43210, U.S.A.
K. Yamanishi	Department of Virology, Research Institute for Microbial Diseases, Osaka University, Japan.

Ablashi/Krueger/Salahuddin (eds) Human Herpesvirus-6

CHAPTER 1

The discovery of human herpesvirus type 6

SYED ZAKI SALAHUDDIN

University of Southern California, School of Medicine, Los Angeles, CA 90033, U.S.A.

1.1. Introduction

This chapter is an effort to summarize the events which led to the discovery of human herpesvirus type 6 (HHV-6). The period of 1984 through 1986 was perhaps one of the most exciting periods in bio-medicine due to the epidemic of acquired immune deficiency syndrome (AIDS). It is important to discuss a segment of this history as it relates to the finding of HHV-6 in the Laboratory of Tumor Cell Biology (LTCB) at the National Cancer Institute (NCI), in Bethesda, MD. HHV-6 was discovered in the latter part of 1984, in 1985 initially reported as human B cell lymphotropic virus (HBLV). This followed the waves of the HIV-1 detection, isolation and other findings. Both 1983 and 1984 were simultaneously exciting and confusing. The initial reports on lymphoadenopathy associated virus (LAV) from the Pasteur Institute in Paris made it impossible not to focus on the new retrovirus, for intellectual as well as for practical reasons. During the latter part of 1983 and early 1984, it began to be clear that this newly discovered retrovirus was present in more patients than only those with lymphoadenopathy. My associates and I, in a subsection of the LTCB, isolated HIV-1 from AIDS patients and from persons who were at high risk for AIDS, e.g., homosexual men, hemophiliacs, etc. (Gallo et al., 1984). We expanded these findings by detection and isolation of HIV-1 from persons at high risk, and from a variety of body fluids and tissues, e.g., peripheral blood, bone marrow, lymph nodes, etc. (Salahuddin et al., 1986). A separate, parallel effort in this area, directed by Drs. Robert C. Gallo and Mikulas Popovic and their associates, focused on the production of HIV-1 and blood testing. This was a very exciting time for LTCB and particularly for Dr. Gallo. Reports from his laboratory in May 1984 established the essential causative relationship to the

newly discovered retrovirus, which, partly due to its tropism for T cells, was named human T lymphotropic virus III (HTLV-III). These findings were a major leap forward not only in advancing our understanding of AIDS, but also raised hope that perhaps the end of the AIDS epidemic was near.

1.2. Discovery of HHV-6

By the end of 1984, involvement with AIDS had led to my increasing interest in malignancies in AIDS patients which were both devastating and not well understood, viz., AIDS-associated lymphoma and Kaposi's sarcoma (KS). These diseases were challenging from a virological point of view and because of the diverse biology involved. Kaposi's sarcoma and lymphoma involve not only a variety of cell types, but were also thought to be mediated by different viruses. For this chapter, I will only discuss our experiences with specimens from AIDS patients with lymphoma because it was these experiments which led to the discovery of human herpesvirus-6 (HHV-6).

In case of AIDS-associated lymphoma, the prevailing wisdom was that, since this was a B cell disease, the Epstein–Barr virus (EBV) must play a major role in combination with the immune deficit. It was also thought that the EBV found in this disease might be different from the commonly isolated variety, the P3HR-1 or B95-8 type. In fact, Dr. Ablashi and I looked into this possibility and were surprised to find that AIDS patients generally had high titers of EBV antibodies as well as a higher incidence of transforming virus in circulation in the plasma (Ablashi et al., 1987a). When it came to lymphoma in AIDS, the findings were indistinguishable from those of nonlymphoma cases or from normal homosexual males. However, when the leukocytes from the peripheral blood were activated, inducing them to divide, some of the cells ballooned and formed syncytia. These were called 'juicy cells' by my associate, Susie Sturzenegger, due to their distinct appearance. To an experienced eye, these cells were distinguishable from those infected by HIV-1. We struggled with these pictures in our minds, and assumed that this might be a cytopathic effect (CPE), perhaps due to a new transmissible agent. I decided to transmit this agent, not knowing whether it was the same in each case or different to normal leukocytes obtained from human cord blood. Transmission to cord blood leukocytes was important to keep them isolated from other adventitious agents, which may be present in cells from adult donors (e.g., EBV, HCMV, etc.), and to separate them from HIV-1. The phenomenon of 'juicy cells' was reproducible in the cord blood leukocytes. These transmissions were done by a variety of methods; cell-free culture supernatant, co-culture of donor and target cord blood leukocytes and by cell lysate of donor cells to cord blood leukocytes. The last method was used repeatedly to inactivate any HIV-1 present in the system and transmit only the intracellular virus, if any. We were indeed successful in eliminating HIV-1 from the cell culture systems. The subsequent

immunological assays showed that the phenomenon of 'juicy cells' was transmissible without positivity for any of the HIV-1 antigens. At this point, I was convinced that I was dealing with a transmissible virus which needed further characterization. This phase took considerable time due to the fact that the work on HIV-1 infection of monocytic cells and the developmental work on Kaposi's sarcoma had priority. By this time, we had slipped into 1985.

Specimen of our transmitted cord blood leukocytes were maintained in culture and the reproducible phenomenon, some cells consistently becoming 'juicy' and the rapid death of cells in the same culture, continued. The 'juicy cells' also died within 10–15 days of culture. This helped us in studying the host range of the virus. While this virus was easily transmissible to cord blood leukocytes, it was very difficult to establish infection of established cell lines. Analysis of the primary cells from patients gave indications that the 'juicy cells' were, perhaps, immature B cells. This formed the basis for the nomenclature human B cell lymphotropic virus (HBLV). Soon after the initial publications (Josephs et al., 1986; Salahuddin et al., 1986), due to the efforts of Dr. Paulo Lusso, it was established that the cord blood cells were T cells rather than B cells (Lusso et al., 1987). In the meantime, Downing et al. (1987) and Tedder et al. (1987) also wrote in *Lancet* that the target cells for the new virus were T cells. We agreed and immediately proposed a correction and the renaming of the virus as HHV-6 (Ablashi et al., 1987b). It must be pointed out, however, that *in vitro* the prototype isolate of HHV-6 infects not only T cells but also B cells (Ablashi et al., 1987b, 1988). At this point, we concluded that we had a transmissible agent, that this was a lytic agent and it might be any of the known or unknown viruses, e.g., EBV, HSV, CMV, or something new. Now that I had eliminated the human T cell lymphotropic viruses I and II (HTLV I and II) and HIV-1 by immunological assay (IFA), it was essential to eliminate the herpesviruses. Specimens were sent to Dr. Bernhardt Kramarsky for electron microscopic analysis. His result showed that the agent I had been working with was a herpesvirus, morphologically indistinguishable from other members of the family. I was, however, very impressed by the following observations:

(a) the abundance of particles in the various stages of maturation;
(b) the abundance of particles released outside the cells (Salahuddin et al., 1986; Biberfeld et al., 1987);
(c) remarkably well-preserved morphology of the released particles;
(d) the excellent condition of the cells during the initial stages of virus maturation and release;
(e) evidence of mitotic division during the early stages of virus production;
(f) unusual release of virus particles from the cell surface;
(g) CPE associated only with the late phase.

Clearly, this virus deserved a closer look. My initial hope was that this could be the agent responsible for the AIDS-associated lymphoma. Although the virus had unusual features, the virological properties did not fit the disease, their predominant T cell tropism.

I had known Dr. Ablashi for a number of years for his work with EBV and other nonhuman primate herpesviruses. He was conveniently located in the same building. When I requested his help in the characterization of this virus he joined the effort enthusiastically. I also approached Dr. Steven Josephs of the LTCB and showed him the data I had developed so far. He was interested and agreed to analyze the virus molecularly. My involvement in virology was limited to retroviruses, an organism with roughly 10 kb genome. Herpesviruses are generally 12–20 times larger, with attending complexity and, therefore, offered a challenge of insurmountable proportions for us. Further disappointment came when I mentioned the data to some colleagues who knew DNA viruses. They could not believe that a member of the herpes family had escaped detection for so long. The last herpesvirus (EBV) was isolated some 20 years ago by Prof. M. Anthony Epstein, from African Burkitt's lymphoma cells.

I discussed the preliminary findings of this virus with Dr. Gallo. He agreed with me that I should invite Dr. Ablashi for collaborative studies on this virus and Dr. Josephs for molecular analysis. The rest is well documented and published. Both these men have contributed enough to justify their being called co-discoverers of HHV-6. It took more than 7 months to prove that this virus was a novel human herpesvirus. Dr. Gary Pearson of Georgetown University, Washington, DC, kindly provided us with some reagents for EBV and HCMV and was a source of information and encouragement throughout this period, and has continued to be since.

Dr. Peter Biberfeld, from the Karolinska Institute, Stockholm, Sweden, joined the laboratory in 1985, initially to isolate viruses which might be the causative agent for KS. We had many discussions, and I shared the data on our new herpesvirus with him. He indicated that he would be interested in contributing to the morphological analysis of the virus and immunocytochemistry of the target cells. This contribution resulted in studies that were published as a part of our initial discovery and as an exclusive electron microscopic study (Biberfeld et al., 1987).

Dr. Gallo was interested in knowing whether this newly discovered virus had association with any disease. He initiated a major sero-epidemiological study. Dr. Ablashi was given the responsibility of accomplishing this objective. Dr. Paul Levine of the Epidemiology Subdivision of NCI was supposed to coordinate the efforts of all the outside collaborators such as Drs. Mark Kaplan (North Shore Community Hospital, NY), Steven Strauss (NIH, Bethesda, MD) and Tony Komaroff (Brigham and Women's Hospital, Harvard University, Boston, MA). All the specimens were directed to Drs. Ablashi and Saxinger of the LTCB, who arranged to perform the serological assays under our standardized protocol, developed by Drs. Ablashi, Saxinger, Biberfeld and me. Some assays were done in my laboratory as a measure of system control, but the bulk of them was performed on contract. As it became evident that HHV-6 was a ubiquitous virus and without a clear relationship to any disease, interest in the virus began to diminish. We continued, however, to pursue studies related to the biological aspects as much as we were able to. We were lucky to find Dr. Balachandran (University of Kansas

Medical Center, Kansas City, KS) as a collaborator during this period. I have enjoyed knowing him since. Dr. Balachandran is an excellent immunologist and virologist with expertise in immunochemistry. He developed a number of monoclonal antibodies to HHV-6 (Balachandran et al., 1989) which have helped not only in diagnostic work, but also in the analysis of many isolates of HHV-6 into different subgroups (Ablashi et al., 1991). He has been invaluable in this area.

During 1986, we initiated collaborative studies with Prof. G.R.F. Krueger of the University of Cologne, Cologne, Germany, to explore the role of HHV-6 in pathogenesis. Professor Krueger contributed a number of scientific publications on the role of HHV-6 in malignant and non-malignant diseases, including EBV and CMV negative infectious mononucleosis (Bertram et al., 1989; Krueger et al., 1990).

A lot of effort on the part of many investigators has been published since our original report. I am also most grateful to all of those who helped with these studies and who have remained unnamed. I am referring to the support personnel whose names seldom appear in scientific papers.

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Ablashi/Krueger/Salahuddin (eds) Human Herpesvirus-6

CHAPTER 2

The epidemiology of human herpesvirus-6

PAUL H. LEVINE[1], RUTH JARRETT[2], and DUNCAN A. CLARK[2]

[1] *Viral Epidemiology Section, Environmental Epidemiology Branch, National Cancer Institute, National Institutes of Health, Bethesda, MD* 20892, *U.S.A., and* [2] *Leukaemia Research Fund Virus Centre, Department of Veterinary Pathology, University of Glasgow, Glasgow G61 1QH, U.K.*

2.1. Introduction

Human herpesvirus-6 (HHV-6) was first isolated from 6 patients with lymphoproliferative disease or immunosuppressive disorders (Salahuddin et al., 1986). Since that time, HHV-6 has been studied in a number of laboratories using a variety of techniques to investigate its epidemiology and determine its role in human disease. At the molecular level, HHV-6 is more closely related to cytomegalovirus than the other 5 characterized human herpesviruses (Lawrence et al., 1990); the degree of homology with the more recently identified HHV-7 (Frenkel et al., 1990) is at present unclear. The Epstein–Barr virus (EBV) has provided much of the impetus to recent studies of HHV-6 epidemiology and in this chapter we will note the apparent similarities and differences between these 2 viruses.

2.2. Methodologic considerations

Sero-epidemiological studies of HHV-6 have utilized an indirect immunofluorescence assay (IFA) as the 'gold standard' since the first HHV-6 serology reports used an assay based on the techniques applied to the study of EBV (Henle and Henle, 1966). A number of studies have shown that there is no cross-reactivity with other herpesviruses in the HHV-6 IFA; the HSB_2 cell line used for the HHV-6 assay is free of EBV as well as other human herpesviruses and retroviruses (Ablashi et al., 1988).

IFA is not as sensitive as other assays, such as the enzyme-linked immunosorbent assay (ELISA) (Halprin et al., 1986; Saxinger et al., 1988), but has a particular

advantage in the area of specificity. An experienced reader using appropriate positive and negative controls can readily determine if the pattern of cells reacting with the test serum is comparable to the pattern of cells known to be infected with HHV-6 (see Fig. 2.1). Several problems are inherent in the IF assay, however. In addition to being relatively insensitive, the end-point dilution can depend on many factors, including the source of antigen, the conjugate and the microscope. Nevertheless, we have found the test to be highly reproducible when samples have been exchanged between different laboratories.

Further details regarding the various laboratory tests currently in use are described more completely elsewhere (Balachandran, Chapter 9, this volume). In the context of this chapter, however, it is important to emphasize the number of possible reasons that laboratories report different seroprevalence rates, even when they are using the same system. The starting serum dilution used is the most important factor affecting the 'seroprevalence' (see Table 2.1). As would be expected, workers using 1 : 10 as the starting serum dilution reported higher seroprevalence rates than those using 1 : 40. It is, therefore, better to regard quoted figures as indicating the percent positive at a particular serum dilution rather than the seroprevalence. The viral strain and the cells in which the virus is propagated may also affect the results. Most groups have propagated the virus in either cord blood lymphocytes or continuous T cell lines such as HSB_2 or J JHAN. Chronically infected cell lines as an antigen generally appear to be more sensitive than HHV-6-infected cord blood in the IF system. This may be because IL-2, which has been shown to have an inhibitory effect on the replication of HHV-6 in thymocytes (Roffman and Frenkel, 1990), is used in maintaining cord blood lymphocytes in

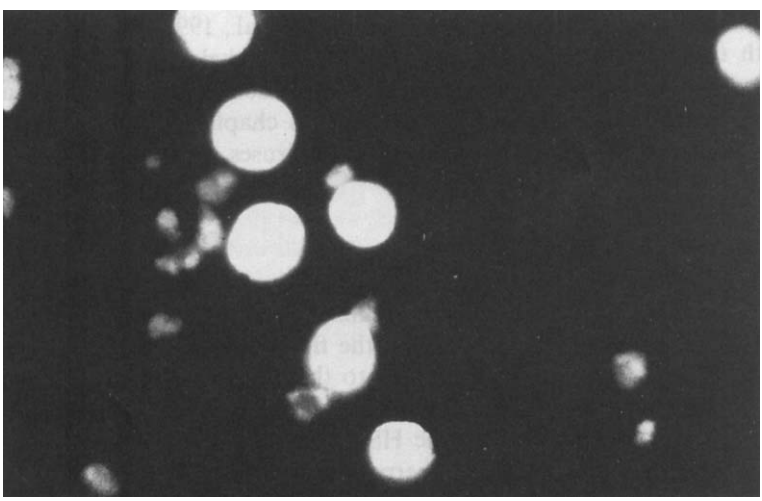

Fig. 2.1. Detection of HHV-6 antigens by indirect immunofluorescence in the HSB_2 cell line. (Photograph provided courtesy of Dr. D.V. Ablashi.)

TABLE 2.1.
Comparison of reported HHV-6 seropositivity rates measured using different assay systems

Study	Assay	Viral strain	Propagation	Initial serum dilution	Age[a]	Geographical locale	Percent seropositive
Briggs et al. (1988)	IFA[c]	AJ	J JHAN	1:50	Adults[a]	UK	52
Brown et al. (1988)	IFA	DV	HSB_2	1:10	> 12 yr[a,c]	USA	67
Pietroboni et al. (1988a)	IFA			1:10	> 15 yr[a]	Australia	94
Ablashi et al. (1988)	IFA	GS					
Krueger et al. (1988)	IFA	GS	HSB_2	1:20	18–52 yr[a]	Germany	63
				1:40			26
Linde et al. (1988)	IFA	GS	HSB_2	1:10	> 1 yr[a]	Sweden	85
Yoshikawa et al. (1989)	IFA	FG-1	CBLs[c]	1:10	15–27 yr[a]	Japan	76
Clark et al. (1990)	IFA	AJ	J JHAN	1:40	> 15 yr	UK	55
Levy et al. (1990a)	IFA	SF	MT-4 or PBMCs[c]	1:8	Adults[a]	California	97
						China (PRC)	100
						England	67
						Scandinavia	88
						Dominican Rep.	91
						Haiti	100
Rodier et al. (1990)	IFA	GS	HSB_2	1:20	Adults	Djibouti, Africa	71
				1:40[b]			44
Enders et al. (1990)	IFA	St.W	J JHAN	1:16	> 10 yr[a]	Germany	70
		U1102 Japan	CBLs	1:16			44
Levine et al. (1991)	IFA	HSB_2		1:10	20–49 yr	Ghana	100
	IFA			1:10	20–49 yr	Malaysia	48
	IFA			1:10	20–49 yr	USA	96
Okuno et al. (1989)	ACIF[c]	Z-29	MT-4	1:10	> 10 yr[a]	Japan	81
Yanagi et al. (1990)	ACIF	Z-29	CBLs	1:10	> 14 yr[a]	Japan	92
				1:40[b]			58
Saxinger et al. (1988)	ELISA[c]	GS	HSB_2	1:400	6–74 yr	USA	97

[a] Denotes that only part of the age distribution of the study is included here.
[b] Signifies that the results at this serum dilution have been calculated from results given in the text.
[c] IFA = indirect immunofluorescence assay; ACIF = anticomplementary immunofluorescence; ELISA = enzyme-linked immunosorbent assay; yr = years; CBLs = cord blood lymphocytes; PBMCs = peripheral blood mononuclear cells.

culture. Recent studies suggest that different virus strains may replicate more efficiently in different cell culture systems (Levy et al., 1990a); the amount of viral antigen present may, therefore, vary with different virus/cell line combinations. This is borne out by the study of Enders et al. (1990, Table 2.1). Different strains of the virus have also been shown to have different biologic, immunologic and molecular properties (Ablashi and Salahuddin, Chapter 7, this volume; Balachandran et al., personal communication) although it is not yet clear whether such differences will affect the sero-epidemiological findings.

A number of other serologic assays have been applied to HHV-6 studies, including radioimmunoprecipitation and Western blot, but the most widely used, other than IFA, has been the ELISA (Saxinger et al., 1988). As noted above, the ELISA is more sensitive than IFA but, thus far, there has been some difficulty in defining 'true negatives'. With the potential availability of serum from uninfected individuals in Asia available for test evaluation (see below) and the concomitant use of equally sensitive and specific (but also labor intensive and expensive) assays such as radioimmunoprecipitation, this may not be a problem in the near future. Preliminary data using standard sera from a recently established reagent bank suggest that the type of antigen (whether whole virus or a cell lysate) used in ELISA may affect the apparent prevalence and titer of antibody (Levine, unpublished data). Different viral strains used in the ELISA, however, produce very similar results (Corrigan, personal communication).

If the EBV model is to prove a parallel to HHV-6 in the field of sero-epidemiology, it is quite likely that the different assays, and even the same assays using different antigens, are detecting diverse responses to viral infection. Future assays directed at specific HHV-6 antigens may help to clarify the role of this virus in disease. In such a rapidly evolving field, therefore, we will emphasize those findings that have been reported in multiple laboratories which provide a reasonably consistent picture of HHV-6 epidemiology.

While most of our current information is derived from serologic studies, other techniques, such as virus isolation, *in situ* hybridization, Southern blot analysis and the polymerase chain reaction (PCR), have added to our knowledge of the epidemiology of HHV-6 (see below).

2.3. Demographic patterns of virus infection

2.3.1. Sero-epidemiologic studies

HHV-6 clearly is a ubiquitous virus, more than half of almost every population reported thus far being seropositive using recent techniques (Table 2.1). In most of these studies, females have slightly higher titers than males (Fig. 2.2) (Briggs et al., 1988; Clark et al., 1990), a finding reported for a number of other viruses including EBV (Biggar et al., 1981). Unlike EBV, however, the antibody titers appear to decline in older age groups (Yanagi et al., 1990), a finding reflected as declining prevalence in some studies (Fig. 2.3) (Enders et al., 1990; Levy et al., 1990a,b).

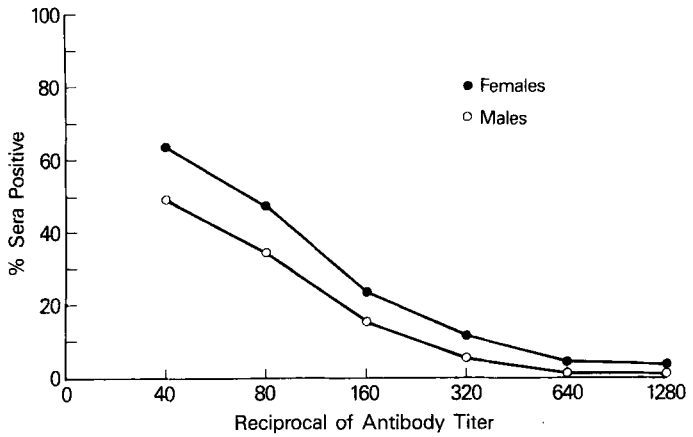

Fig. 2.2. Relationship of HHV-6 antibody titers to sex. (Data derived from immunofluorescence studies of Clark et al., 1990.)

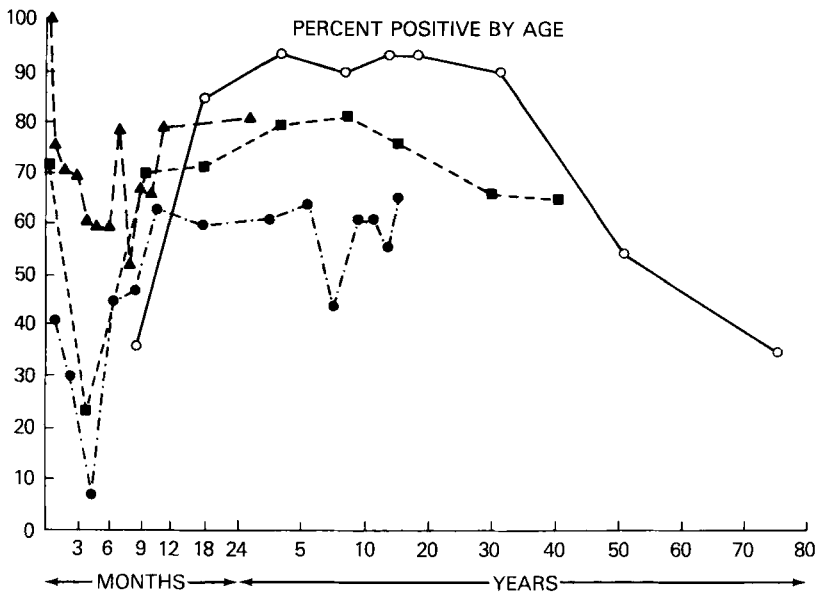

Fig. 2.3. Relationship of HHV-6 antibody prevalence to age. A results from Levy et al. (1990a); B from Enders et al. (1990); C from Briggs et al. (1988); D from Knowles and Gardner (1988); and E from Brown et al. (1988).

Maternal antibodies are usually present at birth and decline in the first few months of life. Seroconversion appears to occur most commonly between 6 and 18 months of age and, most probably as a consequence of recent infection, the highest titers are seen in the young age group (Levy et al., 1990b; Yanagi et al., 1990).

Little data exist regarding differences between various racial/ethnic groups at the present time. Levy et al. (1990b) did not find a significant difference in seropositivity rates when they compared results from different ethnic and racial groups (Table 2.1). However, a difference has been observed in several studies using sera provided by the Burkitt Tumor Project in Accra, Ghana (Neequaye et al., 1987). A pilot study of 158 age and sex matched healthy donors from Ghana, Malaysia and the U.S.A. (Levine et al., 1992a) revealed a higher percentage of positive donors in Ghana and the U.S.A. as compared with Malaysia (Table 2.1). In addition, the geometric mean titer (GMT) of the Ghanaian seropositive donors (1:58.4) was significantly higher than that of seropositive U.S.A. Caucasians (GMT = 1:27.5), Malaysian Indians (GMT = 1:16.2) and Malaysian Chinese (GMT = 1:13.3). Further geographic comparisons are in progress and will need to investigate children and older adults to improve the interpretation of these geographic differences.

2.3.2. Non-serologic approaches to virus detection

Since the initial isolation of HHV-6 from peripheral blood of 6 patients (Salahuddin et al., 1986), the virus has frequently been isolated from the blood of HIV-infected individuals (Downing et al., 1987; Tedder et al., 1987; Lopez et al., 1988; Pietroboni et al., 1988b) and infants with roseola infantum (Yamanishi et al., 1988; Enders et al., 1990). In addition, 2 groups have reported that HHV-6 can be isolated from the saliva of the majority of healthy individuals (Pietroboni et al., 1988a; Harnett et al., 1990; Levy et al., 1990b). The successful isolation of HHV-6 from saliva appears to be dependent on the methodology used as other groups have failed in their attempts to isolate the virus from this source (Yanagi et al., 1990).

Using PCR, HHV-6 DNA sequences have been detected in peripheral blood and saliva samples from the majority of healthy individuals (Gopal et al., 1990; Jarrett et al., 1990). Furthermore, HHV-6-specific DNA sequences and proteins have been localized in major and minor salivary glands and bronchial tissue using *in situ* hybridization and immunohistochemical staining with HHV-6 monoclonal antibody (Fox et al., 1990a; Krueger et al., 1990).

There is, therefore, good evidence that HHV-6, like EBV, persists following primary infection and that the salivary glands are a site of replication. It would appear likely that saliva is an important vehicle for transmission of HHV-6.

2.4. Etiologic implications of clinical studies

The ubiquitous nature of HHV-6 and the fact that the majority of healthy persons are seropositive makes the interpretation of HHV-6 serology in health and disease difficult. The identification of HHV-6 DNA sequences in tissue samples from healthy persons and the frequent isolation of the virus from such people also limit the conclusions that can be taken from similar findings in disease.

At the present time, the strongest evidence for a role of HHV-6 in particular conditions comes from persons who seroconvert at the time of their illness. This would seem to have its drawbacks since IgM antibodies to HHV-6 are detected not only in primary infection but also in reactivation (Fox et al., 1990b) and it is unclear if viral reactivation has any clinical relevance. The significance of elevated IgG antibody titers is also unclear. HHV-6 IgG antibody levels can be elevated following primary EBV and CMV infection (Linde et al., 1988, 1990). This does not necessarily indicate reactivation of HHV-6 since measles virus antibody levels are also elevated in persons with primary EBV infection (Linde et al., 1990). It is possible that polyclonal B-cell proliferation can account for these higher antibody titers in the absence of reactivation.

Case-control studies may prove valuable in highlighting an association of HHV-6 with disease, although again there may be an inability to distinguish between primary infection and reactivation. The higher antibody titers found in young children and also in females indicate the need to adjust for age and sex in analyses of serologic data.

2.4.1. Exanthem subitum (roseola infantum)

Exanthem subitum (roseola infantum) or sixth disease is the only illness at the present time that is generally accepted as being caused in virtually all cases by HHV-6. This etiologic link, first documented by Yamanishi et al. (1988), was based on viral isolation and serology and has been supported by further studies (Takahashi et al., 1988; Enders et al., 1990). The epidemiology of roseola is of interest since this gives us insight into the epidemiology of HHV-6 as well. Exanthem subitum is the most common exanthem seen in infants under 2 years of age. Although a year-round incidence has been observed (Zahorsky, 1913), literature reviews suggest the lowest incidence is in December (Breese, 1941; Clemens, 1945; Juretić, 1963). It would appear that close contact favors onset of disease since outbreaks occur where infants share close quarters (Cushing, 1927; Barenberg and Greenspan, 1939; James and Freier, 1948/1949). The peak prevalence of roseola infantum is from 7 to 13 months of age and the disease is rarely seen after the age of 4 years (Cherry, 1987); the striking similarity between the age incidence of roseola infantum and the age at which HHV-6 seroconversion occurs provides supportive evidence for the viral role in this disease. The disease has been reported in adults during an outbreak (James and Freier, 1948/1949), but other agents such as coxsackie and echoviruses can provide a similar presentation (Cherry, 1987), so that such occurrences (and indeed all studies of roseola) should now be associated with specific serologic markers. Clinicians should also be aware of 'exanthem subitum without rash', an incongruous term but a syndrome also apparently due to primary HHV-6 infection (Suga et al., 1989). The presence of maternal antibody to HHV-6 probably is protective since the disease is rarely, if ever, seen before the age of 6 months. The incubation period in epidemics has been reported to be 5–15 days (Cushing, 1927; Barenberg and Greenspan, 1939; Breese, 1941) and there appears to be no predilection by sex (Cherry, 1987).

2.4.2. Infectious mononucleosis (IM)

The second illness where evidence points to an etiologic relationship is infectious mononucleosis (IM). Most cases of IM are clearly caused by EBV (Henle et al., 1968; Niederman et al., 1968), but some EBV-negative cases have been associated with IgM antibody to HHV-6 (Bertram et al., 1989; Irving and Cunningham, 1990; Steeper et al., 1990). The associations with exanthem subitum and IM demonstrate the parallel between HHV-6 and EBV. Most people are infected in childhood and infancy and apparently have a subclinical or self-limiting disease but the few who escape infection until adolescence or adulthood could have more serious consequences such as IM.

2.4.3. Hepatitis

An acute self-limiting case of hepatitis has been reported in a 65 year old woman with a previous diagnosis of non-A, non-B hepatitis in the course of a primary HHV-6 infection (Irving and Cunningham, 1990). Evidence for the diagnosis of primary HHV-6 infection included a rise in IgM antibody to HHV-6 from undetectable levels 18 days after the onset of the symptoms to a titer of 80 at 40 and 67 days post-onset. IgG antibody to HHV-6 rose from 8 to 256 and no IgM antibody to CMV or EBV could be detected. Other reports of HHV-6-associated hepatitis in adults (Dubedat and Kappagoda, 1989; Ward et al., 1989; Steeper et al., 1990) and neonates (Asano et al., 1990; Tajiri et al., 1990) emphasize the importance of considering HHV-6 as a potential etiologic agent in cases of non-A, non-B hepatitis.

2.4.4. Chronic fatigue syndrome/myalgic encephalitis (CFS/ME)

Antibody titers to HHV-6 are elevated in chronic fatigue syndrome (CFS) (Levine et al., 1989). Because CFS/myalgic encephalitis (ME) is a syndrome designating a variety of illnesses (Levine, 1992) with apparent links to EBV (Jones and Straus, 1987), enteroviruses (Archard et al., 1988) and other putative viral and nonviral infections (Cluff et al., 1959; Levine et al., 1989; DeFreitas et al., 1991), it is likely that only a relatively small proportion of CFS/ME cases could be directly attributed to HHV-6. In one outbreak of CFS (Holmes et al., 1987), elevated antibody titers were reported to EBV, rubella and other viruses and, therefore, it is likely that the elevated HHV-6 antibody titers in CFS either reflect secondary viral reactivation or polyclonal B cell activation and enhanced antibody response in the absence of viral replication.

2.4.5. Hematopoietic malignancies

There have been a limited number of studies investigating HHV-6 serology in lymphoid malignancies. Increased HHV-6 seroprevalence and higher antibody titers have consistently been found in Hodgkin's disease (HD, Ablashi et al., 1988; Biberfeld et al., 1988; Clark et al., 1990). Other malignancies in which elevated

HHV-6 antibody levels have been identified include African BL, acute lymphoblastic leukemia (ALL, Ablashi et al., 1988), acute myeloid leukemia and low-grade non-Hodgkin's lymphoma (Clark et al., 1990). Only one of these reports was of a case-control study (Clark et al., 1990). This report also included adjustments for age and sex in the statistical analyses which, as discussed earlier, may be confounding variables. Interestingly, a more recent case-control study of HD and ALL, utilizing both an ELISA and IFA, found that the high titers in ALL were due to the young age of ALL patients and showed there was no association with the disease (Levine et al., submitted). This highlights the confounding effect age has on analyses of HHV-6 serology. This study also showed lower titers in the HD patients than controls by both assays, all HD patients being untreated, therefore emphasizing the possible importance of therapy as well as disease stage and histological subtype on interpretation of serologic data (Levine et al., 1992b).

The relevance of raised antibody titers in particular malignancies is unclear. Elevated antibody titers to EBV have also previously been reported in lymphoproliferative neoplasms (Henle et al., 1971, 1973a; Levine et al., 1971a,b). Reactivation of these viruses may occur more frequently in patients as a consequence of their malignancy including subsequent treatment for the condition.

As regards HHV-6, molecular studies do not support an etiological role in acute myeloid leukemia and the majority of non-Hodgkin's lymphomas (Jarrett et al., 1988; Josephs et al., 1988). It has been hypothesized that Hodgkin's disease in young adults may result from an abnormal response to late infection by a common virus (Gutensohn and Cole, 1980). HHV-6 antibody levels have been reported to be higher in young Hodgkin's disease patients who are likely to have had reduced social contact early in life, perhaps delaying the time of exposure to common infectious agents (Clark et al., 1990). Again molecular studies would suggest against the involvement of HHV-6 in the etiology of Hodgkin's disease (Jarrett et al., 1988; Josephs et al., 1988), but HHV-6 serology in such persons may act as a marker for delayed infection by another virus involved in the initiation of this disease, or be a reflection of the disease process (Levine et al., 1992b).

2.4.6. Autoimmune disorders

Kawasaki disease is an acute febrile vasculitis of infancy and early childhood marked by prolonged fever, rash and other characteristic features (Rowley et al., 1988). One study has shown increased seroprevalence and higher HHV-6 IgG antibody titers in this condition (Okano et al., 1989). However, only 1 of 18 seropositive patients in this report was positive for IgM and negative for IgG antibody, characteristics of a primary infection. Also, 4 of the 22 patients were HHV-6 seronegative suggesting that the increased titers may have been a consequence of the illness rather than HHV-6 directly causing Kawasaki disease. Marchette et al. (1990) also failed to note an association of HHV-6 with Kawasaki disease in Hawaii.

Sjögren's syndrome has also been associated with HHV-6 (Fox et al., 1989). This is an autoimmune condition characterized by lymphocytic infiltration of salivary

and lacrimal glands. Using a combination of assays consisting of Western blots and radioimmunoprecipitation of antigen, Ablashi et al. (1988) reported an increased HHV-6 seroprevalence in Sjögren's syndrome. HHV-6 DNA sequences and viral antigens have been detected in the major and minor salivary glands of persons without Sjögren's syndrome (Fox et al., 1990a; Krueger et al., 1990) suggesting salivary glands are a normal site of HHV-6 replication. Whether HHV-6 plays a role in Sjögren's syndrome is unknown, but the identification of HHV-6 DNA sequences in tumor tissue from 2 patients with lymphoma occurring in the context of Sjögren's syndrome (Jarrett et al., 1988; Josephs et al., 1988) makes this an interesting possibility.

2.4.7. AIDS

HHV-6 has frequently been isolated from patients with acquired immunodeficiency syndrome (AIDS) (Salahuddin et al., 1986; Downing et al., 1987; Tedder et al., 1987; Lopez et al., 1988; Levy et al., 1990b) and *in vitro* studies suggest that HHV-6 enhances cell death of human immunodeficiency virus (HIV) infected cells, possibly by transactivation of the viral long terminal repeat (Lusso et al., 1988; Ensoli et al., 1989; Horvat et al., 1989). No serologic studies of HHV-6 involving AIDS patients and controls have suggested a clinically important relationship, however, and at least one with negative results has been published (Spira et al., 1990).

2.5. Discussion

In the 5 years since HHV-6 was first isolated, information has been accumulating rapidly in regard to the distribution and pathogenicity of this ubiquitous virus. Some studies report contradictory findings, but it is apparent that infection with HHV-6 usually occurs in early childhood, the virus is pathogenic and is the primary etiologic agent of exanthem subitum, and that antibody titers are determined in part by age (lower in adults than children) and sex (lower in males than females). Other illnesses, such as infectious mononucleosis and hepatitis, are less frequent outcomes resulting from HHV-6 infection, and the link to cancer and AIDS remains tenuous. Furthermore, there appears to be population-based differences in frequency of exposure and antibody titers that remain to be explored. The frequent detection in saliva, suspected as being the primary vector of transmission, suggests similarities to the fifth human herpesvirus isolated, EBV.

The parallels between HHV-6 and EBV are of great importance in understanding the gaps in our current information about HHV-6. Both have been documented as causing human disease, HHV-6 being the primary cause of roseola and EBV being the primary cause of IM. EBV produces tumors in nonhuman primates (Epstein et al., 1973; Sundar et al., 1981) and appears to be a necessary factor in most cases of BL (De-Thé et al., 1978; Barriga et al., 1988) and NPC (Old et al., 1966; Henle et al., 1973b; Raab-Traub et al., 1987). HHV-6 has been detected in

some human tumors (Jarrett et al., 1988; Josephs et al., 1988) but in a less consistent fashion and the serologic associations with cancer have an uncertain significance. Recently, animal studies have suggested an oncogenic potential for HHV-6 (Razzaque, 1990) but, unlike EBV, tumors have not been produced by inoculation solely with active virus.

Another important parallel between EBV and HHV-6 are the assays currently being utilized for sero-epidemiology. If the immune response to EBV is relevant to HHV-6 (and there are obvious differences, such as the rising titers with age for EBV and the declining titers for HHV-6), there is still much to be learned about possible neutralizing or membrane-associated antibodies, antibodies to early antigens, etc.

Herpesviruses are more complex than retroviruses and the time-scale for understanding disease associations is therefore likely to be much longer. There was a 4 year interval between the discovery of EBV (Epstein et al., 1964) and its first association with IM (Henle et al., 1968). Another 10 years passed until a prospective study showed it to be the likely cause of BL (De-Thé et al., 1978), and it took 23 years from the first isolation to show it to be present in all cases of NPC, not just the poorly differentiated cases (Raab-Traub et al., 1987). We are still in the early years of HHV-6 research and the next decades should provide interesting findings.

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Ablashi/Krueger/Salahuddin (eds) Human Herpesvirus-6

CHAPTER 3

Target cells for infection

PAOLO LUSSO

Laboratory of Tumor Cell Biology, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892, U.S.A.

3.1. Introduction

Human herpesvirus-6 (HHV-6) was first identified in 1986 by S.Z. Salahuddin, D.V. Ablashi, R.C. Gallo and colleagues in peripheral blood mononuclear cells (PBMCs) of 6 patients affected by various lymphoproliferative disorders (Salahuddin et al., 1986). The main common feature among these patients was a status of profound immune suppression, either inherent to the underlying disease or of iatrogenic origin. The novel herpesvirus was able to replicate in primary human umbilical cord blood lymphocytes and was provisionally designated human B lymphotropic virus (HBLV). Consistent with the isolation from peripheral blood and the growth in lymphocytes, hematopoietic cell lineages were the first to be investigated as potential targets for HHV-6 infection. However, the original studies on the viral tropism were mainly based on the characterization of limited numbers of infected cells obtained directly from patients blood, whereas the propagation of HHV-6 in culture was still quite inefficient. A critical technical advancement was achieved with the establishment of optimized culture conditions for the propagation of the virus in human mononuclear cells (Lusso et al., 1987), whereby purified populations of infected cells could be obtained. Subsequent immunological and molecular studies conclusively demonstrated that, although cells belonging to other lineages are infectable, $CD4^+$ T lymphocytes represent the major *in vitro* target for HHV-6 infection (Fig. 3.1).

Following the initial isolation, HHV-6 has been recovered again from cultured peripheral blood cells, albeit mostly from immunocompromised individuals (Downing et al., 1987; Tedder et al., 1987; Agut et al., 1988; Lopez et al., 1988; Becker et al., 1989). By molecular analysis with the polymerase chain reaction

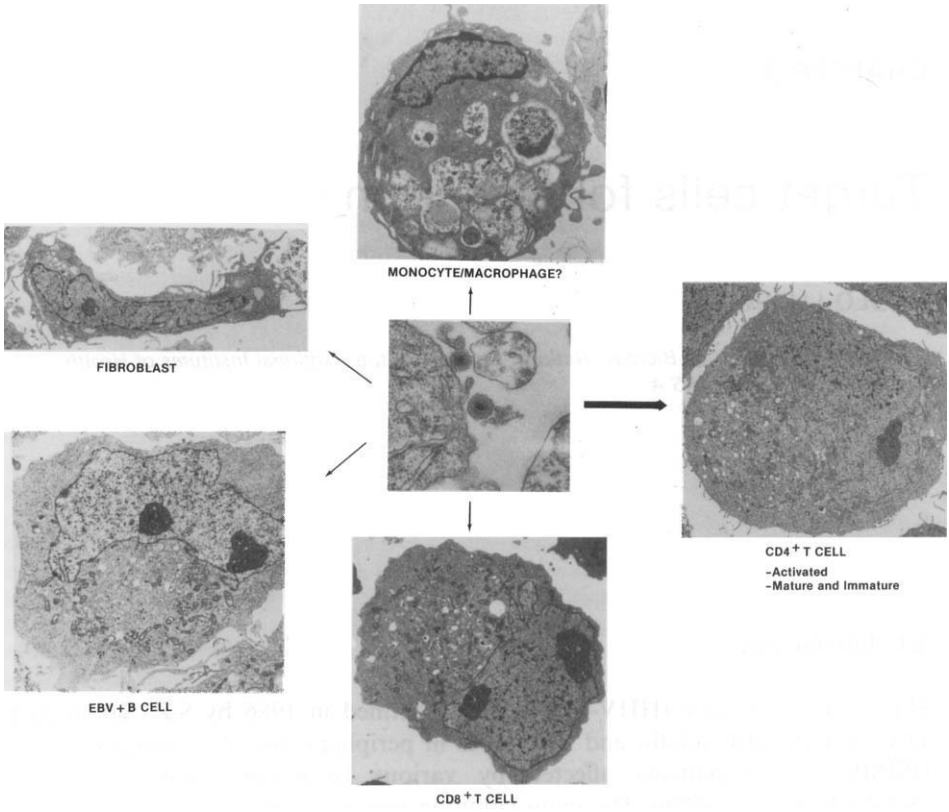

Fig. 3.1. *In vitro* cellular tropism of HHV-6.

(PCR), however, HHV-6 is detected very frequently in peripheral blood of both normal and diseased individuals (Buchbinder et al., 1988; Gopal et al., 1990) and it has been suggested that circulating lymphocytes may represent a site for viral latency. Thus, a discrepancy seems to exist between the PCR findings and the considerable difficulties experienced by many investigators in isolating the virus from peripheral blood. A likely explanation for this phenomenon is the ability of immune mechanisms harbored in peripheral blood to suppress virus expression both *in vivo* and *in vitro*. These are mainly constituted by specific MHC-restricted cytotoxic T lymphocytes (CTLs) and nonspecific, non-MHC-restricted anti-viral effectors, e.g., natural killer (NK) or lymphokine-activated killer (LAK) cells. In contrast to peripheral blood, a generous source of HHV-6 isolates has been human saliva (Pietroboni et al., 1988; Levy et al., 1990b), where anti-viral cellular immunity is less represented. The HHV-6 recovered from saliva is likely to be shed by parenchymal epithelioid cells within the salivary glands, which were demonstrated to harbor the virus and to express viral antigens *in vivo* (Fox et al., 1990).

In most of the cellular systems studied to date, HHV-6 induces a dramatic cytopathic effect, often leading to massive intracultural cell death. In a limited

number of cell types, slower kinetics of cytopathicity can be observed (see below), but no models of persistent, noncytopathic, or latent HHV-6 infection have been described so far. The cytopathic effect induced by HHV-6 in mononuclear cells is closely correlated with the extent of virus replication and presents some typical features. 2–4 days post-infection, a proportion of the cells starts to exhibit peculiar cytomorphological alterations, becoming homogeneously rounded, enlarged in size and refractile. Each cell usually contains 1–2 nuclei, while giant multinucleated syncytia are rarely, if ever, observed. Intranuclear and intracytoplasmic inclusion bodies can be frequently seen. Once the morphological changes have occurred, HHV-6-infected cells have a limited life expectancy in culture (2–5 days).

Most of the initial studies on the cellular tropism of HHV-6 were performed using the prototype isolate named HHV-6_{GS} (Salahuddin et al., 1986). The recent characterization of novel HHV-6 strains has indicated that a certain degree of heterogeneity exists in the cellular host range and other biologic features among different isolates (see Ablashi and Salahuddin, Chapter 7, this volume). However, such differences are mostly restricted to the ability to infect established cell lines of neoplastic origin, whereas the growth characteristics in normal mononuclear cells are rather conserved among the various HHV-6 isolates.

3.2. Role of cellular activation

Cellular activation is required to induce the susceptibility of T lymphoid cells to HHV-6 (Lusso et al., 1989b). To maintain lymphoid cells in a quiescent state, it is critical to avoid any possible exposure to foreign antigens: the simple culture at 37°C in the presence of bovine serum may be sufficient to trigger cellular activation. When freshly isolated human PBMCs are infected with HHV-6 in the complete absence of activatory signals, no infection can be documented, even if the cells are subsequently activated. By contrast, PBMCs pre-activated *in vitro* for at least 4 h at 37°C, as well as T lymphocytes propagated in culture for up to one month in the presence of interleukin-2 (IL-2), can be efficiently infected by HHV-6 even using low virus titers. The activation signal for T lymphocytes can be provided by any of several stimuli, such as phytohemagglutinin (PHA), the combination of phorbol myristate-13-acetate (PMA) and a calcium ionophore (e.g., A23187), mitogenic anti-CD3 or anti-T cell receptor (TCR) monoclonal antibodies (mAb) in the presence of accessory cells, allogeneic stimulation, etc. These studies strongly suggest that the putative receptor for HHV-6 is not expressed on the surface membrane of resting lymphocytes and should be looked for among the activation-associated antigens. In addition, activation seems to be required for the efficient replication of HHV-6 in T cells (Frenkel et al., 1990).

3.3. $CD4^+$ T lymphocytes

Following initial debate over the lineage origin of HHV-6-infectable cells, extensive immunological and molecular characterizations have demonstrated that $CD4^+$ T

lymphocytes represent the predominant target cell for HHV-6 infection both *in vitro* (Lusso et al., 1987, 1988) and *in vivo* (Takahashi et al., 1989). However, the CD4 glycoprotein, which serves as the major receptor structure for human immunodeficiency virus (HIV), another virus with a tropism for $CD4^+$ T lymphocytes, is not implicated in the receptor mechanism for HHV-6 (Lusso et al., 1989b).

To identify the target cell for infection by HHV-6, strain GS, mononuclear cell populations from diverse normal human tissues, i.e., umbilical cord blood, adult peripheral blood, bone marrow, thymus, lymph node and tonsil, were studied (Lusso et al., 1988). After infection, the cells were cultured in the absence of exogenous IL-2 since *in vitro* proliferation studies suggest that HHV-6-infected cells do not respond to this cytokine. Instead, IL-2 can suppress the replication of HHV-6 in cord blood mononuclear cells *in vitro* (Roffman and Frenkel, 1990; Lusso et al., unpublished findings). Infected cultures were monitored for the appearance of the typical morphologic changes and for the presence of intracellular HHV-6 antigens by indirect immunofluorescence analysis using specific mAbs developed by Balachandran et al. (1989). The efficiency of infection, as measured by the ratio between the number of cells initially exposed to the virus and the absolute number of cells expressing HHV-6 antigens at day 8 post-infection, varied among different mononuclear cell sources. Cord blood was more efficient than peripheral blood, most likely reflecting the low proportion of anti-viral effectors in the newborn rather than a higher proportion of susceptible cells. A similar finding was reported for another HHV-6 strain, Z-29 (Black et al., 1989). Thymic mononuclear cells were among the most efficient in supporting virus replication.

Table 3.1 summarizes the immunophenotypic characteristics of HHV-6-infected cells in human cord blood cultures. The vast majority of these cells expresses phenotypic features of activated $CD4^+$ T lymphocytes, exhibiting CD2, CD4, CD5, CD7, CD71 and, in a limited percentage, CD8, CD15 and class II MHC antigens (DR). It is noteworthy that the CD3/TCR complex and the IL-2 receptor (CD25) are not detectable on the surface membrane of terminally infected cells. The absence of the surface CD3/TCR complex, together with the frequent co-expression of CD4 and CD8, could indicate a condition of cell immaturity (cortical thymocyte phenotype), but more likely represent phenotypic alterations induced by specific virus–cell interactions. Indeed, the expression of mRNA of the α- and β-chains, but not of the γ-chain, of the TCR points to a mature stage of differentiation of HHV-6-infected cells. Consistently, studies with T cell clones (see below) conclusively demonstrated that mature T cells are infectable by HHV-6. However, at least 2 lines of evidence suggest that also immature T cells are susceptible to HHV-6: (a) after removal of the $CD3^+$ fraction from enriched T cell populations, efficient HHV-6 replication is still detectable, and (b) infection of thymic mononuclear cells (containing more than 80% immature elements) yields high levels of infection. Further studies on enriched cellular subpopulations from human cord blood demonstrated that virtually all HHV-6-susceptible cells are contained in the CD2 $(pan\text{-}T)^+$ mononuclear cell population (Lusso et al., 1988).

The cellular host range of an HHV-6 strain derived from an infant with exanthem subitum (HST) has also been studied in detail. Infected cells were

TABLE 3.1.
Phenotypic profile of mononuclear cells from normal human umbilical cord blood 8 days post-infection by HHV-6 *in vitro*

Antigen	mAb	Positive cells
T cell associated:		
CD1	OKT6	< 1%
CD2	OKT11	> 95%
CD3	OKT3	< 1%[a]
CD4	Leu3a	85%
CD5	Leu1	90%
CD7	Leu9	72%
CD8	Leu2a	15%
TCR	WT31	< 1%[a]
T cell activation markers:		
MHC class II	OKDR	15%
CD15	LeuM1	15%
CD25 (IL-2 R α-chain)	Becton Dickinson	< 1%
IL-2 R β-chain	Endogen	< 1%
CD38	OKT10	> 95%
CD71	OKT9	> 95%
	Ta1	> 95%
B cell associated:		
CD10, CD19, CD20, CD21, SIg, CIg	OKBcALL, Leu12, Leu16 CR2, antiIgG/IgM	< 1%
Recognition molecules:		
MHC class I	Various	> 95%
β_2-microglobulin	Various	> 95%
CD11a	LFA-1 (α-chain)	> 95%
CD18	LFA-1 (β-chain)	> 95%
CD54	ICAM-1	> 95%
CD58	LFA-3	> 95%
CD49a	VLA-1	> 75%
Others[b]	Various	< 1%

[a] A weak signal was observed only at the cytoplasmic level when acetone-fixed cells were stained.
[b] LeuM2, LeuM3, LeuM5, OKM1, OKM5, MY7, MY9, DRC, Ki1, Leu7, Leu11b, Leu19, CR1, CR3, HPCA-1.

characterized as $CD4^+$ T cells, while the $CD8^+$ fraction was very poorly, if ever, infectable (Takahashi et al., 1989). In contrast with the findings obtained with the GS strain, the CD3 and CD25 antigens were expressed on the surface membrane of infected lymphocytes. This discrepancy may be related to the different virus strain used or, alternatively, to the different timing chosen for phenotypic analysis following exposure to the virus. Indeed, the CD3/TCR complex and the IL-2

receptor are progressively down-regulated in HHV-6-infected cells, as demonstrated in $CD3^+$ T cell clones (Lusso et al., 1991). Another report describing the *in vitro* cellular tropism of HHV-6, strain Z-29, surprisingly identified the susceptible T cell population within the $CD1^+$ mononuclear cell subset (Lopez et al., 1988).

It is noteworthy that the predominant $CD4^+$ T lymphotropism of HHV-6, together with its direct cytocidal effect, represented the first observations suggesting a possible role of HHV-6 as a co-factor in AIDS (Lusso et al., 1989a). The interactions between HHV-6 and HIV will be discussed in detail by Lusso et al. (Chapter 10, this volume).

3.4. $CD8^+$ T lymphocytes

Despite the predominant tropism for $CD4^+$ T cells, expression of the CD8 antigen in a fraction of infected lymphocytes was recognized since the original studies on the cellular host range of HHV-6. In subsequent investigations, human peripheral blood T cells were infected with the GS strain after fractionation of the $CD4^+$ and $CD8^+$ subpopulations. Although the time-course of HHV-6 infection was more rapid in enriched $CD4^+$ T cells, consistent levels of infection were achieved also in the $CD8^+$ cultures (Lusso et al., 1991), as also reported by Levy et al. (1990a) using the SF isolate obtained from the saliva of an HIV-infected individual. In contrast, Takahashi et al. (1989), using the HST isolate, were able to detect only a very limited, if any, expression of HHV-6 in enriched normal peripheral blood $CD8^+$ T cells.

A clonal approach was also employed in our laboratory to more definitively assess the relative susceptibility of the $CD4^+$ and $CD8^+$ T cell subsets to HHV-6. A number of T cell clones obtained by the limiting dilution technique from normal adult PBMCs as described by Moretta et al. (1983) were expanded in the presence of IL-2. Both $CD4^+$ and $CD8^+$ T cell clones were productively infectable by HHV-6, exhibiting similar kinetics of virus expressions and cytopathic effects. Since the clones were constituted by fully differentiated T cells, this study also provided definitive evidence that mature T cells are susceptible to HHV-6.

3.5. B lymphocytes

Most investigators have been unable to document HHV-6 replication in normal human B lymphocytes. When PBMCs were depleted of T lymphocytes using a pan-T marker (e.g., CD2) and then activated with PHA, LPS or other B cell mitogens, virtually no signs of productive infection could be documented (Lusso et al., 1988). Consistently, HHV-6 was not identified *in vivo* in peripheral blood B cells of infected individuals (Takahashi et al., 1989). However, a number of EBV-transformed B lymphoblastoid cell lines can be infected by HHV-6 with variable efficiency. For example, an immature B cell clone (ET_{62}), obtained by limiting dilution from the cell line WIL2, is highly susceptible to productive infection by

HHV-6, whereas only a minority of the cells were infectable in the parental cell line. Another example of EBV^+ B cell line infectable by HHV-6 is Raji, which contains a defective EBV genome. We can hypothesize that the putative receptor(s) for HHV-6 is (are) induced on the surface of normal B cells following EBV infection or as a consequence of *in vitro* immortalization. Alternatively, the simultaneous presence of EBV in the cell may create a favorable milieu for HHV-6 expression, possibly overcoming putative intracellular restriction factor(s).

3.6. Mononuclear phagocytes

The role of mononuclear phagocytes in HHV-6 infection is still unclear. No evidence for virus replication is seen when freshly elutriated monocytes from normal peripheral blood or purified cultures of monocyte-derived macrophages are exposed *in vitro* to HHV-6, strain GS. Only in rare circumstances, after *in vitro* infection of unfractionated PBMCs, electron microscopy has demonstrated the presence of HHV-6 particles within cells exhibiting a macrophage-like morphology. However, it is difficult, on a mere morphological basis, to rule out a simple uptake by the macrophages of viral particles released by surrounding lymphocytes. Consistent with the lack of infection of primary mononuclear phagocytes, a number of permanent cell lines of pro-monocytic origin (e.g., U937, THP-1) are not productively infectable by any of the HHV-6 strains tested (Tedder et al., 1987; Black et al., 1989; Lusso, unpublished findings). Only one HHV-6 isolate (SF), obtained from an HIV-infected individual, was suggested to replicate, albeit at a low level, in primary human macrophage monolayers (Levy et al., 1990a).

Despite the limited infection observed in mature monocytic cells, immature myeloid/monocytic precursors may be infectable *in vivo*, as suggested by the transient neutropenia frequently associated with the acute phase of exanthem subitum. Consistent with this observation, an immature megakaryoblastoid cell line (HEL) was found relatively susceptible to infection by $HHV\text{-}6_{GS}$ (Ablashi et al., 1988b) (Table 3.2). Preliminary data from our laboratory suggest that the ability to form bone-marrow-derived colonies is reduced by exposure of PBMCs to HHV-6 prior to seeding into agar plates in the presence of the relevant colony stimulating factor (see also Carrigan, Chapter 21, this volume).

Recently, Kondo et al. (1991) have reported the detection of latent HHV-6 in circulating monocyte/macrophages from exanthem subitum patients during the convalescence phase. These results strongly suggest that cells of the mononuclear phagocytic system are an important target for HHV-6 infection *in vivo*.

3.7. Fibroblasts

Both primary and immortalized human fibroblasts are infectable, albeit with a low efficiency, by $HHV\text{-}6_{GS}$. Consistent with the slow time-course of infection, both the

TABLE 3.2.
Susceptibility of continuous cell lines to the GS strain of HHV-6

Cell line	Lineage origin	Infectable cells (%)
Raji	B lymphoid	5–10
McM1	B lymphoid	< 1
WIL2	B lymphoid	1–5
ET 62	B lymphoid	> 90
HSB_2	T lymphoid	> 90
MOLT-3	T lymphoid	10–30
MOLT-4	T lymphoid	40–60
CEM	T lymphoid	10–20
Jurkat	T lymphoid	> 90
SupT1	T lymphoid	70–90
Reh	T lymphoid	40–50
ST4	T lymphoid	> 90
H9	T lymphoid	< 1
Hut78	T lymphoid	10–30
MT-2	T lymphoid	5–20
U937	Pro-monocytoid	< 1
THP-1	Pro-monocytoid	< 1
K562	Myeloid/erythroid	< 1
HEL	Myeloid/megakaryoblastoid	15–40
HTB-14	Glioblastoid	2–8
A204	Rabdhomyoblastoid	1–5
HeLa	Epithelioid	1–2

yield of extracellular virus and the cytopathic effects induced by the virus are limited. Starting 8–15 days post-infection, rare cytopathic figures similar to those induced by human cytomegalovirus (HCMV) can be detected in primary human lung fibroblasts passaged several times *in vitro* (Lusso et al., unpublished findings and Chapter 10, this volume). By immunofluorescence analysis with mAbs or human positive sera, both cytoplasmic and nuclear positivity was observed, but the proportion of positive cells remained lower than 5%. Moreover, the course of infection in fibroblasts was not progressive and both the cytotoxic figures and the immunofluorescence positivity slowly declined to eventually disappear 4–6 weeks after exposure to the virus.

In contrast to $HHV\text{-}6_{GS}$, neither the Z-29 nor the SF isolates seemed to yield significant levels of virus replication in fibroblasts (Black et al., 1989; Levy et al., 1990a).

3.8. Other cell lineages

Epithelial cells were recently suggested as a possible target cell for HHV-6 (Komaroff, 1990), but no conclusive data have been reported so far. Nonetheless,

the presence of HHV-6 in the specialized epithelium of the salivary glands (Fox et al., 1990) provides compelling evidence that this lineage is susceptible to the virus, at least *in vivo*. It is interesting to notice that the Epstein–Barr virus (EBV), a herpesvirus which may be involved as a tumorigenic factor in nasopharyngeal carcinoma, also exhibits a dual tropism for lymphocytes and epithelial cells *in vivo*. In our laboratory, very limited virus replication was observed when a neoplastic epithelial cell line (HeLa) was exposed *in vitro* to the HHV-6_{GS} strain.

Little information is currently available regarding muscle cells, both *in vivo* and *in vitro*. Only occasional HHV-6-replicating cells could be observed in our laboratory after infection of a neoplastic skeletal muscle cell line (A204).

A number of cell lines of neural origin were analyzed for their susceptibility to HHV-6_{GS} (Ablashi et al., 1988b). For example, a glioblastoma cell line, HTB-14, exhibited a persistent low-level infection with little evidence for cytopathicity (Table 3.2). By contrast, several other viral strains were found to be unable to replicate in cells of neural origin. Only Levy et al. (1990a) have reported a low, albeit persistent, level of HHV-6_{SF} replication in a neuroblastoma cell line (SK-N-MC).

Recent findings in our laboratory indicate that Natural Killer (NK) cells represent another possible target for HHV-6. A number of $CD3^-CD56^+$ clones derived from normal adult peripheral blood and displaying cytolytic activity against the K562 cell line were productively and cytopathically infected by HHV-6, strain GS. This observation provides the first evidence that NK cells, which may represent the first line of defense against viruses *in vivo*, can be targeted and killed by a viral agent. This is also a possible mechanism whereby HHV-6 may reduce the efficacy of the natural antiviral immunity.

3.9. Established cell lines

Several neoplastic cell lines, mostly exhibiting a $CD4^+$ T cell phenotype, are permissive to productive infection by HHV-6 and some of them have been utilized for the large-scale propagation of the virus (Ablashi et al., 1988a,b). The principal cell lines susceptible to the HHV-6_{GS} strain are presented in Table 3.2, the most efficient virus-producers being HSB_2, Jurkat, and SupT1. HSB_2 exhibits a very early T cell phenotype ($CD2^-CD3^-CD4^-CD5^+CD7^+$). SupT1, which displays a $CD4^+CD8^+$ phenotype, appears to be more resistant than other cell types to the cytopathic effect of HHV-6: significant levels of viral replication can persist for prolonged periods in these cells without a consistent loss of viability of the culture that ultimately may completely clear the infection after several passages.

It is noteworthy that different HHV-6 isolates display different growth characteristics in established cell lines. For example, the AJ strain from Gambia (Tedder et al., 1987) grows well in J-JHAN and C8166, apparently with 'syncytia' formation, the UI102 strain from Uganda (Downing et al., 1987) in J-JHAN and HSB_2 (Wyatt et al., 1990), while the Z-29 strain from Zaire (Lopez et al., 1988) is unable to grow significantly in any of the T cell lines tested, with the exception of MOLT-3

TABLE 3.3.
Susceptibility of PBMCs from different species to infection by HHV-6_{GS} *in vitro*

Species	No. of subjects tested	Efficiency of infection[a]
Guinea pig (*Cavia porcellus*)	4	–
Chicken (*Gallus domesticus*)	12	–
Duck (*Anas platypedes*)	10	–
Rabbit (*Oryctolagus cuniculi*)	6	–
Cat (*Felix catus*)	2	–
Marmoset (*Callithrix jacchus jacchus*)	4	–
Tamarin (*Sanguinis labiatus*)	4	–
Macaque (*Macaca mulatta*)	13	–
Chimpanzee (*Pan troglodytes*)	7	0.38
Gibbon ape (*Hylobates lar*)	7	–
African green monkey (*Cercopithecus aethiops*)	11	–
Human (*Homo sapiens*)	> 500	0.45

[a] Calculated as the ratio between the absolute numbers of PBMCs exposed to HHV-6 and the absolute numbers of infected cells at day 8 post-infection. No IL-2 was added to the cultures following the exposure to the virus.

(Ablashi, unpublished observations) and the HTLV-I-transformed MT-4 (Black et al., 1989). The latter is also the only cell line sustaining the replication of HHV-6_{SF}, a strain that is unique in its ability to replicate better in lymphocytes derived from peripheral blood than in those derived from cord blood (Levy et al., 1990a).

Although conclusive studies are still lacking, it is possible that, similar to what can be observed in primary cell cultures, the cell cycle timing as well as the effect of activatory stimuli may significantly modulate the susceptibility to HHV-6 in established cell lines. In addition, virus transmission to continuous cell lines appears to be more efficient by cell-to-cell contact rather than by cell-free transmission. For example, a periodic co-culture of infected and uninfected cells represents the optimal way to maintain long-term viral expression in cell lines such as HSB_2 or Jurkat, which undergo a relatively rapid cytopathic effect.

3.10. Nonhuman models

The susceptibility to HHV-6 of PBMCs from several nonhuman species is summarized in Table 3.3. Among a considerable number of animals tested, including 6 nonhuman primate species, only chimpanzees were found to be susceptible to productive infection by HHV-6 *in vitro* (Lusso et al., 1990). This cross-species restriction is similar to the behavior of human CMV. As observed in humans, T lymphocytes are the predominant target cells for infection by HHV-6 in chimpanzee peripheral blood, although no significant preference for either $CD4^+$ or $CD8^+$ cells was seen, most likely reflecting the lower CD4/CD8 T cell ratio in these animals ($\sim$1.0). These results suggest that the chimpanzee may represent an interesting model system for the study of HHV-6 infection *in vivo*.

3.11. Conclusions

Further studies, particularly on normal primary cells, are warranted to identify the whole spectrum of the cellular tropism of HHV-6. This information will provide critical indications about the pathogenic consequences of chronic HHV-6 infection as well as suggestions about the possible sites acting as reservoirs for the virus *in vivo*. By inference from other herpes viral systems, it is likely that, after primary infection, HHV-6 is not completely cleared by the immune system, but may persist in a latent form in selected tissues. Unfortunately, no models are currently available of persistent non-cytopathic infection. The acquisition of additional knowledge on the virus–cell interactions will be crucial to address a number of pathophysiological questions related to HHV-6 infection.

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Ablashi/Krueger/Salahuddin (eds) Human Herpesvirus-6

CHAPTER 4

In vitro propagation systems for HHV-6

D.V. ABLASHI[1] and C.L. HUNG[2]

[1] *Laboratory of Cellular and Molecular Biology, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892, U.S.A.*, and [2] *Cell Science Institute, Pharmacia Diagnostic, Inc., Silver Spring, MD 20904, U.S.A.*

4.1. Introduction

The first isolation of HHV-6, previously known as HBLV, was made in 1986 in Dr. Robert Gallo's laboratory from the peripheral blood lymphocytes of AIDS patients and patients with other lymphoproliferative disorders including T and B cell lymphomas, acute lymphocytic leukemia and immunoblastic lymphoma (Salahuddin et al., 1986). The success in isolating HHV-6 could be attributed largely to our experience in establishing short- and long-term cultures of hematopoietic cells *in vitro*. For HHV-6 isolation, cultured peripheral blood lymphocytes were either PHA-stimulated and carried in the presence of IL-2 and fetal calf serum or co-cultivated with mitogen-stimulated human umbilical cord blood mononuclear cells. However, HHV-6 isolation from collagen vascular diseases (Table 4.1) did not require PHA stimulation with cord blood mononuclear cells (Krueger et al., 1991). Figures 4.1 and 4.2 show that large refractile cells appeared between 3–5 days, which were generally short-lived and mono- or binucleated. Many investigators had observed similar phenomena in their cultures but had not detected HHV-6 because they attributed their observations to Epstein–Barr virus (EBV) infection. Since our first report, investigators have begun to study such enlarged cells, resulting in reports of many new HHV-6 isolates throughout the world, derived from healthy individuals and patients with various diseases (Table 4.1). In the early stages, HHV-6 could only be cultured successfully in cord blood mononuclear cells to obtain sufficient quantities of infectious virus for epidemiological and molecular studies. Interestingly, unstimulated cord blood mononuclear cells could not be infected with HHV-6 with any of the isolates (Table 4.2). Using the indirect immunofluorescence assay (IFA) and HHV-6 antibody-positive serum, only very

TABLE 4.1.
Reports of HHV-6 isolations (see also Ablashi et al., 1991a,b)

Reference	Source/Patient
Salahuddin et al. (1986)	Aids, other lymphoproliferative and malignant diseases
Downing et al. (1987)	HIV +
Tedder et al. (1987)	HIV +
Becker et al. (1989)	Hairy cell leukemia/HIV +
Feorino et al. (1987)	HIV +
Lopez et al. (1988)	HIV +
Yamanishi et al. (1988)	Infants with roseola
Kikuta et al. (1989)	Children with exanthum subitum
Ablashi et al. (1988)	Chronic fatigue syndrome
Kaplan et al. (1988)	AIDS
Agut et al. (1988)	HIV-1, -2, AIDS
Pietroboni et al. (1988)	Normal donor's saliva
Carrigan et al. (1990)	Chronic lymphopenia, Legionnaires disease
Ward et al. (1989)	Liver, kidney, bone marrow transplant
Krueger et al. (1990)	Collagen vascular diseases, CFS and atypical lymphoproliferation
Levy et al. (1990)	Saliva, HIV +
Levy et al. (1990)	Saliva, healthy normals

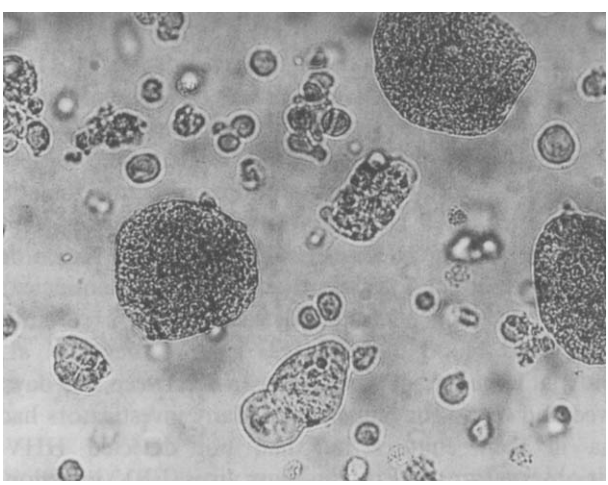


Fig. 4.1. *In vitro* culture of peripheral blood lymphocytes from a patient showing large cells.

few HHV-6 antigen-expressing cells could be detected in contrast to mitogen-stimulated mononuclear cells in which approximately 75% of the cells expressed antigens by 7 days post-infection. Frenkel et al. (1990) reported similar findings using 2 different isolates and suggested that T cell activation was required for replication of HHV-6. The virus did not replicate in quiescent peripheral blood

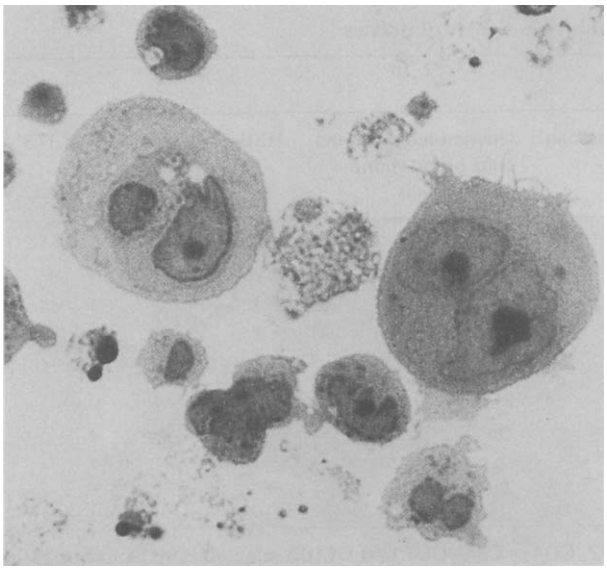


Fig. 4.2. *In vitro* culture of human cord blood mononuclear cells infected with HHV-6 (GS isolate) showing 2 large cells which are binucleated.

lymphocytes but replicated efficiently following exposure to the polyclonal mitogen PHA. They further showed the virus could not replicate in purified T cells treated with PHA in the absence of accessory cells unless exogenous IL-2 was added to the medium. Their studies further concluded that HHV-6 replication requires full progression of the cell cycle, a finding which may have implications for the pathogenesis of HHV-6 in humans. Still, there are some HHV-6 isolates which can only grow efficiently in cord blood mononuclear cells. It is important to find a continuous source of virus for which it is essential to identify and culture continuous cell lines that can support productive HHV-6 replication. This is particularly important since cord blood mononuclear cells and adult peripheral blood lymphocytes are known to be contaminated with other endogenous agents (i.e., cytomegalovirus [CMV], EBV).

Our earlier attempts to infect various hematopoietic cell lines of B and T cell origin and adherent cells (i.e., fibroblasts and epithelium) were not encouraging (Salahuddin et al., 1986). Similar difficulties were experienced by other investigators, thus hampering the progress of molecular studies of HHV-6 which require large quantities of purified virus. In 1987, we showed that some B and T cell lines and a cell line of neural origin, free of herpesviruses and retroviruses, could be infected with the GS strain of HHV-6 (Ablashi et al., 1988). Among these lines, the most productive infection was observed in the HSB_2 cell line, an immature T cell line expressing CD38 receptors. Moreover, HSB_2 cells expressed CD7, CD5 and CD15 receptors (Ablashi et al., 1988). The electron micrograph of HSB_2 cells

TABLE 4.2.
Infection of human cells *in vitro* with various HHV-6 isolates

HHV isolates[a]	Cell identification[b]				
	PHA-stimulated human cord blood mono-nuclear cells	Unstimulated human cord blood mono-nuclear cells	HSB_2[c]	SupT1[d]	MOLT-3[e]
GS	3+	±	3+	3+	±
Davilla	3+	±	3+	3+	±
CO3	3+	±	3+	3+	+
CO5	3+	±	2+	3+	±
CO8	3+	±	2+	3+	+
DA	3+	±	3+	3+	2+
Z-29	3+	±	−	−	3+
DC	3+	±	−	−	+
BA	3+	±	−	−	+
OK	3+	±	+	−	+

[a] The other isolates, i.e., CO1–CO2, CO4 > CO6, CO7 and U1102 infected HSB_2 and SupT1 in the same way as GS isolate.

[b] 3+: formation of large cells, showing ≥ 90% antigen expression with monoclonal antibodies (9A5D12, 7A2) and HHV-6_{GS} antibody-positive human serum.
2+: formation of medium to large cells, showing between 70 and 80% antigen expression with monoclonal antibodies and human serum.
+: very few large cells, showing between 2 and 5% antigen-positive cells with monoclonal antibodies and human serum.
±: occasional cells positive with monoclonal antibodies and human serum and, rarely, formation of large cells.
−: HHV-6 monoclonal antibodies and HHV-6 antibody-positive human serum did not react with uninfected cells when tested by IFA.

[c] HSB_2: immature T cell line.

[d] SupT1: immature T cell line.

[e] MOLT-3: mature T cell line.

infected with the DA strain of HHV-6 showed an abundance of extracellular virions, a majority of which were infectious particles (Fig. 4.3). Approximately 10^4 logs of infectious virus were obtained from HSB_2-infected cells in a period of 14 days. Productive infection of HHV-6 was also obtained with other isolates biologically similar to GS isolate (U1102, CO1–CO8, Davilla). Despite our success in replicating the GS strain of HHV-6 in HSB_2 cells, other isolates of HHV-6 such as Z-29, a CDC isolate (Table 4.1) from an AIDS patient from Zaire (Lopez et al., 1988) and an SF isolate from saliva of an HIV-1 positive patient (Levy et al., 1990) did not infect HSB_2 cells. Thus, our search for other cells capable of replicating Z-29 and SF and other refractory HHV-6 isolates continued.

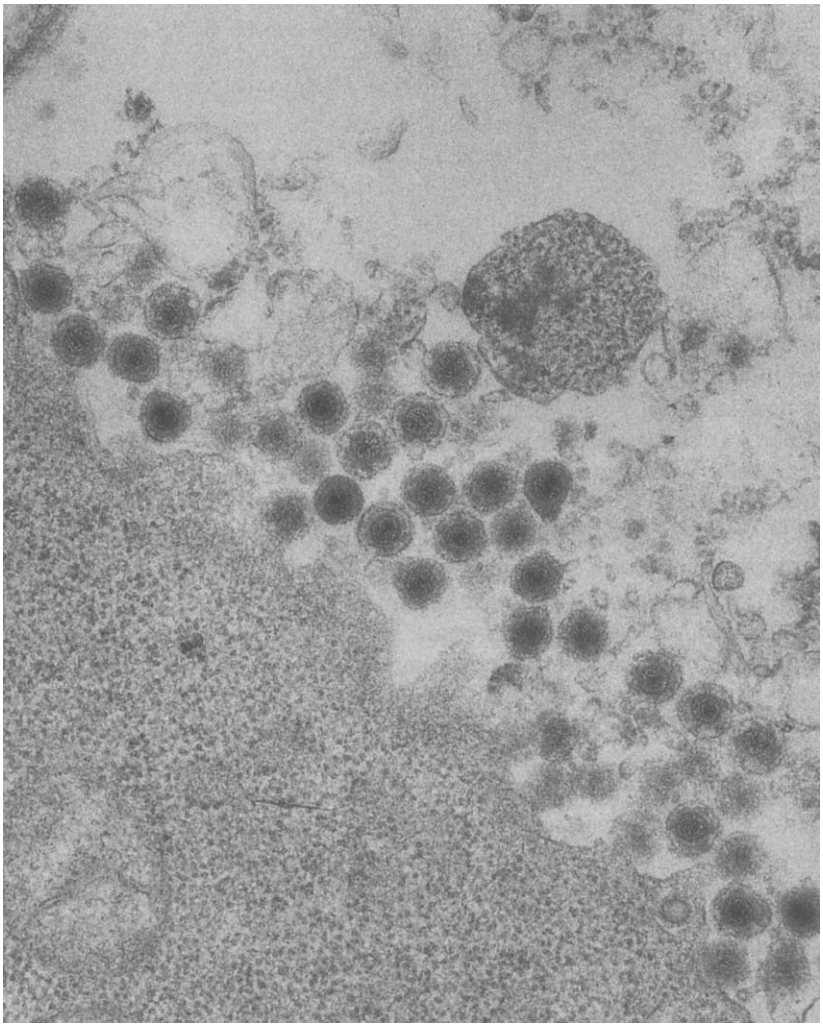


Fig. 4.3. Electron micrograph of HHV-6 (DA isolate) infected HSB_2 cells showing extracellular virions.

4.2. Propagation and productive infection of HHV-6 in continuous cell lines and primary cells

Downing et al. (1987) showed that cord blood mononuclear cells infected with their HHV-6 isolate after mitomycin-C treatment, co-cultivated with T cell lines (CEM, H9, Jurkat), B cell lines (Ramos, Raji) and monocyte macrophages (U937 and HL60) were infectable. The infected cells showed cytopathic effects (CPE) in

5–50% of the cell population, detected by IFA, by 1 week post-infection. However, no follow-up studies were carried out to show whether any of these cell lines could provide HHV-6 in quantities sufficient for biologic and molecular study. Tedder et al. (1987) also reported that their HHV-6 isolate U1102 infected varieties of B and T cells, and that CPE was observed between 3 and 6 days in the HFB-1, WMPT, K652, J JHAN and LV lines. Secondly, adherent cells such as HEL and HEB showed CPE between 6 and 21 days post-infection. They were unable to infect HL60 and U937, cell lines of monocytic origin. Molecular work on HHV-6 (U1102) by Honess and his associates was conducted with a virus produced in a T cell line (J JHANO). Becker et al. (1989) (Table 4.1) reported that their HHV-6 isolate could infect 2 cell lines (T191, a simian T lymphotropic virus type I transformed human cord blood cell lines, and T488, a CD8-positive cell line established from HTLV-I-transformed human cord blood cells), but did not infect Raji cells. Levy et al. (1990) reported a new strain of HHV-6 (HHV-6_{SF}) from the saliva of an HIV-infected person (Table 4.1). The host range of HHV-6_{SF} showed that peripheral blood mononuclear cells were more susceptible to viral infection than cord blood mononuclear cells. HHV-6_{SF} also infected chimpanzee primary T cells and could be propagated in MOLT-4 cells. None of the B and T monocyte/macrophage cell lines were susceptible to infection. Adherent cell lines of human and animal origin likewise did not support productive infection. Lusso et al. (1990) were able to infect primary chimpanzee cells with the GS strain of HHV-6. These data suggest that even though HHV-6 isolates can infect continuous cell lines of B and T cell origin, the yield of virus was extremely low and no infectivity titers were presented. Moreover, the data also shows that propagation of HHV-6 varied considerably between isolates.

Our experience working with different HHV-6 isolates over the years indicated that biological manipulations are necessary for productive infection of cells with HHV-6. Even though the GS strain of HHV-6 can readily infect HSB_2 cells (Table 4.2), productive virus infection is dependent upon 5 factors. (1) The ratio between infected and uninfected cells should be 1:9. If the number of uninfected cells is much greater, the yield is poor since there are too few infected cells to be surrounded by uninfected cells to cause efficient cell to cell contact for infection. (2) The treatment of cells with polybrene modifies the cell surface, thereby allowing more virus adherence and adsorption. Normally, treatment of cells for 2 h with polybrene (5 μg/ml) is sufficient for virus infection. (3) Incubation of cells with virus suspension for approximately 2 h at 37°C is sufficient for 90% virus adsorption by the cell. (4) Growth of the cells after infection should be slowed. This can be achieved by reducing the serum content and/or lowering incubation temperature. (5) The use of hydrocortisone in infected cultures usually resulted in earlier CPE and an increase in infectious virus titers (Salahuddin et al., 1986; Ablashi et al., 1988).

Morphologically, the infection of cells with HHV-6, regardless of the strain of cell type, leads to double or triple enlargement of cells (Fig. 4.1). Thus, infection can be easily tracked by observing the appearance of large cells in the culture following infection. Soundly, infection can be confirmed by testing cells by IFA using HHV-6

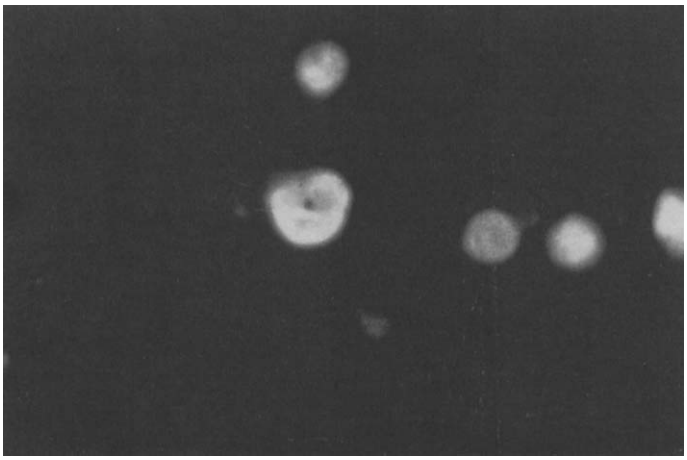
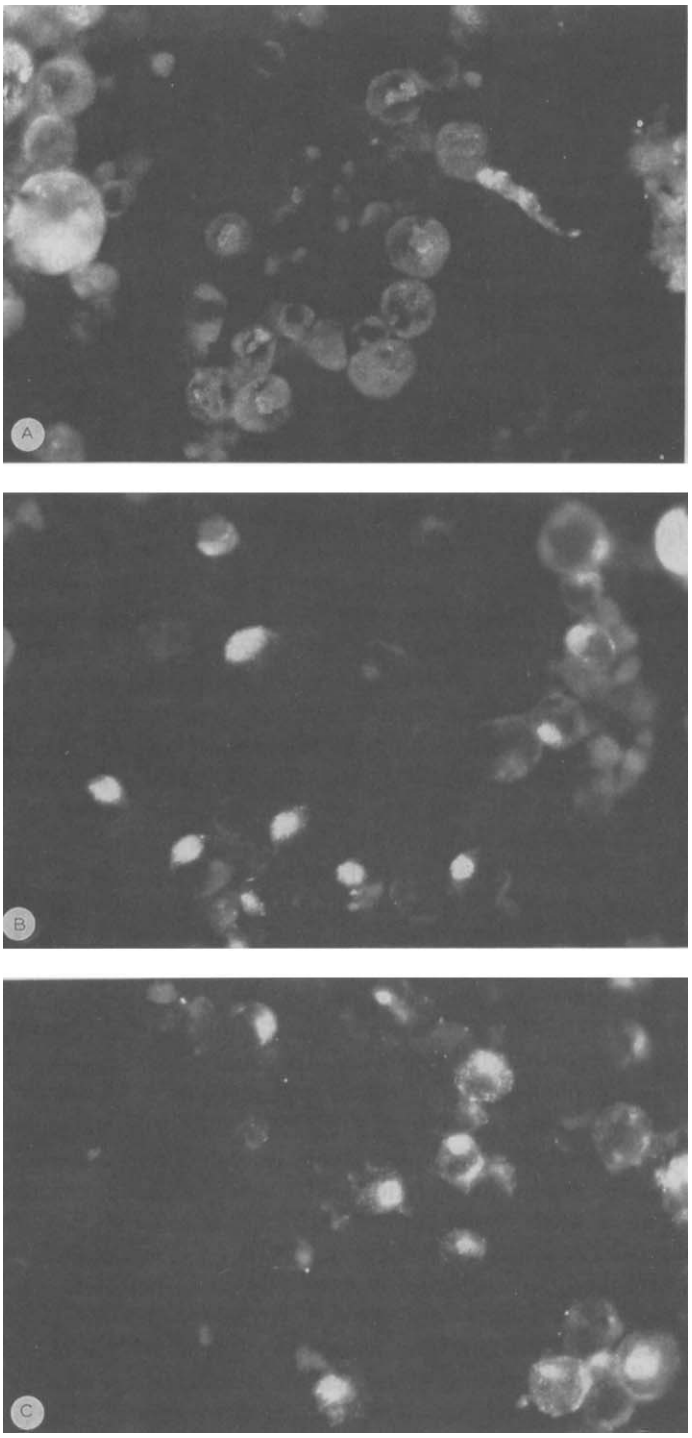


Fig. 4.4. Indirect immunofluorescence staining of HHV-6-infected HSB_2 cells stained with a human serum containing HHV-6 IgG antibody. The stained cells show diffuse staining of the entire cell.

antibody-positive serum, as shown in Fig. 4.4. The specificity of the test is dependent on the availability of HHV-6 (GS isolate) monoclonal antibody (Balachandran et al., 1989) (Fig 4.5) since the majority of human sera contains not only antibodies to HHV-6 but to other human herpesviruses as well. If the cells are infected with 1 : 100 $TCID_{50}$ of virus, the infection proceeds rapidly and by day 14 most of the culture contains approximately 80% antigen-positive cells as evidenced morphologically and by IFA. At this time, cell supernatants can be obtained from the culture and frozen at $-70°C$ or in liquid nitrogen in 20% fetal calf serum. Freezing the virus at higher temperatures ($-20°C$) and without preservative could result in a loss of titer over a period of time. A continuous harvest of virus and infected cells can be achieved by the continuous addition of uninfected cells into the culture. Table 4.2 shows that HSB_2, and SupT1 (an immature T cell line from a non-Hodgkin's T cell lymphoma) have been found to be productively infectable with HHV-6 isolates GS, U1102, DA and other isolates similar to GS (CO1 to CO8). Isolates similar to Z-29 (OK, DC, BA) can be propagated in MOLT-3 cells, a mature T cell line, for productive infection (Ablashi et al., 1991a).

Even though Tedder et al. (1987) were able to infect adherent cells, no evidence of productive infection was presented. In our own laboratory, 3 different cell lines of human fibroblast origin were used for infection with the GS strain. No discrete CPE was observed. In some areas of the culture, shrinkage of cells followed by degeneration were evident. These cells contained HHV-6 antigen as detected by IFA with monoclonal antibody, and electron microscopy revealed a few virus particles. Approximately 1 log of virus was recovered from the culture. Upon reinfection of fibroblast cells with the fibroblast-recovered virus, a very low level of infection was observed, suggesting that fibroblast cells do not support productive infection. Moreover, infection of fresh human monocyte/macrophages with the GS


A
B
C

strain showed occasional enlarged cells. No viral antigens could be detected in these cells, but 30% of the cells contained HHV-6 DNA using a ZVH-14 probe (Josephs et al., 1986). Thus, monocytes/macrophages may be involved in HHV-6 pathogenesis by harboring the latent HHV-6 genome and transmitting it to the central nervous system (CNS) or other organs by fusing with other cells and inducing lesions in susceptible cells. Krueger et al. (1992) showed that established cell lines from Hodgkin's disease expressed HHV-6 antigens (gp85, gp105 and gp116) after infection with HHV-6. After infection, the cells and the expression of p41 rapidly deteriorated and virus particles were detected in the debris and giant cells in the culture were free of virus. Fox et al. (1990) showed that epithelial cells from the salivary gland contained HHV-6 DNA and were sometimes antigen-positive with monoclonal antibodies, suggesting that HHV-6, like CMV, may be latently carried in the salivary gland. Krueger et al. (1990) also showed that specimens from lip salivary glands and transbronchial biopsies from patients with Sjögren's syndrome, AIDS patients and healthy donors contained HHV-6 DNA. These biopsies also exhibited HHV-6 antigen using monoclonal to p41 of the GS strain of HHV-6. The HHV-6-positive cells were epithelial, suggesting that HHV-6 *in vivo* does infect epithelial cells and that the virus hibernates in the epithelial cells of the salivary gland and is frequently shed in the saliva. Since the cells containing HHV-6 in Sjögren's syndrome patients were inflammatory in nature, the virus may contribute to the pathogenesis of this syndrome. This will be further discussed by DeClerck et al. (Chapter 22, this volume).

To date, even though CD4, CD8, CD38 and cells with other receptors are readily infectable *in vitro* with HHV-6, none of these epitopes are apparently identified with HHV-6 receptors because infection of these cells could not be blocked using specific monoclonal antibody to these epitopes. It is important to find the receptor for HHV-6 infection in order to understand the mechanism of HHV-6 infection and to define the biological conditions necessary for productive infection.

We have been able to infect MOLT-3 cells with the GS strain of HHV-6 prior to treatment with PHA; however, the amount of virus produced is extremely lower than obtained from HSB_2-infected cells. This suggests either that mitogen could be inducing cellular receptors essential for infection with the GS strain or that this treatment could activate cell metabolism supporting virus replication of the GS strain.

Fig. 4.5. (a) Immunofluorescence staining of HHV-6-infected HSB_2 cells stained with HHV-6 (GS isolate) monoclonal antibody 9A5D12 (p41). The staining is confined to the nucleous of the infected cell. (b) Immunofluorescence staining of HHV-6-infected HSB_2 cells stained with HHV-6 monoclonal antibody to gp116/64/54 (6A5G3). The staining is acentric. (c) Immunofluorescence staining of HHV-6-infected HSB_2 cells stained with monoclonal antibody to HHV-6 gp105 (7A2); 1 large stained cell showing granular nucleated staining.

4.3. Concluding remarks

Even though HHV-6 is closely related to human CMV, its cell tropism is different. Secondly, HHV-6 isolates do not necessarily infect the same cell type. Continuous passage of virus in one cell line could lead to polymorphism. The possibility that isolates like U1102 and GS prefer immature T cells for replication and productive infection, and that other isolates (Table 4.1), like Z-29 and SF, prefer mature T cells, should be considered. Other isolates like OK (from exanthem subitum, bone marrow transplant patient, leukopenia) may have different target cells for productive infection because the MOLT-3 did not support productive infection by these strains of HHV-6 (Table 4.2). Identification of receptors and biological conditions for productive infection of cells which could support replication of all HHV-6 isolates would be an important step in delineating HHV-6 biology and its role in pathogenesis.

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Ablashi/Krueger/Salahuddin (eds) Human Herpesvirus-6

CHAPTER 5

Infection, latency and immortalization of human cells with HHV-6

JANOS LUKA

University of Nebraska Medical Center, Omaha, NE 68105, U.S.A.

5.1. Introduction

Human herpesvirus-6 (HHV-6), discovered in 1986 by Salahuddin et al. (1986), has been classified as a lymphotropic herpesvirus. The virus readily infects mitogen-activated human cord blood and peripheral blood mononuclear cells *in vitro* (Salahuddin et al., 1986; Lopez et al., 1988; Levy et al., 1990). Certain T cell lines can also be infected with the virus (Ablashi et al., 1987); however, nearly every strain of HHV-6 isolated so far requires a distinct type of T cell line for replication (Ablashi et al., 1988). This lack of a common cell line for propagation of the virus has hampered the progress of research. In the past, at least 7 strains of HHV-6 have been tested and found to infect human fibroblast (Luka et al., 1990, 1992c). This observation may be useful for the propagation of the various viral strains for biologic, immunologic and molecular work. It has also been reported that HHV-6 may transform 3T3 fibroblast, indicating that the virus may have oncogenic properties (Razzaque, 1990). DNA sequence analysis indicated that some of its genes have strong homologies to CMV (68%) and, to a lesser degree, to EBV (48%) (Lawrence et al., 1990; Littler et al., 1990). These properties of the virus and the strong homology to CMV may indicate a close relationship between CMV and HHV-6. Interestingly, HHV-6 can also productively infect human B cell lines containing latent EBV genome, but cannot replicate in the few EBV genome-negative B cell lines tested so far (Ablashi et al., 1990). We recently demonstrated that, at least in some cases, the apparent nonpermissivity of B and T cell lines to HHV-6 infection can be explained by the establishment of HHV-6 latent viral genome in these cell lines (Gubin et al., 1992; Luka et al., 1992a). In our laboratory, we successfully established several cell lines containing HHV-6 genome by infection

of peripheral and cord blood mononuclear cells *in vitro* (Luka et al., 1992c). The purpose of our study was to explore the possible interaction between HHV-6 and other herpesviruses, especially EBV and CMV, and to characterize the latency established in B and T cell lines by HHV-6. In this chapter, we present the *in vitro* infection and replication of human cells and latency of HHV-6 in order to explore target cells for permissive replication and to shed some light on how HHV-6, under the right circumstances, could be capable of immortalizing T cells.

5.2. Infection of human T cell lines with HHV-6 isolates and establishment of latency

Several strains of HHV-6 have been isolated in various laboratories around the world. In our studies, we routinely used 4 strains: 2 isolated in Nebraska (GD, KS), 1 (Z-29) isolated from an HIV-positive AIDS patient from Zaire (Lopez et al., 1988), and the prototype isolate (GS) isolated from a patient with acute lymphocytic leukemia (Salahuddin et al., 1986). As has been documented previously, these strains require either HSB_2 or MOLT-3 cell lines for their replication (Ablashi et al., 1988), but all 4 strains can replicate in human foreskin or lung fibroblast (Luka et al., 1990, 1992a). Because of some of the disadvantages associated with the use of fibroblast to propagate the virus, we searched for a T cell line in which all 4 strains may replicate. Development of a tetraploid cell line (HSB_2M) from the HSB_2 cell line gave us the possibility to do so. This cell line can be infected with all 4 strains, and on average, 60% of the cells produced virions. Looking for cell surface markers on uninfected or virus-infected cells, we demonstrated that several changes occurred in both HSB_2 and HSB_2M cell lines under the course of viral infection (Table 5.1) (Gubin et al., 1992; Luka et al., 1992b). Control UV-inactivated virus did not induce these changes, indicating that biologically active viral DNA is necessary for the expression of new cellular markers. Infection of the HSB_2 cell line with Z-29, GD or KS strains, which are unable to replicate in this cell line, however, induced surface markers which were different from those associated with the lytic virus infection. Of interest is that only one of the markers, CD4, was transiently induced in the lyticly infected cells, while it was permanently expressed in the apparently uninfected cells. This gave us the idea that these cells may harbor latent viral genome. By *in situ* hybridization, using the pZVH 14 plasmid (Josephs et al., 1986), and by PCR, using primers derived from the sequences published (Lawrence et al., 1990; Littler et al., 1990), we demonstrated the presence of HHV-6 genome in these cell lines (Fig. 5.1). After infection with all 4 strains of the virus, the HSB_2M cell line showed 30–60% of the cells expressing viral antigen, as detected by immunofluorescence, using monoclonal antibodies. By rescuing and cultivating the apparently uninfected cells, we selected a new cell line (HSB-M), which contains latent HHV-6 genome. Some of these cell lines were nonproducers, expressing various cell receptors (Table 5.2), while some expressed late viral antigens in 3–7% of the cells. We tested several agents (Table 5.2) for induction of the lytic cycle of the

TABLE 5.1.
Cell surface markers in HHV-6-infected cells

Marker	Day 0	Day 1	Day 3	Day 4	Day 7
HSB_2 cells, lytic cycle					
%CD1	0.38	1.54	5.4	25.08	17.67
%CD2	0.24	1.20	2.46	7.93	4.30
%CD3	0.20	0.74	0.99	1.78	1.96
%CD4	0.24	0.52	2.50	14.36	20.69
%CD5	76.07	96.61	67.24	82.77	57.43
%CD7	99.37	98.63	81.34	75.35	29.02
%CD8	0.40	0.80	0.86	3.22	2.70
%CD18	99.53	97.99	73.82	81.87	52.81
%CD38	10.12	31.04	23.10	57.41	44.86
%HLA-DR	0.40	0.40	0.53	1.02	1.06
%ICAM	1.21	78.69	14.6	70.25	37.70
HSB_2M cells, lytic and latent infection					
%CD1	4.03	14.60	22.04	53.65	28.20
%CD2	13.10	49.76	39.57	64.69	40.50
%CD3	0.62	5.44	0.70	6.90	0.70
%CD4	2.48	9.48	4.26	29.08	24.32
%CD5	32.90	92.93	72.50	95.05	84.30
%CD7	53.13	42.40	35.30	47.42	13.23
%CD8	0.54	3.84	0.90	14.18	4.48
%CD18	87.39	83.15	57.20	85.56	50.61
%CD38	85.73	98.30	71.35	96.17	72.20
%HLA-DR	1.52	0.18	0.86	1.22	0.64
%ICAM	53.77	36.12	18.53	27.39	11.93

virus in these T cell lines. We found 3 agents, tetraphorbol ester (TPA), hydrocortisone (HC) and phytohemagglutinin (PHA), which were able to induce the lytic cycle of the virus in these T cell lines. TPA can also increase the infectivity of HHV-6 in permissive cells (Gubin et al., 1991). Interestingly, some of the latent cell lines could be induced by these inducers separately, while in some cell lines a combination of the TPA and hydrocortisone was necessary. One latent cell line, developed by co-cultivation of HHV-6-infected HSB_2 cells with fibroblast, could be induced only by hydrocortisone, but not by TPA or a combination of the two. These results indicated that the latent viral genome in both cell lines is possibly under different cellular controls. Since the detection method for HHV-6 activation was based on the increase of the late antigen-positive cells detected by monoclonal antibodies, it was not possible to find whether an abortive activation (early antigen expression only) of the lytic cycle occurred in some of the cell lines. While most of the latent cell lines expressed CD4 markers on their surface continuously, the induced cells lost this marker rapidly.

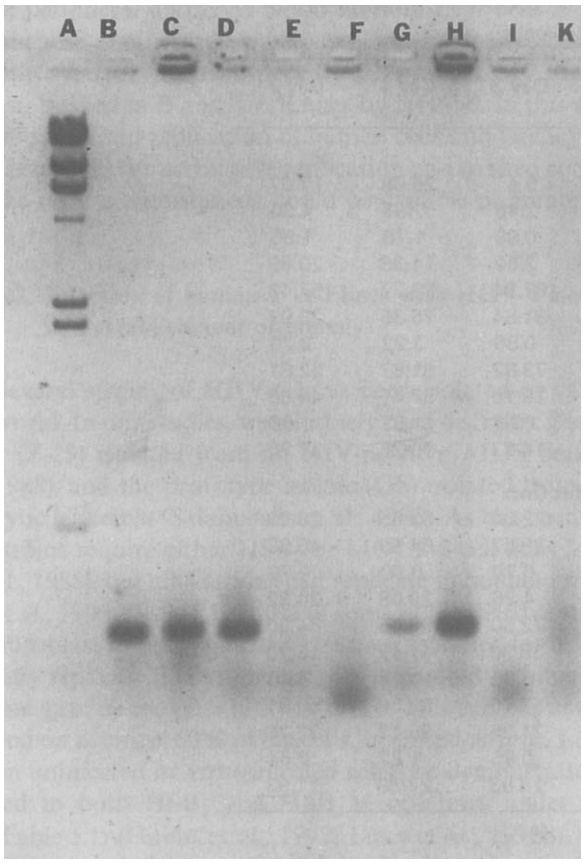

Fig. 5.1. Detection of HHV-6 genome in latently infected cell lines by PCR (A) lambda HindIII marker; (B) HSB-M latently infected cell line (HHV-6); (C) JMH-1, HHV-6 immortalized PBL; (D) CBH-1, HHV-6 immortalized PBL; (E) Raji, EBV genome positive cell line; (F) HSB_2, HHV-6 genome negative T cell line; (G) HSB-ML, latently infected cell line (HHV-6); (H) HSB_2 cell line infected with HHV-6 (at day 5); (I) Ramos, EBV genome negative B cell line; and (K) Ramos, infected with HHV-6 at day 3 after infection.

5.3. Immortalization of T lymphocytes with HHV-6

The virus isolated from the latently infected HSB-M cell line has been used to infect isolated mononuclear cells from peripheral and human cord blood. Several cell lines have been established from this infection containing latent HHV-6 genome (Fig. 5.2). As shown in Table 5.3, the surface markers indicated that these cell lines were T cells, and possibly of thymic origin. Interestingly, every cell line expressed CD4 markers, and some of the cells were double positive for CD4 and CD8. These results indicated that HHV-6 can immortalize, and possibly transform, human T cells under the right biologic conditions.

TABLE 5.2.
Induction of HHV-6 lytic cycle in latently infected cells

Cell line	No. HHV-6 genome	% late ag. pos. noninduced	% late ag. positive after induction with		
			TPA	HC	TPA/HC
HSB_2	0	0	0	0	0
HSB-M	100	3–7	17	21	43
HSB-ML	40	< 1	6	12	8
HSB-MD	30	< 1	2	5	7
HSB-F	30	< 1	4	28	14

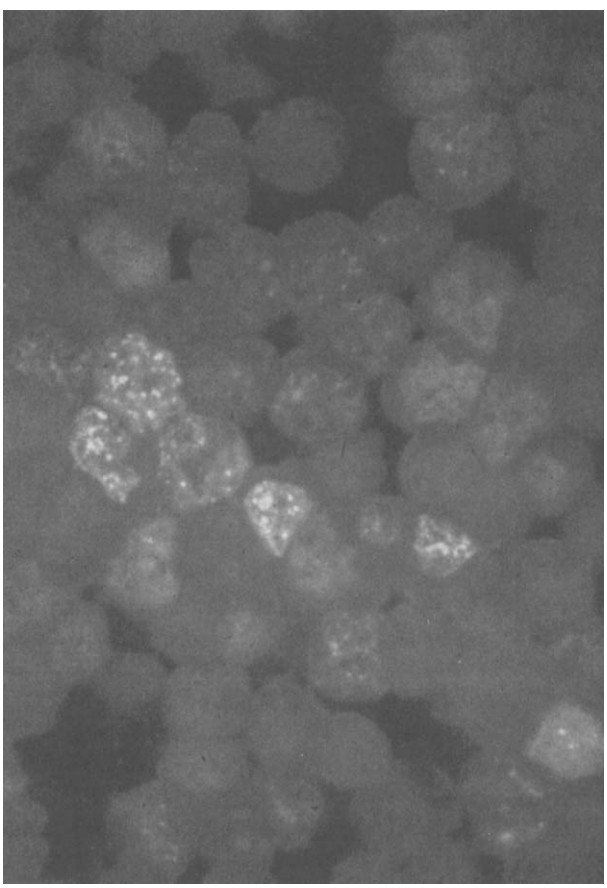

Fig. 5.2. *In situ* hybridization of JMH-1 cell line with pZVH14 probe. The cells were mixed with the HHV-6 genome negative HSB_2 cell line (50%) for internal control.

TABLE 5.3.
Latently infected cell lines; HSB-M (*in vitro* infected tetraploid HSB_2M), JMH-1, JMH-2 and JMH-3 (*in vitro* HHV-6 immortalized PBL)

Markers	HSB-M	JMH-1	JMH-2	JMH-3
CD1	32.0	93.9	99.4	96.2
CD2	50.6	73.6	87.1	71.8
CD3	15.1	16.7	27.1	72.3
CD4	26.4	89.8	41.5	85.9
CD5	71.4	94.0	99.9	96.4
CD7	7.9	14.2	54.5	74.9
CD8	1.0	0	63.0	0
CD18	5.7	90.6	100	95.9
CD38	54.53	94.2	100	96.4
CD54	1.7	78.2	24.3	67.5
CD4/CD8	–	0	32.4[a]	0
HLA-DR	1.4	0	0	0
TdT	++			++

[a] Dual positivity for CD4/CD8 in the JMH-2 cell line.

Preliminary experiments indicated that these CD4-positive cell lines can be infected with HIV, and, after infection, both HIV and HHV-6 virions are released from the cells. Further studies will be necessary to demonstrate whether T cells latently infected with HHV-6 *in vivo* could play a role in the development of AIDS.

5.4. Infection of human fibroblasts with HHV-6

HHV-6 has been shown to infect human fibroblast, but the reproductivity of this infection was variable. We undertook certain studies to resolve this problem. By testing various fibroblast lines, we found that the best permissive cell lines for virus replication were of foreskin origin. We also found that fibroblast was most susceptible to virus infection 3–7 days after sub-culturing the cells. No infection of the cells occurred after day 9, or after the cells became confluent. Similar phenomena also exist in the CMV system (Thiele, personal communication).

The HIV-infected fibroblast could be stained for HHV-6-associated antigens at day 2, and cell-free particles could be detected at day 5 post-infection (Fig. 5.3). The virus-containing supernatant, however, never infected the original T cell lines used for the propagation of the virus. Surprisingly, the virus purified from the fibroblast supernatant was able to infect the T cell lines. This indicated that the fibroblast may produce a factor which could inhibit the infection or replication of HHV-6 in T cell lines. To test this possibility, we co-cultivated MRC-5 cells with HSB_2 cells and used the GS strain of virus for infection. The cells were tested for virus

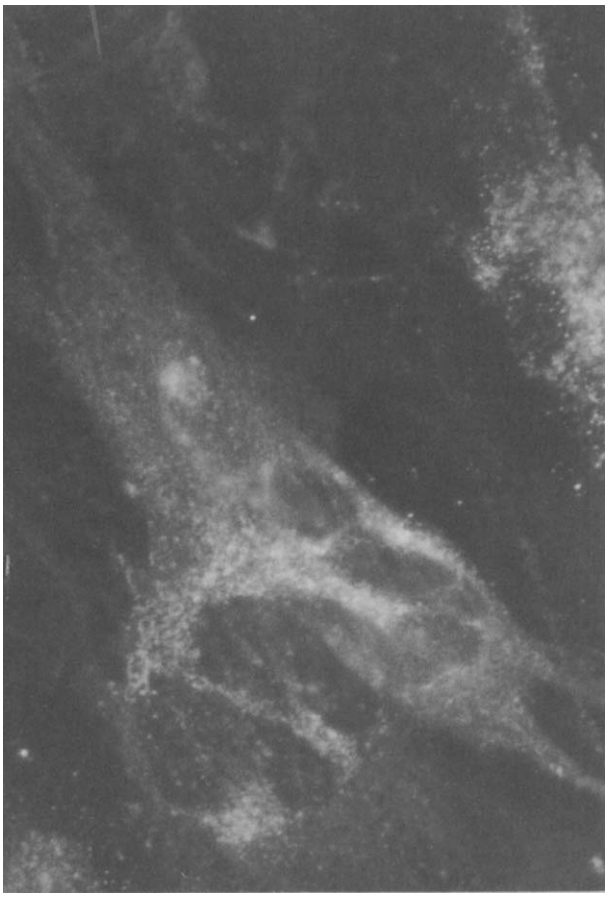

Fig. 5.3. Immunofluorescence assay of HHV-6 infected MRC-5 fibroblast with monoclonal antibody (H-AR-2) to gp110 late HHV-6 antigen.

replication 5, 7 and 14 days after infection with HHV-6 by immunofluorescence. While a low percentage of the fibroblast was positive for viral antigen by immunofluorescence at those times, none of the HSB_2 cells were positive. Two weeks post-infection, the slow-growing HSB_2 cells were removed from the fibroblast and cultivated alone. The cells were tested by *in situ* hybridization and by PCR for the presence of HHV-6 genome. Both methods indicated that latent HHV-6 genome was present in this cell line. As mentioned earlier, this cell line (HSB-F) could be induced by hydrocortisone to express late viral antigen. In this cell line, however, TPA inhibited the induction of lytic cycle. These results indicated that human fibroblast could produce a factor which inhibits lytic infection of T cell lines by HHV-6. This latency is possibly induced by inhibiting the proliferation and/or differentiation of the T cell lines in response to the viral infection, and it is currently under investigation.

5.5. Conclusions

Human herpesvirus-6, originally named human B lymphotropic virus (HBLV), has shown to be tropic for T cells, some B cells and fibroblasts. The infection of B cell lines with HHV-6 is described by Lusso et al. in Chapter 10, this volume, on the interaction of HHV-6 with other viruses. The virus has been identified by its lytic infection of human cord and peripheral blood cells. This lytic activity of the virus, at least in these cells, requires mitogen stimulation. The virus readily infects and replicates in some T cell lines and fibroblasts *in vitro*. In other cell lines, however, the virus replication is suppressed and a latency is established. Latent viral genome can be detected in T, B and fibroblast cell lines, which survive the virus infection. This latency *in vitro*, and immortalization of peripheral blood lymphocytes and cord blood mononuclear cells, suggest that the virus may be latent in similar cell types *in vivo*. Reactivation of the latent virus *in vivo* may explain the presence of large amounts of virus in various immunological disorders and the associated high antibody titers against this virus. Further studies may indicate whether the latency of HHV-6 could be associated with neoplasia of T cell origin, and whether the immortalized CD4-positive cells may play a role in the development of AIDS.

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Ablashi/Krueger/Salahuddin (eds) Human Herpesvirus-6

CHAPTER 6

Electron microscopy of human herpesvirus-6 (HHV-6)

BERNHARD KRAMARSKY[1] and CHRISTIAN SANDER[2]

[1] *Pharmacia Diagnostics Inc., Columbia, MD 21046–1297, U.S.A*, and* [2] *Institute of Pathology, University of Cologne, 5000 Cologne 41, Germany, and Laboratory of Pathology, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892, U.S.A.*

6.1. Introduction

Human herpesvirus-6 (HHV-6), which was originally isolated from peripheral blood lymphocytes of patients with AIDS and other lymphoproliferative disorders (Salahuddin et al., 1986), was subsequently isolated from the blood and saliva of others, including healthy individuals and patients with other diseases such as chronic fatigue syndrome, bone marrow transplant recipients, roseola infantum and autoimmune diseases (Downing et al., 1987; Tedder et al., 1987; Ablashi et al., 1988a; Lopez et al., 1988; Pietroboni et al., 1988; Yamanishi et al., 1988; Krueger and Sander, 1989; Ablashi and Hung, Chapter 4, this volume).

6.2. Ultrastructure

HHV-6 is a herpesvirus, i.e., an enveloped DNA virus with an icosahedral capsid made up of 162 capsomeres (Wildy et al., 1960; Biberfeld et al., 1987) (Fig. 6.1). This virus infects mainly cells of lymphocytic lineage (Josephs et al., 1988; Lusso et al., 1988). Its ultrastructure and morphogenesis closely resemble those of cytomegalovirus (Smith and De Harven, 1973; Biberfeld et al., 1987; Brinkmann et al., 1990) (Table 6.1). In infected cells, HHV-6 is found at various stages of its morphogenesis. Empty and DNA-filled nucleocapsids are observed in the nucleus (Fig. 6.2). These become coated with a fibrous material, designated the tegument by Roizman and Furlong (1974), at the nuclear side of the nuclear membrane (Fig. 6.3) and while

* Present address: Advanced Biotechnologies Inc., Columbia, MD 21046, U.S.A.

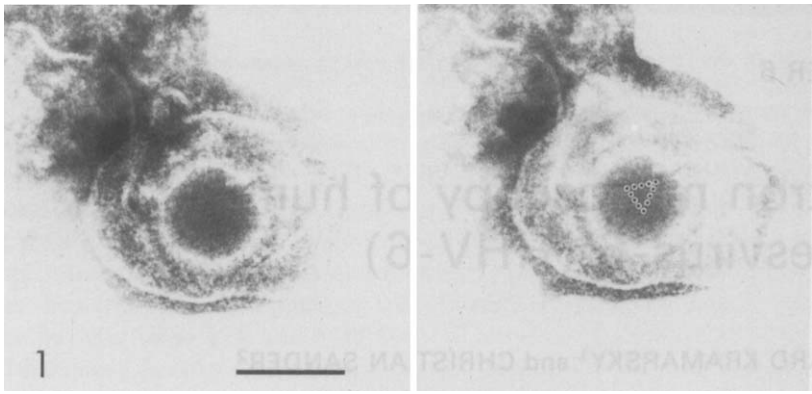


Fig. 6.1. Electron micrograph of a negative stained HHV-6 virion. The right-hand duplicate of the virion is marked to show the triangular facet of the icosahedron. The bar represents 100 nm.

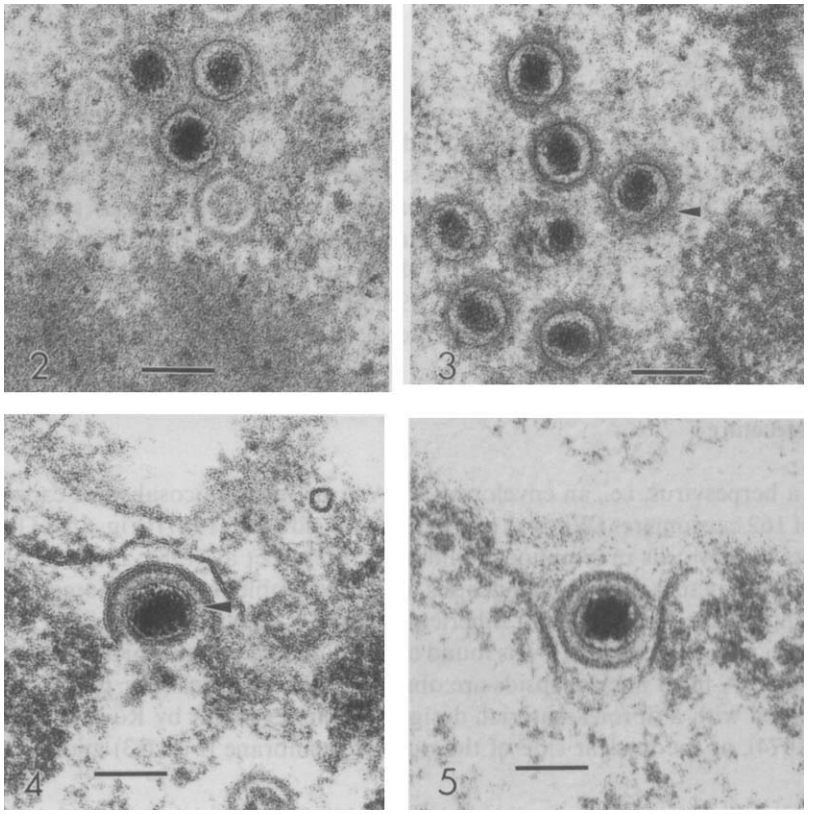


TABLE 6.1.
Ultrastructural comparison of HHV-6 with other herpesviruses

Feature	HHV-6	CMV[a]	HSV-1
Diameter of enveloped virion	160–200 nm	174 nm	150–200 nm
Diameter of the capsid	95–105 nm	106.4 nm	95–110 nm
Diameter of the nucleoid	60–80 nm	64.3 nm	50–70 nm
Symmetry of the capsid	Icosahedral	Icosahedral	Icosahedral
No. of capsomeres in the capsid	162	162	162
Thickness of the tegument	Dense prominent 25–40 nm	Dense prominent 24.4 nm	Often indistinct 20–40 nm

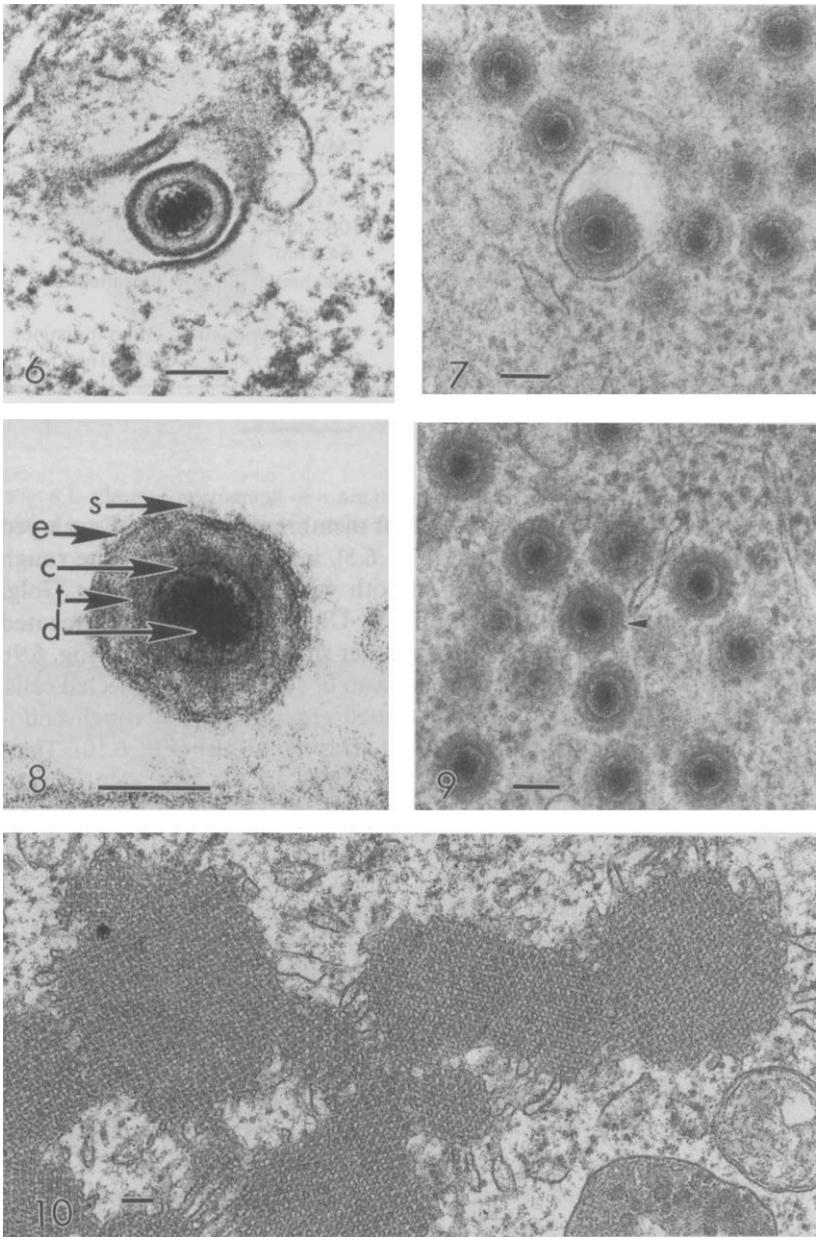
[a] Smith and De Harven (1973).

budding into the cisterna formed by the 2 nuclear membranes (Fig. 6.4). Enveloped HHV-6 virions are found in this cisterna (Fig. 6.5), in the cisterna of the rough endoplasmic reticulum (Fig. 6.6), in clear smooth vesicles formed by the Golgi (Fig. 6.7) and in the extracellular areas (Fig. 6.8). Unenveloped tegument-coated nucleocapsids are found free in the cytoplasm either singly or in clusters (Fig. 6.9). Large inclusion bodies are present in the cytoplasm of some HHV-6 infected cells. These inclusions are situated within the distended cisternae of the rough endoplasmic reticulum and are made up of a parallel array of tubules (Fig. 6.10). Their significance has not been established.

6.3. Virus replication

While all isolates of HHV-6 grow well in human cord blood mononuclear cells, the isolates fall into 2 major groups with respect to their ability to grow in T lymphoblastic cell lines. Members of 1 group, which includes the original isolate, GS, as well as Davilla, CO1 through CO8 and U1102, grow well in HSB_2 cells (Ablashi et al., 1988b) and in SupT1 cells (Ablashi and Hung, Chapter 4, this volume). Members of the second group which include Z-29, DC, OK, and BA, grow well almost exclusively in MOLT-3 cells (Ablashi and Hung, Chapter 4, this volume). The rate of viral replication and production of cytopathic effects also vary between the 2 groups. Members of the first group replicate rapidly and cause a

Figs. 6.2–6.5. Electron micrographs of HHV-6 infected cells. Thin section preparations. The bar represents 100 nm.
Fig. 6.2. Nucleocapsids not coated with tegument inside the nucleus of an infected cell.
Fig. 6.3. Nucleocapsids partially coated with tegument (arrowhead) at the margin of a nucleus.
Fig. 6.4. A virion budding at the inner nuclear membrane into cisterna of the nuclear envelope. The tegument coating (arrowhead) is visible on the enveloped side of the nucleocapsid.
Fig. 6.5. A virion free in the cisterna of the nuclear envelope.



Figs. 6.6–6.10. Electron micrographs of HHV-6 infected cells. Thin section preparations. The bar represents 100 nm.

Fig. 6.6. A virion in the cisterna of the rough endoplasmic reticulum.

Fig. 6.7. A virion in a clear vesicle, probably of Golgi origin.

Fig. 6.8. An extracellular HHV-6 virion. d: DNA core; c: capsid; t: tegument; e: envelope; s: surface spikes.

Fig. 6.9. A cluster of nucleocapsids in the cytoplasm of a cell. Note that all nucleocapsids are coated with tegument (arrowhead).

Fig. 6.10. A large inclusion body in the distended cisternae of the rough endoplasmic reticulum. The inclusion is made up of a parallel array of tubules.

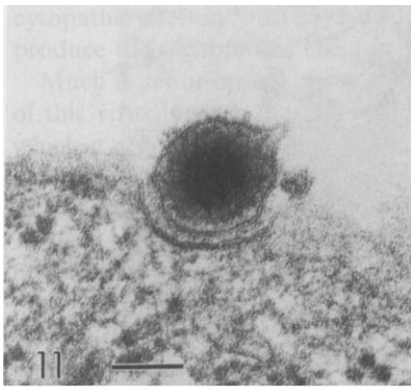


Fig. 6.11. A HHV-6 virion in an early stage of endocytosis. Note that the endocytic pit is not coated with clathrin.

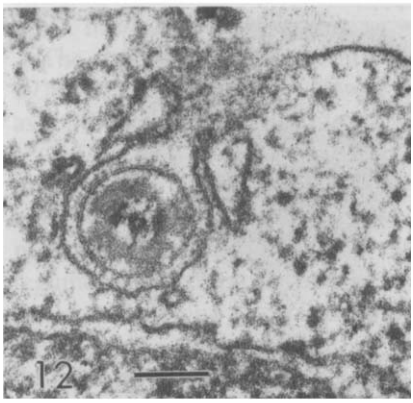


Fig. 6.12. A HHV-6 virion at a latter stage of endocytosis. The virion is completely engulfed. The endocytic pit again is not coated.

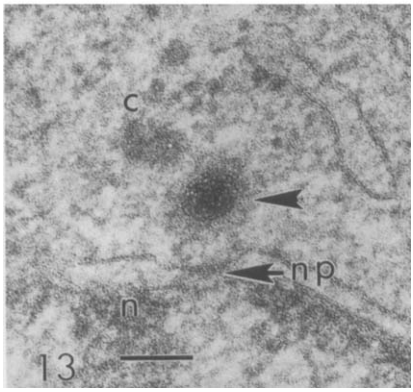
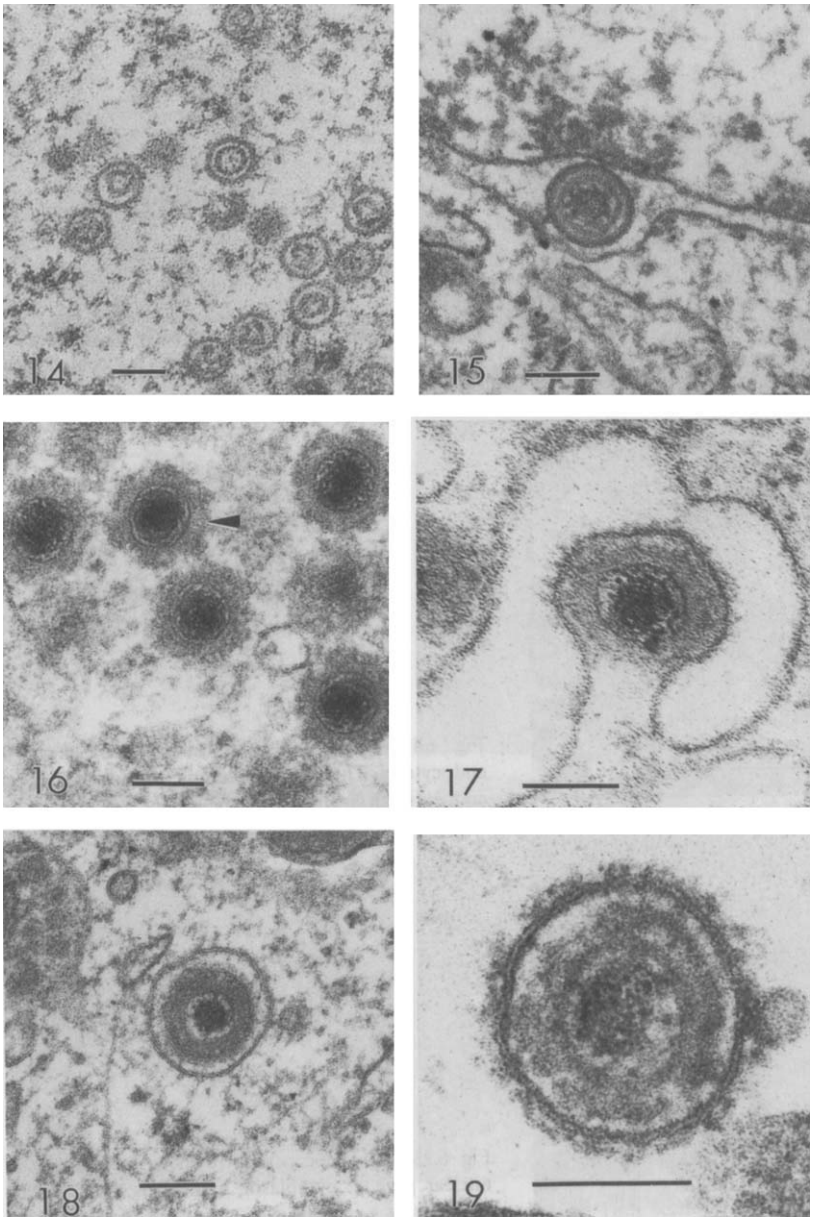


Fig. 6.13. A nucleocapsid (arrowhead) adjacent to a nuclear pore. c: cytoplasm; n: nucleus; np: nuclear pore.

Figs. 6.11–6.13. Early stages of the internalization and uncoating of HHV-6 in lymphoblastic cells. Thin section preparations. The bar represents 100 nm.



Figs. 6.14–6.19. Morphogenesis of HHV-6-GS in HSB_2 cells. Thin section preparations. The bar represents 100 nm.

cytopathic effect in 5–10 days, while those of the second group replicate slowly and produce their cytopathic effect in 10–15 days.

Much is yet uncertain about the replicative cycle of HHV-6. The mode of entry of this virus into the host cell has not been clearly established. The receptor to which it attaches to the cell membrane is not the CD4 receptor to which HIV-1 binds. The virus enters into uncoated pits (Figs. 6.11 and 6.12) but entry via coated pits or by fusion with the cell membrane has not been ruled out.

Even when the virus is taken in via the uncoated or coated pits, the viral membrane must fuse with the membrane of the endocytic vesicle so that the nucleocapsid may enter into the cytosol. The coated nucleocapsid can then migrate toward the nucleus.

Figure 6.13 shows a coated nucleocapsid adjacent to a nuclear pore. The actual entry of the nucleocapsid into the nucleus and the further uncoating of the viral DNA has not been observed.

The progeny virus is assembled like cytomegalovirus (Smith and De Harven, 1973). In HSB_2 cells infected with the GS strain of HHV-6 morphogenesis proceeds as follows: on day 3 after infection, uncoated capsids appear in the nuclei of about 1% of the cells (Fig. 6.14). By day 6, uncoated capsids are observed in almost all infected cells. Many of the capsids contain a nucleic acid core and are, therefore, nucleocapsids. Enveloped virions are seen budding into or located in the perinuclear space delineated by the two nuclear membranes (Fig. 6.15). Unenveloped tegument-coated nucleocapsids are seen free in the cytosol. Some of these coated nucleocapsids bud into clear Golgi vesicles. Enveloped virions are also observed free inside Golgi vesicles (Figs. 6.17 and 6.18). By days 8–10, tegument-coated nucleocapsids accumulate in the cytosol of some cells (Fig. 6.16) and numerous enveloped virions are found in the intercellular spaces (Fig. 6.19).

Uninfected HSB_2 cells have the appearance of typical pre-T lymphocytes with a fairly large nucleus and sparse cytoplasm with few organelles. By days 6–8 and, more conspicuously, by days 8–10 after infection, cells have become enlarged lymphoblasts with a considerable increase in cytoplasm and in cytoplasmic organelles (Sander et al., 1990) (Fig. 6.20). Shortly thereafter, lytic degeneration of the blast cells takes place.

Fig. 6.14. Third day after infection. Nucleocapsids in the nucleus of a cell. Note the absence of tegument coating.

Fig. 6.15. Sixth day after infection. An enveloped virion in the cisterna formed by the two membranes of the nuclear envelope. Note the smooth (spikeless) surface of the virion (see also Figs. 4–6).

Fig. 6.16. A cluster of nucleocapsids in the cytoplasm of a cell. All nucleocapsids are coated with tegument (arrowhead).

Fig. 6.17. A virion budding into a Golgi vesicle. Note the spikes at the surface of the virion.

Fig. 6.18. A virion free inside a Golgi vesicle. The viral surface is covered with spikes.

Fig. 6.19. An extracellular HHV-6 virion. The viral envelope is covered with spikes.

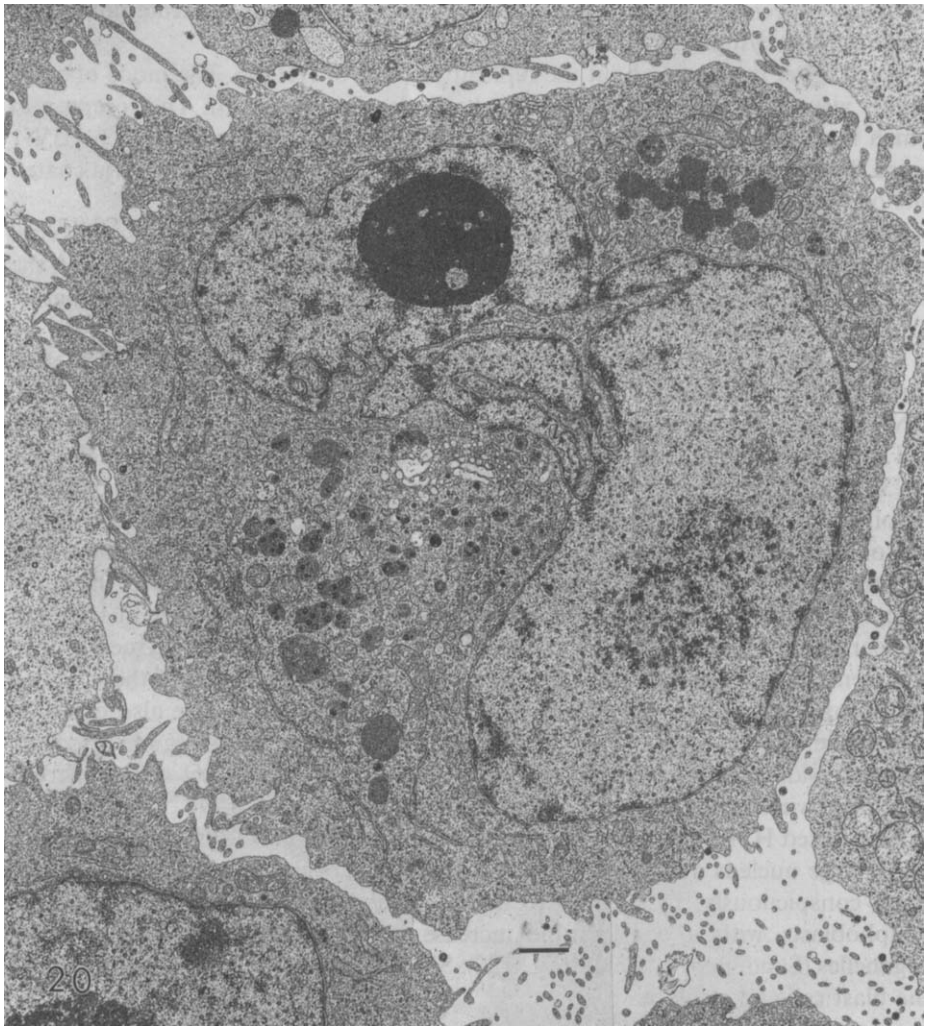


Fig. 6.20. Tenth day after infection. A low magnification view of a HSB_2 cell infected with $HHV\text{-}6_{GS}$. Note the enlarged cytoplasm and the abundance of cytoplasmic organelles. The bar represents 1 μm.

6.4. Summary and discussion

It thus appears that HHV-6 morphogenesis proceeds as follows: capsids appear in the nucleus and are filled with DNA. They then move toward the nuclear envelope. While adjacent to the nuclear membrane, but still in the nucleoplasm, they acquire a coating designated the tegument by Roizman and Furlong (1974) (Fig. 6.3). This accumulation of tegument is sometimes also seen during viral budding from the nucleus into the cisterna formed by the 2 membranes of the nuclear envelope

(Fig. 6.4). By budding at the nuclear membrane, the nucleocapsids become enveloped. The cisterna formed by the 2 nuclear membranes is contiguous with that of the rough endoplasmic reticulum. The enveloped HHV-6 virion can thus migrate through this cisterna to the outer regions of the cell and can then be expelled from the cell.

Alternatively, the virion can fuse with the outer nuclear membrane, losing its envelope. This must occur frequently since, unenveloped, tegument-coated nucleocapsids are frequently seen free in the cytoplasm, either singly or in clusters (Figs. 6.9 and 6.16).

Some of these cytoplasmic nucleocapsids then bud into smooth vesicles, where they again acquire an envelope, this time covered with surface spikes (Figs. 6.7, 6.17 and 6.18). Because the enveloped virions in the cisternae of the rough endoplasmic reticulum are not covered with surface spikes, while those in the smooth (Golgi) vesicles and the extracellular virions are so covered, we believe that the pathway via the cytosol and the Golgi vesicles is the one which produces infectious virus. This is in agreement with the observations of Roffman et al. (1990). Budding at the cell margin has not been observed in the case of HHV-6, but cannot be ruled out.

The time involved in each step of the morphogenesis of HHV-6 varies, depending on the virus isolate and the host cell. GS and related isolates of HHV-6 replicate more rapidly than Z-29 and members of that group. Generally, replication in cord blood lymphocytes occurs more rapidly than in the continuous cell lines.

The rate of cytopathic changes of cells infected with HHV-6 parallels that of virus maturation (Sander et al., 1990). Giant cells (Fig. 6.20) with greatly increased cytoplasm and cytoplasmic organelles appear at about the same time as the intracytoplasmic nucleocapsids and extracellular enveloped virus. The giant cells do not divide and die after a few days.

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Ablashi/Krueger/Salahuddin (eds) Human Herpesvirus-6

CHAPTER 7

Biology and strain variants

D.V. ABLASHI[1] and S.Z. SALAHUDDIN[2]

[1] *Laboratory of Cellular and Molecular Biology, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892, U.S.A., and* [2] *University of Southern California, School of Medicine, Los Angeles, CA 90033, U.S.A.*

7.1. History and introduction

The first HHV-6 isolate, also known as HBLV, was reported from patients from the U.S.A. with AIDS and other lymphoproliferative disorders (Salahuddin et al., 1986). In a group of 6 patients, 2 HHV-6 isolates, i.e., GS and Davilla, were first compared biologically, immunologically and by molecular analysis and were found to be similar (Josephs et al., 1988). In 1987 and 1988, 3 independent isolates were reported from AIDS patients from Zaire and Uganda (Downing et al., 1987; Tedder et al., 1987; Lopez et al., 1988). When the isolate from Zaire, designated Z-29, was compared by molecular analysis to the GS isolate, differences were apparent (Josephs et al., 1988); no other comparison, however, was attempted. Neither was a comparison published of the isolates of Downing and Tedder. The Uganda HHV-6 isolate, designated as U1102, upon comparison with Z-29, showed differences by restriction analysis, usually Sal 1 digestion and PNF1010 probe of Z-29. Two monoclonal antibodies to HHV-6 GS strain (gp82/105 and p180) were not reactive with Z-29 isolate but reacted with U1102 isolate (Wyatt et al., 1990). Yamanishi et al. (1988) reported isolation of HHV-6 from infants with exanthem subitum. No comparison of this isolate with others was attempted. Kikuta et al. (1989), however, reported polymorphism among 5 isolates from exanthem subitum and GS isolate, suggesting molecular differences with the exanthem subitum isolates. The DNA from 5 isolates of exanthem subitum were hybridized with pZVH-14 of GS isolate. The restriction pattern with BamH1, EcoR1 and HindIII were not identical.

In 1990, Levy et al. reported another HHV-6 isolate from the saliva of an AIDS patient. This isolate, designated HHV-6_{SF}, in comparison with Z-29 isolate, showed differences by restriction analysis. The infectivity of this isolate to various

primary and continuous cell lines was different from that observed with the GS isolate by others. Carrigan et al. (1990) also reported 2 isolates from leukopenia (DC) and bone marrow (BA) transplant patients. No comparison with these 2 or with other isolates has been reported. Huang et al. (1990) reported isolation of HHV-6 from peripheral blood lymphocytes of a patient with fatal haemophagocytic syndrome. These investigators used U1102 for the analysis of antibody profile and no differences were observed; they did not, however, compare this isolate with Z-29. Besides the HHV-6 isolates here mentioned, other HHV-6 isolates have been reported from normal donors and from patients with other diseases (see Table 7.1, and Chapter 4, this volume, on *in vitro* propagation systems). No characterization of various isolates has been reported. Based on the history of RNA and DNA viruses, it is not uncommon to find strain differences. Sometimes the differences are minor and may show very little change in their immunological and biological properties. Although the majority of herpesvirus isolates such as herpes simplex I and II, cytomegalovirus (CMV), varicella-zoster and Epstein–Barr virus (EBV) may show differences in their biological behavior, they infect the same cell type. All CMV isolates readily infect fibroblast cells and similarly all strains of EBV infect B cells. Since HHV-6 is a T tropic virus (Lusso et al., 1988), all its isolates differ in biological behavior (see Chapter 4, this volume, on *in vitro* propagation systems), and at present it is difficult to assess how such differences contribute to pathogenesis.

Since there has been no uniform study reported comparing the HHV-6 isolates biologically, immunologically or by molecular analysis, we undertook this study to compare 15 different isolates obtained from different sources, to detect differences and similarities which may be utilized for sero-epidemiologic studies and for

TABLE 7.1.
Source of HHV-6 isolates for comparison of biologic, immunologic and molecular analysis properties[a]

Isolate's designation	Source/Patient	Reference
GS	Acute lymphocytic leukemia, T cell type	Salahuddin et al. (1986)
Davilla	Immunoblastic lymphoma unknown-AIDS	Salahuddin et al. (1986)
Z-29	AIDS patient from Zaire	Lopez et al. (1988)
DC	Chronic lymphopenia	Carrigan et al. (1990)
BA	Bone marrow transplant	Carrigan et al. (1990)
OK	Exanthem subitum	Kikuta et al. (1989)
DA	Chronic fatigue syndrome (CFS)	Ablashi et al. (1988)
CO1 and CO3	Atypical lymphoproliferation and systemic lupus erythematosus (SLE)	Krueger et al. (1989, 1991)
CO2	Collagen-vascular disease	Krueger et al. (1991)
CO3 and CO4	SLE	Krueger et al. (1991)
CO5	Healthy individual	Krueger et al. (1991)
CO6	CFS	Krueger et al. (1991)
CO7	Rheumatoid arthritis	Krueger et al. (1991)

[a] All isolates were made from the *in vitro* cultured peripheral blood mononuclear cells.

association with any particular disease state (Ablashi et al., 1991). One may assume that the differences among the isolates may have profound effects on disease. For example, the infection of specific target cell populations may stimulate certain cytokine production which may contribute to pathogenesis (Flamand et al., 1990).

7.2. Comparison of HHV-6 isolates

The 15 HHV-6 isolates used in our study are listed in Table 7.1. All isolates infected the human umbilical cord blood mononuclear cells after cell stimulation. Their biological behavior in continuous T cell lines, however, varied (see Table 7.2 and Chapter 4, this volume, on *in vitro* propagation systems for HHV-6). The possibility that HHV-6 isolates may possess different cell receptor epitopes for infection cannot be overlooked. It is also interesting to note that some isolates are more lytic, since very few cells survive with some isolates and a high percentage of cell viability is seen with other isolates. The surviving cells in the culture have not been fully examined for HHV-6 DNA. There is preliminary evidence that some surviving cell populations contain HHV-6 genome and do not produce virus, suggesting viral latency. There is no data available concerning whether more or less cytopathic strains of HHV-6 are better candidates for latency. Takahashi et al. (1989) showed that lymphocyte population $CD4^+$ T cells were HHV-6 positive in exanthem subitum patients. Their later studies suggest that in the acute phase of the disease, $CD4^+$ T cells as well as monocytes/macrophages contained HHV-6 DNA. When patients recovered, the monocytes/macrophages contained HHV-6 DNA, but no viral DNA was detected in the lymphocyte population. Levy et al. (1990) also found that HHV-6_{SF} isolate can infect monocytes/macrophages but no data was presented to indicate whether these cells produce virus. These observations suggest that HHV-6 induces latency in monocytes/macrophages. There is no data at present concerning whether all HHV-6 isolates or only specific isolates are capable of infecting monocytes/macrophage populations. Since various HHV-6 isolates show differences in their infection of T lymphocytic cell lines (Ablashi et al., 1991), it may not be a general property of all isolates to be able to infect the monocyte/macrophage population equally. Neither is it known whether there is a sub-population of monocytes/macrophages which possess receptors for HHV-6 infection.

Our data in Figs. 7.1 and 7.2 show that GS and GS-type isolates (CO5) are more lytic than Z-29 and DC (isolate from leukopenia patients). If one compares the rate of infection by these 4 isolates, using 100 $TCID_{50}$ infectious dose, it can be concluded that by day 14, the cells infected by GS, CO5 and Z-29 isolates contain between 75–90% antigen positive cells (Figs. 7.1 and 7.2). Only 5% of cells were infected with DC isolate, suggesting either poor infectivity of the cell line used or that this isolate is less cytopathic than the others (Abalshi et al., 1991). The electron microscopy of cells infected with the DC isolate further confirmed that there were more defective virions produced and only a very few complete extracellular virions were detected. Thus it is evident that certain HHV-6 isolates are not only less

TABLE 7.2.
Reactivities of various HHV-6 isolates to monoclonal antibodies (GS strain) by indirect immunofluorescence assay[a]

Monoclonal antibody identification[b]	Protein (P) molecular weight	HHV-6 Isolates								
		GS (Salahuddin)	Davilla (Kaplan)	CO5[c] (Krueger)	CO8 (Krueger)	DA (Ablashi)	Z-29 (CDC)	DC (Carrigan)	BA (Carrigan)	OK (Kikuta)
9A5D12	P41	+	+	+	+	+	+	+	+	+
7A2	gp102	+	+	+	+	+	+	+	+	+
12B32G4	P135	+	+	+	+	+	+	+	+	+
6A5G3	gp116/gp64/gp54	+	+	+	+	+	+	+	+	+
2D6	gp82/gp105	+	+	+	+	+	−	−	−	−
13D6	gp82/gp105	+	+	+	+	+ (wk)	−	−	−	−
4A6	P180	+	+	+	+	+	−	−	−	−

[a] Balachandran et al. (1989).
[b] Other isolates, i.e., CO1–CO4, CO8 and U1102 had similar reactivities with monoclonal antibodies as GS.
[c] CO5 is an HHV-6 isolate from peripheral blood lymphocytes of a normal healthy donor and CO8 is another HHV-6 isolate from a SLE patient. All monoclonal antibodies were tested at 1:10 dilution on cells infected with various HHV-6 isolates. ± (WK) reactivity at 1:10 dilution.

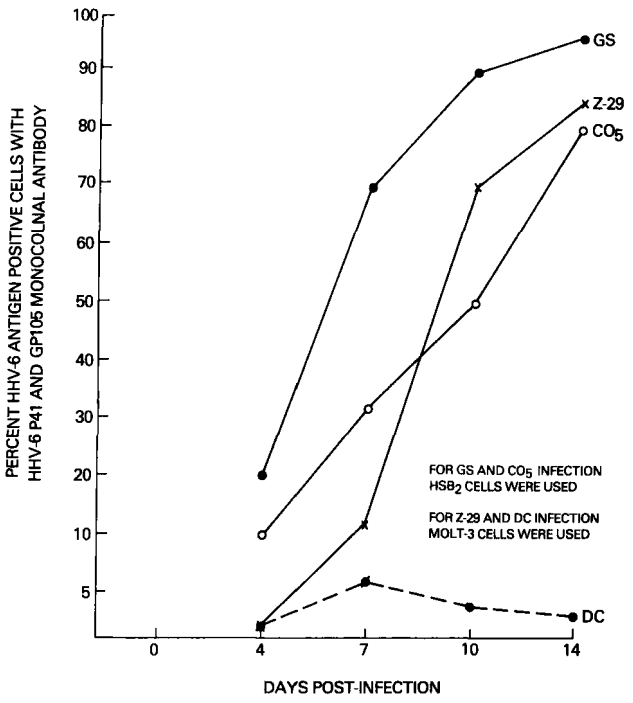

Fig. 7.1. Infection of human T cell lines with HHV-6 isolate (GS, Z-29, CO5 and DC) expressing HHV-6 viral antigens as detected by HHV-6 monoclonal antibodies to GS isolate.

cytopathic, but also cell associated. All strains of humans CMV are known to be cell associated. Thus, some HHV-6 strains may be biologically similar to CMV. Whether such isolates (Z-29, DC, OK, BA) are good candidates for cell transformation or latency is not known.

Further immunologic analysis of the 15 isolates showed that in the IFA some monoclonal antibodies (Balachandran et al., 1989) made against the GS strain proteins (Table 7.2) were reactive with all isolates (see Fig. 4.5 in Chapter 4, this volume, on *in vitro* propagation systems for HHV-6), regardless of their origin, but other monoclonal antibodies failed to react with Z-29, DC, BA and OK isolates. These data further show that epitopes to certain monoclonal antibodies against the GS strain are either modified or absent with Z-29, DC, BA and OK isolates (Ablashi et al., 1991). Levy et al. (1990) also found that a monoclonal antibody prepared against 1 exanthem subitum HHV-6 isolate reacted by IFA with his HHV-6 isolate, suggesting that the SF isolate and exanthem subitum isolates are closely related. When he compared SF with Z-29 by restriction enzyme analysis, he found them distinct. Based on our IFA data, some monoclonal antibodies against the GS isolate (Table 7.2) did not react with cells infected with SF isolate; it is possible that SF is another HHV-6 variant. Further molecular analyses using GS and other isolates are needed to confirm to which group of HHV-6 isolates the SF isolate belongs.

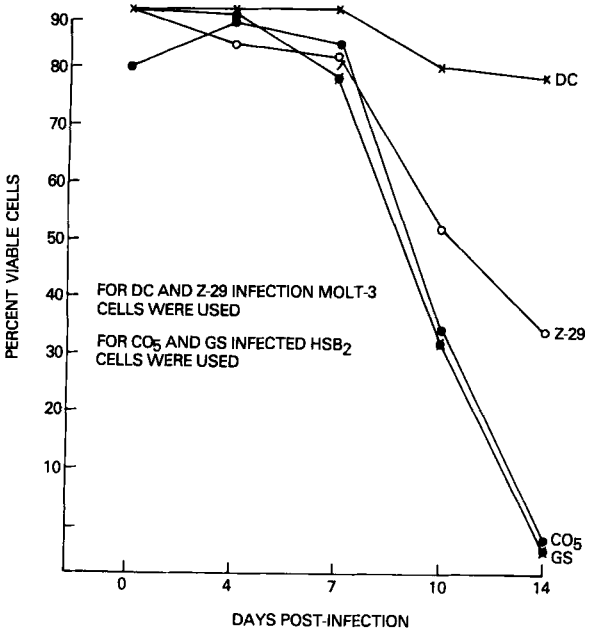

Fig. 7.2. Comparison of cell viability of cell lines infected with HHV-6 isolates (GS, Z-29, CO5 and DC).

Immunoprecipitation data using a monoclonal antibody 2D6 to GS strain (gp82/105) did not react by IFA to Z-29, DC, BA or OK. The GS and GS-type isolates CO1–CO6 (Table 7.2) immunoprecipitated strongly labeled 82k polypeptide and a diffusely labeled 105k polypeptide. Two additional minor polypeptides with molecular weights 64k and 58k were also immunoprecipitated. The Z-29 and similar isolates (DC, BA, OK) immunoprecipitated 2 polypeptides with molecular weights of 72k and 70k, and an additional 78k polypeptide was also precipitated (Fig. 7.3). Thus, immunoprecipitation data show differences in reactivity with HHV-6 isolates. The absence of reactivity with a monoclonal antibody suggests differences in the antigen make up of these isolates. The absence of reactivity with a monoclonal antibody could be due to the loss or modification of an epitope or to the absence of a reacting protein. The finding of Wyatt et al. (1990), using 2D6 and 13D6 monoclonal antibody to gp82/105 to differentiate U1102 and Z-29, further support that the U1102 isolate may be similar to GS. Our unpublished data show that there is only 1.5% difference between the GS and U1102 isolates. Using ZVH-14 probe of HHV-6_{GS} (Josephs et al., 1986), DNA from U1102 showed hybridization and all monoclonal antibodies reacted with U1102 infected HSB_2 cells. U1102 could also readily infect SupT1 cells. It is evident that monoclonal antibodies are extremely useful in differentiating HHV-6 isolates as well as in identifying candidate HHV-6 isolates.

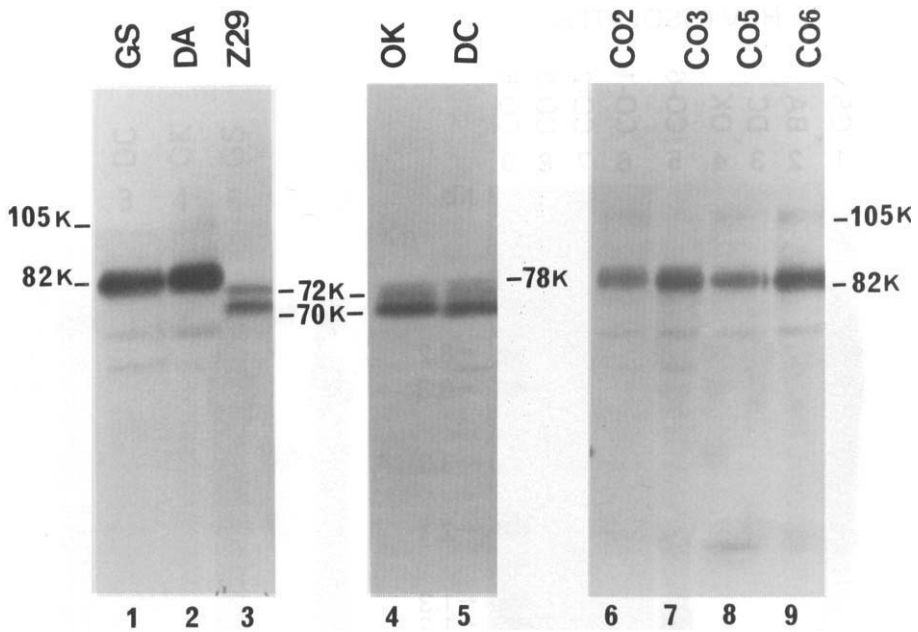

Fig. 7.3. Radio-immunoprecipitation of various proteins with different HHV-6 isolates with HHV-6 monoclonal antibodies to GS isolate.

To further support the biologic and immunologic analyses in differentiating HHV-6 isolates, 15 isolates were analyzed by restriction enzyme (Ablashi et al., 1991). High molecular weight DNA was extracted from these isolates. Southern blots were prepared. Each lane contained 15 μg DNA treated with either HindIII, EcoR1 or BamH1. The EcoR1 restriction DNA of all 15 isolates using the ZVH14 probe (Josephs et al., 1986) showed the same restriction enzyme banding pattern as the GS strain, with the exception of OK, which contained a 3.3 kb band instead of the conserved 1.2 and 2.1 kb bands detected in all the other isolates (Fig. 7.4). Using HindIII (Fig. 7.5) and BamH1 (Fig. 7.6) the isolates can be placed into two distinct groups according to the banding patterns. The first group was similar to the GS strain (CO1–3, CO5–6) and contained a single HindIII band at 7 kb (Fig. 7.5) and a doublet at 22 kb with BamH1 (Fig. 7.6). The second group consisting of isolates similar to Z-29 (DC, BA, OK) contained a HindIII fragment of 23 kb and one of 7.0 kb (Fig. 7.5). A single band at 30 kb was evident with BamH1 (Fig. 7.6). The DA isolate (Ablashi et al., 1988) from a chronic fatigue syndrome patient contained 3 bands with HindIII at 22, 8.7 and 7.0 kb (Fig. 7.5) and appeared to be a mixture of GS- and Z-29-like patterns. Thus it is possible that this isolate is in a different class. This isolate reacts with all the monoclonal antibodies (Table 7.2) and has the ability to infect productively HSB_2, SupT1 and MOLT-3 cell lines (see Table 4.2 in Chapter 4, this volume, on *in vitro* propagation systems). The restriction enzyme pattern of 15 isolates with HindIII and BamH1 are consistent with biologic

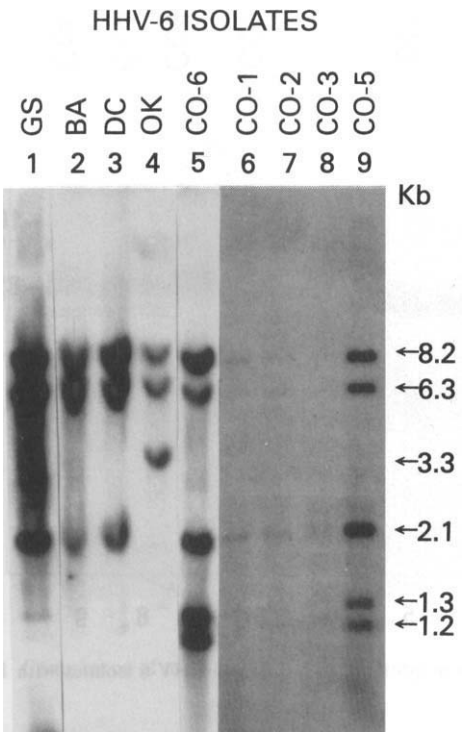

Fig. 7.4. ECOR1 digests: the ECOR1-restricted DNAs from various HHV-6 isolates were run on 0.8% agarose gels, transferred to nytran filters. The filters were hybridized to radiolabeled PZVH-14 probe of HHV-6_{GS}. The isolates of HHV-6 examined were GS, BA, DC, OK, CO6, CO1, CO2, CO3 and CO5.

findings and monoclonal antibody reactivities. These data raise the possibility that a divergence of the 2 groups of isolates coincides with the restriction enzyme as well (Ablashi et al., 1991).

It remains to be determined whether specific groups are etiologically associated with disease subsets. It can be concluded that the 15 isolates may be placed in 1 of 2 groups, a GS-type (group A) or a Z-29-type (group B). As more isolates are becoming available, similar studies are necessary to discover whether they still fit into the patterns presented here. Since there are only HHV-6 isolates from exanthum subitum reported from the Far East, and no isolate has been reported from Eastern European countries or from North America, it would be important to find whether a prevalence of HHV-6 antibody in these countries will vary if sera are tested against the isolates from those countries, rather than against GS, U1102 or Z-29 isolates. Preliminary data suggest certain sera immunoprecipitated polypeptides of 2 different isolates, suggesting infection by 2 different HHV-6 strains (Balachandran, unpublished findings) Taken together, the data on biologic and immunologic findings suggest that one can differentiate between the isolates on the

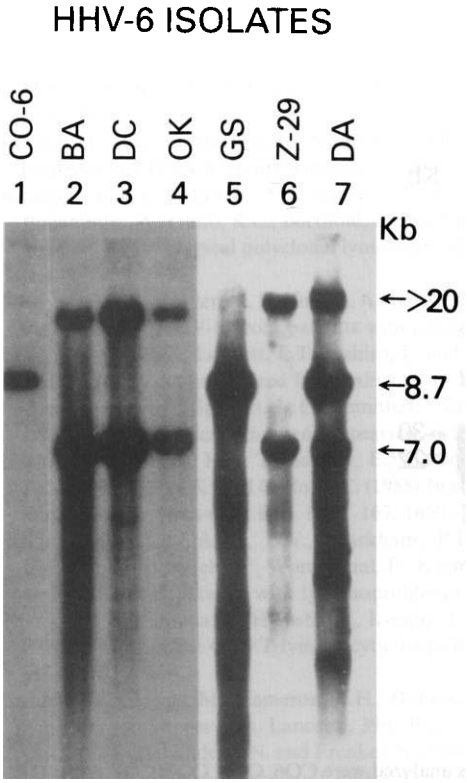

Fig. 7.5. HindIII digested HHV-6 DNAs. The isolates of HHV-6 analyzed were CO6, BA, DC, OK, GS, Z-29 and DA.

basis of their tropism to different cell lines and by their reactivity to monoclonal antibodies (Ablashi et al., 1991). Since monoclonal antibodies have also been generated against proteins of Z-29 and exanthem subitum isolates, it would be important to know whether the reactivities of these antibodies to different isolates fall into similar patterns, as observed with monoclonal antibodies against GS isolate. Our data also suggest that the GS strain of HHV-6 is a powerful inducer of IL-1β, TNF-α (Flamand et al., 1991) and hydrocortisone receptor (Lunardi-Iskander and Ablashi, unpublished findings). The question remains to be answered whether the induction of cytokines is related to more cytolytic strains or if it is a general property of HHV-6. Induction of cytokines by HHV-6 is important because this is one way the virus may be contributing to pathogenesis. It is not known how much a particular isolate is related to certain disease manifestations. Since the initial isolation of HHV-6 in 1986 was due to improved cell culture techniques and more sensitive immuno-virological techniques, considerable progress has been made in our understanding of HHV-6.

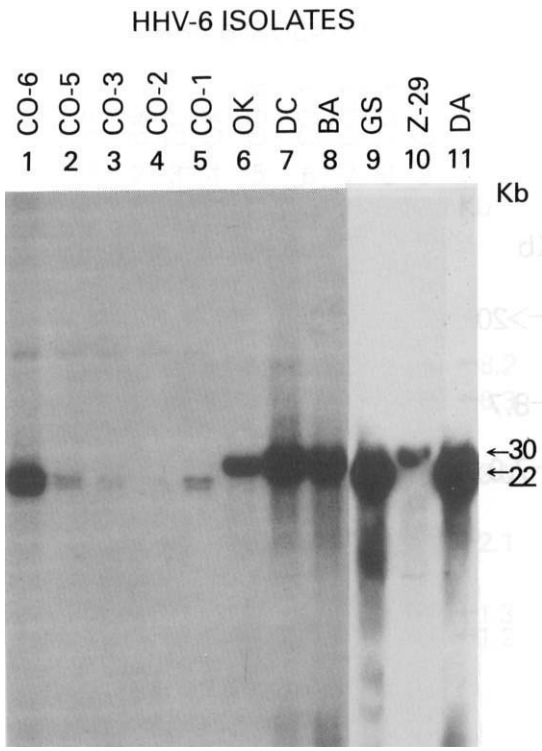

Fig. 7.6. BamH1 digestes HHV-6 DNAs. The strains analyzed were CO6, CO5, CO3, CO2, CO1, OK, DC, BA, GS, Z-29 and DA.

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Ablashi/Krueger/Salahuddin (eds) Human Herpesvirus-6

CHAPTER 8

Molecular biology of human herpesvirus 6 (HHV-6)

ZWI N. BERNEMAN[1] and DHARAM V. ABLASHI[2]

[1] *Laboratory of Tumor Cell Biology, and* [2] *Laboratory of Cellular and Molecular Biology, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892, U.S.A.*

8.1. HHV-6: molecular evidence for a new human herpesvirus

At the same time that human herpesvirus 6 (HHV-6) was first reported (Salahuddin et al., 1986), the first probe specific for that virus, pZVH14, was also published (Josephs et al., 1986). This 8.7 kb clone only hybridized to DNA and RNA of cells infected with HHV-6 and not to the genomic DNA of the other known human herpesviruses – herpes simplex virus (HSV), Epstein–Barr virus (EBV), human cytomegalovirus (HCMV), varicella-zoster virus (VZV) – or *herpesvirus saimiri* of squirrel monkeys, which also induces T cell lymphomas in marmosets and owl monkeys (Ablashi et al., 1979). Similar hybridization results with the pZVH14 probe were obtained with other HHV-6 isolates (Downing et al., 1987; Tedder et al., 1987; Agut et al., 1988; Lopez et al., 1988; Becker et al., 1989). Restriction enzyme analysis of purified viral DNA also showed that the HHV-6 pattern was markedly different from that of the other human herpesviruses (Lopez et al., 1988). This firmly established that HHV-6 – formerly called human B lymphotropic virus or HBLV – is a new member of the human herpesvirus family. Very early, it also became possible to amplify specific HHV-6 sequences using the polymerase chain reaction (PCR) technique (Buchbinder et al., 1988).

8.2. Molecular heterogeneity of HHV-6

Restriction polymorphism has been noted when comparing different HHV-6 isolates (Josephs et al., 1988a; Jarrett et al., 1989; Kikuta et al., 1989). It now appears that at least 2 groups – which we propose to call A and B – can be

TABLE 8.1.
Categorization of different HHV-6 strains using restriction analysis polymorphisms

Group A	Group B
GS	Z29
Davilla	HST, KBT, KSM, KWG, SUZ
U1102	OK
AJ	KF, BA
SIE, TAN	SF
CO1, CO2, CO3, CO5, CO6	VW, AW, BOU, MAR, BLE, MBE

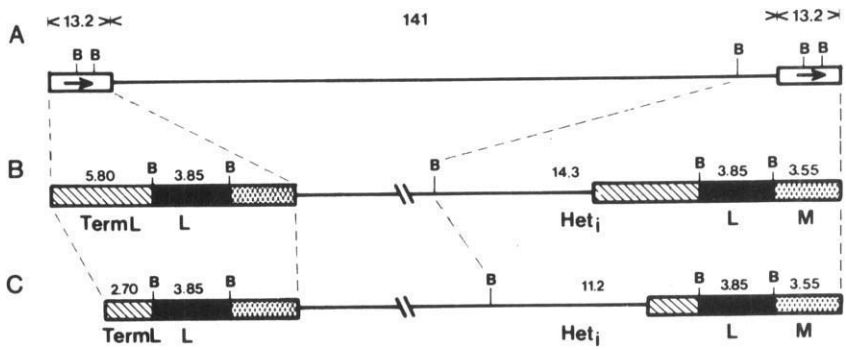
distinguished in the HHV-6 isolates, based on biological, immunological and molecular characteristics (Wyatt et al., 1990; Ablashi et al., 1991; Aubin et al., 1991; Schirmer et al., 1991). HHV-6 group A includes strains that disclose a higher cytopathic effect than group B. Pooling of restriction analysis data from different molecular studies allows to group the strains as follows (Table 8.1) (Downing et al., 1987; Ablashi et al., 1991; Aubin et al., 1991; Schirmer et al., 1991; Berneman et al., unpublished). Group A includes strains GS (Salahuddin et al., 1986), Davilla (an AIDS patient derived isolate, Dr. Marc Kaplan, North Shore University Hospital, Long Island, New York), U1102 (Downing et al., 1987), AJ (Tedder et al., 1987), SIE (Agut et al., 1988), TAN (Agut et al., 1989), CO1, CO2, CO3, CO5, CO6 (Krueger et al., 1991); Group B includes strains Z29 (Lopez et al., 1988), HST, KBT, KSM, KWG, SUZ (Yamanishi et al., 1988), OK (Kikuta et al., 1989), KF or DC, BA (Carrigan et al., 1990; Russler et al., 1991), SF (Levy et al., 1990), VW, AW (Schirmer et al., 1991), BOU, MAR, BLE and MBE (Aubin et al., 1991). There also seem to be restriction pattern differences within each group. Up to now, all the HHV-6 probes tested hybridize to DNA of strains belonging to both groups. Most of the available sequence data originate from 2 strains belonging to group A HHV-6, namely GS and U1102. In a 163 basepair (bp) region, no difference was noted between 2 strains of group A (SIE and TAN), or between 2 strains of group B (HST and MBE). In the same segment, a 4% nucleotide difference was observed between group A (strain SIE) and group B (strain HST) (Aubin et al., 1991). Sequence analysis comparison within a 465 bp fragment of the DNA polymerase gene of the U1102 and AJ strains – both of which presumably belong to group A (Downing et al., 1987) – showed differences in 1% of the nucleotides (Teo et al., 1991). Not unexpectedly, and as far as we can tell from the present data, a higher nucleotide divergence seems to exist between strains belonging to different HHV-6 groups than between strains within the same group.

8.3. General molecular structure of the HHV-6 genome

The general features of the HHV-6 genome are summarized in Table 8.2. HHV-6 has a double-stranded DNA genome. Its size has been estimated to be 170 000 bp

TABLE 8.2.
General characteristics of the HHV-6 genome

Linear double-stranded DNA genome
Size; 161 500–170 000 basepairs
40–43% G + C content
Coding capacity for >70 proteins
One unique long (U_L) segment flanked by direct repeats (DR) at both termini
Length heterogeneity of the terminal DR
Integration in host genome: unknown
Circularization of < 10% of the genomic population
Concatemeric dimers of intranuclear genomes
Contains repetitive sequences
Genomic inversion not apparent
Closest phylogenetic relative: HCMV
U_L of HHV-6 colinear with the U_L of HCMV
Restriction site polymorphism among different isolates
Polymorphic variants occur upon passage
Encodes for virus-specific DNA polymerase and alkaline DNase
No virus-specific thymidine kinase activity detectable



Model of HHV-6 genomic architecture. The HHV-6 genome consists of a 141-kb unique segment bracketed by a directly repeated sequence element. (A) Schematic diagram of the longer form of the genome. (B) and (C) Enlargements of the structures of the direct repeat elements in the longer and shorter forms of the genome, respectively. The locations of relevant BamHI sites are indicated by B, and relevant BamHI fragments are identified by fragment name. Fragment lengths are in kb. The orientation of the genome shown here is inverted relative to that presented previously (Pellett et al., 1990) because in this orientation homologous regions of HHV-6 are colinear with those of the prototype arrangement of CMV (T. Dambaugh et al., unpublished data).

Fig. 8.1. Schematic representation of the HHV-6 overall genomic structure. Reprinted from *Virology* with permission of Dr. P. Pellett (Lindquester and Pellett, 1991) and the Academic Press, Inc.

in the GS strain (Josephs et al., 1988a), 161 500 bp in the U1102 strain (Martin et al., 1991a), and either 162 000 or 168 000 bp in the Z29 strain (Lindquester and Pellett, 1991). The overall G + C content of the Z29 strain genome was calculated to be 43% (Lindquester and Pellett, 1991). This value is in agreement with a value

of 40% found earlier (Downing et al., 1987), of 41% found for 21 858- and 4 407-bp-long regions of the U1102 strain genome (Lawrence et al., 1990; Teo et al., 1991) and of 40.3% and 39.9% in 7 981- and 8 717-bp-long segments of the GS strain genome, respectively (Josephs et al., 1991a). This is the lowest G + C content found so far for human herpesvirus genomes.

The HHV-6 genome appears to consist of a 141 000 bp unique long segment (U_L), flanked on both sides by the same terminal repeat, in the same, i.e., direct, orientation (Lindquester and Pellett, 1991; Martin et al., 1991a). This is illustrated

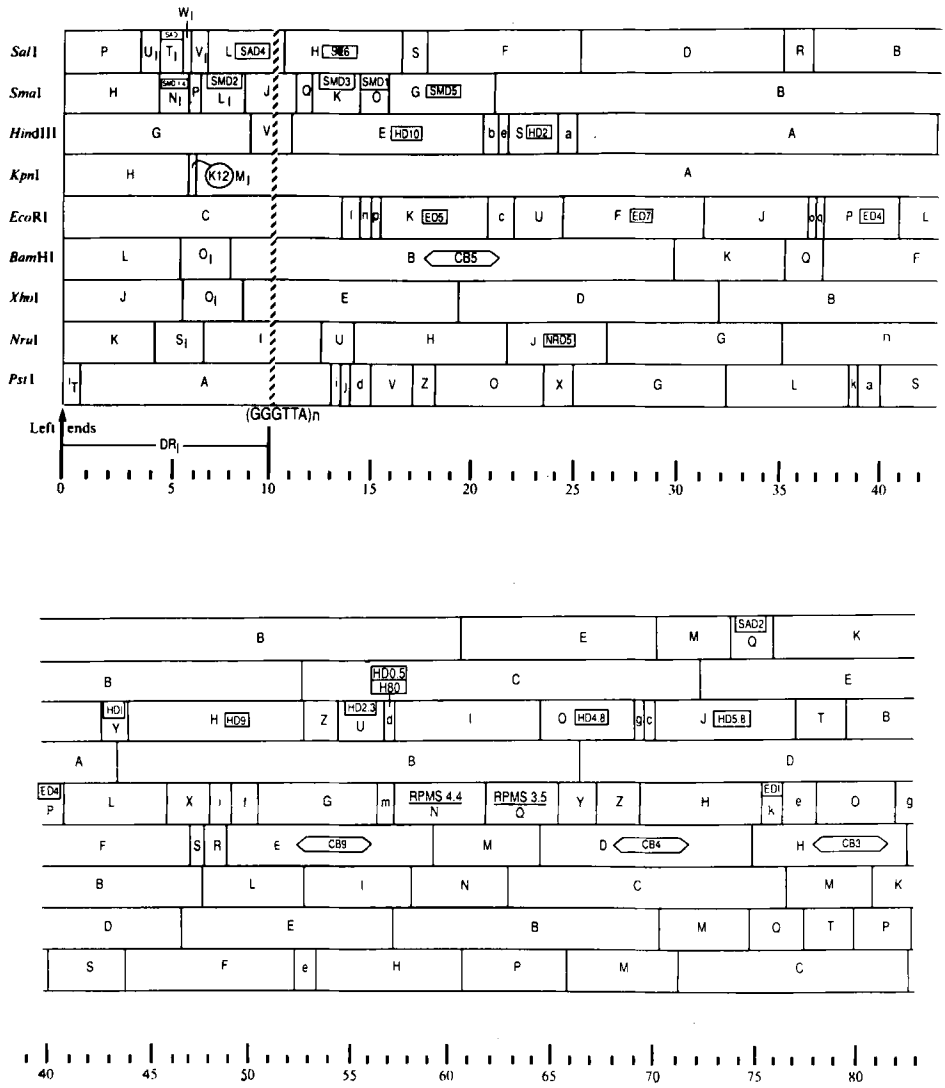
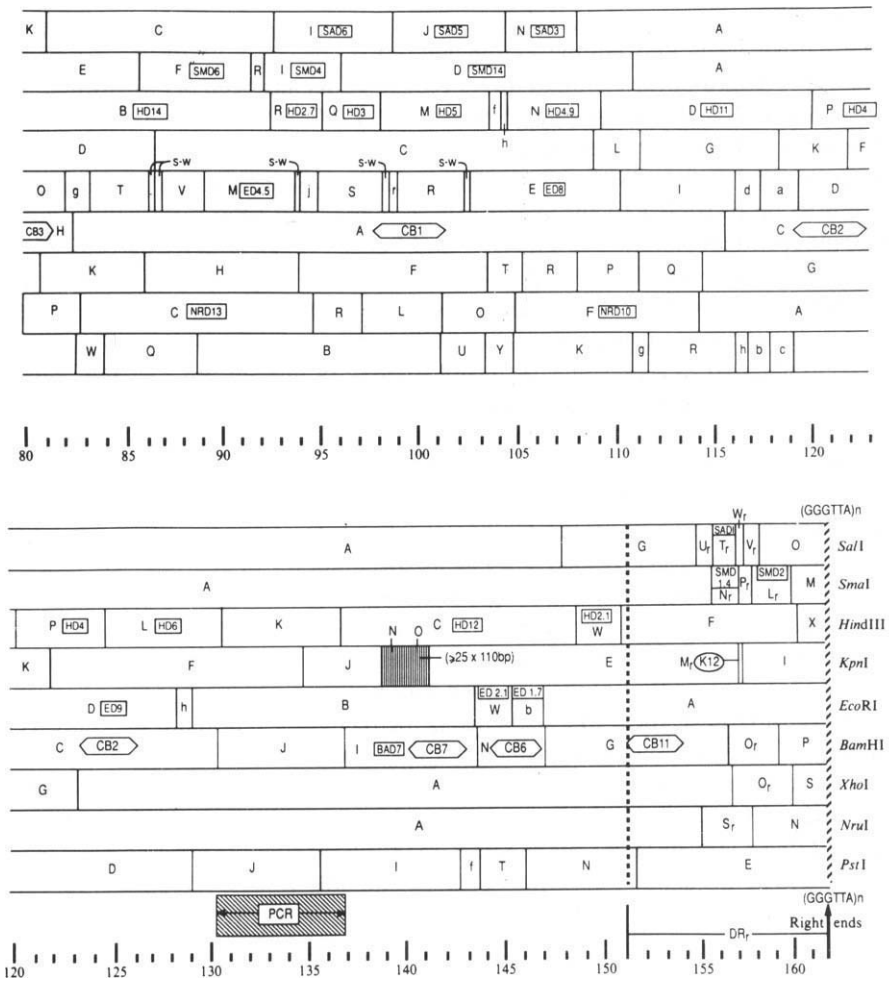


Fig. 8.2A.



The position of cleavage sites for each of the nine mapping enzymes within the unit-length genome of HHV-6 U1102. The designation of each restriction fragment is shown. The names of fragments isolated as stable clones in bacterial vectors are indicated as follows: plasmid clones within rectangles ( ), cosmid clones within lozenges ( ), M13 clones contained within a circle (○) and clones isolated in lambda are underlined. The region of the genome which was unstable in bacterial vectors and was characterized as a PCR product is shown in the hatched box. The position of the KpnI repeat sequences is also illustrated within a box. The position of the KpnI-N fragments relative to the KpnI-O fragments has not been determined (see text). The right end of the terminal duplications (marked) coincides with the proposed location in HHV-6 U1102 of the reiterated $(GGGTTA)_n$ motif found in the GS strain of the virus. The broken line indicates the junction between the unique sequence and the left end of DR_R. Subscripts l and r indicate fragments contained entirely within the left and right terminal repeats. i_T is the terminal PstI fragment.

Fig. 8.2. Restriction map of the U1102 HHV-6 genome. The HD9 region corresponds to the region identified by the pZVH14 probe, the CB5 clone is homologous to pZVB70. Reprinted from *J. Gen. Virol.* with permission of Dr. M. Martin (Martin et al., 1991a) and the Society of General Microbiology, United Kingdom.

in Fig. 8.1. The structure of the HHV-6 genome appears to be similar to that of channel catfish virus and equine cytomegalovirus. In the Z29 strain, a heterogeneity in the length of the terminal direct repeats (DR) has been demonstrated. In one form, the DR is 10 100 bp long, in the other 13 200 bp. This results in a length heterogeneity of the overall genome, respectively 162 000 and 168 000 bp (Lindquester and Pellett, 1991). The variation in the DR elements of HHV-6 does not seem to be restricted to the Z29 strain, but has also been observed in more than 20 independent isolates (Lindquester and Pellett, 1991). Different groups have published extensive restriction maps of the entire HHV-6 genome (Martin et al., 1991a; Neipel et al., 1991b; Teo et al., 1991). This is illustrated in Fig. 8.2.

Most of the purified virus particles have a linear genome. Less than 10% seems to have circularized in a plasmid-like fashion, directly linking the 'left' DR (DR_L) with the 'right' DR (DR_R) (Lindquester and Pellett, 1991). The nuclei of infected cells also seem to contain concatemeric dimers of the linear genome, linking the DR elements tail-to-head, e.g., DR_{L1}–U_{L1}–DR_{R1} · DR_{L2}–U_{L2}–DR_{R2} (Martin et al., 1991a).

The HHV-6 genome contains 60 copies of the tandem repetitive sequence GGGTTA. This sequence is also present at the junction of the internal repeats flanking the short and long unique segments (IR_S–IR_L) of the genome of Marek's disease virus, a herpesvirus causing T cell lymphomas in chicken (Kishi et al., 1988). In the latter, the GGGTTA repeat is part of a sequence that is similar in structure, but not in sequence, to that of the *a* sequence of herpes simplex virus type 1 (Kishi et al., 1991). In HSV-1, the *a*-like sequence is the recognition domain for cleavage/packaging of the DNA into the nucleocapsid (Deiss and Frenkel, 1986). The GGGTTA tandem repeats are located at or near the 'right' end of the DR. Interestingly, GGGTTA tandem repeats are also the main feature of eukaryotic telomeric DNA (Moyzis et al., 1988). Other repetitive sequences are also present in the HHV-6 genome: a 1 500-bp-long TC/G repeat, and at least 25 tandem repeats of a 110-bp-long sequence, containing a unique Kpn I site (Fig. 8.2) (Martin et al., 1991a).

8.4. Characterization, cloning and sequencing of HHV-6: homology and colinearity with HCMV

Although HHV-6 was found to be significantly different from the other herpesviruses, as to warrant the designation as a novel virus, very soon a homology with HCMV was reported (Efstathiou et al., 1988). This was later confirmed, when a 21 858-bp DNA sequence of the HHV-6_{U1102} strain genome was published (Lawrence et al., 1990; Littler et al., 1990). This sequence stretch included genes coding among others for the alkaline exonuclease, phosphotransferase (Chee et al., 1989) and major capsid protein of HHV-6, as well as the conserved spliced gene of unknown function. Table 8.3 gives an overview of the homology between the putative HHV-6 and HCMV proteins with known or presumed function. No functional study with HHV-6 proteins has yet been published, and the function of

TABLE 8.3.
Homology between putative HCMV and HHV-6 proteins

Putative function	HCMV ORF[a]	% Identity	No. of amino acids[b]	Reference
Large structural phosphoprotein	HCMVUL32	25	72	[1]
		23.2	284	[2]
G-protein-coupled receptor homologue	HCMVUL33	26.7	110	[1]
		24.3	300	[2]
Early-late nonstructural DNA-binding phosphoprotein	HCMVUL44	41.8	282	[3]
Large tegument protein	HCMVUL48	14.8	2077	[4]
DNA polymerase	HCMVUL54	45.8	1012	[5]
Major DNA binding protein	HCMVUL57	51.1	70	[1]
		41.1	552	[2]
Transactivator	HCMVUL69	45.6	50	[1]
		29.6	257	[2]
DNA replication	HCMVUL70	25.3	466	[2]
Glycoprotein H	HCMVUL75	23.9	685	[4]
Major capsid protein	HCMVUL86	43.8	1345	[6,7]
		40	145	[1]
Phosphotransferase	HCMVUL97	27	561	[8,7]
Alkaline exonuclease	HCMVUL98	37	488	[7]
Helicase	HCMVUL105	35	72	[1]

[a] ORF, open reading frame.
[b] The number of amino acids examined depends on each individual study and covers the whole or only a part of the putative HHV-6 protein.

[1] Neipel et al. (1991b).
[2] Berneman et al. (unpublished).
[3] Chang and Balachandran (1991).
[4] Josephs et al. (1991a).
[5] Teo et al. (1991).
[6] Littler et al. (1990).
[7] Lawrence et al. (1990).
[8] Chee et al. (1989).

these peptides has been deduced from the homology with HCMV, which itself was often derived from the homology primarily with HSV-1 and also with EBV and VZV (Chee et al., 1990a). A homology with HCMV was also demonstrated, regarding the early-late, nonstructural DNA-binding phosphoprotein recognized by the HHV-6-specific 9A5D12 monoclonal antibody, which reacts with all HHV-6 strains tested so far (Chang and Balachandran, 1991). A significant homology was also noted between putative HHV-6 proteins and the glycoprotein H and putative large tegument protein (Josephs et al., 1991a), and the large structural pp150 phosphoprotein of HCMV (Neipel et al., 1991b; Berneman et al., unpublished). The HHV-6 equivalent of the latter protein has recently been shown to encode the immunodominant antigen of HHV-6, recognized by all reactive human sera (Neipel et al., 1991a). This corresponds to the highly immunogenic

character of the HCMV homologue (pp150) (Jahn et al., 1987). HCMV encodes for 3 proteins, which contain 7 putative transmembrane domains, similar to the family of eukaryotic cell-encoded G protein-coupled receptors (GCR) (Chee et al., 1990b). This is a group of conserved receptors, including rhodopsin, beta-adrenergic and serotonin receptors, as well as oncogenes. The discovery of HCMV GCR homologues suggested that HCMV may have captured an eukaryotic GCR gene during evolution. It also suggested a new pathway in the induction of disease by that virus. This could be valid for HHV-6 as well, since a homologue of the HCMV GCR homologue in the U_L region was also found (Neipel et al., 1991b; Berneman et al., unpublished). This could be one candidate gene that could play a role in the putative oncogenic transformation mediated by HHV-6 (see Section 8.7).

Using biochemical markers, it was demonstrated that HHV-6 encoded for specific DNA polymerase and alkaline DNase, but not thymidine kinase, deoxyuridine triphosphate nucleotidohydrolase (dUTPase) or uracil-DNA glycosylase (UNG) (Williams et al., 1989; DiLuca et al., 1990). The DNA polymerase gene of HHV-6 has been cloned and, again, its sequence discloses a significant homology with HCMV (Teo et al., 1991). In HSV-1, the DNA polymerase is 1 of 7 genes necessary and sufficient for the origin of replication (ori)-dependent viral DNA synthesis (McGeogh et al., 1988; Wu et al., 1988). Those genes and their HCMV and HHV-6 homologues are listed in Table 8.4 (Chee et al., 1990a). The putative HHV-6 helicase, major DNA-binding protein and helicase/primase complex-comprising protein are suggested from the homologies in Tables 8.3 and 8.4 (Neipel et al., 1991b; Berneman et al., unpublished).

The extensive sequence maps (Martin et al., 1991a; Neipel et al., 1991b; Teo et al., 1991), as well as the available sequence information and comparison with HCMV (see above), have disclosed that the alignment of the HHV-6 genes is similar to, i.e.,

TABLE 8.4.
Genes essential for the *ori*-dependent replication of HSV-1 and their HCMV and HHV-6 homologues

HSV-1 ORF[a]	Function of HSV-1 protein	HCMV ORF	Reference of HHV-6 homologue[b]
UL5	Helicase	HCMVUL105	[1]
UL8	Part of helicase/primase complex		
UL9	Ori-binding protein		
UL29	Major DNA-binding protein (*dbp*)	HCMVUL57	[1, 2]
UL30	DNA polymerase (*pol*)	HCMVUL54	[3]
UL42			
UL52	Part of helicase/primase complex	HCMVUL70	[2]

[a] ORF, open reading frame.
[b] [1] Neipel et al. (1991b).
[2] Berneman et al. (unpublished).
[3] Teo et al. (1991).
The information for the blank spaces has not been found or is not available at present.

co-linear with, their equivalents in the unique long (U_L) segment of HCMV. This confirms the close phylogenetic relationship of the 2 viruses. Homologies with the other human herpesviruses proteins have also been described in the above-mentioned studies, but they were always less pronounced than the homology between HHV-6 and HCMV.

Classically, the herpesviruses are divided into 3 categories: the alpha- or neurotropic herpesviruses (HSV, VZV), the beta- or salivary gland herpesviruses (HCMV) and the gamma- or lymphotropic herpesviruses (EBV, *herpesvirus saimiri*, Marek's disease virus, herpesvirus of turkeys) (Chee and Barrell, 1990). HHV-6 productively infects T lymphocytes, and would thus seem to belong to the gamma-herpesviruses. Nevertheless, the sequence and mapping data strongly support its classification as a beta-herpesvirus.

8.5. Homology between HHV-6 and other viruses: homology between the putative HHV-6 PH protein and the AAV-2 *rep* gene

A 24% identity at the protein level between a putative HHV-6 protein (called PH) and the *rep* protein of adeno-associated virus type 2 (AAV-2) has been reported (Thomson et al., 1991). AAV-2 is a human helper-dependent parvovirus, which can only replicate if it co-infects the target cell with an adenovirus or a herpesvirus. The *rep* gene shows a significant homology to the T antigens of polyomavirus and SV40 and the E1 proteins of papillomaviruses. The *rep* gene is essential for the replication of AAV-2 and transactivates homologous and heterologous promotors. It also inhibits transformation induced by papillomaviruses (reviewed by Thomson et al., 1991). The homology between the AAV-2 *rep* and the HHV-6 PH gene products could have arisen from the capture by HHV-6 of the *rep* gene by nonhomologous recombination with AAV-2 (Thomson et al., 1991).

8.6. Molecular interactions between HHV-6 and HIV-1

Human immunodeficiency virus type 1 (HIV-1) and HHV-6 infect the same type of cell, namely the $CD4^+$ T lymphocyte. The 2 viruses can productively infect the same cell, resulting in accelerated cell death (Lusso et al., 1989). Moreover, HHV-6 expression transactivates HIV-1 long terminal repeat (LTR) directed gene expression, as assayed by the expression of the chloramphenicol acetyltransferase (CAT) assay (Ensoli et al., 1989; Horvat et al., 1989, 1991). The transactivation of HIV-1 by HHV-6 occurs with both types of HHV-6 strains, as exemplified by the GS and Z29 strains (Horvat et al., 1991). The HIV-1 LTR region inducible to transactivation by HHV-6 appears to map to the NF-kappaB motifs of the HIV-1 enhancer region (Ensoli et al., 1989; Horvat et al., 1991), which are also the binding site of nuclear protein(s) specifically activated or induced by HHV-6. Several HHV-6 fragments are able to induce HIV-1 LTR-directed CAT gene expression:

the pZVB70, pZVB10 and pZVH14 clones (Horvat et al., 1991). The transactivation by the first 2 clones is totally dependent on a functional NF-kappaB site, while pZVH14 may also use other region(s) in the HIV-1 LTR.

Also, the region containing the putative immediate early genes of HHV-6 has been amplified by PCR and cloned (Martin et al., 1991b). Those clones are also able to transactivate HIV-1 LTR.

Lately, the attention has been drawn on the observation that the transactivation by HHV-6 is dependent not on the promotor, but on the reporter gene used to demonstrate transactivation (Campbell et al., 1991). A similar observation was made earlier for the EBV BMLF1 gene, which encodes an immediate early transactivator (Markovitz et al., 1989). According to this study, if HHV-6 increases HIV-1 expression, it may do so at the post-transcriptional rather than at the transcriptional level, e.g., by stabilizing mRNA (Campbell et al., 1991).

Infection with HHV-6 is also able to induce the expression of CD4 molecules on the surface of $CD4^-CD8^+$ lymphocytes, rendering them susceptible to infection by HIV-1 (Lusso et al., 1991). The induction of CD4 occurs at the transcriptional level and simultaneously with HHV-6 expression, suggesting possible transactivation of the CD4 gene by HHV-6 viral proteins or by HHV-6-induced cellular proteins.

8.7. Does HHV-6 have transformating properties?

Since HHV-6 was first isolated from patients with lymphoproliferative disorders (Salahuddin et al., 1986), the question is whether the virus is associated with these diseases. Southern blot hybridization of 179 DNA samples, extracted from various malignant and reactive lesions, only revealed positivity in 3 cases (Josephs et al., 1988b) (Table 8.5): 1 African Burkitt's lymphoma, that was also EBV-positive; and 2 EBV-negative B cell lymphomas, 1 follicular large cell lymphoma and 1 diffuse small cleaved cell lymphoma arising in a patient with Sjögren's syndrome. Figure 8.3 shows that, in the latter patient, HHV-6 sequences were apparent in involved lymph nodes, but not in an early parotid gland biopsy taken when the condition was still in the Sjögren's syndrome pre-lymphoma phase. Another series of 117 DNA samples revealed hybridization in 2 cases: 1 case of EBV-negative T zone lymphoma arising in a patient with angioimmunoblastic lymphadenopathy and 1 case of diffuse large cell B lymphoma in a patient with a pre-existent Sjögren's syndrome (Jarrett et al., 1988). In those 2 series, Hodgkin's disease lymph node DNA was negative for HHV-6 sequences by Southern blot hybridization with the pZVH14 probe.

In another recent series, 3 out of 25 cases with Hodgkin's lymphoma were positive by PCR for HHV-6 sequences in the tumoral lymph nodes, while none of the 41 non-Hodgkin's lymphoma cases was positive (Torelli et al., 1991). Moreover, 2 of the Hodgkin's disease lymph nodes also tested positive by Southern blot hybridization with the pZVH14 probe. Interestingly, all 3 positive cases belonged to the same nodular sclerosis-lymphocyte depletion histological subtype. These provocative and interesting studies raise the distinct possibility that HHV-6 is

TABLE 8.5.
Detection of HHV-6 sequences in tumors by Southern blot hybridization with pZVH14 probe. Reprinted from *Leukemia* with permission of Macmillan Press (Josephs et al., 1988b)

Pathology of tissue[a]	No. positive/total	
B cell		3/82
Follicular (small cell) lymphoma	0/37	
Follicular (large cell) lymphoma	1/4	
Diffuse (large cell) lymphoma	0/6	
Diffuse (small cleaved cell) lymphoma	1/6	
Burkitt's (African) lymphoma	1/7	
CLL (diffuse well-differentiated lymphoma)	0/22	
T cell		0/22
Diffuse (small and large cell) lymphoma	0/22	
ALL	0/1	
Myeloid		0/15
AML and AMML	0/10	
CML	0/5	
Hodgkin's disease		0/8
Lymphadenopathy		0/38
Angioimmunoblastic lymphadenopathy	0/7	
Reactive hyperplasia	0/31	
Kaposi's sarcoma		0/14
HIV related	0/7	
African endemic	0/7	

[a] CLL, chronic lymphocytic leukemia; ALL, acute lymphocytic leukemia; AML, acute myelogenous leukemia; AMML, acute myelomonocytic leukemia; CML, chronic myelogenous leukemia; HIV, human immunodeficiency virus.

associated with a minority of lymphomas, although it is still unclear at this stage whether the virus plays the role of a driver or passenger in the pathogenesis of these tumors.

In vitro data suggest that HHV-6 could contain sequences with transforming potential, since transfection of NIH3T3 cells with the pZVH14 clone has been reported to transform those cells (Razzaque, 1990). Although HHV-6 clearly behaves as a lytic virus *in vitro*, the NIH3T3 transformation data should draw our attention to the transforming potential of HHV-6. Clearly, more work and more definitive information are needed in this still controversial field.

8.8. Molecular evidence for HHV-6 in other diseases

The role of HHV-6 in retinitis is being studied (see Qavi et al., Chapter 20, this volume, on HIV-1 and HHV-6 infections of human retina and cornea). The data

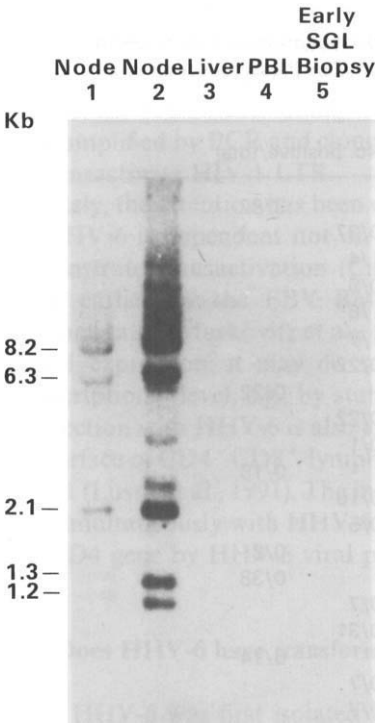


Fig. 8.3. Detection of HHV-6 sequences in DNA from B cell lymphoma and control tissue from a Sjögren's syndrome patient. Southern blot hybridization of Eco RI-digested DNA with the pZVH14 probe. Reprinted from *Leukemia* with permission of Macmillan Press (Josephs et al., 1988b). Lane 1: abdominal node; lane 2: thoracic node; lane 3: liver; lane 4: peripheral blood lymphocytes; and lane 5: early salivary gland biopsy.

indicate that HHV-6 DNA sequences were detected in several retinal lesions from AIDS patients, by PCR and dot blot hybridization. Using ^{35}S-labeled RNA probes derived from the pZVH14 probe, HHV-6 transcriptional activity was detected in 2 out of 7 retinas containing HHV-6 and HIV-1 DNA sequences. This suggests that HHV-6, like HCMV, may enhance the susceptibility for HIV-1 replication in retinal cells.

The HHV-6 viral load in chronic fatigue syndrome was studied by short-term cultures of peripheral blood mononuclear cells. HHV-6 antigens and sequences could be demonstrated in 3 out of 7 patients, but not in healthy controls (Josephs et al., 1991b). This suggests that at least some patients may carry a higher viral burden than normal. Whether this is a cause or effect or epiphenomenon remains to be determined.

8.9. Human herpesvirus 7 (HHV-7)

Less than 4 years after the first report of HHV-6, another new human $CD4^+$ T lymphotropic herpesvirus was reported (Frenkel et al., 1990). The first strain described (RK) was isolated from a healthy individual. Restriction analysis of viral DNA revealed a pattern distinct from HHV-6. Hybridization with 11 HHV-6 DNA probes revealed hybridization with only 6 of the probes tested, resulting in a maximal homology of 31 kb out of the 75 kb tested (41.3%). We have obtained an independent strain of HHV-7 (JI) from a patient with chronic fatigue syndrome (Berneman et al., 1991). Hybridization with HHV-6 probes spanning 97.7 kb of the HHV-6 genome reveals hybridization with at most 37.6 kb (38.5%). On the other hand, all those probes hybridize to different HHV-6 strains, from both groups A and B, even after high stringency washes. These results show that the molecular divergence between HHV-6 and HHV-7 is larger than between different HHV-6 strains. They also support the classification of HHV-7 as a virus distantly related to, but significantly different from, HHV-6.

8.10. Future prospects

Since its first description 4 years ago, a lot has been learned about the molecular structure of HHV-6. Different important observations have been made, among others: the homology with HCMV, the strain variations along 2 groups, the possibility of gene capture from viral and eukaryotic genomes. Currently, different groups in England, Germany and the U.S.A. are in the process of sequencing the whole virus. At present, at least half of the HHV-6 genome has already been sequenced. In addition to providing valuable information regarding the phylogenetic relationship between the different herpesviruses, the HHV-6 sequencing project will make possible to devise rational antiviral treatments, which could be directed against essential viral genes involved in replication, transactivation or signal transduction. This may become necessary, since HHV-6 is increasingly linked with serious diseases, as can be gathered from the present volume. Finally, the era of gene therapy is dawning, and herpesvirus vectors are appearing on the horizon. The T cell tropic character of HHV-6 may become a handle through which T cell disorders, like AIDS, could be treated. This, of course, would require a thorough molecular knowledge of HHV-6. Considering the progress that has been made, it can be projected without exaggeration that the complete genome will be sequenced within the next 5 years. We have only started to grasp the significance of it all.

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Ablashi/Krueger/Salahuddin (eds) Human Herpesvirus-6

CHAPTER 9

Proteins of human herpesvirus-6

N. BALACHANDRAN

Department of Microbiology, Molecular Genetics and Immunology, The University of Kansas Medical Center, Kansas City, KS 66103, U.S.A.

9.1. Introduction

Identification and characterization of viral proteins and defining their functions are essential for the complete understanding of viral replication, interaction of virus with target cells and for the development of specific antiviral therapy, diagnostic reagents and a protective vaccine. Herpesviruses contain double-stranded DNA with the capacity to code for more than 50 proteins. In an infected cell, these viral proteins are synthesized in a cascade manner, which is generally believed to be regulated at the transcription level. After more than two decades of intensive work on the 5 human herpesviruses, namely HSV-1, HSV-2, VZV, CMV and EBV, we are beginning to understand the role of viral proteins in the complex biology of these viruses. Even within these viruses, the pace of information is linked to several parameters such as the ability of virus to grow in cell lines, the rapidity of growth and the availability of mutants and other reagents. Although human herpesvirus-6 (HHV-6) was isolated more than 4 years ago, only a little is known about HHV-6 proteins. This lack of knowledge is due to the difficulty in growing HHV-6 *in vitro* and due to the lack of specific reagents to analyze HHV-6 proteins. This limitation hampers our attempt to write a cohesive chapter on HHV-6 proteins. In an attempt to catalogue the descriptive data on HHV-6 proteins, we have tried to compensate this deficiency by incorporating data from our ongoing studies.

9.2. Initial identification of HHV-6 proteins

With an estimated 170 kb DNA, HHV-6 should code for more than 70 proteins. As yet, a productive HHV-6 infection in monolayer cell lines has not been established.

Only suspension cultures of human peripheral blood lymphocytes (PBL), cord blood lymphocytes (CBL) and T cell lines are available for viral propagation and for the analysis of viral proteins. Even though only a limited number of papers has been published on HHV-6 proteins, comparison of the reported molecular weights of HHV-6 proteins is hampered by the use of different gel systems and different strains of HHV-6. Shiraki et al. (1989) have analyzed the virion proteins of the HHV-6-related isolate HST from exanthem subitum, and have identified at least 29 polypeptides ranging from 280k and 30k. Among these, the 225k, 120–110k, 84–76k, 70–65k, 60k and 53k polypeptides were identified in the envelope fraction. In an initial SDS-PAGE analysis, total cell extracts of uninfected and HHV-6 strain GS [HHV-6_{GS}] infected HSB_2 cells labeled with [^{35}S] methionine or with [^{3}H] glucosamine at 6 days post-infection were used (Balachandran et al., 1989). In this study, more than 20 polypeptides ranging from 180k to 31k and 8 glycopolypeptides specific for HHV-6-infected cells were identified. Identification of proteins specific for HHV-6-infected cells in these total cell extracts was complicated by the fact that several proteins present in the infected cells were also seen in the uninfected cells. This could be attributed to the absence of synchronized infection, replicating uninfected cells, absence of host protein shut off by HHV-6 and/or due to induction of host protein synthesis by virus. Hence the identification of HHV-6 proteins from infected cells depends upon radioimmunoprecipitation (RIP) and Western blot (WB) assays using human sera, polyclonal rabbit or mouse sera and by monoclonal antibodies (MAbs).

9.3. HHV-6 proteins specific for infected cells recognized by polyclonal rabbit antibodies

In an initial attempt to identify HHV-6 proteins, Ablashi et al. (1988a) have used polyclonal rabbit antibodies against purified HHV-6_{GS} in immunoprecipitation reactions with [^{35}S] methionine and cysteine labeled HHV-6_{GS}-infected HSB_2 cells. Although several polypeptides were detected in the autoradiograph, except for the 200k, 180k and 55k polypeptides, the molecular weights of the other polypeptides were not identified. We have raised polyclonal rabbit antibodies against HHV-6_{GS}-infected cells, which were expressing viral antigens in more than 90% of the cells (day 6–8 post-infection (PI)). This antiserum was absorbed with uninfected cells and was used in subsequent assays. More than 33 [^{35}S] methionine-labeled polypeptides specific for HHV-6-infected cells with approximate molecular weights ranging from 180k to 31k were immunoprecipitated by this serum and among these, the 180k, 135k, 125k, 120k, 116k, 105k, 82k, 77k, 68k, 64k, 50k, 41k and 33k polypeptides were relatively prominent (Balachandran et al., 1989).

To determine the kinetics of synthesis of these proteins, we have standardized a synchronized infection. High titer virus for these experiments was obtained by collecting infected cells at the peak of cytopathic effect (day 6–12 PI). 10^7 cells suspended in 1 ml of medium were frozen and thawed twice. Cell debris was removed by centrifugation and the supernatant fluid was used as the virus

inoculum. 10^6 uninfected HSB_2 cells were infected with HHV-6_{GS} at an moi of 10 $TCID_{50}$/cell. After virus absorption for 2 h (time 0), infected cells were labeled with [^{35}S] methionine for different lengths of time. Infected cells were also incubated with 300 µg/ml of phosphonoacetic acid (PAA), which has been shown to inhibit viral DNA synthesis (DiLuca et al., 1990). Electron microscopic examination of infected cells after 72 h PI revealed large quantities of enveloped virus particles in cytoplasmic vacuoles and in extracellular compartments. Even with this synchronized infection, identification of proteins specific for HHV-6-infected cells in the total cell extracts at different time PI was obscured by host cell proteins. Hence we used these infected cells in RIP assay with the rabbit anti-HHV-6_{GS} (Fig. 9.1).

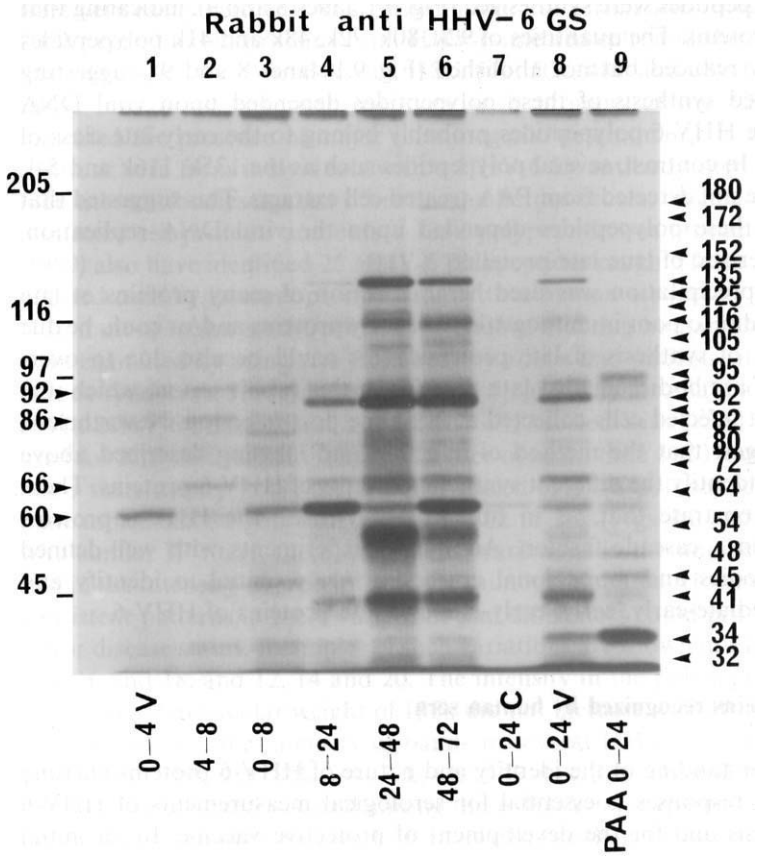


Fig. 9.1. Kinetics of synthesis of HHV-6_{GS} proteins. Uninfected HSB_2 cells (C, lane 7) and HHV-6-infected cells (V, lanes 1–6, 8 and 9), labeled with [^{35}S] methionine, were used for immunoprecipitation reactions with polyclonal antibodies against HHV-6_{GS}-infected cells. HSB_2 cells were infected with HHV-6 at an moi of 10 $TCID_{50}$/cell and after 2 h absorption, cells were washed (time 0) and labeled with [^{35}S] methionine between 0–4, 4–8, 0–8, 0–24, 24–48 and 48–72 h post-infection. Infected cells were also incubated with 300 µg phosphonoacetic acid (PAA) between 0 and 24 h post-infection (lane 9). Numbers indicate approximate molecular weights (kDa) of prominent HHV-6 polypeptides immunoprecipitated. Samples were analyzed on 9% acrylamide cross-linked with N,N'diallyltartaradiamide. Standard pre-stained and unstained molecular weight markers were included in parallel lanes.

Polypeptides immunoprecipitated by the rabbit anti-HHV-6_{GS} antibody show different kinetics of synthesis and some of the polypeptides were synthesized early in infection. A 95k polypeptide was detected as early as 0–4 hour post-infection, continued to be synthesized until 24 hours and was undetectable afterwards (Fig. 9.1, lanes 1 to 6). Another polypeptide with an approximate molecular weight of 86k was also detected by 4 h PI and was undetected after 8 h PI. Some of the polypeptides such as the 45k, 34k and 32k were synthesized as early as 8 h PI and continued to be detected at a constant level throughout the observed period of 72 h PI. In contrast, the synthesis of 135k, 92k, 70k, 41k polypeptides first detected between 8 and 24 h PI continued to be immunoprecipitated in greater quantities at later time PI. In the presence of PAA, higher amounts of 95k, 64k, 48k, 45k and 34k polypeptides were synthesized (Fig. 9.1, lanes 8 and 9), indicating that these are early proteins. The quantities of 92k, 80k, 72k, 48k and 41k polypeptides were significantly reduced but not abolished (Fig. 9.1, lanes 8 and 9), suggesting that the continued synthesis of these polypeptides depended upon viral DNA replication. These HHV-6 polypeptides probably belong to the early-late class of HHV-6 proteins. In contrast, several polypeptides such as the 135k, 116k and 54k polypeptides were not detected from PAA-treated cell extracts. This suggested that the synthesis of these polypeptides depended upon the viral DNA replication, which is characteristic of true late proteins.

Since immunoprecipitation was used here, detection of many proteins at late time PI could be due to poor immunogenicity of early proteins and/or could be due to a higher level of synthesis of late proteins. This could be also due to over-representation of antibodies against late proteins in this rabbit serum, which was generated against infected cells collected at late time post-infection. Nevertheless, these results suggest that the method of infection and labeling described above could be used to identify the different synthetic patterns of HHV-6 proteins. These results also demonstrate that, as in other herpesviruses, the HHV-6 proteins are synthesized in a cascade fashion. Additional experiments with well-defined polyclonal antibodies and monoclonal antibodies are essential to identify and classify the immediate-early, early, early-late and late proteins of HHV-6.

9.4. HHV-6 proteins recognized by human sera

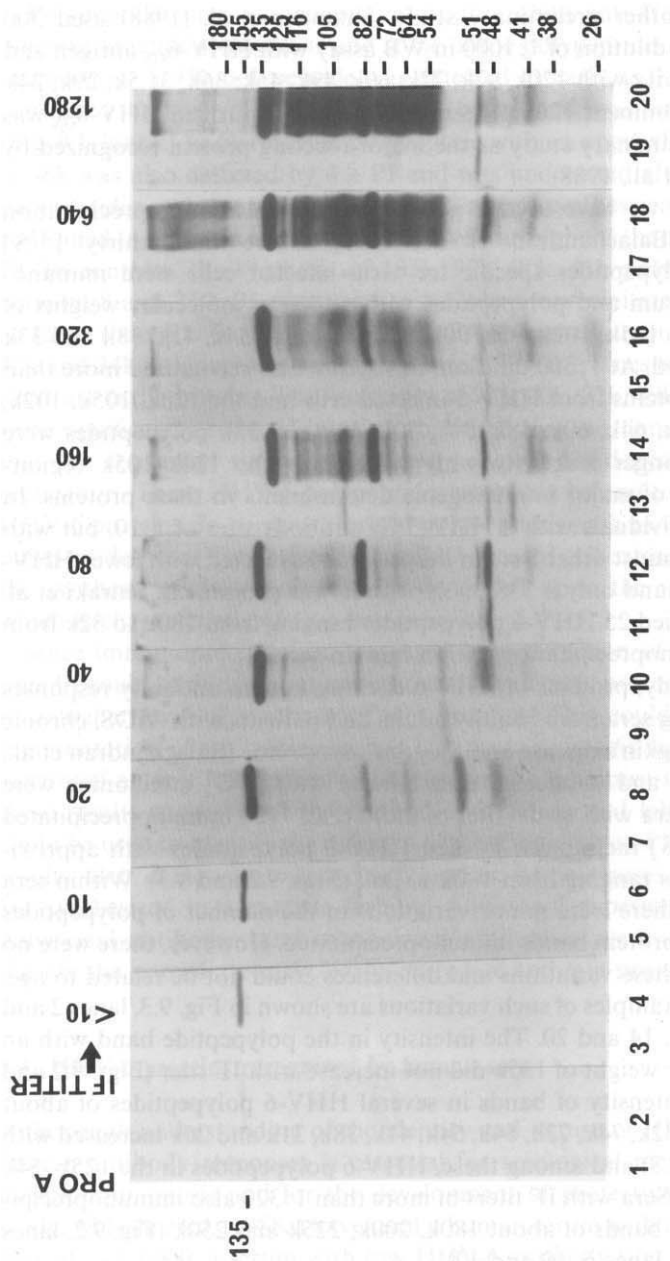
A thorough understanding of the identity and nature of HHV-6 proteins eliciting human antibody responses is essential for serological measurements of HHV-6 infection, diagnosis and for the development of protective vaccine. In an initial study, Ablashi et al. (1988a) have used a human serum with an HHV-6 IF antibody titer of $>1:6000$, a serum with low HHV-6 antibody titer and a negative serum. Although several proteins were immunoprecipitated by these sera, this study did not describe the molecular weights and the identity of these proteins. Three human sera from patients with Sjögren's syndrome were also tested by these authors in WB reactions. A number of virus-specific bands were detected and among them only the polypeptides with an approximate weights of 88k, 72k, 68k and 58k

were identified. In another preliminary study, Saxinger et al. (1988) used 300 normal donor sera at a dilution of 1:1000 in WB assay with HHV-6_{GS} antigen and reported weaker reactivity with 120k, 97k, 77k, 60k, 49k, 45k, 36k, 31.5k, 29k, 24k and 18.5k bands. A prominent 120k protein from gradient-purified HHV-6_{GS} was also identified in a preliminary study as the major reacting protein recognized by human sera (Josephs et al., 1988).

In our initial study, we have used a patient's serum in immunoprecipitation and WB reactions (Balachandran et al., 1990). More than thirty [^{35}S] methionine-labeled polypeptides specific for virus-infected cells were immunoprecipitated by this serum and polypeptides with apparent molecular weights of 180k, 135k, 120k, 116k, 105k, 102k, 95k, 90k, 82k, 74k, 64k, 54k, 41k, 38k and 33k were very easily detected. At 1:500 dilution, this serum also recognized more than 20 Western blotted proteins from HHV-6-infected cells and the 120k, 105k, 102k, 95k, 90k, 82k, 77k, 74k, 64k, 60k, 58k, 54k, 50k, 48k and 38k polypeptides were easily recognized. Stronger reactivity with proteins in the 120k–105k regions indicated the presence of major immunogenic determinants in these proteins. In contrast, sera from individuals with a HHV-6 IF antibody titer of 1:10, but with high titer antibodies against other human herpesviruses, reacted with fewer HHV-6-infected cell proteins and only a 135k polypeptide was prominent. Shiraki et al. (1989) also have identified 25 HHV-6 polypeptides ranging from 280k to 32k from infected cells by immunoprecipitation with a human serum.

In a more recent study, proteins of HHV-6 eliciting human antibody responses were examined by using sera from healthy adults and patients with AIDS, chronic fatigue syndrome, Hodgkin's disease and Sjögren's syndrome (Balachandran et al., 1991). HHV-6-infected and uninfected cells labeled with [^{35}S] methionine were used for RIP assays. Sera with an IF titer of more than 1:20 immunoprecipitated more than twenty [^{35}S] methionine-labeled HHV-6 polypeptides with approximate molecular weights ranging from 180k to 26k (Figs. 9.2 and 9.3). Within sera with similar IF titers, there were minor variations in the number of polypeptides and in the intensity of protein bands immunoprecipitated. However, there were no consistent patterns in these variations and differences could not be related to age, sex or disease status. Examples of such variations are shown in Fig. 9.3, lanes 2 and 8; 4, 16 and 18; and 12, 14 and 20. The intensity in the polypeptide band with an approximate molecular weight of 135k did not increase with IF titer (Figs. 9.2 and 9.3). In contrast, the intensity of bands in several HHV-6 polypeptides of about 125k, 110k, 105k, 92k, 82k, 74k, 72k, 64k, 54k, 41k, 38k, 31k and 26k increased with IF titer (Figs. 9.2 and 9.3) and among these, HHV-6 polypeptides in the 125k–54k range were prominent. Sera with IF titers of more than 1:320 also immunoprecipitated faint polypeptide bands of about 180k, 200k, 225k and 250k (Fig. 9.2, lanes 16, 18 and 20; Fig. 9.3, lanes 6, 10 and 12).

All the sera from the limited number of patients tested also immunoprecipitated more than 20 HHV-6 polypeptides (Fig. 9.3). There was no preferential increase in reactivities with any specific HHV-6 polypeptide for any certain patient group. Similar to sera from healthy adults, the intensity of bands increased with IF titer and the HHV-6 polypeptides in the 125k–54k range were prominent. The 135k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125
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polypeptide was recognized by all sera tested and these reactivities could not simply be due to the abundance of this protein in the infected cells, as the intensity of this protein band did not correlate with IF titer of the sera. This 135k polypeptide most probably represents the major capsid protein of the virus (Littler et al., 1990) and is probably the 120k polypeptide identified by Josephs et al. (1988). Detection of this protein in immunoprecipitation reactions by human sera may be of limited diagnostic value, since it may represent persistence of these antibodies following HHV-6 infection and/or may represent antibodies against other herpesviruses cross-reacting with HHV-6.

Yoshida et al. (1989) have examined serum samples from acute and convalescent phases of an infant with roseola in RIP assay with HHV-6 Y-3 isolate from a roseola patient. Acute phase serum immunoprecipitated a 140k polypeptide and convalescent serum reacted with > 200k, 140k, 120k, 62k, 41k, 39k and 34k polypeptides from infected cells and 200k, 140k, 120k, 95k, 85k, 78k, and 34k polypeptides from purified virus. Our preliminary studies show that sera from healthy children react with HHV-6 polypeptides in a manner similar to that described above for adult sera. Both the number of HHV-6_{GS} polypeptides immunoprecipitated and the increase of protein band intensity with increasing IF titer were also observed with pediatric samples. Further studies using sequential serum samples from children with roseola and other conditions and healthy adults are necessary to define the progression of responses to the various HHV-6 proteins, their persistence and the diagnostic parameters that may be meaningful for interpreting these serological reactions.

The HHV-6 polypeptides recognized by human sera in these studies represent soluble proteins enriched by the detergent extraction procedure and many insoluble proteins may have been removed from the antigen preparation. The use of protein A precludes the detection of polypeptides recognized by IgM antibodies. Similarly, the use of infected cells collected at late time (> 6 days) PI have probably biased these results towards the detection of late viral proteins. Hence HHV-6 polypeptides detected in these studies may not fully represent HHV-6 early viral proteins and probably represent a minimum estimate for the number of HHV-6 proteins eliciting antibody responses in humans. Despite these considerations, these data demonstrated that antibody responses in individuals were directed against several HHV-6 proteins and that the increase in IF titer was correlated with the increase in the quantity of HHV-6 proteins immunoprecipitated. The number of patient sera included in the reported studies was limited. No specific increases

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Fig. 9.2. SDS-PAGE analysis of [^{35}S] methionine-labeled uninfected and HHV-6_{GS}-infected HSB_2 cell extracts immunoprecipitated by sera from healthy adults. Lanes 1, 3, 5, 7, 9, 11, 13, 15, 17 and 19: uninfected cells. Lanes 2, 4, 6, 8, 10, 12, 14, 16, 18 and 20: HHV-6-infected cells. Lanes 1 and 2: immunoprecipitation with protein A agarose only. Lanes 3–20: immunoprecipitation with sera from healthy adults and protein A agarose. Numbers indicate approximate molecular weights (kDa) of prominent HHV-6 polypeptides immunoprecipitated. IF titer indicates HHV-6 antibody (IgG) titers of sera as measured in an immunofluorescence assay with acetone-fixed HHV-6-infected cells.

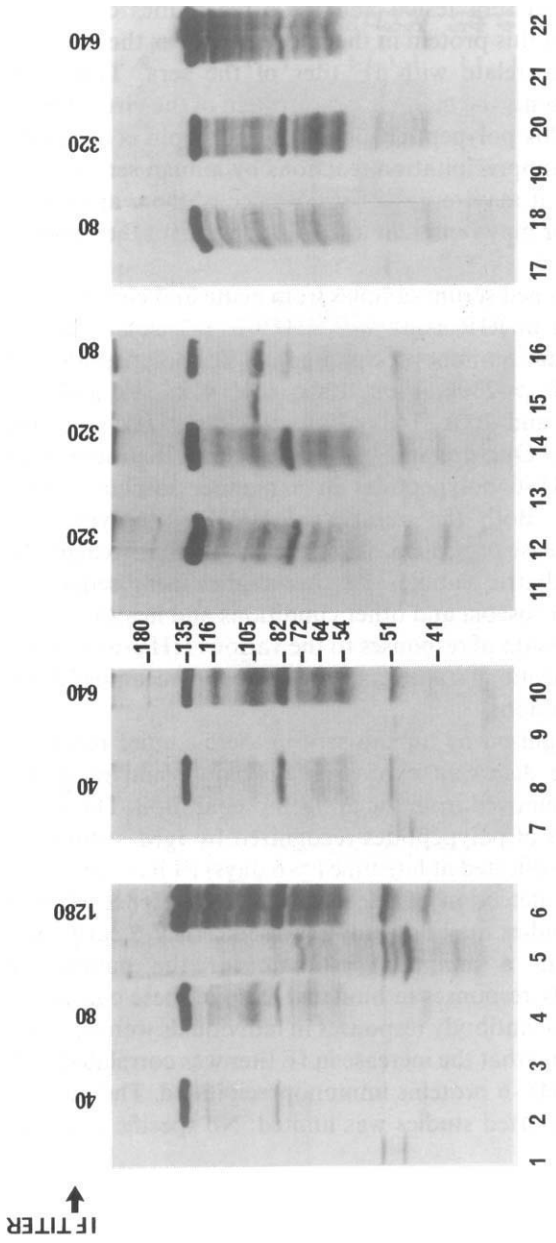

Fig. 9.3. SDS-PAGE analysis of [^{35}S] methionine-labeled uninfected and HHV-6_{GS}-infected HSB_2 cell extracts immunoprecipitated by sera from patients. Lanes 1, 3, 5, 7, 9, 11, 13, 15, 17, 19 and 21: uninfected cells. Lanes 2, 4, 6, 8, 10, 12, 14, 16, 18, 20 and 22: HHV-6-infected cells. Lanes 1–6: sera from patients with CFS. Lanes 7–10: sera from patients with Hodgkin's disease. Lanes 11–16: sera from patients with Sjögren's syndrome. Lanes 17–22: sera from patients with AIDS. Numbers indicate approximate molecular weights (kDa) of prominent HHV-6 polypeptides immunoprecipitated.

were detected in any of the HHV-6 polypeptides unique to individual patient groups (Balachandran et al., 1991). Analysis of HHV-6 polypeptides of different HHV-6 strains recognized by larger numbers of samples from patients with varying conditions is necessary to determine whether or not specific relationships exist between serologic responses and disease status.

9.5. HHV-6 glycoproteins recognized by human sera

Viral glycoproteins associated with virion envelopes of herpesviruses are the primary targets for the host's protective immune response. In an initial study, a patient's serum recognized about 8 glycopolypeptides from HHV-6_{GS}-infected cells (Balachandran et al., 1989). In the subsequent study (Balachandran et al., 1991), 9 glycopolypeptides, gp116, gp105, gp92, gp82, gp64, gp54, gp38, gp34 and gp31 were

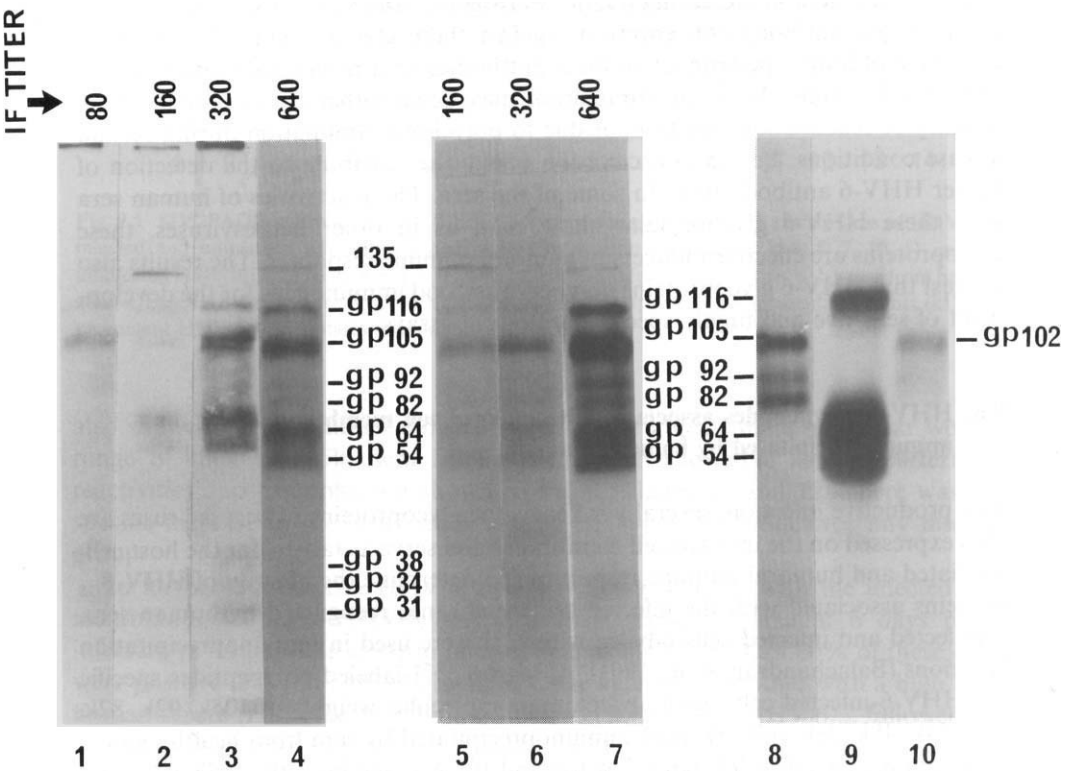

Fig. 9.4. SDS-PAGE analysis of [^{3}H] glucosamine-labeled HHV-6_{GS}-infected cells immunoprecipitated by human sera and by monoclonal antibodies. Lanes 1–4: sera from healthy adults. Lanes 5 and 7: sera from patients. Lane 8: monoclonal antibody 2D6. Lane 9: monoclonal antibody 6A5H7. Lane 10: monoclonal antibody 7A2. No reactivities were seen with uninfected cells. Approximate molecular weights (kDa) of prominent HHV-6 glycopolypeptides (gp) are indicated.

immunoprecipitated by human sera with IF titer of more than 1:80 (Fig. 9.4). Longer exposure of autoradiographs also showed 2 additional 180k and 225k glycopolypeptides. For sera from both healthy adults and patients, the intensity of glycopeptide bands increased with IF titer and there were no differences related to disease status. A sharp polypeptide band with an approximate molecular weight of 135k was also immunoprecipitated by all sera from [^{3}H] glucosamine-labeled infected cell extracts and the intensity of this band did not change with IF titer.

Among the HHV-6 glycopolypeptides immunoprecipitated by human sera, glycopolypeptides gp116, gp105, gp92, gp82, gp64 and gp54 were prominent. The approximate molecular weights of glycopolypeptides immunoprecipitated by human sera were similar to the 3 sets of glycopolypeptides immunoprecipitated by monoclonal antibodies (Fig. 9.4, lanes 8, 9 and 10). Increased intensity of glycopolypeptide bands visualized in the autoradiographs correlated with an increase in IF (IgG) titers of 1:80. The molecular weights of glycopolypeptides were similar to the prominent [^{35}S] methionine-labeled polypeptides (125k–54k) immunoprecipitated by these sera in increasing quantities (Figs. 9.2 and 9.3). This is suggestive of elevated IgG antibody concentration against these glycoproteins. This may be indicative of longer persistence of these antibodies or a prolonged stimulation in certain individuals. Antibody stimulation may occur either by viral replication, primary or reactivation infection, or due to polyclonal stimulation during certain disease conditions. Periodic reactivation could also attribute to the detection of higher HHV-6 antibody titers in some of the sera. The reactivities of human sera with these HHV-6 glycoproteins show that, as in other herpesviruses, these glycoproteins are effective inducers of humoral immune responses. The results also suggest that HHV-6 glycoproteins may serve as good immunogens for the development of sensitive and highly specific serological assays.

9.6. HHV-6 polypeptides associated with infected cell membranes immunoprecipitated by human sera

In a productive infection, several viral envelope glycoproteins of herpesviruses are also expressed on the infected cell membranes and serve as targets for the host cell-mediated and humoral immune responses. To determine the identity of HHV-6_{GS} proteins associated with the infected cell membranes recognized by human sera, uninfected and infected cells labeled with ^{125}I were used in immunoprecipitation reactions (Balachandran et al., 1991). At least 8 ^{125}I-labeled polypeptides specific for HHV-6-infected cells, with approximate molecular weights of 105k, 92k, 82k, 64k, 54k, 48k, 38k and 31k were immunoprecipitated by sera from healthy adults and from patients (Fig. 9.5, lanes 2, 4, 6, 8 and 10). Among these, the 105k, 92k, 82k, 64k and 54k polypeptides were prominent. The 82k and 105k polypeptides were prominent even in samples immunoprecipitated with sera with lower IF titer (Fig. 9.5, lanes 2 and 4). Intensity of these polypeptide bands and others increased with IF titer (Fig. 9.5, lanes 2, 4, 6, 8 and 10). Longer exposure of autoradiographs

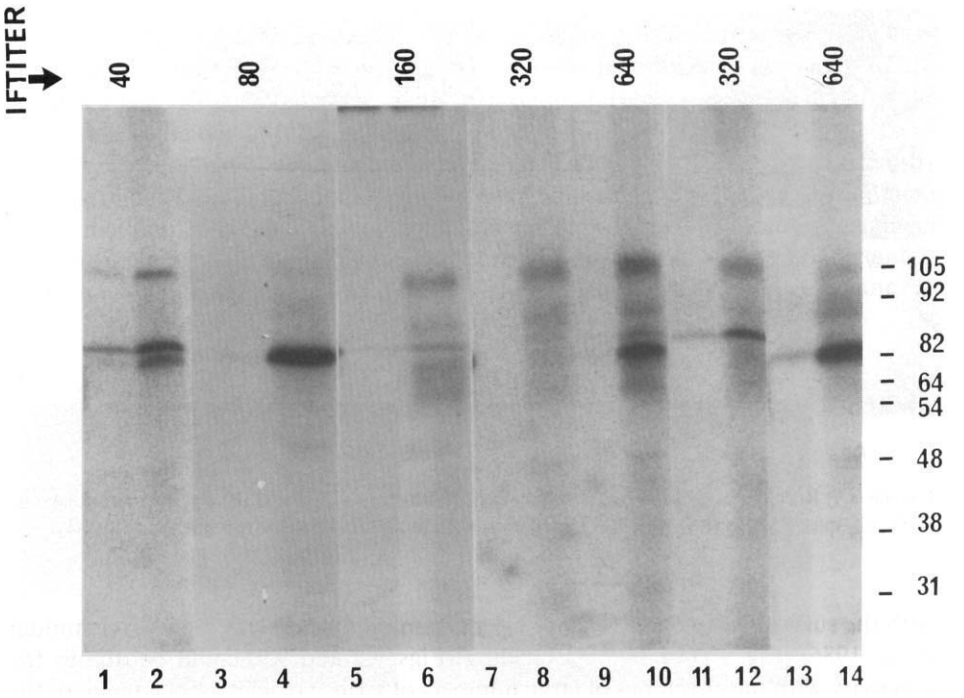

Fig. 9.5. SDS-PAGE analysis of [^{125}I]-labeled polypeptides associated with the surfaces of HHV-6_{GS}-infected and uninfected cells immunoprecipitated by human sera. Lanes 1, 3, 5, 7, 10, 11 and 13: uninfected HSB_2 cells. Lanes 2, 4, 6, 8, 9, 12 and 14: HHV-6-infected cells. Lanes 1–10: sera from healthy adults. Lanes 11–14: sera from patients. Numbers indicate approximate molecular weights (kDa) of prominent HHV-6 polypeptides immunoprecipitated.

also showed two additional diffused bands in the approximate molecular weight range of 180k to 225k. Sera from patients also showed a similar pattern of reactivities and examples are shown in Fig. 9.5, lanes 11 and 12. There was no specific increase in the reactivities of any HHV-6 polypeptides for any disease group.

To further characterize HHV-6 glycoproteins associated with the infected cell membranes, we used HHV-6_{GS}-infected HSB_2 cells collected at 6 days post-infection in an immune electron microscopic assay. More than 80% of these cells were positive for viral antigens by IFA. Cells were reacted either with a high titer human serum (HS) or with rabbit antibodies (RS) against HHV-6_{GS}, followed by appropriate second antibodies conjugated with gold particles (10 nm). Thin sections were made, stained and examined by electron microscope. Both in our studies (Fig. 9.6A and B) and in previous studies by Biberfeld et al. (1987) with human sera, only the envelope of extracellular virus particles was labeled. The outer cell membranes of HHV-6-infected HSB_2 cells and CBL (Biberfeld et al., 1987) uniformly lacked HHV-6 envelope antigens. Reactivities of human sera and MAbs

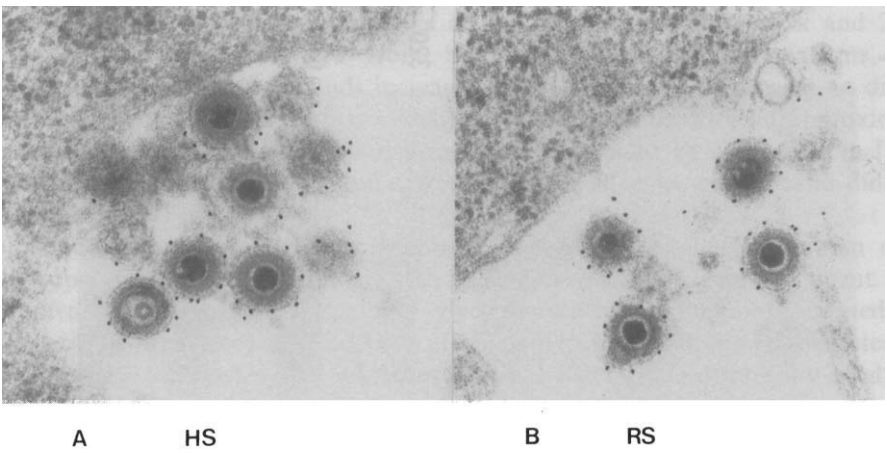

Fig. 9.6. (A) Reactivities of a human serum from a healthy adult (HS) and (B) a rabbit serum against HHV-6_{GS}-infected cells (RS) with HHV-6-infected cells in an immune electron microscopy assay.

with the surfaces of infected cells in surface immunofluorescence assay (Salahuddin et al., 1986) (Fig. 9.7) or by ^{125}I labeling (Figs. 9.5 and 9.9) could be due to the reactivity with the envelopes of large numbers of virus particles associated with the surfaces of infected cells. The absence of HHV-6 glycoproteins on the infected cell membranes at late time post-infection is intriguing. They may be selectively excluded from the infected cell membranes or by a general mechanism also affecting the host cell surface proteins. This is in contrast to other herpesviruses, where viral antigens are frequently detected at the cell surfaces even before the virus assembly and are good targets for lysis with antibody and complement ADCC. Critical evaluations are essential to understand the significance of these phenomena in the biology of HHV-6.

9.7. HHV-6 proteins defined by monoclonal antibodies

Monoclonal antibodies (MAbs) are important tools for the unambiguous identification and characterization of viral proteins. We have raised monoclonal antibodies by fusing Sp2/O Ag.14 myeloma cells with spleen cells from BALB/c mice immunized with three weekly intraperitoneal injections of 3×10^7 infected cells (Balachandran et al., 1989). Several hybridomas secreting HHV-6-specific antibodies were initially selected on the basis of their specific reactivity with HHV-6-infected cells in ELISA and IFA. Culture supernatant fluids from these clones were next used in immunoprecipitation reactions with [^{35}S] methionine-labeled uninfected and infected cells and hybridoma clones that were immunoprecipitating HHV-6-specific polypeptides were cloned twice and rescreened. Each MAb precipitated one or more [^{35}S] methionine-labeled polypeptides from HHV-6-infected

cells (Fig. 9.8) and distinctly different immunoprecipitation patterns were obtained by these MAbs, indicating that antibodies had different specificities. So far, we have identified 12 distinct HHV-6 proteins and several investigators are using MAbs against 7 HHV-6_{GS} proteins that we have developed. Here we describe in detail the reactive specificities of these MAbs.

With acetone-fixed infected cells, MAb 9A5D12 show bright, predominantly granular nuclear and occasional cytoplasmic fluorescence (Fig. 9.7A). A prominent [^{35}S] methionine-labeled polypeptide with an approximate molecular weight of 41k and a minor 110k band were immunoprecipitated by this MAb (Fig. 9.8, lane 2) and the 41k polypeptide was recognized in WB reactions (Fig. 9.9, lane 1).

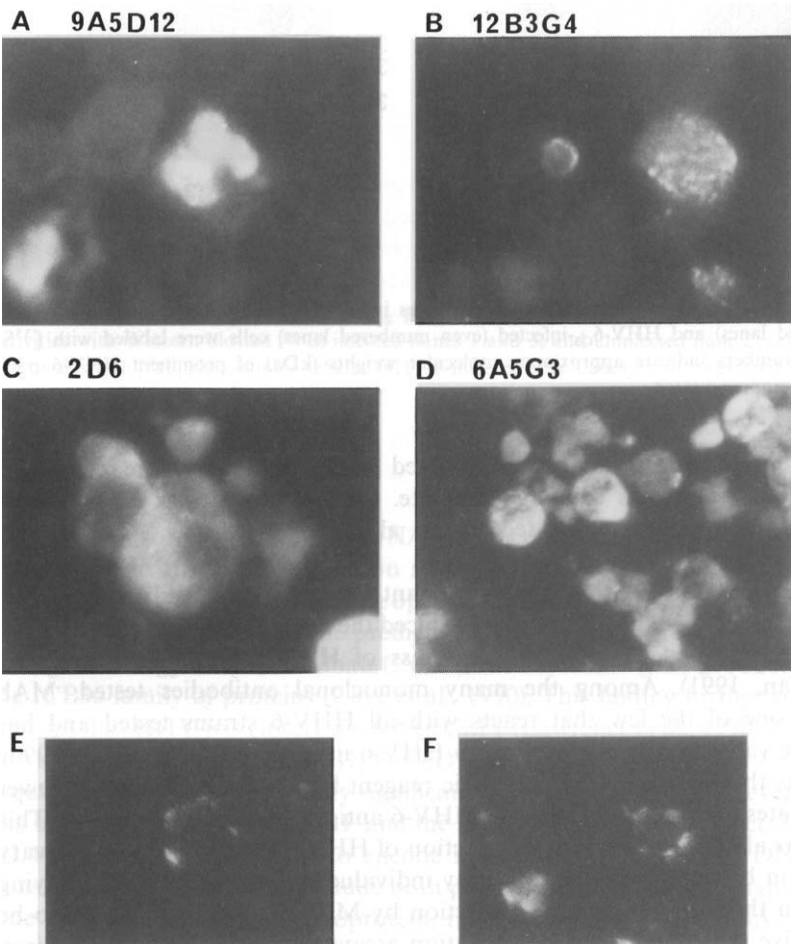

Fig. 9.7. Reactivities of MAbs with HHV-6_{GS}-infected cells in immunofluorescence assay (IFA). HHV-6-infected HSB_2 cells were collected at 3 days post-infection, fixed in acetone (A–D) or unfixed (E and F) and then reacted with MAbs followed by FITC-labeled anti-mouse antibodies.

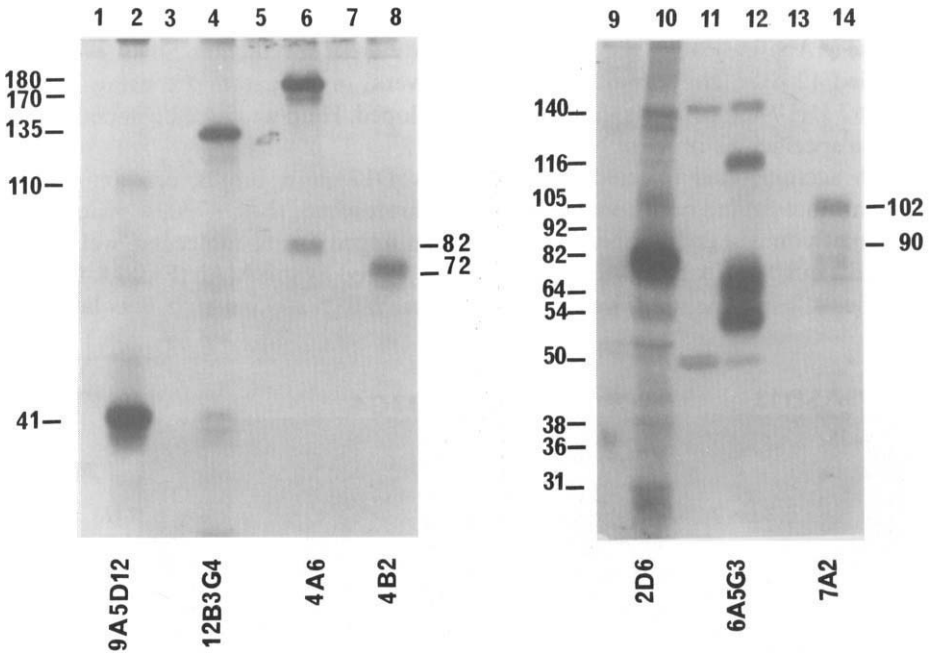

Fig. 9.8. SDS-PAGE analysis of HHV-6_{GS} polypeptides immunoprecipitated by MAbs. Uninfected (odd numbered lanes) and HHV-6_{GS}-infected (even numbered lanes) cells were labeled with [^{35}S] methionine. Numbers indicate approximate molecular weights (kDa) of prominent HHV-6 polypeptides immunoprecipitated.

Both these polypeptides are phosphorylated polypeptides and are conserved among all HHV-6 strains examined to date. Identical molecular weight polypeptides were immunoprecipitated from all HHV-6 strains (Chang and Balachandran, 1991).

HHV-6 P41 is one of the most abundant proteins produced early in the replicative cycle (Fig. 9.11A). Since PAA reduced the quantity of P41 synthesized, it is tentatively assigned to the early-late class of HHV-6 proteins (Chang and Balachandran, 1991). Among the many monoclonal antibodies tested, MAb 9A5D12 is one of the few that reacts with all HHV-6 strains tested and has considerable value in monitoring *in vitro* HHV-6 infection (Krueger et al., 1990). This MAb is therefore an ideal diagnostic reagent for the identification of newer HHV-6 isolates and in the detection of HHV-6 antigen in clinical specimens. This antibody has also been used for the detection of HHV-6 viral antigens in salivary glands and in bronchial glands of healthy individuals, and patients with varying disorders. In these studies, antigen detection by MAb 9A5D12 was found to be more sensitive than the *in situ* hybridization assays (Krueger et al., 1990). Since human sera also reacted with a 41k polypeptide of HHV-6_{GS} (Balachandran et al., 1989, 1991), HHV-6 protein P41 may also be useful in the serological measurement of HHV-6 infection.

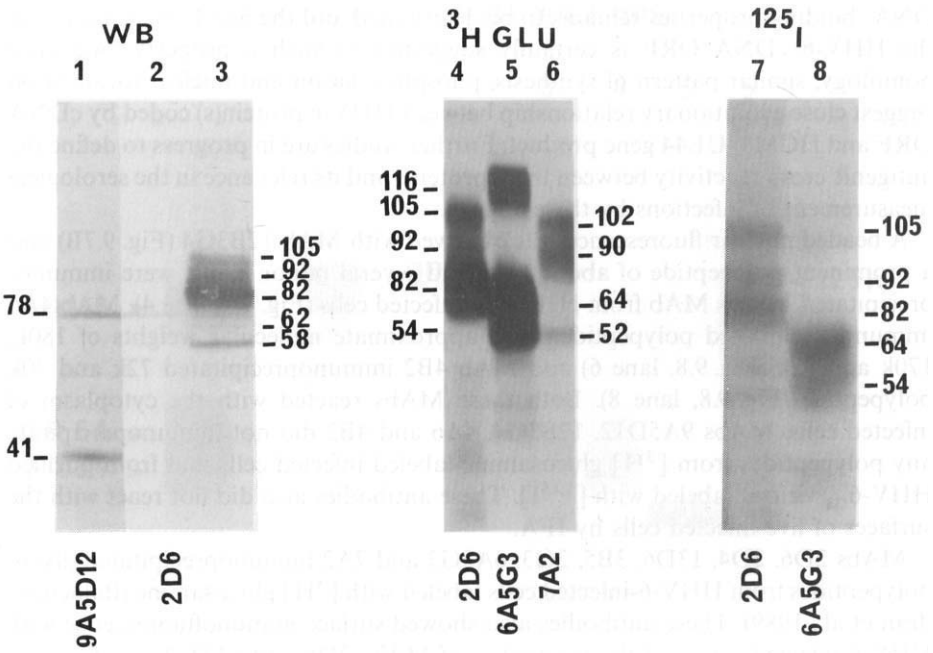

Fig. 9.9 SDS-PAGE analysis of HHV-6_{GS} glycopolypeptides immunoprecipitated by MAbs. Reactivities with Western blotted (WB) infected (lanes 1 and 3) and uninfected (lane 2) cells. Lanes 4–6: reactivities with [^{3}H] glucosamine-labeled HHV-6-infected cells. Lanes 7 and 8: reactivities with [^{125}I]-labeled virion envelope polypeptides.

We have also characterized a cDNA identified from an HHV-6_{GS} cDNA library constructed in the λgt11 expression system. Sequence analysis revealed that the cDNA encodes a single 623AA long open reading frame (ORF) with an open 5′ end. The amino terminal half of the predicted HHV-6 protein from the cDNA shows strong homology with the human CMV (HCMV) UL44 gene product coding for the ICP36 family of proteins (Chee et al., 1990). This finding further supports the concept that HHV-6 is closely related to CMV (Lawrence et al., 1990). Comparison of HCMV UL44 ORF and HHV-6 cDNA ORF with HSV-1, VZV and EBV sequences have not revealed any significant homologies. There are several similarities between HHV-6 cDNA ORF and the HCMV UL44 gene product. The HCMV UL44 gene has been shown to encode for a group of early-late DNA binding nonstructural proteins that include both phosphorylated and glycosylated species. One of these is a 52k phosphoprotein, translated from 4.5 kb RNA (Leach and Mocarski, 1989). HCMV UL44 gene expression has been shown to occur from different TATA boxes, both in early and late replicative cycles of HCMV. Similar to HHV-6 P41, HCMV UL44 gene products are also localized to the nucleus of HCMV-infected cells. Whether the 41k and 110k polypeptides of HHV-6 also have

DNA -binding properties remains to be determined and the highly basic nature of the HHV-6 cDNA ORF is certainly suggestive of such a property. Sequence homology, similar pattern of synthesis, phosphorylation and nuclear localization suggest close evolutionary relationship between HHV-6 protein(s) coded by cDNA ORF and HCMV UL44 gene product. Further studies are in progress to define the antigenic cross-reactivity between these proteins and its relevance in the serological measurement of infections by these viruses.

A beaded nuclear fluorescence was observed with MAb 12B3G4 (Fig. 9.7B) and a prominent polypeptide of about 135k and several minor bands were immunoprecipitated by this MAb from HHV-6_{GS}-infected cells (Fig. 9.8, lane 4). MAb 4A6 immunoprecipitated polypeptides with approximate molecular weights of 180k, 170k and 82k (Fig. 9.8, lane 6) and MAb 4B2 immunoprecipitated 72k and 70k polypeptide (Fig. 9.8, lane 8). Both these MAbs reacted with the cytoplasm of infected cells. MAbs 9A5D12, 12B3G4, 4A6 and 4B2 did not immunoprecipitate any polypeptides from [^{3}H] glucosamine-labeled infected cells and from purified HHV-6_{GS} virions labeled with [^{125}I]. These antibodies also did not react with the surfaces of live infected cells by IFA.

MAbs 2D6, 2D4, 13D6, 3B5, 2G3, 6A5G3 and 7A2 immunoprecipitated glycopolypeptides from HHV-6-infected cells labeled with [^{3}H] glucosamine (Balachandran et al., 1989). These antibodies also showed surface immunofluorescence with HHV-6-infected cells and the reactivities of MAbs 2D6 and 6A5G3 are shown in Fig. 9.7E and F. MAbs 2D6, 2D4, 13D6, 3B5 and 2G3 showed identical reactivities in immunoprecipitation and IF assays and results with MAb 2D6 are shown here. These antibodies reacted with several polypeptides and glycopolypeptides with approximate molecular weights of 105k, 92k, 82k, 62k, 38k, 36k and 31k (Fig. 9.8, lane 10 and Fig. 9.9, lane 4). Lower exposure of the autoradiograph showed 2 polypeptides of about 76k and 84k in the broad prominent 82k region. These glycoproteins were collectively designated as gp82–gp105. MAbs 2D6, 2D4 and 13D6 recognized polypeptides of about 105K, 92K, 82K, 62K and 58K in WB reactions and very weakly with the 38k, 36k and 31k polypeptides (Fig. 9.9, lane 3), indicating common antigenic determinants among these polypeptides. A 78k polypeptide recognized by this and by other antibodies from both uninfected and infected cells was considered as nonspecific (Fig. 9.9, lanes 1–3). MAbs 2D6, 2D4, 13D6, 3B5 and 2G3 immunoprecipitated the 105k, 92k and 82k polypeptides from ^{125}I-labeled virions (Fig. 9.9, lane 7).

Three intensively labeled 116k, 64k and 54k polypeptides and glycopolypeptides were immunoprecipitated by MAb 6A5G3 (Fig. 9.8, lane 12, Fig. 9.9, lane 5 and Fig. 9.10, lane 1). Although this MAb did not react with uninfected cells in IFA or in ELISA, it immunoprecipitated 2 polypeptides of approximate molecular weights 140k and 50k from uninfected and infected cells. The relationship between the host polypeptides and HHV-6 polypeptides is not known at present. From ^{125}I-labeled virions, polypeptides of about 64k and 54k were immunoprecipitated (Fig. 9.9, lane 8 and Fig. 9.10, lane 4) and the glycopolypeptides recognized by MAb 6A5G3 were designated as gp116/gp64/gp54. When nonreduced samples were electrophoresed, the gp64k and gp54k were replaced by a broadly migrating polypeptide of

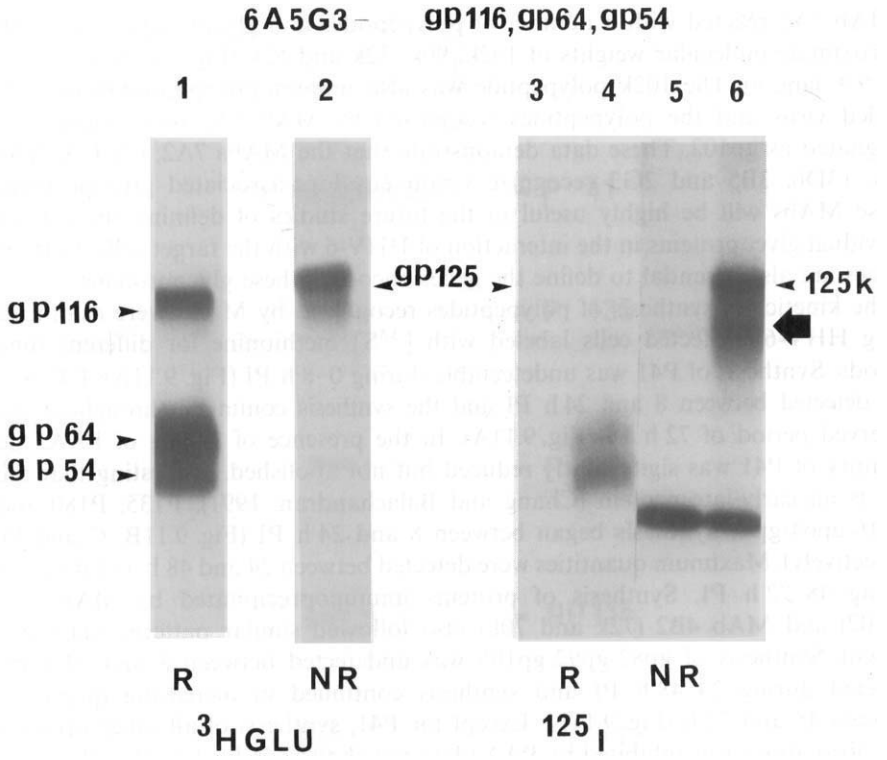

Fig. 9.10. SDS-PAGE analysis of [^{35}S] methionine-labeled cell extracts immunoprecipitated by a serum from healthy adult. Lane 1: uninfected cells. Lanes 2–10: cells infected with different HHV-6 strains.

approximate molecular weight 125k (gp125) and the migration of gp116k remained unchanged (Fig. 9.10, lane 2). This data demonstrated that gp125 is a disulfide linked hetero dimer of gp64k and gp54k. MAb 6A5G3 recognized only the 64k and 54k polypeptides from the ^{125}I-labeled virions and gp116k was not detected (Fig. 9.9, lane 8 and Fig. 9.10, lane 4). No reaction was seen with uninfected cells (Fig. 9.10, lane 3). When nonreduced samples were electrophoresed, the gp64k and gp54k polypeptides were replaced by a broadly migrating polypeptide of approximate molecular weight 125k (Fig. 9.10, lane 6). This also strengthened the notion that 125k is a disulfide-lined heterodimer of virion envelope-associated gp64k and gp54k polypeptides. Preliminary studies suggest a precursor–product relationship among these polypeptides; our data suggest that gp116 is the partially glycosylated precursor molecule which undergoes cleavage. These cleavage products mature into the fully glycosylated gp64 and gp54 molecules, which are incorporated in the virion envelope. In their synthesis and cleavage patterns, the gp114/gp64/gp54 complex of HHV-6_{GS} resemble VZV glycoprotein gB and whether these glycopolypeptides represent gB of HHV-6 remains to be established.

MAb 7A2 reacted with a number of polypeptides and glycopolypeptides with approximate molecular weights of 102k, 90k, 72k and 52k (Fig. 9.8, lane 14 and Fig. 9.9, lane 6). The 102k polypeptide was also immunoprecipitated from ^{125}I-labeled virus and the polypeptides recognized by MAb 7A2 were collectively designated as gp102. These data demonstrate that the MAbs 7A2, 6A5G3, 2D6, 2D4, 13D6, 3B5 and 2G3 recognize virion envelope-associated glycoproteins. These MAbs will be highly useful in the future studies of defining the role of individual glycoproteins in the interaction of HHV-6 with the target cells. Further studies are also essential to define the genes encoding these glycoproteins.

The kinetics of synthesis of polypeptides recognized by MAbs were examined using HHV-6_{GS}-infected cells labeled with [^{35}S] methionine for different time periods. Synthesis of P41 was undetectable during 0–8 h PI (Fig. 9.11A). P41 was first detected between 8 and 24 h PI and the synthesis continued throughout the observed period of 72 h PI (Fig. 9.11A). In the presence of 300 μg of PAA, the quantity of P41 was significantly reduced but not abolished, suggesting that the P41 is an early-late protein (Chang and Balachandran, 1991). P135, P180 and gp116/gp64/gp54 synthesis began between 8 and 24 h PI (Fig. 9.11B, C and D, respectively). Maximum quantities were detected between 24 and 48 h and declined during 48–72 h PI. Synthesis of proteins immunoprecipitated by MAb 7A2 (gp102) and MAb 4B2 (72k and 70k) also followed similar patterns (data not shown). Synthesis of gp82/gp92/gp105 was undetected between 8 and 24 h PI, detected during 24–48 h PI and synthesis continued in increasing quantities between 48 and 72 h (Fig. 9.11E). Except for P41, synthesis of all other proteins described above was inhibited by PAA (data not shown), indicating that these are probably HHV-6 late proteins.

MAbs against HHV-$6_{Z\text{-}29}$ have been also developed by two other groups of investigators. Okuno et al. (1990) have developed MAbs against HHV-$6_{Z\text{-}29}$ infected CBL and have reported neutralization of virus infectivity by two MAbs – OHV-3 and OHV-9. Both these MAbs immunoprecipitated HHV-$6_{Z\text{-}29}$ glycopolypeptides, gp98k and gp92k. The relationship of these glycopolypeptides with the glycoproteins of HHV-6_{GS} that we have described above are yet to be determined. Yamamoto et al. (1990) have purified HHV-$6_{Z\text{-}29}$ and have identified more than 20 bands ranging from 30k to 200k. The 191k, 143k, 108k, 101k, 80k, 57k, 47k and 41k polypeptides were very prominent. They have raised a MAb against the purified HHV-$6_{Z\text{-}29}$ nucleocapsids and have identified a 101k protein in WB. Human sera with HHV-6 antibodies reacted strongly with the 101k protein indicating that this protein could be used as a serologic marker for HHV-6 infection.

Knowledge about the structure of the HHV-6 genome, gene organization, gene expression, regulation, coding proteins and their functions is limited. Recently, a sequence of 21 858 base pairs from the genome of HHV-6 strain U1102 (HHV-6_{U1102}) has been determined (Lawrence et al., 1990). The sequence has a mean composition of 41% G:C and 17 ORFs have been predicted and a little is known about the identity of the proteins coded by these genes. Further development of MAbs and polyclonal antibodies against early and other proteins of HHV-6 and identification of HHV-6 proteins transactivating HIV LTR (Ensoli et al.,

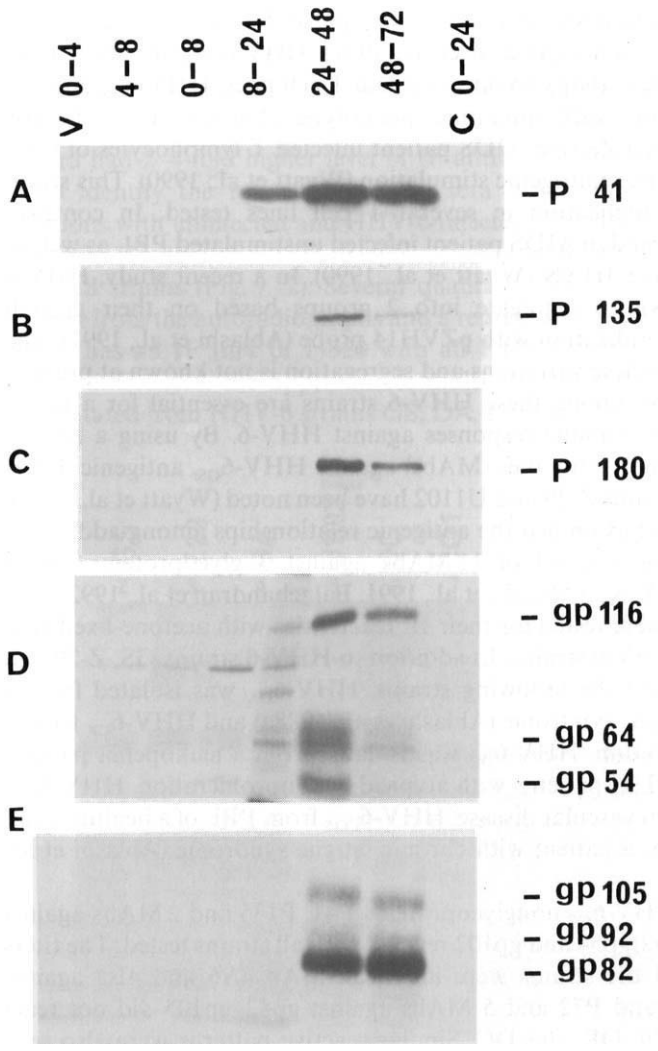

Fig. 9.11. Kinetics of synthesis of HHV-6_{GS} polypeptides immunoprecipitated by MAbs. Details of the experimental procedures are given in the legend of Figure 9.1.

1989; Horvat et al., 1989, 1991) are essential for a better understanding of the structure and function of HHV-6 proteins.

9.8. Antigenic variations among HHV-6 strains

Many of the newer herpes isolates were identified as HHV-6 by their hybridization to a 9 kilobase (kb) HHV-6_{GS} DNA probe pZVH14. Studies from several labora-

tories show DNA restriction site polymorphism and differences in the *in vitro* tropism among HHV-6 strains (Ablashi et al., 1991). HHV-6 strains show considerable variations in their ability to infect human T cell lines. HHV-6_{GS} infected HSB_2 cells, a T cell line with immature phenotype (Ablashi et al., 1988b). HHV-$6_{Z\text{-}29}$ isolated from a Zairian AIDS patient infected T lymphocytes of CBL and PBL and required prior mitogenic stimulation (Wyatt et al., 1990). This strain did not show efficient replication in several T cell lines tested. In contrast, HHV-6_{U1102} from an Ugandan AIDS patient infected unstimulated PBL as well as in T cell lines, HSB_2 and J-JHAN (Wyatt et al., 1990). In a recent study, HHV-6 strains have been shown to segregate into 2 groups based on their growth properties and DNA hybridization with pZVH14 probe (Ablashi et al., 1991). The biological significance of these variations and segregation is not known at present.

Antigenic relationships among these HHV-6 strains are essential for a better understanding of human immune responses against HHV-6. By using a limited number of our monoclonal antibodies (MAbs) against HHV-6_{GS}, antigenic differences between HHV-6 strains Z-29 and U1102 have been noted (Wyatt et al., 1990). In another study, we have examined the antigenic relationships among additional HHV-6 strains by using a panel of 11 MAbs against 3 glycoproteins and 4 nonglycoproteins of HHV-6_{GS} (Ablashi et al., 1991; Balachandran et al., 1992). The MAbs described above were tested for their IF reactivities with acetone-fixed cells infected with different HHV-6 strains. In addition to HHV-6 strains GS, Z-29 and U1102, we have also used the following strains. HHV-6_{DA} was isolated from a patient with chronic fatigue syndrome (Ablashi et al., 1988a) and HHV-6_{OK} from a child with exanthem subitum. HHV-6_{DC} was isolated from a leukopenia patient. HHV-6_{CO1} was from PBL of patients with atypical lymhoproliferation, HHV-6_{CO2} from unclassified collagen vascular disease, HHV-6_{CO5} from PBL of a healthy adult and HHV-6_{CO6} was from a patient with chronic fatigue syndrome (Ablashi et al., 1991).

Two MAbs against HHV-6_{GS} nonglycoproteins P41, P135 and 2 MAbs against glycoproteins gp116/gp62/gp54 and gp102 reacted with all strains tested. The titers of these MAbs with all the strains were identical. MAb 4A6 and 4B2 against nonglycoproteins P180 and P72 and 5 MAbs against gp82–gp105 did not react with HHV-6 strains Z-29, OK and DC. Similar reactive patterns were also seen with viruses grown in PHA-stimulated PBL and CBL. These results demonstrate that HHV-6 strains possess group-common and group-specific epitopes and suggest differences in the antigenic make-up of these strains. MAb reactivities segregated these strains into 2 groups, which were identical to the segregation based on their growth in 2 T cell lines, HSB_2 and MOLT-3, and DNA polymorphism (Ablashi et al., 1991). Based on these properties, we have tentatively assigned HHV-6 strains GS, U1102, DA and CO1–CO8 as HHV-6 group A and HHV-6 strains Z-29, OK and DC as HHV-6 group B.

Human sera also detected antigenic differences among these 2 groups of HHV-6 strains which suggests that these 2 groups are antigenically closely related and yet different. We have analyzed the reactivities of sera from healthy adults (ages 18–50).

Sera showed variations in their IF antibody titer (IgG) with cells infected with HHV-6 strains GS, DA and Z-29. The geometric mean antibody titer (GMT) of sera from adults were 128 for GS, 110 for DA and 100 for Z-29. 81% of adult sera (110/136) had similar antibody titers against all 3 HHV-6 strains and 19% (26/136) of sera had 2–4-fold higher level of IF antibodies against either GS/DA or Z-29.

To identify the reacting proteins, sera were tested in immunoprecipitation reactions with uninfected and HHV-6-infected cells labeled with [^{35}S] methionine. Sera with IF titers of $>1:20$ recognized more than 20 HHV-6 specific polypeptides from all strains (Fig. 9.12). Several qualitative and quantitative differences were evident from the autoradiographs and a representative example is shown here. This serum has an IF titer of 1:320 with all HHV-6 strains tested. Except for some minor differences, the approximate molecular weight of polypeptides immunoprecipitated from HHV-6 strains GS, DA, U1102, CO1, CO2, CO5 and CO6 were

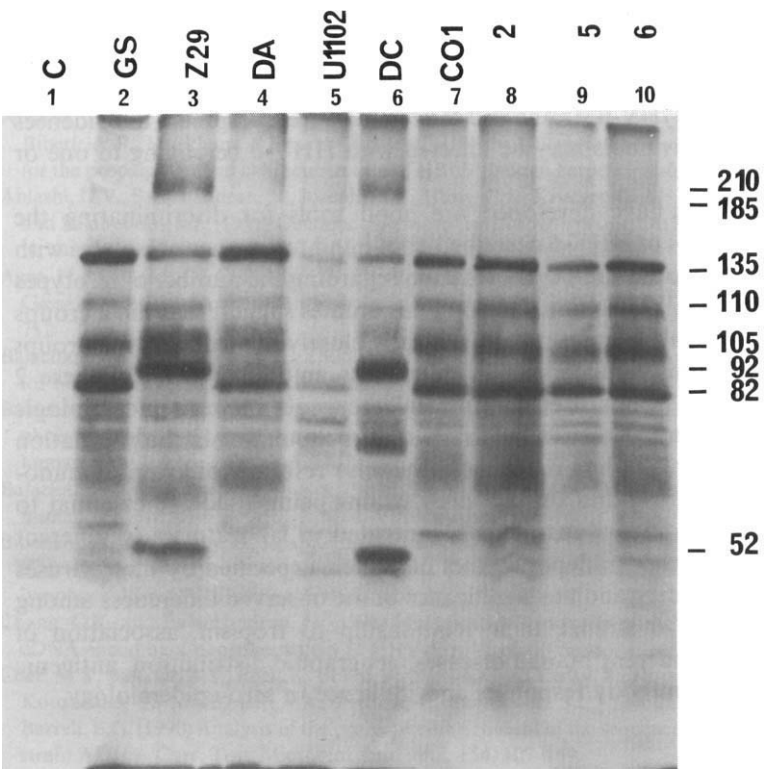

Fig. 9.12. SDS-PAGE analysis of HHV-6_{GS} glycopolypeptides immunoprecipitated by MAb 6A5G3. Immunoprecipitated samples were reduced (R) with 2-mercaptoethanol or nonreduced (NR). Lanes 1 and 2: reactivities with [^{3}H] glucosamine-labeled HHV-6-infected cells. Lanes 3 and 5: reactivities with [^{125}I]-labeled uninfected cells. Lanes 4 and 6: reactivities with [^{125}I]-labeled virion envelope polypeptides.

similar (lanes 2, 3, 5, 7–10). Some of the polypeptides such as 135k, 78k, 72k, 41k and 31k were immunoprecipitated from all strains (Fig. 9.12). Mobilities of many of the polypeptides immunoprecipitated from HHV-6 strains Z-29, DC (Fig. 9.12, lanes 3 and 6) and OK (data not shown) infected cells differed from group A strains polypeptides. These differences were not just due to cell-type differences, as similar protein profiles were also seen with infected CBL and PBL (data not shown).

Polypeptides of HHV-6 group B strains with approximate MWs of 210k and 185k were detected by a number of sera and not in group A strains (Fig. 9.12, lanes 3 and 6). These were readily immunoprecipitated by some sera with Z-29 IF antibody titer of 1:40 or more. Some sera with HHV-$6_{Z\text{-}29}$ antibody titer of 1:640 immunoprecipitated considerable amounts of HHV-$6_{Z\text{-}29}$ and HHV-6_{GS} polypeptides in the 135k–52k region, but only low quantity of 210k and 185k Z-29 polypeptides. Some sera had 4-fold higher Z-29 IF antibody titer and immunoprecipitated considerable amounts of 185k and 210k HHV-$6_{Z\text{-}29}$ polypeptides (Balachandran et al., 1992). In contrast, some sera with HHV-6_{GS} and HHV-6_{DA} antibody titers of 1:640 immunoprecipitated considerable amounts of GS, DA and Z-29 polypeptides in the 135k–52k region, but only a low quantity of 210k and 185k Z-29 polypeptides. These reactivities demonstrated antigenic differences among the 2 groups of HHV-6 strains and the results were interpreted as evidences for the notion that individuals may be infected with HHV-6 belonging to one or both of the groups.

The MAbs that we have developed are good tools for discriminating the members of the 2 groups of HHV-6 described above and testing newer isolates with these MAbs will provide additional information regarding the number of serotypes of HHV-6 in the human population. Our ongoing studies suggest that the 2 groups of HHV-6 strains probably represent antigenically closely related serotype groups of HHV-6. The obvious implications of differences among proteins of these 2 groups are that virus strains used as antigenic reagents in sero-epidemiologic studies must be shown to be identical with the strains circulating in the population being analyzed, since misleading data may otherwise result. Moreover, immunochemical and molecular studies on the virus strains being used are essential to assess the validity of comparisons of data generated in laboratories in different parts of the world. Further in-depth studies of proteins specified by these viruses are essential to fully understand the significance of the observed differences among the 2 groups of HHV-6 strains, their relationship to tropism, association of different groups with different human diseases, geographic distribution, antigenic relationships, human antibody responses and influence in sero-epidemiology.

9.9. Conclusions

Within a short time after the discovery of HHV-6, substantial progress has been made in understanding the biology of HHV-6, sero-epidemiology and the spectrum of associated diseases. However, more work is needed in the area of HHV-6 proteins and, in this chapter, we have catalogued the available information. It is

likely that there will be continued progress in this area over the next several years. Information generated will allow us to establish the role of viral proteins in the replicative cycle of virus, virus infection, latency and other functions, which will eventually give a better understanding of this new member of the herpesvirus family and open up approaches to control HHV-6 infection.

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CHAPTER 10

Interactions between HHV-6 and other viruses

PAOLO LUSSO[1], DHARAM V. ABLASHI[1], and JANOS LUKA[2]

[1] *National Cancer Institute, National Institutes of Health, Bethesda, MD 20892, U.S.A., and* [2] *Department of Pathology and Laboratory Medicine, University of Nebraska Medical Center, Omaha, NE 68105, U.S.A.*

10.1. Introduction

The original isolation of HHV-6 from patients with hematologic malignancies initially led to the hypothesis that this virus could be involved in the pathogenesis of some human lymphoproliferative disorders (Salahuddin et al., 1986). However, the behavior of HHV-6 *in vitro*, namely, the apparent lack of transforming ability and the remarkable cytopathic effect exerted on T lymphocytes, was indicative of an immunosuppressive rather than tumorigenic role *in vivo*. This suggestion is consistent with the fact that, despite its high seroprevalence in the general population (Saxinger et al., 1988), HHV-6 has been most commonly isolated from the peripheral blood of immunocompromised individuals, including patients with acquired immunodeficiency syndrome (AIDS) (Salahuddin et al., 1986; Downing et al., 1987; Tedder et al., 1987; Agut et al., 1988; Lopez et al., 1988; Becker et al., 1989). In addition, using the polymerase chain reaction technique, HHV-6 genetic material was detected with higher frequency in the peripheral blood of AIDS patients than of healthy individuals (Buchbinder et al., 1988). It may also be of interest that HHV-6 isolates have been recently obtained from patients with autoimmune disorders (Hoffmann et al., 1991), another category of individuals with severe immunologic dysfunctions. The association with immune deficiency or dysregulation has undoubtedly marked the history of HHV-6, whose action was visualized in the context of a plethora of other agents of pathogenic or opportunistic nature. This provided a strong motivation to study the interactions between HHV-6 and different viral agents, particularly human immunodeficiency virus (HIV) or other viruses targeting the immune system and potentially implicated in immunodeficiencies.

One of the pathologic hallmarks of AIDS is the depletion of $CD4^+$ T lymphocytes, a subpopulation of T cells playing a crucial role in the generation of both humoral and cellular immune responses. This feature is commonly explained by the consideration that HIV, the causative agent of AIDS and related disorders, is selectively infectious and cytopathic for $CD4^+$ immune cells (Gallo and Montagnier, 1989). However, this mechanism *per se* does not provide a satisfactory explanation for the diverse clinical courses observed in HIV-infected people. It has, therefore, been postulated that other factors may play the role of co-factors in AIDS by enhancing the pathogenic effects of HIV and thereby accelerating the progression of the disease. For example, several DNA viruses (e.g., herpes-, papova-, hepadna-, adenoviruses) and the retrovirus human T cell leukemia virus type I (HTLV-I) *trans*-activate the regulatory sequences contained in the long terminal repeat (LTR) of HIV-1 (Gendelman et al., 1986; Davis et al., 1987; Mosca et al., 1987; Siekevitz et al., 1987; Nabel et al., 1988; Seto et al., 1988; Ensoli et al., 1989). This effect may induce reactivation of dormant HIV infection or augment the level of virus replication *in vivo*. However, most of the above-mentioned viruses do not share with HIV a preferred tropism for $CD4^+$ T cells and this may limit their opportunity to directly interact with HIV itself. Only two viruses among those *trans*-activating the HIV LTR, namely HHV-6 and HTLV-I, are primarily infectious for $CD4^+$ T cells and, therefore, represent particularly suitable candidates to play a role in the course of HIV infection. In addition, several clinical and experimental observations suggest that also human cytomegalovirus (HCMV) and Epstein–Barr virus (EBV), albeit not primarily $CD4^+$ T lymphotropic, may play a part in the pathogenesis of the immune deficit and/or of some opportunistic tumors in AIDS.

Extensive sero-epidemiological studies have been performed to prove the contribution of viruses other than HIV to the development of AIDS. For example, considerable evidence exists that individuals co-infected by HIV-1 and HTLV-I have an accelerated rate of progression toward full-blown AIDS, compared to singly infected patients (Bartholomew et al., 1987; Hattori et al., 1989; Page et al., 1990). In the case of HHV-6, unfortunately, the picture is complicated by the ubiquitous distribution of this virus in the general population. Several authors have found a positive correlation between antibody titers to HHV-6 and the progression of HIV-related disease (Ablashi et al., 1988a; Krueger et al., 1988; Huemer et al., 1989; Levy et al., 1990; Rodier et al., 1990; Scott and Constantine, 1990), whilst others failed to observe any correlation between HHV-6 seropositivity and either the presence of HIV infection or the stage of HIV-related disease (Brown et al., 1988; Fox et al., 1988; Spira et al., 1990). It should be emphasized, however, that the progressive derangement of the $CD4^+$ T cell function in HIV-infected individuals significantly impairs a wide range of immune mechanisms, including the 'helper' T cell-driven antibody production by B lymphocytes. Therefore, particularly in the advanced stages of HIV infection, serology *per se* constitutes a poor index of active HHV-6 replication.

It is likely that only *in vivo* studies, such as the prospective evaluation of HHV-6 viremia in HIV-infected people or the experimental infection with both HHV-6 and HIV in animal models will provide definitive evidence for the role played by

HHV-6 infection in AIDS. In the absence of suitable *in vivo* systems, however, several *in vitro* models have been developed to study the interactions between HHV-6 and HIV-1.

10.2. Interactions between HHV-6 and HIV-1

Several mechanisms exist through which HHV-6 may positively interact with HIV-1. It is possible that HHV-6, like other putative co-factors in AIDS, exploits some of these mechanisms to accelerate the progression of HIV-1 infection toward full-blown AIDS.

10.2.1. Productive co-infection of CD4$^+$ T lymphocytes by HHV-6 and HIV-1: acceleration of the cytopathic effect

The hypothesis that HHV-6 could play a role as a co-factor in AIDS was first formulated after the demonstration that, similar to HIV, HHV-6 has a predominant tropism for CD4$^+$ T lymphocytes and rapidly kills them in culture systems (Lusso et al., 1988). To prove this hypothesis, the initial step was to elucidate whether HHV-6 and HIV can directly interact within individual CD4$^+$ T lymphocytes. A major obstacle that theoretically may prevent two viruses from co-infecting individual cells is interference at the receptor level. In this case, several lines of evidence indicated that HHV-6, unlike HIV, does not utilize CD4 as a receptor on the surface membrane of susceptible cells (Lusso et al., 1989b): (1) competition experiments demonstrated that neither HIV-neutralizing monoclonal antibodies (mAbs) directed to human CD4 (i.e., OKT4a, Leu3a) nor the truncated, soluble form of the CD4 protein (sCD4) are able to block HHV-6 infection; (2) unlike CD4, the putative HHV-6 receptor seems to be expressed on the surface membrane of activated, but not resting T cells (see Chapter 3, this volume; and (3) immature T lymphoid cells, not yet expressing CD4, are infectable by HHV-6 (Schonnebeck et al., 1991), as are human T cell lines chronically harboring HIV-1, whose membrane CD4 is totally down-regulated or complexed with the gp120 envelope glycoproteins of HIV-1 (Lusso et al., 1989b). Therefore, it was evident that HHV-6 and HIV-1 can independently infect the same CD4$^+$ T cell without reciprocal interference. Indeed, CD4$^+$ T cells derived from normal human peripheral blood were found to be susceptible to co-infection by HHV-6 and HIV-1 (Fig. 10.1). Electron microscopic observation of co-infected cultures permitted to verify that both viruses actively replicate within individual cells. The simultaneous presence of HHV-6 and HIV-1 within CD4$^+$ T cells had two dramatic consequences: the expression of HIV-1 had a more rapid time-course in co-infected cultures compared to those infected by either virus alone and even more importantly, the cytopathic effect was significantly accelerated (Lusso et al., 1989a). Conflicting data were subsequently reported by other investigators (Carrigan et al., 1990), who observed a suppression rather than acceleration of HIV-1 replication in the course of HHV-6 co-infection. Surprisingly, however, accelerated kinetics of

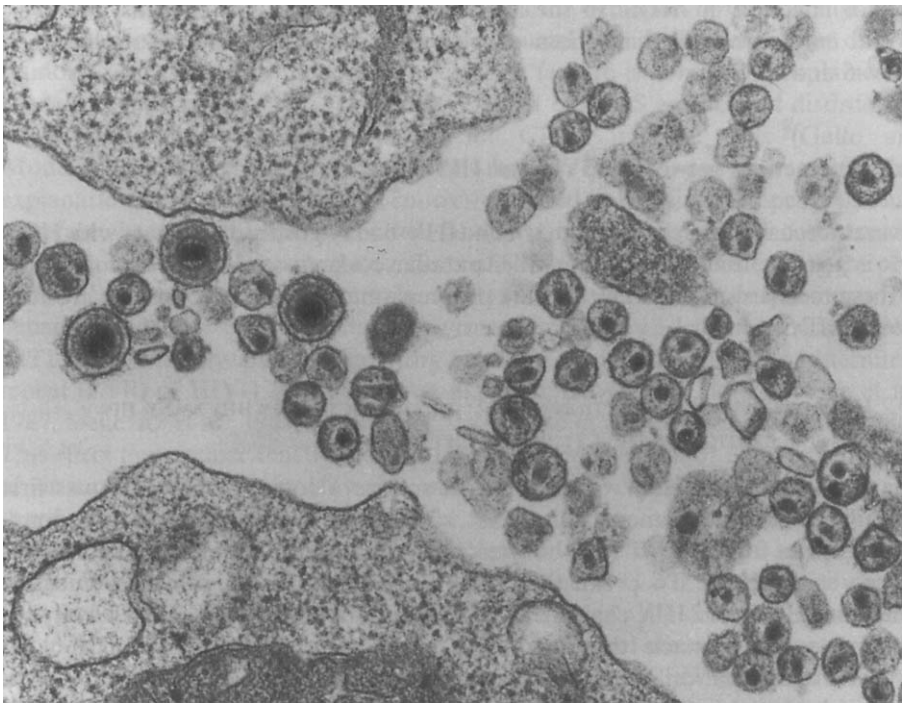

Fig. 10.1. Electron micrograph demonstrating the simultaneous presence of HHV-6 and HIV-1 mature virions in the supernatant fluid of co-infected human peripheral blood T lymphocyte cultures. Magnification 90000 ×. (For printing purposes figure has been reduced.)

cellular death were observed despite the decreased level of HIV-1 expression. These discrepancies may be related to the difference in the viral strains used or in the multiplicity of infection adopted for each virus. Altogether, the described studies indicate that HHV-6 and HIV-1 may directly interact within co-infected cells and that such interaction is predominantly positive.

As previously discussed, *in vivo* studies in animal models will undoubtedly provide critical information to understand the role of HHV-6 in AIDS. In this perspective, several species have been tested in our laboratory for their permissivity to HHV-6 infection. The chimpanzee (*Pan troglodytes*) is the only nonhuman species hitherto recognized to be susceptible, at least *in vitro*, to HHV-6 (Lusso et al., 1990). The characteristics of the infection in the chimpanzee model were similar to those previously described in humans: T lymphocytes not only represented the major target cells for HHV-6 infection but could also be productively co-infected by HHV-6 and HIV-1, resulting in an accelerated cytopathic effect. It is well established that chimpanzees can be infected *in vivo* with HIV-1, although no signs of clinical evolution have been observed in these animals. This fact is possibly related to the protected environment where the animals are kept, which

prevents contacts with most pathogenic or opportunistic agents, including herpesviruses. Chimpanzees may thus represent a suitable experimental animal model to study the interactions between HHV-6 and HIV-1 and the relevance of such interaction to the development of immunodeficiency.

10.2.2. *Trans*-ACTIVATION OF THE HIV-1 LTR BY HHV-6

Several environmental factors, including DNA and RNA viruses, ultraviolet light and antigenic stimulation, are able to activate in *trans* the transcription of HIV by interacting, directly or indirectly, with its regulatory sequences contained in the LTR. *Trans*-activation of the HIV-1 LTR may represent one of the mechanisms whereby HHV-6 accelerates the time-course of HIV-1 expression and cytopathicity in co-infected T lymphocyte cultures. Experiments with recombinant plasmids containing the HIV-1 LTR linked to a reporter gene demonstrated that HHV-6 is a potent *trans*-activator of HIV-1, with an efficiency comparable to that of HIV-1 itself (Ensoli et al., 1989; Lusso et al., 1989a). An additive effect was detected between the *trans*-activation induced by HHV-6 and that induced by *tat*, i.e., the HIV-1-specific *trans*-activating protein. Thus, after the initial triggering of HIV-1 replication, the effect of HHV-6 is amplified by the action of *tat*. Consistently, higher levels of mRNA transcripts specific for HIV-1 can be detected by the *in situ* hybridization technique in co-infected cultures compared to singly infected ones (Ensoli et al., 1989). The original observation that HHV-6 *trans*-activates the HIV-1 LTR was subsequently confirmed (Horvat et al., 1989).

To identify the regions of the HIV-1 LTR that are responsive to HHV-6 *trans*-activation, a detailed analysis was performed using a series of LTR deletion mutants. The elements responsive to HHV-6 were mapped to a region located between position -103 and -48 of the HIV-1 LTR, which includes the enhancer elements (Fig. 10.2). The same HIV-1 enhancer was found to be sufficient to confer

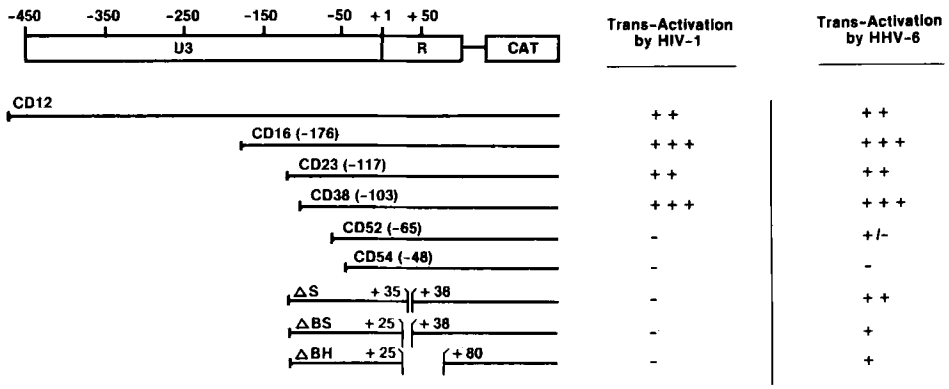

Fig. 10.2. Mapping of the HIV-1 LTR regions responsive to HHV-6 *trans*-activation using a series of LTR deletion mutants linked to the CAT reporter gene.

HHV-6 responsiveness to a heterologous promoter. Interestingly, the *trans*-activation induced by HHV-6 was shown to be independent from the presence of a functional TAR (*tat*-activation region, i.e., the region responsive to *tat* action) (Fig. 10.2). This result can explain the additive effect observed between HHV-6 and HIV-1 and may be relevant to *in vivo* pathogenesis, since a TAR-independent mechanism would allow HHV-6 to rescue, by complementation, putative defective HIV genomes lacking a functional *tat*–TAR activation axis. Finally, DNA-binding studies demonstrated that HHV-6-specific, or HHV-6-induced, nuclear factors bind to selected enhancer motifs within the HIV-1 LTR (Ensoli et al., 1989). These factors, which are currently the object of intensive investigation, most likely represent the final mediators of the HHV-6-induced *trans*-activation. Altogether, these data indicate that HHV-6 is able to activate (or reactivate) the expression of HIV-1 and could thereby enhance the level of replication of HIV-1 in co-infected patients.

10.2.3. Positive regulation of CD4 expression by HHV-6: induction of HIV-1 susceptibility in $CD8^+$ T lymphocytes

Another possible mechanism of interaction between HHV-6 and HIV occurs at the receptor level. The major receptor for HIV is the CD4 antigen (Klatzman et al., 1984), a glycoprotein with molecular weight of 59 kd, which is expressed at high levels by a subset of mature T lymphocytes (the $CD4^+$ subset, which includes T lymphocytes with 'helper-inducer' functional capability), but also, albeit with lesser intensity, by cells belonging to the mononuclear phagocytic system, by selected EBV-transformed B lymphoblastoid cell lines and, possibly, by other cell types. During their intrathymic differentiation, immature T cells transiently acquire a double-positive $CD4^+CD8^+$ phenotype, but subsequently lose one of the two antigens to eventually become single-positive, either $CD3^+CD4^+$ or $CD3^+CD8^+$, immunocompetent T lymphocytes. While in post-thymic $CD3^+CD4^+$ T cells the CD8 genes are unmethylated and their expression can be induced by physiologic factors (e.g., IL-4), in circulating $CD3^+CD8^+$ T cells the expression of CD4 is believed to be repressed by DNA methylation (Richardson et al., 1986).

Infection by HHV-6 was shown to exert a positive regulatory effect on the expression of CD4 (Lusso et al., 1991). After infection with HHV-6, significant upregulation of the expression of CD4 can be observed in cell lines of T lymphoid origin, such as Jurkat. The expression of the early genes of HHV-6 seems to be sufficient to modulate CD4, as demonstrated by studies with phosphonoformic acid, an inhibitor of viral DNA polymerases. The upregulation of CD4 and, possibly, of other membrane markers could not be simply explained by the rigidification of the cell membrane induced by HHV-6 infection (Schonnebeck et al., 1991).

Analysis at the clonal level has demonstrated that both $CD3^+CD4^+$ and $CD3^+CD8^+$ T lymphocytes are infectable by HHV-6. This observation has permitted study of the regulation of CD4 by HHV-6 in cells which totally lack CD4, such as normal $CD3^+CD8^+$ T lymphocytes. Infection by HHV-6 was shown

to induce *de novo* expression of CD4 in $CD8^+$ T cell populations and clones derived from adult peripheral blood. These cells maintain the expression of the CD8 antigen and thus acquire a double-positive 'thymic' $CD4^+CD8^+$ phenotype (Fig. 10.3). Analysis using Northern blot has demonstrated that HHV-6 activates the expression of CD4 at the transcriptional level (Lusso et al., 1991). Analogous results were obtained using the HSB_2 and 67-I cell lines. These observations provided the first immunological and molecular evidence that mature $CD4^-CD8^+$ T lymphocytes can be induced by a naturally occurring agent to reacquire expression of CD4 in the post-thymic life. Similar findings were previously reported only using stimuli not encountered *in vivo*, such as the demethylating agent 5-azacytidine, suggesting that in post-thymic $CD4^-CD8^+$ T lymphocytes CD4 expression is tightly repressed by DNA methylation. The HHV-6-induced activation of CD4, possibly mediated by virus-specific or virus-induced *trans*-activating factors, may thus overcome such a strong mechanism of genetic control.

As mentioned above, the CD4 antigen represents the major receptor structure for HIV on the surface membrane of susceptible cells. It has been demonstrated that the newly acquired CD4, induced by HHV-6 infection, rendered normal $CD8^+$ T cells susceptible to HIV-1 infection. The penetration and reverse transcription of HIV-1 were abrogated by previous treatment of the cells with a neutralizing monoclonal antibody to CD4, thus proving the specificity of this phenomenon.

The increased availability of CD4 receptors and/or the expanded HIV-1 cellular tropism determined by HHV-6 may help to explain the acceleration in both HIV-1 expression and cytopathic effect induction observed during co-infection *in vitro* (see above). However, the ability of HHV-6 to induce *de novo* expression of CD4 may also broaden the range of HIV-susceptible cells *in vivo*, thereby favoring the spread of HIV throughout the organism of co-infected hosts. In addition, the induction of the susceptibility to HIV-1 infection in $CD8^+$ T cells may be relevant to the

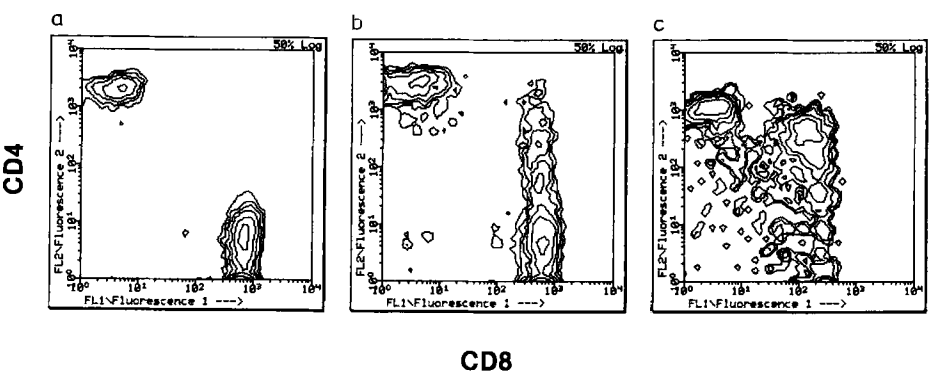

Fig. 10.3. Effect of HHV-6 infection on the expression of surface CD4 and CD8 antigens by human peripheral blood T cells, as assessed by dual-color flow cytometry. (A) Uninfected cells, 6 days after establishment in culture; (B) HHV-6 infected cells, 6 days post-infection and (C) HHV-6-infected cells, 9 days post-infection.

functional defects often observed in this T cell subset in patients with AIDS. However, conclusive *in vivo* data are still lacking and the actual relevance of this phenomenon has yet to be ascertained.

10.3. Interactions between HHV-6 and HTLV-I

Both HHV-6 and HTLV-I exhibit a preferred tropism for $CD4^+$ lymphocytes both *in vitro* and *in vivo*. In contrast to HHV-6, HTLV-I has the ability to transform these cells and is also the causative agent of a malignant lymphoproliferation involving the $CD4^+$ T cell, adult T cell leukemia. One of the characteristics of HTLV-I, at least in its *in vitro* behavior, is the inability to infect and transform cells by cell-free virus transmission (Popovic et al., 1983), whereas cell-to-cell transmission is an efficient process consistently leading to T cell immortalization after a period of 4–6 weeks in culture. This feature represents a substantial obstacle to study the effects of viral interaction, since it is impossible to obtain a suitable uninfected control for HTLV-I-infected cells, whereby comparative studies can be performed. Thus, most results have been obtained using long-term cultured HTLV-transformed cell lines in the absence of adequate controls.

In our laboratory, HTLV-I-transformed T cell lines of both $CD4^+$ and $CD8^+$ phenotype have been successfully superinfected with diverse HHV-6 strains. For example, the $CD8^+$ clone 67-I was productively infected with HHV-6 and exhibited evident signs of cytopathic effect. Electron microscopic observation of dually infected 67-I cultures provided evidence that both viruses can simultaneously replicate within the same cell (Fig. 10.4). It is interesting that the rate of HTLV-I replication within these cultures was not substantially altered by HHV-6 superinfection. This observation is consistent with the finding that HHV-6 does not *trans*-activate the HTLV-I LTR (Ensoli et al., 1989). As described above, expression of the CD4 antigen was induced in 67-I cells after HHV-6 infection.

It has been reported that MT-4, another HTLV-I-transformed T cell line, is susceptible to infection by the Z-29 strain of HHV-6 (Black et al., 1989). However, no data have been presented regarding the simultaneous replication of HHV-6 and HTLV-I in these cells, nor the effects exerted by HHV-6 on HTLV-I expression.

10.4. Interactions between HHV-6 and EBV

HHV-6 shares two major features with the fourth human herpesvirus, EBV: the lymphotropic nature and the possible pathogenetic role in human lymphoproliferative disorders, including B cell non-Hodgkin's lymphoma and Hodgkin's disease. The concept of a possible co-tumorigenic role of HHV-6 has been recently supported by studies in the NIH-3T3 mouse fibroblast system (Razzaque, 1990). It is well established that EBV can infect and immortalize *in vitro* human $CD21^+$ B lymphocytes while, as mentioned above, HHV-6 does not seem to be able to induce cell immortalization, nor infect primary human B lymphocytes (Lusso et al.,

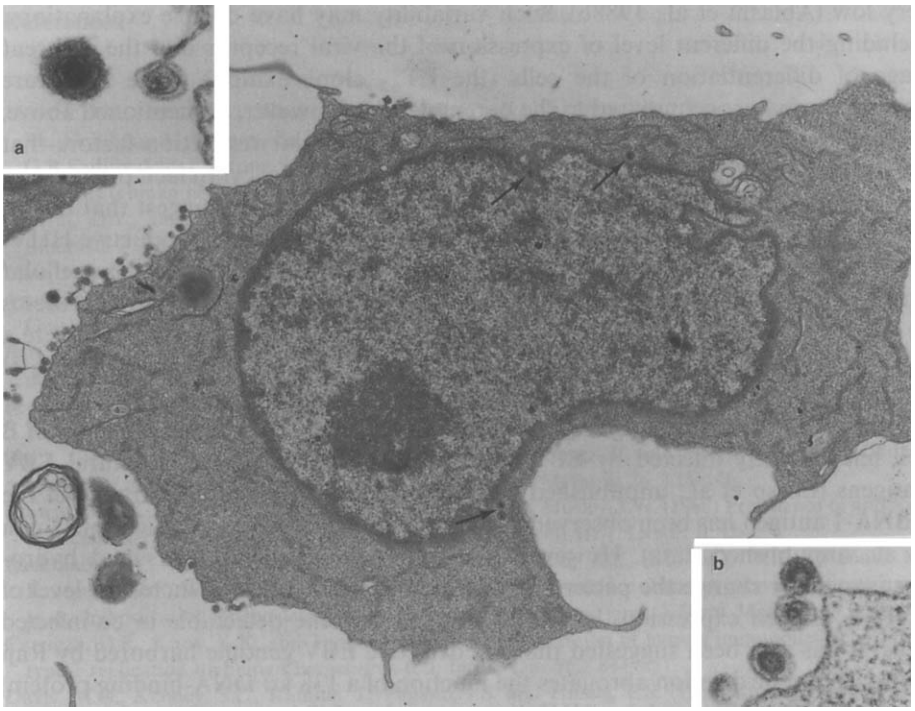

Fig. 10.4. Electron micrograph illustrating a cell from the HTLV-I-transformed $CD8^+$ T cell clone 67-I superinfected by HHV-6, strain GS. (a) Mature HHV-6 and HTLV-I particles; (b) Budding HTLV-I virion. The presence of immature forms of both viruses demonstrates their active replication within individual cells. Magnification 15000× (insert 90000×). (For printing purposes figure has been reduced.)

1988). However, a number of EBV-transformed B lymphoblastoid cell lines can be superinfected by HHV-6 with variable efficiency (Ablashi et al., 1988b). Interestingly, EBV-negative B lymphoid cell lines become susceptible to HHV-6 after infection with EBV. Why B cells acquire the susceptibility to HHV-6 after EBV-transformation is still a matter for speculation. Hypothetically, the membrane receptor for HHV-6 could be induced in normal B cells following EBV infection, in a fashion similar to other cellular activation markers. However, the restriction for HHV-6 infection in EBV-negative cells may also occur at the post-receptor level. In this case, the simultaneous presence of EBV in the same cell could overcome putative cellular factor(s) restricting HHV-6 expression.

The efficiency of HHV-6 infection in EBV-transformed cell lines is remarkably variable. A typical example is the ET_{62} clone, derived in our laboratory by the limiting dilution technique from the WIL2 cell line. While the clone was found to be highly susceptible to HHV-6, the efficiency of infection in the parental cell line is

very low (Ablashi et al., 1988b). Such variability may have diverse explanations, including the different level of expression of the viral receptor and the different stage of differentiation of the cells (the ET_{62} clone exhibits more immature phenotypic features compared to the parental cells). However, as mentioned above, HHV-6 replication could also be limited by intracellular restriction factors that may, therefore, be relevant to the establishment of viral latency. In fact, preliminary results obtained in the laboratory of one of the authors (J.L.) suggest that only a proportion of cells from EBV-transformed B cell lines undergoes productive HHV-6 infection with typical expression of late viral antigens, release of extracellular virions and induction of cytopathic effect. In other cells, by contrast, HHV-6 seems to establish a nonproductive or latent infection and can be induced to complete a lytic infectious cycle by agents such as phorbol esters or hydrocortisone (Luka et al., unpublished results).

In most of the cases analyzed, including ET_{62} cells, HHV-6 superinfection of B cell lines latently infected by EBV fails to induce expression of structural EBV antigens (Lusso et al., unpublished data). On the contrary, disappearance of the EBNA-1 antigen has been observed in the early stages of HHV-6 replication (Luka et al., unpublished data). However, treatment with phorbol esters and hydrocortisone may change the pattern of viral expression: besides an increased level of HHV-6 antigen expression, late EBV antigens become detectable in co-infected cells. It has also been suggested that the defective EBV genome harbored by Raji cells (a genomic deletion abrogates the function of a 138 kd DNA-binding protein) could be complemented by HHV-6, since a low-level expression of late EBV antigens becomes detectable in co-infected Raji cells stimulated by phorbol esters and hydrocortisone (Luka et al., unpublished data).

The increasingly refined methods for the identification of HHV-6 and EBV in patient tissues will certainly contribute to clarify if these herpesviruses can act in cooperation in the pathogenesis of some hematologic malignancies. In this respect, the development of suitable animal models may also provide important clues to understand the combined role of these agents *in vivo*.

10.5. Interactions between HHV-6 and HCMV

It has been recently observed that some isolates of HHV-6 (e.g., GS and some field isolates) can productively infect human fibroblasts (Luka et al., 1990; Lusso et al., unpublished observations). This has enabled investigators to examine the effects of the interaction of HHV-6 with the fifth human herpesvirus, HCMV, which infects fibroblasts with high efficiency. Preliminary results indicate that when the two viruses are used simultaneously for infection, they both replicate within the same culture and, possibly, within the same cells. However, if either virus is added 4 hours or more before the other, no expression of the second virus is detectable in the cultures (Luka et al., unpublished data). Whether this phenomenon indicates the occurrence of viral interference at the receptor level or some other type of negative interaction between HHV-6 and HCMV is unknown.

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Ablashi/Krueger/Salahuddin (eds) Human Herpesvirus-6

CHAPTER 11

Human herpesvirus-6 (HHV-6) and exanthem subitum

HIDEAKI KIKUTA

Department of Pediatrics, Hokkaido University School of Medicine, Sapporo 060, Japan

11.1. Introduction

Salahuddin et al. (1986) initially isolated a novel human herpesvirus (human herpesvirus-6, HHV-6), from patients with lymphoproliferative disorders. Lately, HHV-6 has been isolated from the leukocytes of persons with various illnesses (Downing et al., 1987; Agut et al., 1988; Lopez et al., 1988) and from the saliva of several healthy adults (Pietroboni et al., 1988; Levy et al. 1990). Yamanishi et al. (1988) reported that the isolated virus from exanthem subitum was identical or closely related to HHV-6 antigenically and morphologically and appears to be the agent for exanthem subitum. Exanthem subitum is a common benign infectious disease of infancy characterized by 3 or 4 days of high fever associated with a paucity of physical findings. The temperature falls to normal by crisis coincidental with the appearance of a morbilliform rash that fades within 2 days. We discuss HHV-6 isolation from exanthem subitum and its virus characteristics.

11.2. Detection of HHV-6 from exanthem subitum

We examined HHV-6 in peripheral blood mononuclear cells (PBMCs), saliva and urine from 8 Japanese patients with exanthem subitum. PBMCs were separated by Ficoll–Conray gradient centrifugation. The cells were cultured in RPMI 1640 medium with 20% fetal calf serum (FCS), recombinant interleukin-2 (r-IL2) (Takeda Chemical Industries, Osaka, Japan) and phytohemagglutinin (PHA) (Difco, Detroit) in a CO_2 incubator at 37°C for 10 days. Cord blood mononuclear cells (CBMCs) were separated on Ficoll–Conray gradients and the cells were

cultured in RPMI 1640 medium with 20% FCS, r-IL2 and PHA for 3 days. The cells were then exposed to saliva and urine samples and cultured for 7–10 days. We assessed HHV-6-related antigen expression by anticomplement immunofluorescence and the presence of HHV-6 DNA by Southern blot hybridization.

HHV-6 DNA was detected in 5 of the 8 PBMC DNA from patients with exanthem subitum. Immunofluorescence analysis using mouse monoclonal antibody to cytoplasmic antigen of HHV-6-infected cells (provided by Dr. K. Yamanishi) revealed that antigenically our isolates were HHV-6. No HHV-6 DNA was detected in cell DNA from the eight age-matched uninfected controls. All saliva and urine samples from patients with exanthem subitum were negative for HHV-6 by both Southern blot hybridization and anticomplement immunofluorescence. Levy et al. (1990) reported that HHV-6 was recovered at high frequency from the saliva of healthy individuals. However, our results indicate that the main site of HHV-6 replication in exanthem subitum may be PBMCs.

11.3. Restriction enzyme site heterogeneity among different isolates of HHV-6 from exanthem subitum

DNA extracted from the cultured cells was cleaved with BamHI, EcoRI or HindIII. The DNA (10 μg/lane) was electrophoresed through 0.6% agarose gel and transferred onto nitrocellulose membrane filters. Each filter was hybridized with the ^{32}P-labeled cloned fragment pZVH14 (provided by Dr. R.C. Gallo) of the HHV-6 genome for 48 h at 41°C in 1 × standard saline citrate (SSC; 0.15% sodium chloride and 0.015 M sodium citrate), 50% formamide, 0.5% SDS, and heat-denatured salmon sperm DNA (100 μg/ml). After hybridization, the filters were washed 3 times at room temperature in 0.1 × SSC with 0.1% SDS, then incubated 3 times for 1 h at 50°C. The filters were then dried and exposed to Sakura X-ray film (Tokyo) at − 80°C.

The DNA from infected PBMCs from 5 of the 8 patients with exanthem subitum hybridized with pZVH14. However, the restriction patterns with BamHI, EcoRI, and HindIII were not identical to the prototype virus (Fig. 11.1). The pZVH14 probe is an 8.7 kilobase HindIII fragment of HHV-6 (Josephs et al., 1986, 1988). However, Southern blotting of HindIII DNA digests showed 2 bands in the 5 isolates. Southern blotting of BamHI DNA digests showed 2 bands in the 5 isolates in contrast to 4 bands in the prototype virus. Southern blotting of EcoRI DNA digests showed 4 bands in 3 isolates and 5 bands in 2 isolates in comparison to 5 bands in the prototype virus (Kikuta et al., 1989).

These data show the viability in restriction fragment length polymorphisms in isolates of HHV-6. Becker et al. (1989) isolated new T lymphotropic human herpesviruses. They showed 2 bands by Southern blot analysis of the HindIII DNA digests, as did ours. Ethidium bromide staining of gels prior to blotting revealed the differences (Fig. 11.2). This indicates that restriction site heterogeneity occurs even among different isolates of HHV-6 in Japan. The viability in restriction enzyme

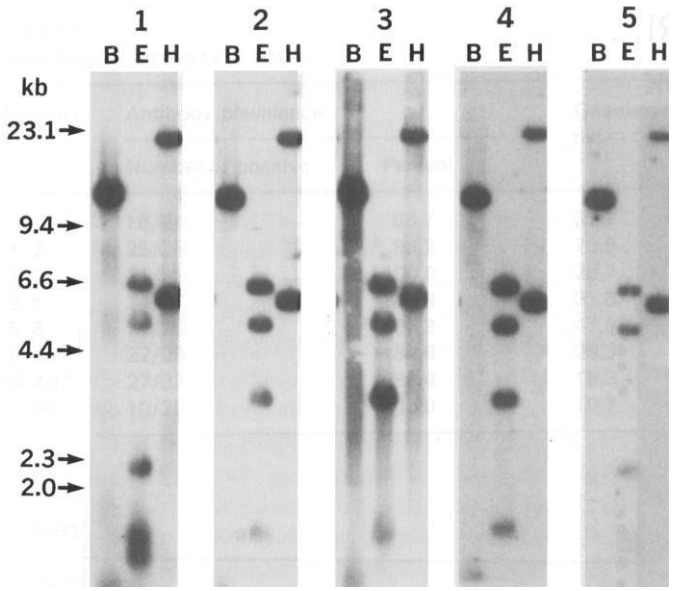

Fig. 11.1. Autoradiography of BamHI, EcoRI, and HindIII DNA digests with ^{32}P-labeled pZVH14. Lanes 1–5: HHV-6-infected cell DNA. B denotes BamHI, E EcoRI, and H HindIII.

patterns of HHV-6 isolates throughout the world remains to be studied; however, our results suggest that some viability exists among strains.

11.4. Antibody prevalence

We have tested 212 children for antibody to HHV-6 with an anticomplement immunofluorescence test on cord blood lymphocytes infected with HHV-6 (OK strain isolated from a patient with exanthem subitum). Propagation of HHV-6 was confirmed by Southern hybridizations of the infected-cell DNA with a pZVH14 probe. Infected cells were harvested at the point of maximal cytopathic effect and fixed on slides with cold acetone. Uninfected cells were used as the negative antigen control. Polyclonal or monoclonal antibodies to herpesvirus type 1, Epstein–Barr virus, and human CMV did not react with HHV-6-infected cells.

The cell smears were incubated with serially diluted serum at 37°C for 30 min. The lowest serum dilutions were 1:5. After being washed with phosphate buffered saline (PBS), guinea pig complement (Cedarlane Laboratories Ltd., Hornby, Ontario, Canada) was added. The preparations were maintained at 37°C for 15 min. After additional washing with PBS, they were incubated with fluorescein isothiocyanate-conjugated goat anti-guinea pig C_3 (Cappel Cooper Biomedical, Malven, PA) for another 30 min. After another washing with PBS, all the stained

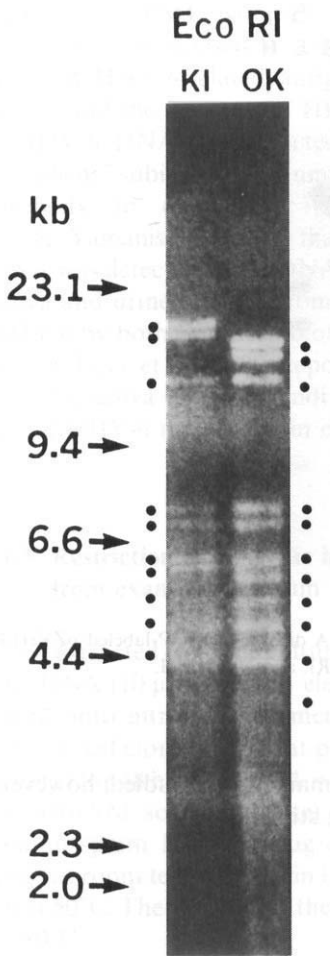

Fig. 11.2. Ethidium bromide staining of 2 EcoRI HHV-6 DNA digests. The dots indicate the EcoRI DNA digests by ethidium bromide staining.

preparations were mounted in PBS:glycerin (1:1) and examined under a Nikon Universal Research Microscope.

The antibody-positive rate of children aged from 0 to 12 months was 66.7%. The prevalence of antibody was highest in serums from children aged from 1 to 3 years. Children aged 1 to 2 years had the highest geometric mean titers of HHV-6 antibody. The antibody titer in girls was higher than that in boys in the group 1–2 years of age. In the group more than 40 years of age, there were consistent declines in seropositivity and geometric mean titers of HHV-6 antibody (Table 11.1, Fig. 11.3).

TABLE 11.1.
Prevalence of antibody to HHV-6

Age (yr)	Antibody prevalence		Geometric mean titer
	Number of positive	Percent	
<1	16/24	66.7	31.7
1–2	25/26	96.2	79.8
2–3	31/31	100.0	26.7
3–5	24/27	88.9	32.8
5–8	24/26	92.3	47.4
8–15	22/26	84.6	26.3
15–40	27/32	84.4	19.3
>40	10/20	50.0	10.7

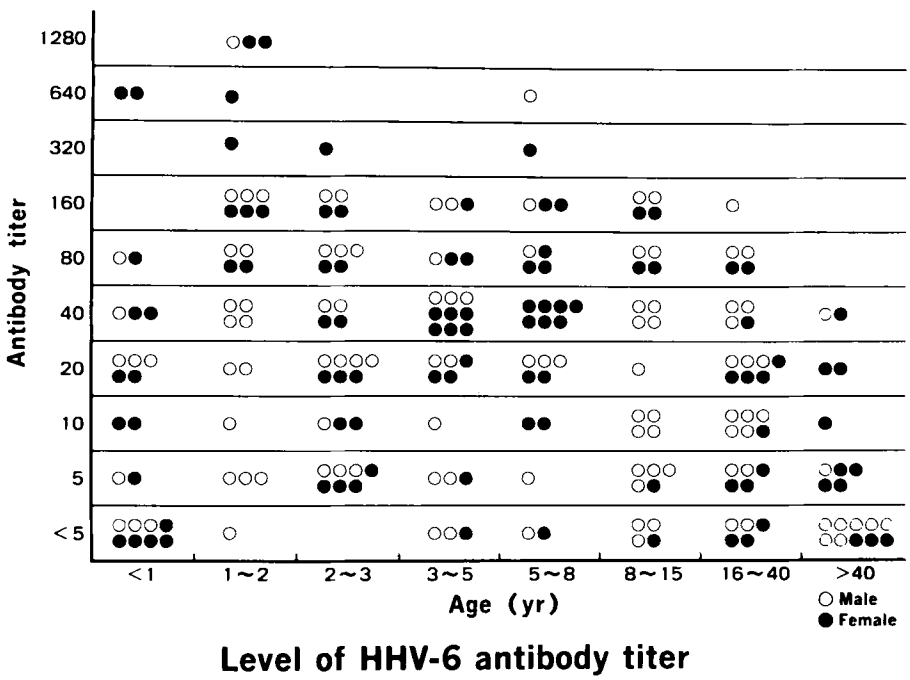

Fig. 11.3. Titer of antibody to HHV-6.

Almost all children had the antibody against this virus after 12 months of age. Our antibody prevalence data support the previous suggestion (Briggs et al., 1988; Knowles and Gardner, 1988) that HHV-6 is a ubiquitous virus acquired very early in life, perhaps around the time of birth or shortly thereafter. In addition, the serologic response to HHV-6 in females may differ from that in males.

11.5. Replication of HHV-6

We examined the replication of HHV-6 (OK strain) in PBMCs from healthy adults and CBMCs by anticomplement immunofluorescence and Southern blot hybridization. The effect of some chemicals on HHV-6 replication was also assessed in adult PBMCs and in CBMCs. Cells were exposed to HHV-6 at a multiplicity of infection (moi) of 10^{-2} $TCID_{50}$/cell at 37°C for 1 h. The infected cells were then cultured in a humidified atmosphere of 5% CO_2 at a density of 2×10^6 cells/ml in RPMI 1640 medium supplemented with 20% FCS and 5 ng/ml of monoclonal antibody OKT3 (IgG2) (Ortho Diagnostic Systems, Raritan, NJ), monoclonal antibody to CD3, 5 μg/ml of PHA, 5 ng/ml of TPA (12-*o*-tetradecanyl-phorbol-13-acetate), or 1×10^{-6} M of calcium ionophore A23187.

In several experiments, HHV-6-inoculated PBMCs from adult humans have been compared with CBMCs. In the absence of monoclonal antibody to CD3, a low induction rate of HHV-6 antigen occurred in CBMCs, whereas adult lymphocytes showed no positive cells (Table 11.2). In contrast, treatment with monoclonal antibody to CD3 caused a dramatic increase in viral replication. The average values were 73.8% in the 3 CBMC specimens and 52.2% in the 8 PBMC specimens. HHV-6 replication was confirmed by Southern blot hybridization. PHA enhanced replication of HHV-6 in CBMCs but not in PBMCs. Infection of CBMCs by HHV-6 was productive; however, little or no infection was observed with PBMCs from adults exposed to the virus even if stimulated by PHA.

TABLE 11.2.
Percentage of HHV-6 antigen-positive cells[a] in peripheral blood mononuclear cells (PBMCs) and cord blood mononuclear cells (CBMCs) in the presence or absence of monoclonal antibody to CD3 or phytohemagglutinin (PHA)

Specimen	HHV-6 antigen-positive cells		
	Medium	anti-CD3 (5 ng/ml)	PHA (5 μg/ml)
PBMCs			
1	<0.1	65.8	<0.1
2	<0.1	27.3	<0.1
3	<0.1	50.4	<0.1
4	<0.1	67.8	<0.1
5	<0.1	68.9	<0.1
6	<0.1	48.5	<0.1
7	<0.1	35.2	<0.1
8	<0.1	53.9	<0.1
CBMCs			
1	5.3	91.2	56.3
2	6.8	78.9	35.1
3	5.8	51.4	36.7

[a] Cells were infected with HHV-6 at an moi of 10^{-2} $TCID_{50}$/cell.

Monoclonal antibody to CD3 strongly enhanced HHV-6 replicative cycles in PBMCs from adults (Kikuta et al., 1990a, b). TPA and calcium ionophore have no effect on HHV-6 replication in PBMCs and CBMCs.

HHV-6 (OK strain) replicates in freshly stimulated human umbilical cord blood; however, HHV-6 replication is usually restricted in adult peripheral blood compared with that in cord blood. The mechanisms that control HHV-6 replication in host cells remain to be determined. Both monoclonal antibody to CD3 and PHA consistently induce polyclonal proliferation of T cells. PHA binds to large numbers of T cell surface glycoproteins; however, the cell surface molecules responsible for the ability of PHA to stimulate T cells have not been defined. The activation of T cells via stimulation of PHA may differ from the antigen-dependent T cell receptor (TCR)-CD3-mediated pathway. This suggests that monoclonal antibodies to CD3 and PHA have different mechanisms in HHV-6 replication. Interactions between HHV-6 and cells are the key to understanding the mechanisms that permit or restrict replication of the virus in the host cell. The cellular and viral factors that promote the lytic cycle have not yet been defined.

Lusso et al. (1988) reported that HHV-6 infects predominantly CD3-depleted immature T cells but not CD3-positive mature T cells. In contrast, Takahashi et al. (1989) reported that most of the cells susceptible to HHV-6 are CD3- and CD4-positive mature T cells. Our results support the premise that the major susceptible cells may be CD3-positive mature T cells.

While the mechanism of enhancement of viral replication by monoclonal antibody to CD3 remains obscure, further examination of the effects of this antibody on HHV-6-infected PBMCs could be useful in the study of the relationship between PBMCs and HHV-6.

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Ablashi/Krueger/Salahuddin (eds) Human Herpesvirus-6

CHAPTER 12

Exanthem subitum and HHV-6 infection

KOICHI YAMANISHI, TOSHIOMI OKUNO, KAZUO TAKAHASHI, KRUAVON BALACHANDRA, KAZUHIRO KONDO, TOSHIO KONDO, and TETSU MUKAI

Department of Virology, Research Institute for Microbial Diseases, Osaka University, Japan

12.1. Introduction

Exanthem subitum or roseola infantum, which was first described by Zahorsky in 1910, is a common disease in infants, characterized by high fever for a few days and the appearance of rash coinciding with subsidence of fever. It has been believed that a virus causes this disease because of evidences of the long incubation time, the leukopenia, the failure to respond to antimicrobial agents and the failure to recover bacteria from the various body fluids. However, attempts to isolate the causative agents have been unsuccessful.

A novel human herpesvirus, now named human herpesvirus-6 (HHV-6), has been isolated independently by several groups from patients with lymphocytic disorders (Salahuddin et al., 1986; Agut et al., 1987; Downing et al., 1987; Tedder et al., 1987; Lopez et al., 1988). Although this virus was initially named 'human B lymphotropic virus', later it was found mainly to infect and replicate in lymphocytes of T cell lineage (Ablashi et al., 1987; Lusso et al., 1987, 1988; Takahashi et al., 1988). Characterization of HHV-6 indicated that it was antigenically and genetically distinct from other human herpesviruses (cytomegalovirus, herpes simplex virus type 1 and 2, varicella–zoster virus, and Epstein–Barr virus) (Josephs et al., 1986; Salahuddin et al., 1986; Lopez et al., 1988). It was not known whether HHV-6 causes disease in humans. We reported that HHV-6 is the causal agent of exanthem subitum (ES) (Yamanishi et al., 1988).

12.2. Clinical features of ES

The clinical appearance of ES has been well described in the pediatric textbooks. The main clinical characteristics are as follows. The onset of the disease is often

abrupt and it lasts for a few days. The typical eruption appears after the decline of the temperature. The most common complication is convulsive seizures. The prognosis is uniformly excellent even for those cases complicated by convulsive seizures.

Although the clinical appearance of ES and hematologic changes of patients have been described in a textbook (Krugman et al., 1985), those by HHV-6 infection were not clear until now because this virus was isolated very recently. We have recently observed patients with ES in Thailand. All patients clinically suspected of ES had symptoms with fever and rash. Among 31 patients whose antibodies had converted to HHV-6 during the convalescent phase, the clinical features were available from 23 cases (Table 12.1). The total clinical course of illness was less than 10 days, and 17 cases had fever (more than 37°C) for 2–4 days, followed by the appearance of rash which lasted for 1–3 days (70%). The characteristics of rash was maculopapular in appearance on the trunk which then spread to face, arms and legs. Lymphadenopathy was seen in 18 patients (86%), being located mostly at suboccipital parts (78%). The other clinical manifestations were diarrhea (52%), vomiting (32%), running nose (55%), cough (45%) and hepatomegaly (37%). Convulsion was occasionally observed in patients (11%)

TABLE 12.1.
Summary of clinical signs in 23 patients

Findings	Incidence % (proportion)
1. Fever	100
Duration 2–4 days	73.9 (17/23)
5–7 days	26.1 (6/23)
2. Rash	
Characteristic : maculopapular	100
Apparent time	
Before subside of fever	30.4 (7/23)
After subside of fever	69.6 (16/23)
3. Associated manifestations	
Lymphadenopathy	85.7 (18/21)
Location: cervical	0
postauricle	22.2 (4/18)
suboccipital	27.8 (5/18)
suboccipital with cervical or/and postauricle	50.0 (9/18)
Hepatomegaly (1–2 cms)	36.8 (7/11)
Diarrhea	52.2 (12/23)
Vomiting	31.6 (6/19)
Running nose	54.5 (12/22)
Cough	45.0 (9/20)
Infected pharynx	10.5 (2/19)
Convulsion	10.5 (2/19)

during the febrile phase. Although meningo-encephalitis by HHV-6 infection has been reported (Ishiguro et al., 1990), it is very rare. Two reports appeared on liver injury in infants followed by HHV-6 infection. The first was a fatal case of fulminant hepatitis probably due to HHV-6. HHV-6 could be isolated from peripheral blood and HHV-6 DNA was also detected in tissue (Asano et al., 1990). The second case was a liver dysfunction followed by ES, and HHV-6 was also isolated in peripheral blood (Tajiri et al., 1990).

In order to examine the hematology in patients, blood was taken during the acute phase (febrile or exanthem phase) from 23 patients, and blood examinations were studied (Table 12.2). Fifteen patients (65%) had more than 9000 per mm^3 of white blood cells which were within the normal range (18100–9100 per mm^3 in children newborn to 4 years old), but the numbers reduced to less than 9000 per mm^3 in 8 (34.8%) of the 23 patients. While 2 of the 4 patients in the group of the febrile phase had neutropenia, all patients of the group of the exanthem phase had neutropenia (less than 40% which was lower than normal range). On the other hand, in all patients in the group of the exanthem phase, the lymphocyte proportion was more than 50%, which was higher than the normal range. The average proportion of neutrophils and lymphocytes in these patients were 15 and 77%, respectively. These results indicated that neutropenia was prominently observed with relative lymphocytosis in patients infected with HHV-6 during the exanthem phase of HHV-6 infection. Hellstrom and Vahlquist (1951) also reported that neutropenia and relative lymphocytosis were recognized by the experimental

TABLE 12.2.
Hematological findings of 23 exanthem subitum patients during febrile and exanthem phase

Findings	Cases of patients	
	Febrile phase	Exanthem phase
White blood cells[a]		
< 9000 per mm^3	1	7
> 9000 per mm^3	3	12
Neutrophils[a]		
< 20%	0	17
20–40%	2	2
> 40%	2	0
Lymphocytes[a]		
< 50%	2	0
50–70%	2	2
> 70%	0	17

[a] Normal values (from newborn to 4 years old):
White blood cells = 18100–9100 per mm^3
Neutrophils = 60–40%
Lymphocytes = 30–50%

inoculation of blood from ES patients in volunteers. This phenomenon may be the result of the immune response.

12.3. Virus isolation

The successful human transmission of the infection by experimental inoculation of blood of ES patients to other infants was reported by Kempe et al. (1950) and by Hellstrom and Vahlquist (1951). As described above, efforts to isolate the causative agents of ES were made but were not successful for a long time.

We also attempted to isolate a causative virus of ES from patients (Yamanishi et al., 1988). Fresh heparinized peripheral blood was taken from ES patients during the febrile phase, and mononuclear cells were separated by using Ficoll–Paque (Pharmacia) gradient centrifugation. Cells were cultured in complete medium (RPMI 1640 medium supplemented with 10% fetal calf serum (FCS), recombinant human interleukin-2 (IL-2) (kindly supplied by Takeda Chemical Industries, LTD), phytohemagglutinin (PHA) at 5 μg/ml (Honen Oil Company) and hydrocortisone at 5 μg/ml) in a CO_2 incubator at 37°C. In total, 4 samples of peripheral blood were collected from patients during the febrile phase (2–3 days after onset of fever), and cultured for 2 weeks. The cytopathic effect was observed at around day 9 after cultivation with the characteristics of balloon-like syncytia (Fig. 12.1) as described elsewhere (Salahuddin et al., 1986). Cells were mounted on the slide, fixed and stained with acute and convalescent sera by anticomplement immunofluorescence (IF) test. While specific staining was not observed with serum of the acute phase, specific fluorescein staining was found with serum of the convalescent phase in both cytoplasm and nucleus. Four paired sera of patients of the acute or convalescent phase were used for antibody tests against the virus isolated from a patient and/or HHV-6 antigen, and antibody titers of all sera from acute phase were less than 1:10. Increased titers of antibody were detected in all sera from the convalescent phase against homologous antigen and HHV-6 (1:20 to 1:320) (Table 12.3). Cells were transferred to umbilical cord blood mononuclear (CBMN) cells at the ratio of 1:5, and cultured further for 1 week. Antigen-positive cells were always observed in cultured cells and the number of antigen-positive cells increased. Then examination of thin sections of lymphocytes infected with this virus by electron microscopy was performed (Fig. 12.2). Viral particles, which were sized at 90–110 nm in diameter with an average diameter of approximately 100 nm and morphologically similar to the herpes-group viruses, were found as nucleocapsids in the nuclei and as enveloped particles, approximately 170–200 nm in size, in cytoplasma and in the extracellular area (mainly extracellular) (Yoshida et al., 1989).

The isolation of HHV-6 from ES patients was reported by other investigators (Asano et al., 1989a; Portolani et al., 1990; Yoshiyama et al., 1990). The rate of virus isolation from ES patients during the febrile and exanthem phases were described by Asano et al. (1989a). The rate of virus isolation from mononuclear cells was 100% on days 0–2, and gradually decreased to 0% on day 8 and thereafter. We

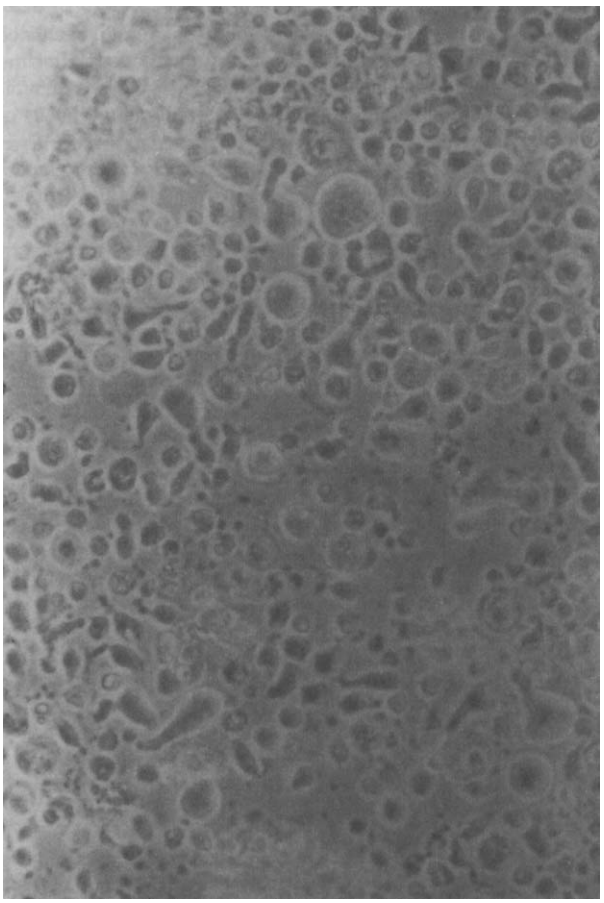

Fig. 12.1. Cytopathic effect of cells infected with HHV-6.

also attempted to compare the rate of virus isolation from ES patients during the febrile and exanthem phases in Thailand (Table 12.4). While the rate of virus isolation during the febrile phase was 71%, that during the exanthem phase was lower (46%). Atypical and inapparent infection of HHV-6 has also been reported by the evidence of the serological and virus isolation from patients (Asano et al., 1989b; Suga et al., 1989; Takahashi et al., 1989.).

The relationship of ES with HHV-6 infection by the serological study has been investigated mainly by researchers in Japan (Yamanishi et al., 1988; Ueda et al., 1989). Ueda et al. (1989) studied antibodies to Japanese HHV-6 in paired serum specimens from the acute and convalescent phases of infants with ES and the data strongly supported the correlation of ES and HHV-6 infection. We also reported (Kondo et al., 1990) that 26 serum samples obtained from ES patients during the acute and convalescent phases were assayed for IgG and IgM antibodies. IgM

TABLE 12.3.
Cultured cells were stained with convalescent serum by anticomplement immunofluorescence technique as described in Methods at day 9 after the primary cultivation and 1 week after the transfer to cord blood. Viral antigen was detected from cultures of patients No. 1–3 on day 9 after primary cell culture without cord blood cells, and viral antigen was detected in cultured cells of patient No. 4 after transfer to cord blood cells

Patient	Age (month)	Days after onset	Antibody titer against		Virus isolation
			Agent	HHV-6	
1. K.Y.	6	2	< 1:10	< 1:10	+
		18	1:320	1:320	
2. O.R.	6	3	< 1:10	< 1:10	+
		13	1:40	1:40	
3. M.N.	6	3	< 1:10	< 1:10	+
		14	1:40	1:40	
4. N.K.	6	3	< 1:10	< 1:10	+
		14	1:20	1:20	

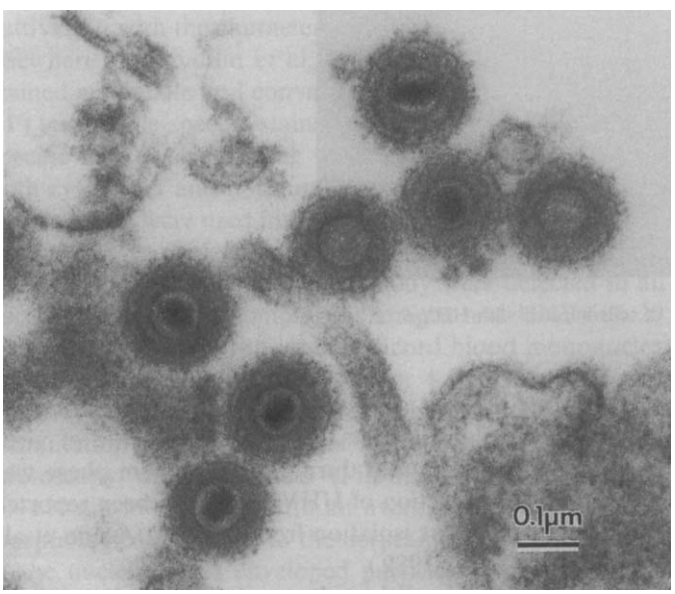

Fig. 12.2. Herpes-like virus particles in nucleus and extracellular area of infected lymphocytes.

antibodies were not detected in the sera until day 4 of onset of disease, but they were detected on day 7 and persisted for 3 weeks. On the other hand, IgG antibodies were first detected on day 7, increasing to more than 1:80 3 weeks after onset of disease and lasted at least until 2 months (Fig. 12.3).

Table 12.4.
Isolation of HHV-6 at different clinical stages of exanthem subitum patients

Clinical stages	Number of patients	Number of HHV-6 isolation	% (proportion)
Febrile phase	7	5	71.4% (5/7)
Exanthem phase	24	11	45.8% (11/24)
Total	31	16	51.6% (16/31)

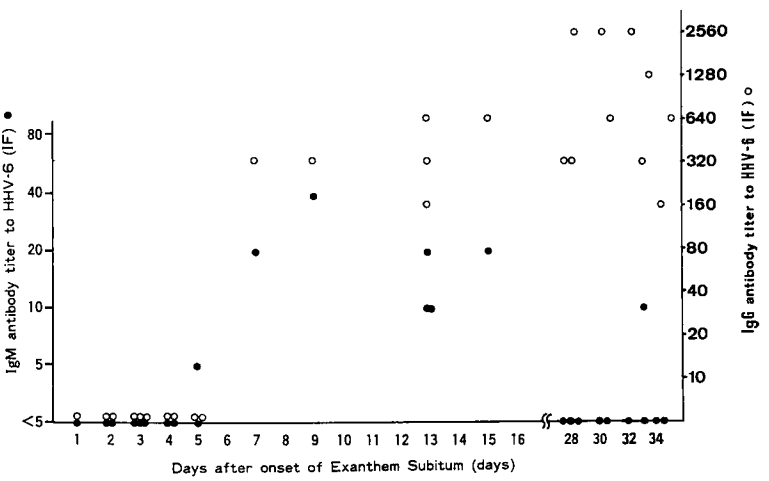

Fig. 12.3. IgG and IgM antibody response to HHV-6 during the acute and convalescent phases of exanthem subitum in patients. Sera were collected from patients with exanthem subitum and the antibody titers were estimated by IF test. ○ denotes IgG and ● IgM.

From these results, HHV-6 can be isolated from ES patients at high frequency, and ES seems to be the primary infection of HHV-6 in our lives.

12.4. Prevalence of antibody to HHV-6

Next, we attempted serological examination with sera from donors aged from under 10 to 59 years old (Okuno et al., 1989) in Japan. In total, 179 sera from donors 10–59 years old were examined, 141 sera showed positive staining, and the positive rate was 79%. The rate of antibody-positive serum in each generation did not differ much even in the age of less than 10 years. Then, we investigated sera from young children under 21 months old in detail. Antibody titers were measured of in total 156 sera. Some children aged from 0 to 5 months old had antibodies and the positive rate was only 13% (8/61). After 6 months of age, the number of children having antibodies gradually increased and the positive rate during the term from

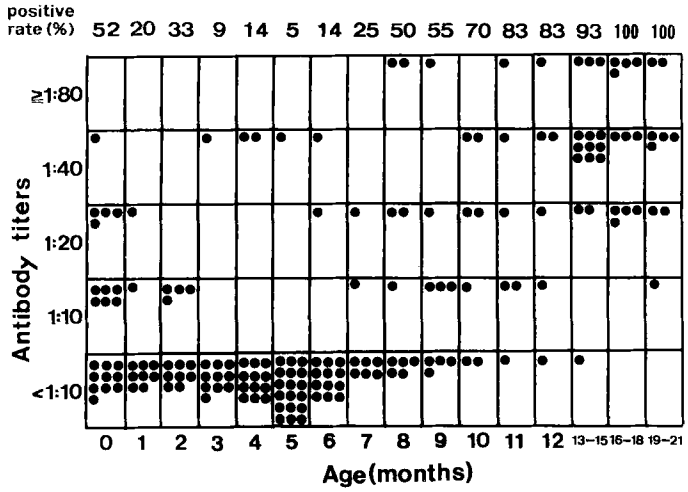

Fig. 12.4. Reciprocal titers of serum dilution expressed as antibody titers.

6 to 12 months was 48% (29/60) and almost all children older than 13 months had antibodies. Furthermore, while the antibody titer of positive sera between 6 and 12 months widely distributed from 1:10 to > 1:80, it was restricted to more than 1:20 after one year old (Fig. 12.4).

The results clearly suggest that almost all children will be exposed to HHV-6 in the latter half of the first year of life and will have antibodies against the virus. The antibody-positive rate decreases up to 6 months after birth, and then starts to increase and the antibodies may last throughout the life. Since almost all pregnant women have antibodies to HHV-6 (unpublished data) and IgG antibody is transferred from mother to child through the placenta, it is believed that children are protected against HHV-6 infection, because the antibodies are detectable during early months after birth in infants.

Similar results of antibody prevalence were also reported by other investigators in Japan and other countries (Brown et al., 1988; Balachandra et al., 1989; Yoshikawa et al., 1989; Enders et al., 1990; Farr et al., 1990).

12.5. Cellular tropism of HHV-6 *in vivo*

HHV-6 has a tropism for mainly $CD4^+$ lymphocytes (Downing et al., 1987; Tedder et al., 1987; Lusso et al., 1988), and also some established cell lines including HL 60 which is established from monocytic leukemia (Downing et al., 1987). We investigated the *in vivo* cellular tropism of HHV-6 in ES patients (Takahashi et al., 1989). Peripheral blood mononuclear cells (PBMCs) of ES patients in the febrile phase were separated and incubated in gelatin-coated dishes for 2 h in Dulbecco's modified minimum essential medium (DMEM) supplemented with 10% FCS.

Nonadherent cells were pipetted off gently several times with DMEM prewarmed at 37°C and adherent monocytes were removed by 5 mM EDTA in phosphate-buffered saline. These monocytes were simply co-cultured, and nonadherent cells were co-cultured in a serial 10-fold dilution with 1×10^6 of PHA-stimulated CBMCs in complete medium. Fourteen days after cultivation, HHV-6-infected cells were detected with convalescent serum of an ES patient by IF tests. HHV-6 was isolated from nonadherent cells but not from adherent monocytes. The frequency of HHV-6-infected cells was at least 10^{-3}. In order to further characterize the *in vivo* cellular tropism of HHV-6, the $CD4^+CD8^-$, $CD4^-CD8^+$ and $CD4^-CD8^-$ (including B cells), $CD3^+CD4^+$ and $CD3^-$ cell subpopulations were sorted out from PBMCs of 6 patients by a cell sorter (FACStar, Becton Dickinson) using fluorescein isothiocyanate (FITC)-conjugated CD4, phycoerythryn (PE)-conjugated CD8 and CD3 monoclonal antibodies. The viable sorted lymphocytes with serial 10-fold dilution were co-cultured with CBMCs and the viral antigens in HHV-6-infected cells were detected as described above. As shown in Table 12.5, HHV-6 was consistently isolated from $CD4^+CD8^-$ cells in 4 patients and from $CD3^+CD4^+$ cells in 2 patients but none was isolated from $CD4^-CD8^+$, $CD4^-CD8^-$, $CD3^-$ cells nor B cells. The frequency of HHV-6-infected $CD4^+CD8^-$ cells was at least 10^{-4} in 3 patients and 10^{-5} in one patient. These results indicate that HHV-6 predominantly infects $CD4^+CD8^-$ and $CD3^+CD4^+$ T lymphocytes with mature phenotypes *in vivo*.

TABLE 12.5.
Isolation of HHV-6 from lymphocyte subpopulations in patients with exanthem subitum

Patient	Subpopulation	Number of cells co-cultured with CBMC			
		10^5	10^4	10^3	10^2
1	$CD4^+CD8^-$	+[a]	+	–	–
	$CD8^+CD4^-$	–	–	–	–
	Others	–	–	–	–
2	$CD4^+CD8^-$	+	–	–	–
	$CD8^+CD4^-$	–	–	–	–
	Others	–	–	–	–
3	$CD4^+CD8^-$	+	+	–	–
	$CD8^-CD4^-$	–	–	–	–
	Others	not done			
4	$CD4^+CD8^-$	+	+	–	–
	$CD8^+CD4^-$	–	–	–	–
	Others	–	–	–	–
5	$CD4^+CD3^+$	+	–	–	–
	$CD3^-$	–	–	–	–
6	$CD4^+CD3^+$	+	+	–	–
	$CD3^-$	–	–	–	–

[a] + denotes positive isolation of HHV-6

Our result that HHV-6 is selectively tropic for $CD4^+$ T lymphocyte *in vivo* may have some implications on the involvement of HHV-6 in the pathogenesis of CD4 T cell disorders such as human immunodeficiency virus (HIV) and human T lymphotropic virus (HTLV) infection. HHV-6 infection or reactivation in the immunosuppressive state of these diseases may modify or exacerbate the disease.

We have also shown that HHV-6 DNA could be detected by polymerase chain reaction (PCR) in PBMCs which were collected during both the acute and convalescent phases of ES patients (Kondo et al., 1990). We further analyzed, in detail, the tropism of this virus by the PCR technique after separation of mononuclear cells into adherent and not adherent cells (Kondo et al., 1991). We then attempted to detect HHV-6 DNA in adherent (monocytes) and nonadherent cells from patients with ES using PCR. Blood samples from patients with ES during the acute and convalescent phases and from healthy adults were collected in heparinized tubes. Mononuclear cells were separated, resuspended ($0.5–2 \times 10^6$ cells/ml) in RPMI 1640 supplemented with 25% horse serum and cultured in plastic plates coated with gelatin. Cultures were incubated for 1 h at 37°C in an atmosphere of 5% CO_2. Then nonadherent cells were collected and added again to gelatin-coated dishes to get purer nonadherent cells. In order to obtain adherent cells, dishes were washed several times with RPMI 1640 prewarmed at 37°C, and adherent cells were incubated in RPMI 1640 supplemented with 25% horse serum. After 12 h incubation, dishes were washed several times with prewarmed RPMI 1640, and phosphate buffered saline (PBS), supplemented with 10 mM EDTA, was added to plates and incubated at room temperature for 20–30 min to detach adherent cells. Cells were then washed with RPMI 1640 3 times by centrifugation and resuspended in RPMI 1640. Cell preparations were tested for the nonspecific esterase-positive cells. By this method, more than 95% of the adherent cells were nonspecific esterase positive, and more than 90% of the nonadherent cells were nonspecific esterase negative. Blood samples were collected during the acute and convalescent phases from 5 patients diagnosed clinically and serologically as having ES. All samples during the acute phase were collected from patients during the febrile phase, and samples during the convalescent phase were obtained from patients after 1.5–2.5 months after the onset of the illness. First, sera collected during the acute and convalescent phases were tested for antibodies to HHV-6 by the IF technique. While antibodies were not detected ($< 1:10$) in sera obtained during the acute phase, the antibody titer became 1:640–1:2560 during the convalescent phase. HHV-6 DNA was detected in both adherent and nonadherent cells collected during the acute phase; predominantly from nonadherent cells in patients 1 and 2 (Fig. 12.5). On the other hand, HHV-6 DNA could be detected mainly in adherent cells collected during the convalescent phase (DNA was detected in only adherent cells in patients 1, 2 and 3), although the amount of DNA appeared to decrease during the convalescent phase. Next, the detection of HHV-6 DNA in cells collected from healthy adults aged 27–47 years was attempted. Thirty blood samples were collected from 20 healthy adults, some being bled 2–3 times at certain intervals. The HHV-6 antibody titer of 30 sera was between 1:160–1:320, which was within normal range. Then, HHV-6 DNA in both

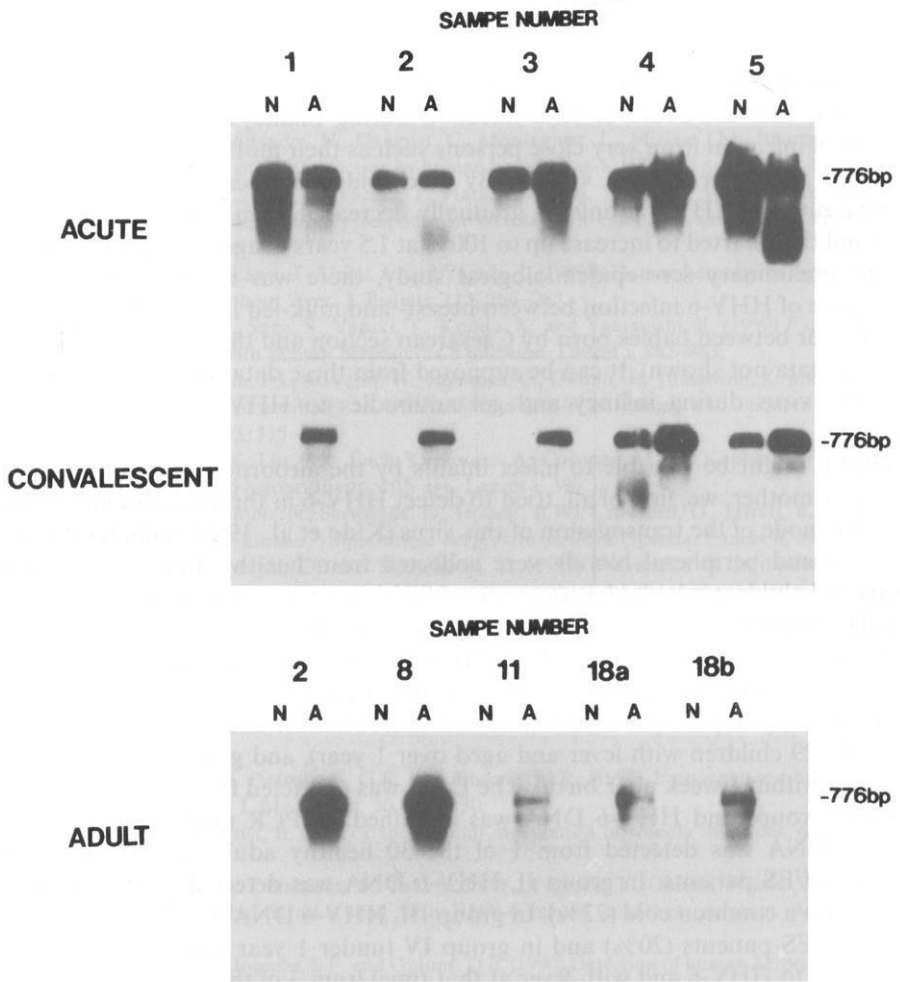

Fig. 12.5. The detection of HHV-6 DNA in peripheral blood mononuclear cells of patients with ES and healthy adults by PCR. N denotes nonadherent cells and A adherent cells.

fractions was examined by PCR. In total, 5 samples became positive; 2 (samples 18a, b) were derived from a person who was bled 3 times, the other 2 (samples 2 and 11) were from a person who was bled twice. Furthermore, DNA was only detected in samples of adherent cells. Since virus isolation was not possible from the peripheral blood of ES patients during the convalescent phase, HHV-6 might remain in a latent state in monocytes. These results suggest that HHV-6 may persist for a long time in monocytes/macrophages in a latent state, although it is not clear whether this cell is the main site of latency *in vivo*.

12.6. Transmission of HHV-6

It is believed that local spread or seasonal outbreak of ES is rare. It might be considered that the route of viral infection during infancy is transplacental or horizontal infection from very close persons such as their mothers or at delivery as described for other human viruses. By sero-epidemiological studies, antibody positive rates to HHV-6 in infants gradually decreased from 0 to 5 months after birth and then started to increase up to 100% at 1.5 years of age as described above. In our preliminary sero-epidemiological study, there was no difference in the prevalence of HHV-6 infection between breast- and milk-fed infants (Takahashi et al., 1988) or between babies born by Caesarean section and those by trans-vaginal delivery (data not shown). It can be supposed from these data that we are infected with this virus during infancy and get antibodies to HHV-6 by two years of age.

Since it might be possible to infect infants by the airborne route from a close sibling or mother, we, first of all, tried to detect HHV-6 in throat swabs and make clear the mode of the transmission of this virus (Kido et al., 1990). Samples (throat swabs or/and peripheral blood) were collected from healthy adults along with adults and children who had a common cold with fever at that time, and they were classified according to the following groups; group I (30 healthy adults aged 27–47 year old including 10 mothers of infants with ES), group II (9 adults having a common cold when samples were collected), group III (10 infants aged 5–9 months old with ES), group IV (10 children with fever at that time and aged under 1 year), group V (29 children with fever and aged over 1 year), and group VI (14 healthy neonates within 1 week after birth). The DNA was extracted from throat swabs of different groups and HHV-6 DNA was amplified by PCR method. In group I, HHV-6 DNA was detected from 1 of the 30 healthy adults (3.3%) including mothers of ES patients. In group II, HHV-6 DNA was detected from 2 of the 9 adults with a common cold (22%). In group III, HHV-6 DNA was detected from 2 of the 10 ES patients (20%) and in group IV (under 1 year old children having antibodies to HHV-6 and with fever at that time) from 3 of the 10 children (30%). On the other hand, the rate of detection of HHV-6 decreased to about 3% (1/29) in over 1 year old children having antibodies to HHV-6 and with fever at that time. However, no DNA was detected from the 14 neonates within 1 week after birth (group VI). A very similar result was reported that HHV-6 could be detected in saliva at the high frequency (Jarrett et al., 1990). Furthermore, two groups reported that HHV-6 was successfully isolated from saliva of normal people who had antibodies to HHV-6 (Pietroboni et al., 1988; Harnett et al., 1990; Levy et al., 1990), and viral antigens or DNA could be detected in salivary glands (Fox et al., 1990; Krueger et al., 1990). From these results, the airborne route may be suggested as a mode of transmission of HHV-6 infection.

In summary, HHV-6 causes exanthem subitum as the primary infection, and this virus may have a cellular tropism of $CD4^+$ lymphocyte. The transmission of this virus is likely airborne early in life mainly from mother to child.

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Ablashi/Krueger/Salahuddin (eds) Human Herpesvirus-6

CHAPTER 13

HHV-6-induced mononucleosis-like illnesses

CHARLES A. HORWITZ,[1] GERHARD R.F. KRUEGER,[2] THERESA A. STEEPER,[1] and GERHARD BERTRAM[3]

[1] *Department of Pathology, Abbott Northwestern Hospital and University of Minnesota Medical School, Minneapolis, MN 55407, U.S.A.,* [2] *Department of Pathology and Laboratory Medicine, University of Texas Medical Center, Medical Science Building, Houston, TX 77030, U.S.A., and* [3] *Ear, Nose and Throat Clinic, Municipal Hospital Dortmund, University Witten-Herdecke, W-4600 Dortmund, Germany*

13.1. Human herpesvirus-6 (HHV-6)

HHV-6 is a recently described enveloped herpesvirus with a diameter of 160–200 nm, an icosahedral nucleocapsid containing 162 capsomeres and a large, double-stranded DNA genome or nucleoid (Josephs et al., 1986; Salahuddin et al., 1986; Ablashi et al., 1988; Krueger and Sander, 1989). The DNA is coiled about a central cylindrical mass having symmetrical subunits reminiscent of cytomegalovirus (CMV) and other herpesviruses. HHV-6 was initially isolated from peripheral blood lymphocytes of 6 immunodeficient patients, including several who were human immunodeficiency virus (HIV) seropositive (Josephs et al., 1986; Salahuddin et al., 1986; Tedder et al., 1987). Subsequent studies characterized the *in vitro* viral–cellular interactions, ultrastructural morphology, and immunologic and molecular make-up of HHV-6 (Ablashi et al., 1988). The virus was initially called human B lymphotropic virus (HBLV), because it appeared selectively to infect B lymphocytes in tissue culture. Subsequently, however, a wider tropism was demonstrated, particularly for T lymphocytes but also for other cells, including megakaryocytes and glial tissue. Its main T cell tropism is directed against lymphocytes bearing a wide range of markers, including CD2, CD4, CD5, CD7, CD8, the transferrin receptor, leukocyte antigen HLe-1, and CD15 (Lusso et al., 1988). More recently, CD38, a marker of immature T cells, was identified from HHV-6-infected lymphocytes derived from a patient with an atypical polyclonal lymphoproliferative process (Krueger and Sander, 1989). Examination of different viral isolates from individual patients demonstrated minor molecular variances indicative of some degree of polymorphism that may explain differences in infectable target cells.

It has also been noted that HHV-6 exerts a pronounced cytopathic effect on host T cells and that co-infection of lymphocytes bearing the CD4 receptor by both HIV and HHV-6 results in enhanced cellular degeneration. This increased cell lysis may be of importance in the progression of AIDS (Lusso et al., 1989). It has also been suggested that prior EBV infection results in the induction of a receptor that is usable by both HHV-6 and HIV for B cell entry and that B lymphocytes not containing the EBV genome are not infectable *in vitro* by HHV-6 (Ablashi et al., 1988). Eventually, HHV-6 infected T cell lines such as HSB_2 that are free of other human herpesviruses were used to develop serologic tests first by IFA and later by ELISA, Western Blot and radioimmunoprecipitation procedures. Presently, well-standardized IFA or ELISA procedures for the detection of IgM and IgG anti-HHV-6 molecules are available for diagnostic use (Salahuddin et al., 1986; Ablashi et al., 1988; Saxinger et al., 1988).

HHV-6 infections are worldwide, and most HHV-6 seroconversions occur between the ages of 6 and 12 months after passively acquired maternal antibodies have waned (Brown et al., 1988). The majority of such childhood infections are silent; however, some are associated with a short-lived febrile illness and rash that are designated clinically as exanthem subitum or infantile roseola (Yamanishi et al., 1988). Antibody prevalence studies have shown that few adults are still susceptible to primary infection (Brown et al., 1988; Linde et al., 1988; Saxinger et al., 1988).

13.2. Infectious mononucleosis-like illnesses

Classically, infectious mononucleosis (IM) refers to an Epstein–Barr virus (EBV) induced illness in young adults associated with characteristic reactive blood smears, exudative tonsillar pharyngitis, prominent posterior cervical lymphadenopathy, and serologically detectable heterophil antibodies with IM-specific differential absorption traits (Lee et al., 1968; Horwitz and Steeper, 1992). Similar illnesses, so-called mononucleosis or IM-like but without heterophil antibodies, can also be caused by EBV and cytomegalovirus (CMV). Less common causes are drugs (including Azulfidine, Dapazone, Hydantoin, Halothane, and para-aminosalicylic acid), adenoviruses, rubella, *Toxoplasmosis gondii* and human immunodeficiency virus (HIV) (Penman, 1969; Evans, 1978; Steeper et al., 1987). Several recent articles on the various heterophil-antibody-negative (Het-Neg) IM-like illnesses have addressed diagnostic problems, including overlapping clinical and serologic features, inconstancy of morphologic and serologic criteria, and technical limitations (Penman, 1969; Klemola et al., 1970; Nikoskelainen et al., 1974; Horwitz et al., 1977; Evans, 1978; Henle et al., 1979; Schmidt, 1984). Practically, the Het-Neg IM-like syndromes must be separated from potentially more serious illnesses such as acute leukemia and malignant lymphoma. Diagnostic uncertainty in these patients has resulted in unnecessary lymph node biopsies, bone marrow aspirations, and other irrelevant tests. Because of the variability in clinical signs and symptoms such as the absence of significant lymphadenopathy in most cases of CMV-induced mononucleosis (CMV-Mono) as well as in some cases of EBV-

IM, the initial suspicion of an IM-like illness may only arise after careful appraisal of a peripheral blood smear for significant numbers of atypical lymphocytes (Horwitz et al., 1977; Henle et al., 1979). In fact, the heterogeneous IM-like illnesses are only held together by similar morphologic features, namely the presence of a significant atypical lymphocytosis that resembles that typically found in heterophil-positive (Het-Pos) EBV-IM.

13.3. HHV-6-induced IM-like illnesses

Because cell cultures of HHV-6-infected and phytohemagglutinin (PHA)-stimulated mononuclear cells showed blast-like cytologic features that were thought to be different from PHA-induced cells, it could be predicted that lymphoproliferative disorders of unspecified type would turn out to be manifestations of active HHV-6 infections (Ablashi et al., 1988; Krueger et al., 1989). Subsequently, Niederman and associates reported data from 3 adults with mild afebrile illnesses associated with persistent cervical lymphadenopathy and high anti-HHV-6 IgG titers (Niederman et al., 1988). Atypical lymphocytes were present in all 3 cases and EBV and CMV were excluded serologically. Other reports confirming the association of HHV-6 with IM-like lymphoproliferative processes have also appeared (Bertram et al., 1989; Irving and Cunningham, 1990; Steeper et al., 1990). Below is a summary of clinical, hematologic, biochemical and serologic data from patients with Het-Neg IM-like illnesses thought to be due to HHV-6. Because dual infections are commonly encountered with herpesvirus infections, unless otherwise designated, 'heterophil-negative, HHV-6-induced IM-like illnesses' refers to only cases where EBV and CMV infections were excluded by appropriate serologic studies (Bertram et al., 1989; Irving and Cunningham, 1990; Linde et al., 1990; Krueger et al., 1991).

13.4. Classification of cases

Mononucleosis-like illnesses due to HHV-6 can be grouped into 4 different categories: (1) patients with tonsillar pharyngitis and cervical lymphadenopathy; (2) patients with short-lived nonspecific viral-type illnesses; (3) patients with viral-type illnesses and very abnormal hepatic function thought to have active viral hepatitis (AVH); and (4) patients with occasional febrile illnesses superimposed on underlying or evolving immune deficiency states. Exanthematous rashes have been noted in some adult patients with active HHV-6 infections, apparently irrespective of group classification (Irving and Cunningham, 1990).

13.5. Clinical data

Group 1 patients presented prominent tonsillar pharyngitis, with or without associated exudative or membranous tonsillitis, and varying degrees of cervical

lymphadenopathy. The cervical nodes range from several millimeters up to 2.0 cm in size and the posterior cervical nodes are often involved, similar to cases of EBV-IM. Occasionally, lymphadenopathy alone is the sole reason for presentation to a clinician. Whereas lymphadenopathy in EBV-IM usually resolves by 4–6 weeks, about 10–15% of HHV-6 patients have persistent or recurrent lymphadenopathy over the next 6–12 months of follow-up. Surgical excision has sometimes been considered in the persistent cases and, in fact, eventually performed in some patients.

Group 2 and 3 cases had illnesses dominated by fevers ranging from 2 to 14 days in duration that were accompanied by varying combinations of myalgias, headaches and abdominal pain. Lymphadenopathy, when present, was unimpressive and confined to anterior cervical lymph nodes. The division of a particular case into group 2 or 3 was dependent on the severity of hepatic dysfunction, in particular whether the transaminase (AST) levels were low or at moderate range (as typically seen in EBV-IM or CMV-Mono) (group 2) or high levels (>900 U/L) (group 3), usually implying significant hepatic necrosis suggestive of AVH. Finally, group 4 patients have underlying or evolving immunodeficiency states and develop super-imposed febrile illnesses and characteristic IM-type blood smears. In one such serially evaluated case, an HIV-positive previously asymptomatic patient presented with persistent fever (>30 days), peripheral and retroperitoneal lymphadenopathy, hepatosplenomegaly, and blurred vision. Diagnostic studies (toxoplasmosis, EBV, etc.) were negative or nondiagnostic, and HHV-6 studies, done in retrospect, were indicative of an active HHV-6 infection (Steeper et al., 1990).

13.6. Hematologic findings

During the first week of illness, similar to other viral processes, a slight leukopenia may be present, while in the second or third week, peak total leukocyte counts are noted that range from within normal limits to 23 200 per mm^3. A relative lymphocytosis is regularly encountered and, in an unspecified number of cases, an absolute lymphocytosis is noted. The T helper/T suppressor cell ratio usually inverts with average values of 0.75 during the acute phase of illness; levels slightly below 1.0 may persist until 6–8 weeks after resolution of symptoms. During this time, CD4 cells are moderately decreased in number (Krueger and Sander, 1989).

As implied in our definition of an IM-like illness, peripheral blood smears fulfill the classical morphologic criteria for IM and include 50% mononuclear cells, at least 10 atypical lymphocytes per 100 WBCs, and marked lymphocytic polymorphism or heterogeneity. Cells identified as atypical lymphocytes have been well described and illustrated by various hematopathologists (Downey and McKinley, 1923; McKenna, 1979). A small percentage of blood smears from HHV-6 cases, in addition to the usual atypical lymphocytes noted above, show more plasmacytic cells than one normally encounters in EBV-IM. When smears are specifically analyzed for the presence of immature plasmacytic forms (so-called plasmablasts

and proplasmacytes), plasmacytic lymphocytes, and mature plasma cells and then compared to EBV-IM and IM-like illnesses due to CMV and HIV, the plasmacytic features appear more pronounced in the HHV-6 cases (Table 13.1). Thus, prominent plasmacytic features (i.e., $\geqslant 5$ immature plasma cells per 100 WBCs) were noted in 3 of the 8 (37%) HHV-6 cases, 1 of the 10 (10%) cases with CMV-mono, and 1 of the 3 (33%) cases with acute HIV seroconversions. Such prominent plasmacytic features were not identified in smears from EBV-IM or healthy controls.

Characteristics favoring plasmacytic (versus lymphocytic) differentiation of reactive mononuclear cells include conspicuous nucleoli, prominent clumping of nuclear chromatin with intervening clear spaces (versus smudged chromatin), round or oval smooth (rather than indented) eccentrically placed nuclei, prominent perinuclear clear zones, and very dense opaque basophilic cytoplasm. Mature plasma cells were found in only one case of the entire study group (Table 13.1, HHV-6 group, and Fig. 13.1). Cells designated by us as plasmablasts and proplasmacytes are well illustrated in several reports (Wolf, 1954; Spriggs and Jerrome, 1967) and in Sandoz's *Atlas of Haematology* (Undritz, 1973). These cells have also been called 'Turk' cells in the older literature; this latter term has fallen into disuse. Subsequent review of conflicting literature on the nature of cells designated as plasmablasts attests to the sparsity of solid data on their origin. They are morphologically similar to 'immunoblasts' encountered in blood smears after sensitization of patients with foreign stimuli (Crowther et al., 1969). Despite the

TABLE 13.1.
Analysis of reactive mononuclear cells, including plasmacytic variants in acute HHV-6, CMV, HIV and EBV-induced illnesses (HHV-6: human herpesvirus-6; HIV: human immunodeficiency virus-1; CMV: cytomegalovirus; Mono: mononucleosis; EBV: Epstein–Barr virus; Het-Pos: heterophil-positive)

	HHV-6 (8 cases)	CMV-Mono (10 cases)	HIV sero-conversions (3 cases)	EBV, Het-Pos (10 cases)	Controls, asymptomic (10 cases)
Atypical lymphocytes (%),[a] Downey-type cells	18.1 (10–31)	30.6 (11–52)	25.3 (20–31)	42.2 (25–80)	1.4 (0–3)
Reactive lymphocytes, plasmacytic features (%)[a]	4.3 (2–7)	2.7 (0–8)	4.3 (3–6)	1.5 (0–5)	1.3 (0–6)
Immature plasmacytic cells (PB + PP)[a,b]	5.0 (1–17)	1.9 (0–7)	4.3 (3–7)	0 (0–1)	0 (0)
# cases with $\geq$ 5%PB + PP per 100 cells (% total cases)	3[c] (37.5)	1 (10.0)	1 (33.3)	0 (0)	0 (0)

[a] Refers to mean percentage of reactive cells per 100 WBCs, numbers in parentheses to range.
[b] Plasmablasts (PB) and proplasmacytes (PP), while part of the spectrum of reactive mononuclear cells are separated from reactive lymphocytes for analysis (see text).
[c] Mature plasma cells were identified in only 1 of 8 HHV cases (see Fig. 13.1) and in none of the smears from CMV, HIV, EBV or control cases.

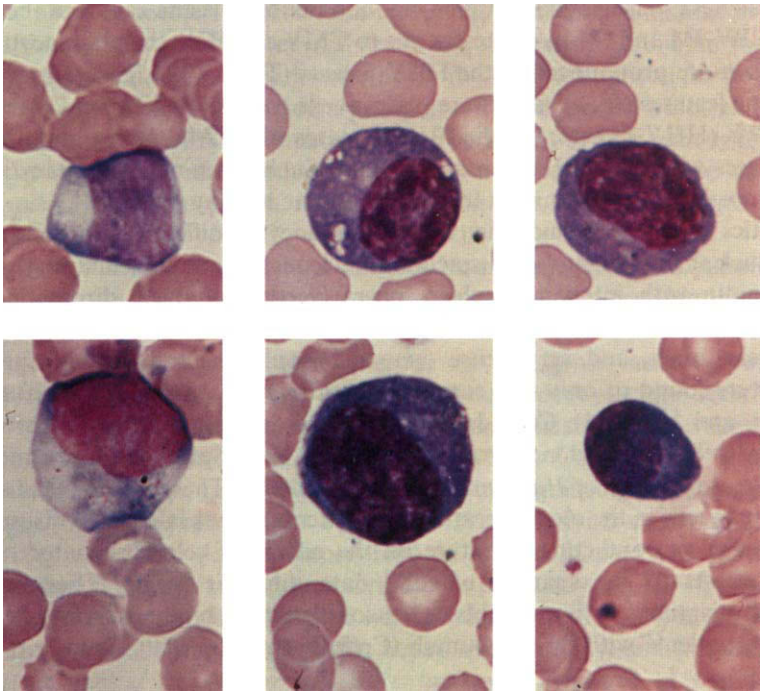
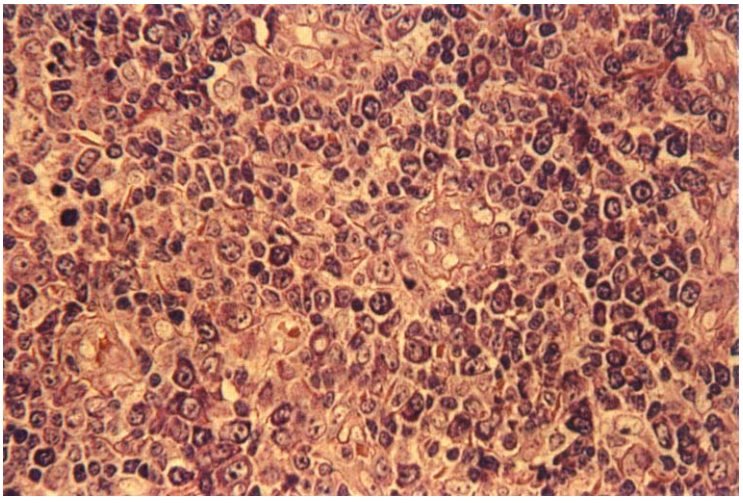


Fig. 13.1. Composite slide from case # 6, a 27 year-old female with an active HHV-6 infection showing both atypical Downey-type lymphocytes (left one-third of composite) and cells of plasmacytic type. Immature plasmacytic cells are noted in the central and upper right frames and a mature plasma cell in the lower right frame. The Downey-type cells have smudged nuclear chromatin and are indented by surrounding erythrocytes, whereas the plasmacytic cells have more distinct nuclear chromatin clumping, rounder, smoother and more eccentrically placed nuclei, and more basophilic cytoplasm (Wright–Giemsa stain, magnification 512×). (For printing purposes figure has been reduced.)



uncertain origin of these cells, they, along with proplasmacytes and other lymphocytes with plasmacytoid features, are also often found transiently in significant numbers in patients with exanthematous infections such as rubella, and in some cases of AVH due to the hepatitis A or B virus. In summary, patients with HHV-6-induced illnesses have blood smears fulfilling IM morphologic criteria, and a significant percentage of such smears also show reactive plasmacytic features of the type typically associated with viral exanthematous illnesses of childhood and AVH.

13.7. Histologic findings

Although HHV-6 and EBV infect different lymphocytic populations, the histopathologic changes in lymph nodes and tonsils during the acute phase of either EBV-IM or an HHV-6-induced IM-like illnesses are similar. In brief, the nodal architecture is distorted by a prominent paracortical (interfollicular) nodal expansion that is composed of polymorphous populations of transformed large lymphocytes and immunoblasts. The former cells show a wide range of nuclear configurations and have small peripherally located nucleoli and pale amphophilic cytoplasm. The cells with immunoblastic features have large vesicular nuclei, prominent central nucleoli, and intense abundant basophilic or amphophilic cytoplasm. Moderate numbers of admixed plasma cells are seen in about one-third of the cases, and paracortical clusters of histiocytes and Reed–Sternberg-like cells are also seen in some cases. Figure 13.2 illustrates a characteristic paracortical expansion by large lymphoplasmacytic and immunoblastic cells in a tonsil removed from a patient with an acute HHV-6-induced illness.

13.8. Liver function

Some degree of hepatic dysfunction is encountered in most but not all cases of HHV-induced IM-like illnesses. Low or moderate peak aspartate aminotransferase (AST) activity (usually < 500 U/L) and bilirubin values (< 2.5 mg/dl) are encountered in group 1, 2 and 4 cases, while higher AST levels (> 930 U/L) and bilirubin values that vary from 2.5 to 12.0 mg/dl are seen in group 3 cases. Patients with viral-type illnesses and very high AST levels (generally > 1000 U/L) are often clinically thought to have AVH, and serologic studies for the various hepatitis-specific viruses (hepatitis A, B, C, etc.) are usually performed. Liver biopsies have not yet been reported in group 3 HHV-6 patients, so it is unknown whether the dominant pathologic pattern is significant parenchymal necrosis, as is typical for AVH, or sinusoidal/portal lymphocytic inflammation characteristic of active EBV

Fig. 13.2. Lymphoplasmacytic and immunoblastic hyperplasia of paracortical area derived from enlarged tonsil removed from a patient with an acute HHV-6-induced IM-like illness (Giemsa, magnification 375×). (For printing purposes figure has been reduced.)

and CMV infections. Moderately elevated lactate dehydrogenase (LDH), alkaline phosphatase (AP), and gamma-glutamyl transpeptidase (GGT) activity are also commonly encountered in HHV-6 cases (Krueger et al., 1991). Also, very high LDH values (>1000 IU/ml) have been described in some group 3 cases in the absence of overt clinical hemolysis (Steeper et al., 1990).

13.9. Additional standard serologic data

Antinuclear antibody studies (ANA) are usually negative. In a single HHV-6 case where moderate ANA titers were noted, they persisted at low to moderate levels for 15 months of clinical follow-up and were not associated with evidence of multisystem disease. Studies of rheumatoid factors, anti-i cold agglutinins and serologic tests for syphilis are negative and IM-type heterophil antibodies are not detectable except in an occasional case where they may be indicative of residual agglutinins from an earlier EBV infection and incidental to the current HHV-6-induced illness.

TABLE 13.2.
Routine laboratory data in acute HHV-6-IM-like illnesses compared to EBV- and CMV-induced cases (AL: atypical lymphocytes; E Sed R: erythrocyte sedimentation rate; ND: no data; AST: aspartate aminotransferase; GGTP: gamma-gutamyl transpeptidase; LDH: lactate dehydrogenase; CA: cold agglutinins; HHV-6: human herpesvirus-6; EBV: Epstein–Barr virus; CMV: cytomegalovirus; CMV-Mono: CMV mononucleosis; IM: infectious mononucleosis)

	23 HHV-6 IM-like illnesses[a]	125 EBV-IM	82 CMV-Mono
≥ 10 ALs per 100 WBCs	100% (23/23)	100%[a]	100%[a]
Leukocytosis (> 10000 per mm^3)	63% (12/19)	75%[b]	5%[a]
Platelet count (< 100000 per mm^3)	6.7% (1/15)	15%[b]	13%[a]
Elevated E Sed R (mm/h)	100% (11/11)	61%[b]	65%[a]
Presence IM-type heterophil test	4.7% (1/23)	95%[a]	0%[a]
Elevated AST	77% (13/17)	97%[a]	88%[a]
peak levels < 500 U/L	(10/13)	90%[a]	85%[a]
peak levels ≥ 930 U/L	(3/13)	< 1%[a]	< 1%[a]
Elevated bilirubin (≥ 2.0 mg%)	18% (3/17)	17%[a]	3%[a]
Elevated GGTP	75% (12/16)	91%[a]	76%[a]
Elevated LDH (≥ 240 U/L)	80% (12/15)	97%[a]	88%[a]
peak LDH > 1000 U/L	(2/15)	1%[a]	2%[a]
significant hemolysis	(0/15)	1%[a]	2%[a]
Antinuclear antibodies	10% (1/10)	15%[b]	5%[a]
Rheumatoid factors	0% (0/10)	10%[b]	10%[b]
Serologic tests for syphilis	0% (0/10)	10%[b]	10%[b]
Anti-i CAs	0% (0/10)	30%[a]	< 1%[a]

[a] Only serially studied Minneapolis or Cologne–Dortumund cases used in calculations.
[b] Estimate based on medical literature.

Table 13.2 summarizes routine laboratory data from 23 patients with HHV-6 IM-like illnesses and provides a comparison to similar data from EBV-IM and CMV-Mono.

13.10. Viral serology

IgM antibodies to HHV-6 are usually detectable during the acute phase of illness by indirect immunofluorescence (IFA) (Saxinger et al., 1988; Krueger et al., 1989; Steeper et al., 1990). Peak titers noted during the first 2 weeks of illness vary from 1:10 to 1:160 (IFA) with mean levels of about 1:40. There is a gradual decline in IgM response that is apparent by 60 days and usually negative ($<1:10$) by 9–12 months. Enzyme-linked immunoassay (ELISA) methods can also be used to detect IgM responses; however, lower sensitivity ($\pm 75\%$) is encountered over IFA-IgM-positive sera. Brisk IgG anti-HHV-6 responses, including 4-fold titer rises, are seen in the vast majority of HHV-6 cases with mean peak convalescent titers often to levels of $\geq 1:320$. Declines to IFA titer levels of 1:20–1:80 (levels typically associated with latency) generally occur by 18 months after onset. With ELISA IgG methods, significant changes in absorbance (i.e., relative value [RV] changes of ≥ 1.00 on serial samples) are only noted in three-quarters of cases. Thus, in some patients with 4-fold titer change by IFA, either negative IgG ELISA absorbance values (<1.00) or positive constant levels are noted on serial testing. The reasons for discrepancies between IFA and ELISA are unknown. Overall, the IgG titer

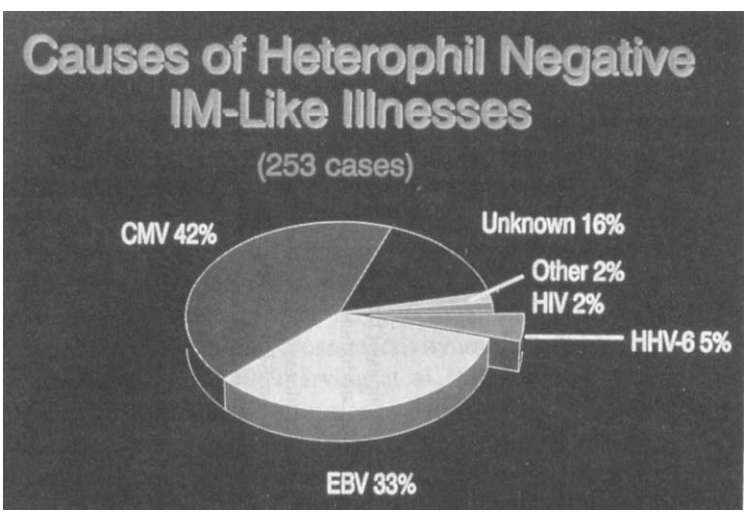


Fig. 13.3. Pie chart showing the various causes and relative incidence of 253 heterophil-negative IM-like illnesses. HHV-6 makes up 5% of the cases.

TABLE 13.3.
Representative clinical and laboratory data from 5 patients with active HHV-6 infections (LN: lymphadenopathy; ST: sore throat; NEM: no evidence of malignancy; CMV: cytomegalovirus; EBV: Epstein–Barr virus; HHV-6: human herpesvirus; TOXO: toxoplasmosis; IFA: indirect immunofluorescence; ELISA: enzyme-linked immunospecific assay; AST: aspartate aminotransferase; LDH: lactate dehydrogenase; Bili: bilirubin; WNLs: within normal limits; nd: not done)

Case	Clinical case data	Date of Test	TOXO IgG	EBV IgM	CMV IgM	IFA-HHV-6		ELISA HHV-6	
						IgG	IgM	IgG	IgM
#4	3M: prominent cervical LN; few symptoms, slow resolution (×6 months)	11/19/84	<20	<10	<16	80	10	2.52 (+)	0.70 (−)
		12/3/84	<20	<10	<16	160	20	1.57 (+)	1.10 (+)
#13	20M: severe non-exudative tonsillitis, fever, headache; cervical LN, splenomegaly. Duration of illness: 7 days. Peak AST: 2× normal	1/2/86	<8	<10	<16	2580	80	nd	nd
		2/1/86	<8	<10	<16	320	160	nd	nd
		6/1/86	<8	<10	<16	640	160	nd	nd
		10/1/86	<8	<10	<16	640	320	nd	nd
		1/1/87	<8	<10	<16	160	<5	nd	nd
		6/10/87	<8	<10	<16	40	<5	nd	nd
#3	14F: febrile illness (10–14 d), myalgias, headache, abdominal pain; AST, Bili-WNLs	4/22/76	<8	<10	<16	20	10	4.02 (+)	3.20 (+)
		6/19/76	<8	<10	<16	160	20	2.55 (+)	2.14 (+)
#6	27F: febrile illness (×3–4 days), headache, myalgias, no ST, minimal cervical LN, plasmacytic blood smears. Peak AST: 930, Bili 4.5	4/19/75	<8	<10	<16	20	10	0.50 (−)	2.6 (+)
		5/15/75	<8	<10	<16	160	10	1.46 (+)	9.8 (+)
#8	33M: febrile illness (×30 d) in previously asymptomatic HIV-positive patient. Enlarged retroperitoneal nodes; hepatosplenomegaly. Peak AST: 247, Bili 0.8	4/7/87	<20	<10	<16	20	20–40	0.41 (−)	1.6 (+)
		5/15/87	<20	<10	<16	160	20–40	1.33 (+)	0.6 (−)
		2/1/89	nd	nd	<16	10	<10	0.42 (−)	0.42 (−)
		4/26/89	nd	nd	<16	40	<10	0.26 (−)	0.16 (−)

changes by IFA and positive IgM data on one or more samples by either IFA or ELISA supports an interpretation of an active HHV-6 infection. Approximately 5% of heterophil-negative IM-like illnesses are due to isolated active HHV-6 infections (see Fig. 13.3). Representative data from 5 patients undergoing active HHV-6 infections, including examples from each clinical category (1–4) noted earlier, is presented in Table 13.3. The details from 4 of these cases were included as part of an earlier publication and are used with permission of the editors (Steeper et al., 1990).

13.11. Specificity of IgM anti-HHV-6 responses

A variety of interfering substances, including rheumatoid factors, antinuclear antibodies, heterotopic cross-reactions, and unusual persistence of IgM antibodies, can result in serodiagnostic problems (Schmidt, 1984). The presence of IgM responses implies an active viral process but does not always imply an ongoing primary infection. This is well-known for CMV and rubella and also appears to be true for HHV-6; i.e., the HHV-6 IgM antibodies may be only a reflection of herpesvirus carrier-state reactivation (Hanshaw et al., 1972; Horwitz et al., 1977; Schmidt, 1984). Interference by rheumatoid factor and antinuclear antibodies can be best avoided by separating serum IgM from IgG and IgA prior to specific IgM testing. A variety of separation methods have been described, including density gradient columns, pre-absorption of test serum with staph protein A, and pre-treatment of serum with anti-IgG. An IgM-specific test performed on the IgG-absorbed and separated supernate significantly decreases the likelihood of both false-positive and false-negative test results. These principles as applied to HHV-6 have clearly resulted in increased specificity (Saxinger et al., 1988; Irving and Cunningham, 1990) and make IgM/IgG separation prior to specific IgM testing a necessity.

13.12. Specificity of IgG anti-HHV-6 responses

Initially, there was concern about serological cross-reactions by IFA between CMV and HHV-6 (Huemer et al., 1989). However, subsequent serum absorption studies with either CMV or HHV-6 that removed only homologous IgG titers represented data making cross-reactivity unlikely (Ablashi et al., 1988; Lopez et al., 1988; Okuno et al., 1989; Irving et al., 1990; Linde et al., 1990). Also, the poor correlations of titer and prevalence data between HHV-6 IgG and other herpesvirus IgG levels in sera from routine blood donors and renal transplant recipients did not support the thesis of cross-reactivity (Chou and Scott, 1990; Fox et al., 1990). These studies and others further suggested that some of the HHV-6 responses resulted from reactivation of the HHV-6 carrier state by viruses such as EBV and CMV (Ablashi et al., 1988; Irving et al., 1990).

13.13. Dual infections

More recently, it has been shown that many patients with acute EBV or CMV infections in fact have simultaneous dual infections with HHV-6 (Irving et al., 1990; Krueger et al., 1991) and caution is urged in interpreting HHV-6 data without knowledge of CMV and EBV data. In a recent study, the effect of primary EBV infections on HHV-6, CMV and measles IgG immunoglobulin titers was evaluated (Linde et al., 1990). Based on 2–3 year follow-up, approximately 75% of IgG anti-CMV, anti-HHV-6, and anti-measles levels showed significant titer changes. On the other hand, patients with CMV-Mono showed significant titer changes in only HHV-6 IgG levels (i.e., not with anti-EBV IgG or anti-measles IgG). The authors concluded that elevated HHV-6, CMV and measles IgG titers in EBV-IM were probably due to polyclonal activation of B cells by either lymphokines or EBV itself, while in CMV-Mono the elevated HHV-6 titers were most likely related to HHV-6 carrier-state activation by CMV. In another study, dual herpesvirus infections were studied in patients with clinical IM syndromes (Krueger et al., 1991). In documented primary EBV infections, serologic data suggestive of active HHV-6 infection was encountered in up to 40% of cases and in patients with active CMV infections in about 15% of cases. Only 5% of the aforementioned study group had non-EBV, non-CMV illnesses due to active HHV-6 infections. Interestingly, hepatic dysfunction has been noted to be more significant in patients with dual EBV/HHV-6 infections than in those patients with either infection alone. This study also identified biphasic transaminase (SGOT, SGPT) peaks during the acute-illness phase in a small percentage of patients with dual EBV/HHV-6 infections, as has been noted for HBV with delta-virus superinfection. In summary, since HHV-6 can be reactivated by other herpesviruses or be part of dual viral infections, it is important to rule out EBV and CMV hyperactivity before accepting HHV-6 as the sole cause of a particular heterophil-negative IM-like illnesses.

13.14. Diagnostic approach

Since HHV-6 is capable of producing a heterophil-negative IM-like illness with reactive blood smears, differential possibilities in such cases are further expanded. Thus, our algorithmic approach has been changed to now include HHV-6 (see Fig. 13.4, revised with permission of the editors, Steeper et al. (1987)). Practically, HHV-6 testing is indicated when EBV studies are negative or nondiagnostic in patients with lymphadenopathy and in primarily febrile patients with reactive blood smears and negative CMV studies. It should also be specifically considered in patients with AVH when standard serologic markers for the various hepatitis-specific viruses (A, B and C) are negative. Whereas the majority of patients with AVH have some reactive blood smear features, no more than 4–6% fulfill IM criteria. It is in the latter subgroup where HHV-6 studies will most likely suggest active infection.

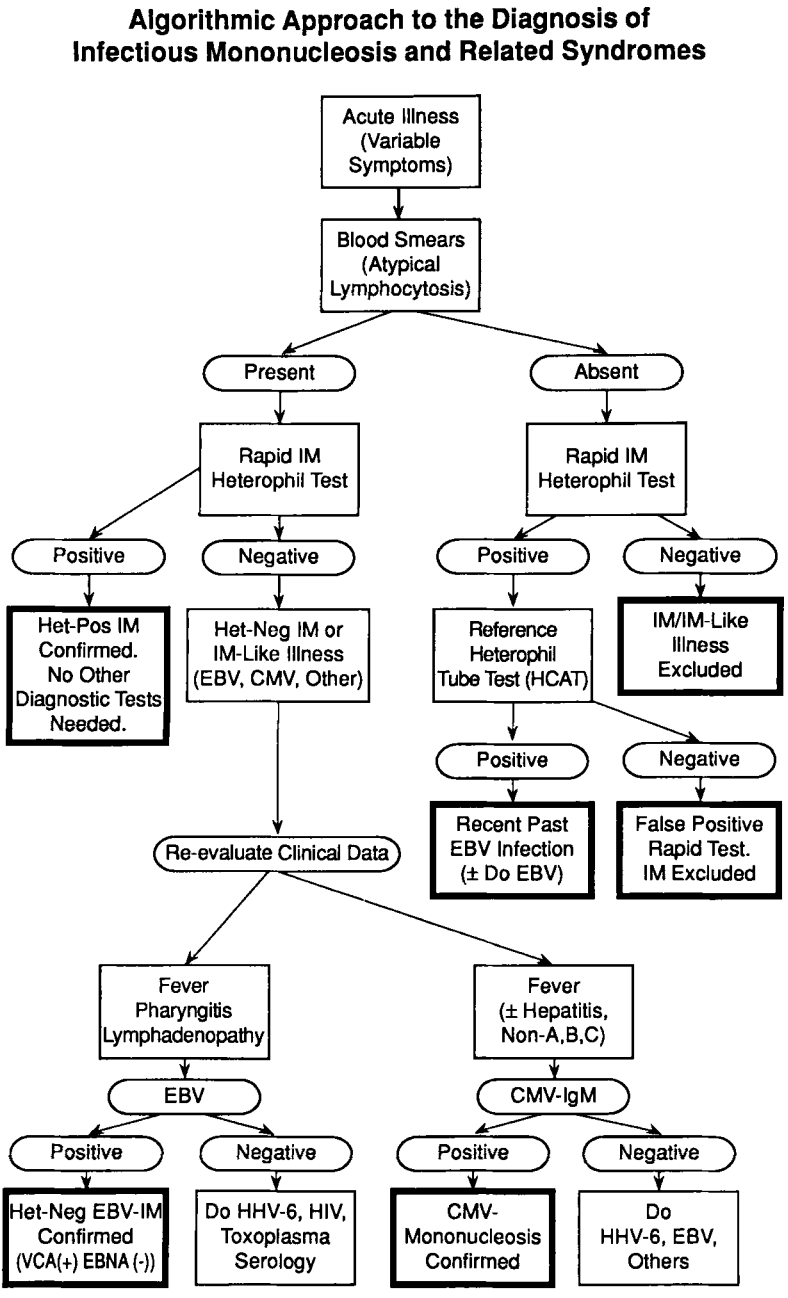


Fig. 13.4. Algorithmic approach to the diagnosis of IM and IM-like heterophil illnesses, including human herpesvirus-6 (HHV-6), by initially using blood smear and rapid heterophil test data.

13.15. Unanswered questions

Overall, questions remain about the natural history of HHV-6 infections. These include the frequency and conditions under which viral reactivation occurs, what laboratory methods can be used to separate primary from reactivated HHV-6 infections, and the frequency with which active HHV-6 infections occur in acute illnesses where blood smears do not fulfill IM criteria and do not appear reactive. Additional future studies may be indicated to determine the frequency of active HHV-6 infections in renal and liver transplant patients, the viral hemophagocytic syndrome, and in cases of the post-viral chronic fatigue syndrome (Komaroff, 1988; Ward et al., 1989; Okuno et al., 1990; Huang et al., 1990), none of which typically show IM-type blood smears. An additional intriguing question deals with the long-term clinical consequences of active HHV-6 infection, particularly what percentage of patients develops persistent atypical polyclonal lymphoproliferative processes or malignant lymphoma (Krueger et al., 1989).

Acknowledgment

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Ablashi/Krueger/Salahuddin (eds) Human Herpesvirus-6

CHAPTER 14

Kikuchi's disease (histiocytic necrotizing lymphadenitis)

MASAHIRO KIKUCHI[1], YOSHIAKI SUMIYOSHI[1] and YOUICHI MINAMISHIMA[2]

[1] *First Department of Pathology, Fukuoka University, School of Medicine, Fukuoka 814-01, Japan, and* [2] Department of Microbiology, Miyazaki Medical College, Miyazaki, Japan

14.1. Introduction

Kikuchi's disease was first reported in 1972 as a lymphadenitis with focal proliferation of reticular cells accompanying numerous nuclear debris (Kikuchi, 1972). Since then, many cases have been reported in Japan (Fujimoto et al., 1972; Kikuchi et al., 1977, 1986, 1990; Kikuchi, 1978; Fujimori et al., 1981). Recently, some reports of this lesion appeared in Europe, the United States, and Asia (Pileri et al., 1982; Turner et al., 1983; Ali and Horton, 1985; Chan and Saw, 1986; Chamulak et al., 1990). Histologically, the lesion shows focal proliferation of transformed lymphocytes, mainly T cell lymphocytes, with numerous histiocytes and nuclear debris, mainly in the paracortex (Kikuchi et al., 1977, 1986, 1990). Electron microscopy demonstrates a high incidence of tubuloreticular structures in lymphocytes, histiocytes, and vascular endothelial cells in the affected areas (Imamura et al., 1982; Eimoto et al., 1983). Because of the clinical features and histological findings as well as the patterns of elevated serum levels of 2′5′-oligoadenylate synthetase in the early stage of disease, a viral nature of the lesion is highly suspected (Kikuchi et al., 1990).

Human herpesvirus-6 (HHV-6) has been isolated from patients with lymphoproliferative disorders, patients with acquired immunodeficiency syndrome (AIDS), and normal adults (Krueger and Sander, 1989). Recently, Yamanishi et al. (1988) discovered that HHV-6 is a causal agent of exanthem subitum. Tropism analysis using cord blood lymphocytes has suggested that HHV-6 causes a predominant CD4, mature T cell lymphocyte tropism (Takahashi et al., 1989). Kikuchi's disease demonstrates aggregates of numerous CD4-positive cells in the affected areas (Turner et al., 1983; Kikuchi et al., 1986), and some authors have

suggested a relation between HHV-6 infection and this disease (Eizuru et al., 1989; Kurata et al., 1989). In this chapter, we analyze relation of this disease to HHV-6.

14.2. Materials and methods

Nineteen patients with Kikuchi's lymphadenitis were selected from the files in the Department of Pathology, Fukuoka University. Lymph node specimens were removed and fixed in formalin or B-5 solution, embedded in paraffin, and stained by hematoxylin-eosin, Giemsa, periodic acid-Schiff (PAS), silver impregnation for reticulin fibers, or immunohistochemistry. Parts of the specimens were fresh-frozen in liquid nitrogen and stored at $-80°C$ until sectioning. Immunohistochemistry was performed on paraffin-embedded and frozen sections with the use of the alkaline phosphatase-conjugated avidin–biotin complex method. The antibodies applied on the paraffin-embedded sections were L26 for B cells, UCHL-1 for T cells, and lysozyme for histiocytes, and on the frozen sections, CD3, CD4, CD8, and CD15. Against HHV-6, the samples were stained with a mouse monoclonal antibody, OHV-1, against cytoplasmic antigen of HHV-6-infected cells. Cord lymphocytes infected with HHV-6 and uninfected human embryonic lung cells were used as a positive control and a negative control, respectively, and antibodies for HHV-6, supplied by Drs. Yamanishi and Kurata (Osaka University) using imprints obtained at the biopsies, were also stained with these antibodies using the same method. Clinical data were obtained from the clinician. In addition to patients with Kikuchi's disease, groups with 10 patients in each who had malignant lymphoma, nonspecific lymphadenitis, tuberculosis, reactive paracortical hyperplasia, probably due to viral infections, or malignant lymphoma, were compared.

14.3. Results

The patients with Kikuchi's disease (age range 7–47 years; median, 20 years) had a 6–143 day history of lymphadenopathy. The ratio of males to females was 1:2.2 showing female predominance. All lymph nodes examined were obtained from the cervical area. Histologically, the affected nodes represented foci of transformed lymphocytes including immunoblasts, scattered histiocytes with or without phagocytosis, necrobiotic cells, and nuclear debris. Necrotic foci were present in almost all specimens, but were obvious in one, which demonstrated fibrin thrombi in the small blood vessels in the affected foci. No, or only few plasma cells and granulocytes were present in the affected foci in all specimens (Figs. 14.1 and 14.2). The nodal parenchyma showed usually diminished lymph follicles with some swollen reticulum cells scattered in the enlarged paracortex. Mild pericapsular infiltration of lymphocytes was found only in 2 specimens.

Immunologically, the proliferated lymphocytes represented a positive reaction for the T cell marker, UCHL-1, but only a few cells reacted with the B cell marker, L26. T cells were composed of CD4 or CD8; the ratio for both cells was between

Fig. 14.1. Kikuchi's disease. Case 10. Partially affected foci in the paracortex. The enlarged Paracortex has diminished lymph follicles in the neck lymph node. HE stain, magnification 20×. (For printing purposes figure has been reduced.)

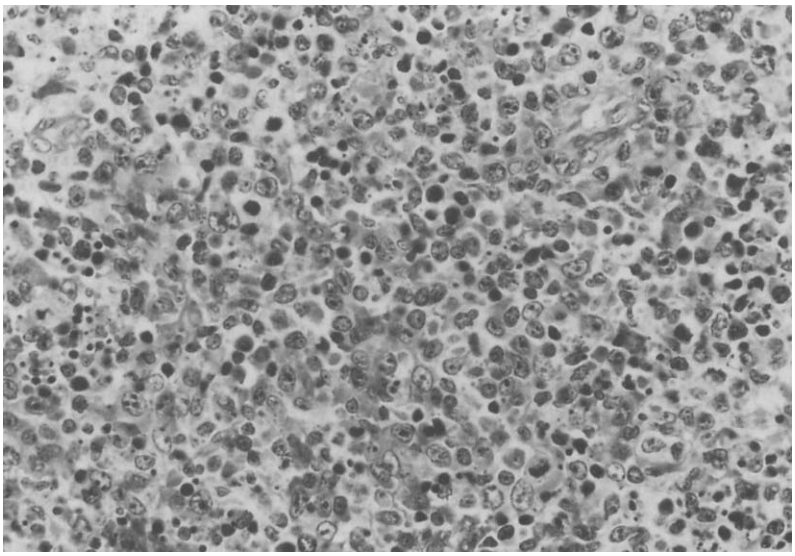

Fig. 14.2. Kikuchi's disease. Case 10. High magnification of the affected foci in Fig. 14.1 shows proliferation of many transformed lymphocytes including immunoblasts, histiocytes, and some nuclear debris. HE stain, magnification 600×. (For printing purposes figure has been reduced.)

1:3 and 3:1. CD15-positive histiocytes were distributed through the affected foci also and occupied about 20–30% of the cells.

Reactivity for HHV-6 antigens is tabulated in Table 14.1. 17 of the 19 patients were positive for HHV-6: 13 of the 19 on frozen sections and 12 of the 19 by imprints. The HHV-6-positive cells were sporadic and a few were demonstrated in the affected foci on histological examination. In 3 patients, positive cells were detected in the parenchyma outside the affected foci. The numbers of positive cells were fewer than the affected foci. The positive cells had a diffuse cytoplasmic reaction and were scattered sporadically. They demonstrated histiocytic or lymphoid features and were 8–10 μm in diameter, with oval nuclei and obvious cytoplasm (Fig. 14.3). A positive reaction of the membrane was detected usually in transformed larger lymphoid cells. The imprints of these positive specimens revealed the same reaction of the cytoplasm or membrane as was shown on the sections (Fig. 14.4). As controls, patients with several reactive and neoplastic diseases were studied. On frozen sections from 4 of the 10 patients with nonspecific

TABLE 14.1.
HHV-6 antigen in lymph nodes of Kikuchi's disease (Mem.: membrane-bound immunoreaction; Cyt.: cytoplasmic immunoreaction; Toxo.: toxoplasma; EBVCA: Epstein–Barr virus capsular antigen; CM.: cytomegalovirus; N.D.: not done.)

Case no.	Age (Yr)	Sex	Location	Duration (days)	HHV-6				Serological test
					Tissue		Imprint		
					Mem.	Cyt.	Mem.	Cyt.	
1	7	M	neck	6	+	–	+	+	N.D.
2	12	F	neck	49	+	–	+	+	N.D.
3	14	F	neck	16	–	–	–	–	N.D.
4	14	F	neck	15	–	–	+	+	N.D.
5	16	F	neck	24	–	–	+	+	Toxo.(–)
6	16	F	neck	8	+	–	+	–	N.D.
7	18	F	neck	24	+	–	+	–	N.D.
8	20	M	neck	9	+	–	+	+	N.D.
9	20	F	neck	26	–	–	+	–	N.D.
10	24	M	neck	12	+	–	–	–	N.D.
11	24	M	inguinal	29	–	–	–	–	N.D.
12	26	F	neck	54	+	–	–	–	N.D.
13	26	M	neck	23	+	–	–	–	N.D.
14	26	F	neck	30	+	–	–	–	N.D.
15	29	F	neck	49	+	+	+	+	N.D.
16	32	M	neck	26	–	–	+	–	N.D.
17	34	F	neck	17	+	–	+	–	Toxo.(+), EBVCA(+)
18	44	F	neck	12	+	–	–	–	Toxo.(–), EBVCA(–), CM(–)
19	47	F	neck	143	+	–	+	+	N.D.

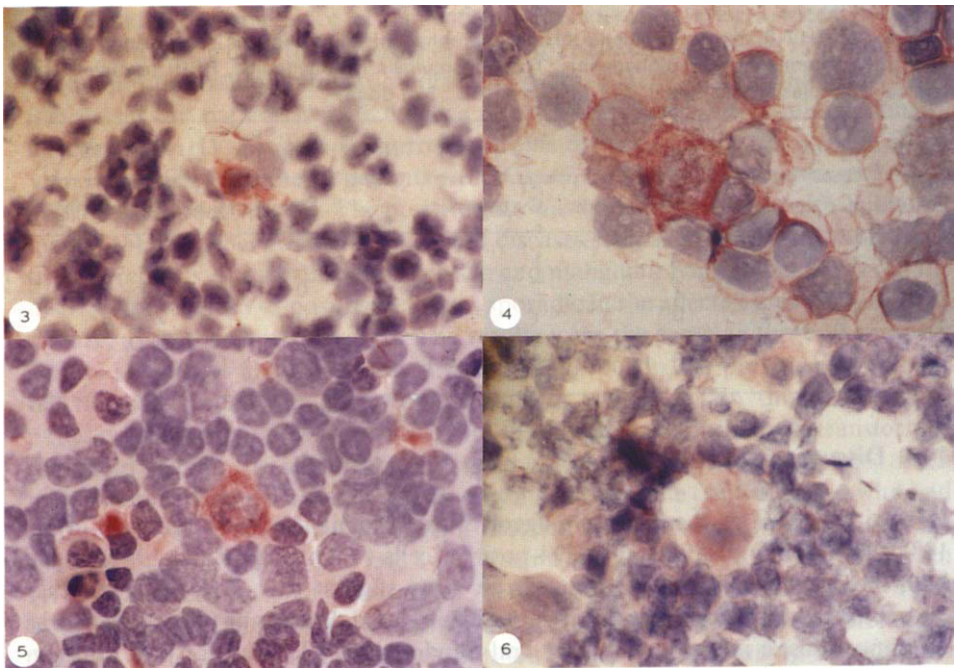

Fig. 14.3. Kikuchi's disease. Case 15. An HHV-6-positive cell shows diffuse cytoplasmic reaction in the affected foci. Frozen tissue, magnification 1350×. (For printing purposes figure has been reduced.)
Fig. 14.4. Kikuchi's disease. Case 8. An HHV-6-positive cell shows a fine granular intracytoplasmic reaction and some weakly positive cells with a membrane-bound reaction. Imprint, magnification 1350×. (For printing purposes figure has been reduced.)
Fig. 14.5. Nonspecific lymphadenitis. A positive cell in the cervical lymph node. Imprint, magnification 1350×. (For printing purposes figure has been reduced.)
Fig. 14.6. Malignant lymphoma, pleomorphic type. One weakly positive histiocyte with a cytoplasmic fine granular reaction is seen in the cervical lymph node. Frozen tissue, magnification 1350×. (For printing purposes figure has been reduced.)

lymphadenitis, 2 of the 10 with tuberculous lymphadenitis, 1 of the 10 with reactive paracortical hyperplasia probably due to viral infection, and 2 of the 10 with malignant lymphoma, HHV-6 antigens were present. In 3 patients with nonspecific lymphadenitis (3 on tissue, 3 on imprint), 3 with tuberculosis (1 on tissue, 2 on imprint), 1 with reactive paracortical hyperplasia, and 2 with malignant lymphoma, the specimens demonstrated reactivity in the cytoplasm. The positive cells were distributed sporadically. In 1 patient with nonspecific lymphadenitis the reaction was membranous in transformed lymphocytes and cytoplasmic in macrophages. The positive cells in malignant lymphoma had histiocytic features in the tumor tissue. The results are tabulated in Table 14.2.

TABLE 14.2.
Positive for HHV-6 antigen (Kikuchi: Kikuchi's lymphadenitis = histiocytic necrotizing lymphadenitis; Non: nonspecific lymphadenitis; Tbc: tuberculosis lymphadenitis; RPH: reactive paracortical hyperplasia; ML: malignant lymphoma; N.D.: Not done)

	No. of cases	Positive cases	Tissue		No. of cases	Imprint	
			Membranous	Cytoplasmic		Membranous	Cytoplasmic
Kikuchi	19	17	13	1	19	12	7
Non	10	4	1	2	3	0	3
Tbc	10	2	0	1	3	0	2
RPH	10	1	0	1	N.D.	N.D.	N.D.
ML	10	2	0	2	N.D.	N.D.	N.D.

14.4. Discussion

HHV-6 is known as the causative agent of exanthem subitum (Yamanishi et al., 1988). Predominant affinity of CD4-positive cells (Takahashi et al., 1989) and a high incidence of latent infection of the virus suggest that HHV-6, like other herpesviruses, may cause peculiar lymphocyte-related diseases or play some role as an opportunistic infection (Krueger and Sander, 1989). In addition to exanthem subitum, an association of HHV-6 with some lymphadenopathies including AIDS and Kikuchi's disease, as well as rejected kidneys of renal transplantation, has been reported (Okuno et al., 1990), but the real role of the virus has not been clarified. Kikuchi's disease has characteristic clinical findings of localized lymphadenopathy on the neck, leukopenia, fever, occasional skin rash, and natural healing within several months. It usually affects young females from 15 to 35 years of age (Kikuchi et al., 1990). From these clinical manifestations, some protozoa or microorganisms, such as toxoplasma (Kikuchi et al., 1977), *Yersinia enterocolitica* (Feller et al., 1983), Epstein–Barr virus (Takada et al., 1980), and other viruses were suspected as causative agents. Elevated serum levels of 2′5′-oligoadenylate synthetase in the early stage of disease and the frequent appearance of tubuloreticular structures in the lymphocytes and other cells in the affected foci intensified the suspicion of viral infection (Kikuchi et al., 1990). Niederman et al. (1988) reported on 3 young adult patients with HHV-6 infection who manifested cervical lymphadenopathy and leukopenia early in the illness, which persisted for up to 3 months. Their clinical features were similar to those of Kikuchi's disease.

Using anticomplement immunofluorescence methods, Eizuru et al. (1989) found that 17 of the 18 patients with Kikuchi's disease and 6 of the 8 patients with other reactive lymphadenopathies or malignant lymphomas tested positive for HHV-6 antigen. Kurata et al. (1989) also reported on 5 of the 17 patients with Kikuchi's disease who had clear involvement of HHV-6. However, they could not clarify whether or not HHV-6 is associated with Kikuchi's disease. Yanagi et al. (1990) reported a high prevalence of over 85% of the antibody to HHV-6, with the highest geometric mean titer in Japanese patients in the 1–2 year age group, and

postulated a rare incidence of reinfection or reactivation after 3 years of age. It seems from these data that the role of HHV-6 in Kikuchi's disease as the primary infection is doubtful. Examination of serum titers of HHV-6 of IgG and IgM types is necessary in patients with Kikuchi's disease, to connect activation or primary infection of HHV-6 with an occurrence of this disease.

In this series, 17 of the 19 patients with Kikuchi's disease (13 on tissue and 12 on imprint) had infected cells. The positive rate was higher in patients with Kikuchi's disease than in other control groups with diseases such as nonspecific lymphadenitis, tuberculosis, paracortical hyperplasia, and malignant lymphoma. However, the distribution of HHV-6-positive cells was sporadic in the affected areas in Kikuchi's disease, as in the control groups. The results were the same as those of previous studies (Eizuru et al., 1989; Kurata et al., 1989), but in the previous studies the reaction was usually in the cytoplasm and/or the nuclei. In this series, in 13 patients, the reaction was positive on the surface membrane of transformed lymphoid cells in the sections and/or on the imprints (Figs. 14.3 and 14.4). Such membranous reaction was detected in only 1 patient in the control group who had nonspecific lymphadenitis. Cytoplasmic reaction was a common positive feature in the control groups (Figs. 14.5 and 14.6). The membranous reaction was obvious in HHV-6-infected cultured cord cells. As described previously (Kikuchi, 1972; Kikuchi et al., 1986, 1990), Kikuchi's disease represents distinctive histological features, such as proliferation of transformed lymphocytes including considerable numbers of immunoblasts, histiocytes, and nuclear debris, with necrobiotic or necrotic foci in the affected areas, usually in the paracortex. HHV-6-positive cells were distributed usually in cellular areas with numerous lymphocytes and histiocytes, but not in the necrotic foci, and only a few individual cells showed a positive reaction. The sporadic distribution of HHV-6-positive cells in Kikuchi's disease, as in the disease represented by the control groups, suggests an occult infection of the virus in these patients, but distribution of HHV-6 antigen on the surface of lymphoid cells in Kikuchi's disease might be related to proliferation of this virus on the lymphoid cells; however, the role of the virus is uncertain. The frequent presence of HHV-6 in the affected foci and the membranous reaction in the positive cells indicate a possible role of HHV-6 in the proliferation of transformed lymphocytes with nuclear debris. Examination of these values is necessary in patients with Kikuchi's disease, to connect activation or primary infection of HHV-6 to an occurrence of this disease. Examination of serum titers of HHV-6 is very important to detect the relation of HHV-6 to Kikuchi's disease. From the patterns of accumulations of CD4-positive cells and histiocytes with no proliferative activity and CD8-positive cells with proliferative activity, the numerous nuclear debris and the presence of HHV-6 antigens on the surface of some transformed lymphoid cells and a few histiocytes suggest aggregations of CD4- and CD8-positive cells and histiocytes due to positive chemotaxis from some agents, following destruction of these cells by proliferating CD8-positive cells. HHV-6-infected cells might be activated by these processes, resulting in an appearance of HHV-6 on the surface of some transformed lymphocytes. Such an immunological sequence was speculated in patients with AIDS and cytomegalovirus infection (Grundy et al., 1987).

14.5. Summary

Antigens against HHV-6 were detected in 17 of the 19 patients with Kikuchi's disease. The antigen-positive cells were more frequently encountered in Kikuchi's disease than in other reactive lymphadenopathies and malignant lymphoma. The distribution of positive cells, lymphocytes, or histiocytes was sporadic as with the other diseases, but the membranous reaction in transformed lymphocytes was characteristic in Kikuchi's disease. Frequent appearances and membranous distribution of the antigen suggest some role of HHV-6 in the activation or extension of Kikuchi's disease.

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Ablashi/Krueger/Salahuddin (eds) Human Herpesvirus-6

CHAPTER 15

HHV-6 in atypical polyclonal lymphoproliferation (APL) and malignant lymphomas

GERHARD R.F. KRUEGER[1], DHARAM V. ABLASHI[2], STEVEN F. JOSEPHS[3] and NARAYANASWAMY BALACHANDRAN[4]

[1] *Department of Pathology and Laboratory Medicine, University of Texas Medical Center, Medical Science Building, Houston, TX 77030, U.S.A.,* [2] *Laboratory of Cellular and Molecular Biology, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892, U.S.A.,* [3] *Laboratory of Tumor Cell Biology, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892, U.S.A., and* [4] *Department of Microbiology, Molecular Genetics and Immunology, The University of Kansas Medical Center, Kansas City, KS 66103, U.S.A.*

15.1. Introduction

Human herpesvirus-6 (HHV-6) is the second member of the human herpesvirus family with pronounced lymphotropism; it resembles in many clinical aspects the Epstein–Barr virus (EBV; HHV-4) although the lymphoid target cell for infection and replication differs in both viruses (Ablashi et al., 1988; Krueger and Sander, 1989; Lusso et al., 1989; Takahashi et al., 1989). The effect on the lymphoid system by HHV-6 – similar to other lymphotropic viruses – is a dual one: (a) response to the antigen as in other viral infections, and (b) modulation of such response by direct infection of cells of the immune system (Lennert et al., 1981; Krueger et al., 1983b; Krueger et al., 1991c). Both EBV and HHV-6 may cause latent, lytic and apparently transforming infections (Razzaque and Josephs, 1989; Razzaque, 1990). Both viruses are ubiquitous with high antibody prevalence in the population and both apparently reside in salivary gland and bronchial epithelial cells during latency (Wolf et al., 1981; Fox et al., 1990; Krueger et al., 1990). Conditions are not adequately identified yet which govern the HHV-6 infection of epithelial or lymphoid cells, and which determine whether the infection causes latency or viral replication. The clinical pathology, however, is widely influenced by the state of activity of the immune system at the time of active infection (Purtilo et al., 1985; Krueger and Sander, 1989). Since immune reactivity varies with the age of the individual, active HHV-6 infection may also induce quite variable disease patterns

depending upon the time of primary infection or reactivation (Krueger and Sander, 1989).

This phenomenon pertains as well to the lymphoid system for which a number of changes have been described following HHV-6 infection or accompanied by HHV-6 reactivation: transient or persistent lymphocytosis with lymphoblasts (Berthold et al., 1989), heterophil-negative infectious mononucleosis (Niederman et al., 1988; Bertram et al., 1989, 1991; Steeper et al., 1990; Horwitz et al., Chapter 13, this volume), Kikuchi–Fujimoto's disease (Yamanishi et al., 1988; Hoffmann et al., 1991), various kinds of atypical polyclonal lymphoproliferations (Hoffmann et al., 1988; Krueger et al., 1988b, 1989; Eidt et al., 1991), and certain malignant lymphomas (Salahuddin et al., 1986; Krueger and Ablashi, 1987; Jarrett et al., 1988; Josephs et al., 1988a; Krueger et al., 1988b; 1989; Borisch-Chappius et al., 1989; Ellinger et al., Chapter 16, this volume). We will summarize here the current knowledge of HHV-6 and its suggestive relationship to progressive and malignant lymphoproliferative diseases.

15.2. Working definition of HHV-6 reactivation and active infection

Investigations for HHV-6 infection as used in the studies described in this chapter follow a stepwise procedure. Suspicion of virus-associated atypical polyclonal proliferation (APL) or malignant lymphoma is initially based on the routine biopsy diagnosis (or before in case histories with immune deficiency or allotransplantation). This will lead to serological screening of the patient's sera. Indirect immunofluorescence (IFA) IgG serum titers against HHV-6-infected HSB_2 or MOLT-4 cells of 1:640 or above are indicative of a reactivated infection (Krueger et al., 1991a). IgM may or may not be positive (1:20 and above). In patients with immune disturbances, however, even lower titers may become suspicious in conjunction with the case history and with clinical findings (Krueger et al., 1991b). Serologic studies are then supplemented by virus isolation. We routinely use peripheral blood lymphocytes (PBL) as source since productive infection of lymphocytes by HHV-6 appears to be a rare event in healthy persons in contrast to the more frequent virus shedding from saliva (Harnett et al., 1990; Levy et al., 1990; Krueger et al., 1991b). The patients' PBL in culture are checked for HHV-6 antigen expression using monoclonal antibodies (Balachandran et al., 1989) and immunocytological techniques. Supernatants of these cells serve to infect other HHV-6 susceptible cells (e.g., HSB_2 or MOLT-4). In tissue biopsies (optimally frozen sections), HHV-6 is shown by *in situ* hybridization, antigen expression, Southern blotting or polymerase chain amplification reaction (PCR) (Buchbinder et al., 1988; Krueger et al., 1988b). It is advisable, however, to use some way of quantification of the results to distinguish between latent infection and increased expression during viral replication (Krueger et al., 1989). *In situ* techniques (hybridization and antigen expression) have the advantage to permit identification of involved cells. Finally, tissue biopsies or bone marrow aspirates may be used for virus isolation in tissue culture.

15.3. Atypical polyclonal lymphoproliferation (APL)

The term 'atypical polyclonal lymphoproliferation' has been proposed for lymphoproliferative disorders which clinically mimick malignant lymphomas but are missing the criterion of monoclonality and other signs suggesting (but not proving)

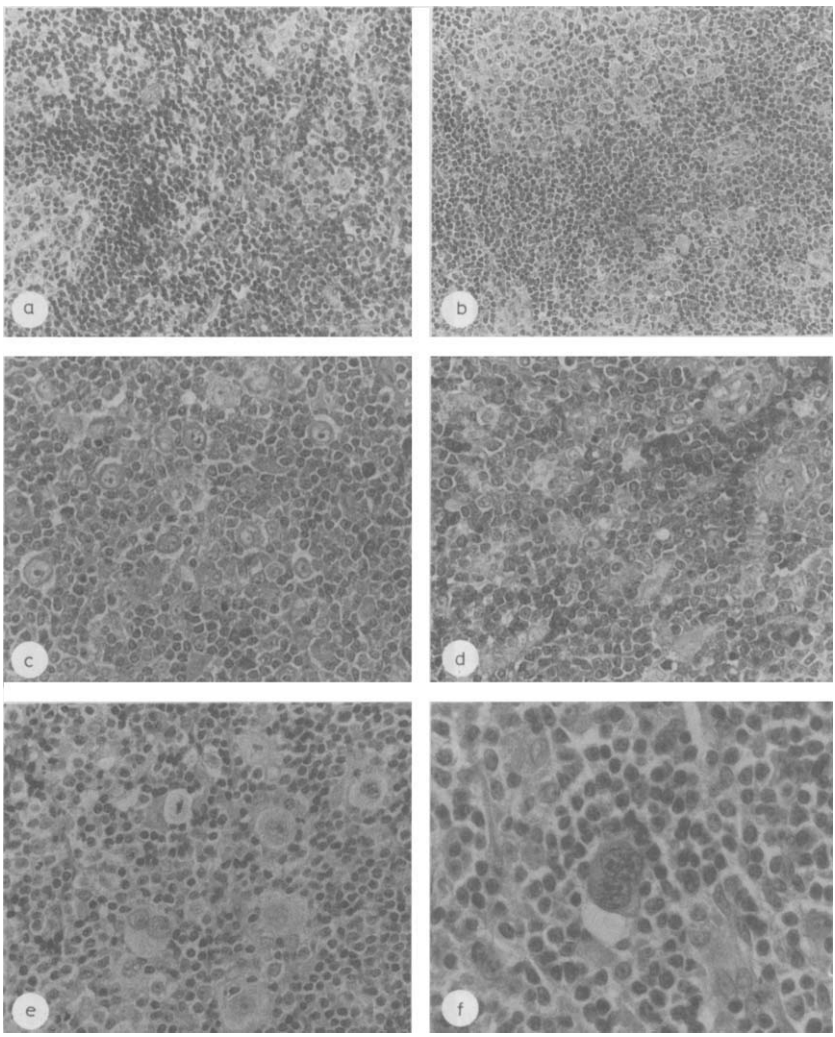


Fig. 15.1. Representative examples of lymphoid changes in herpesvirus-associated atypical polyclonal lymphoproliferation (APL). (a),(b) Focal or diffuse mixed lympho-histiocytic population with effacement of lymph node structure (H&E, magnification 150 ×); (c),(d) increase in immunoblastic and plasmacytoid cells to some extent resembling excessive infectious mononucleosis (Giemsa, magnification 375 ×); (e),(f) histiocytic and lymphoid giant cells (H&E, magnification 375 × and 600 ×). (For printing purposes figure has been reduced.)

malignant transformation such as gene rearrangement (Krueger et al., 1988b, 1989). APL may consist of a morphologically heterogenous group of lesions which can be given different names in conventional pathology (Figs. 15.1–15.3). They have in common (a) that their incidence appears increased in patients with some kind of immune disturbance, (b) that they are frequently associated with persistently active infections by lymphotropic viruses, (c) that they may progress to overt

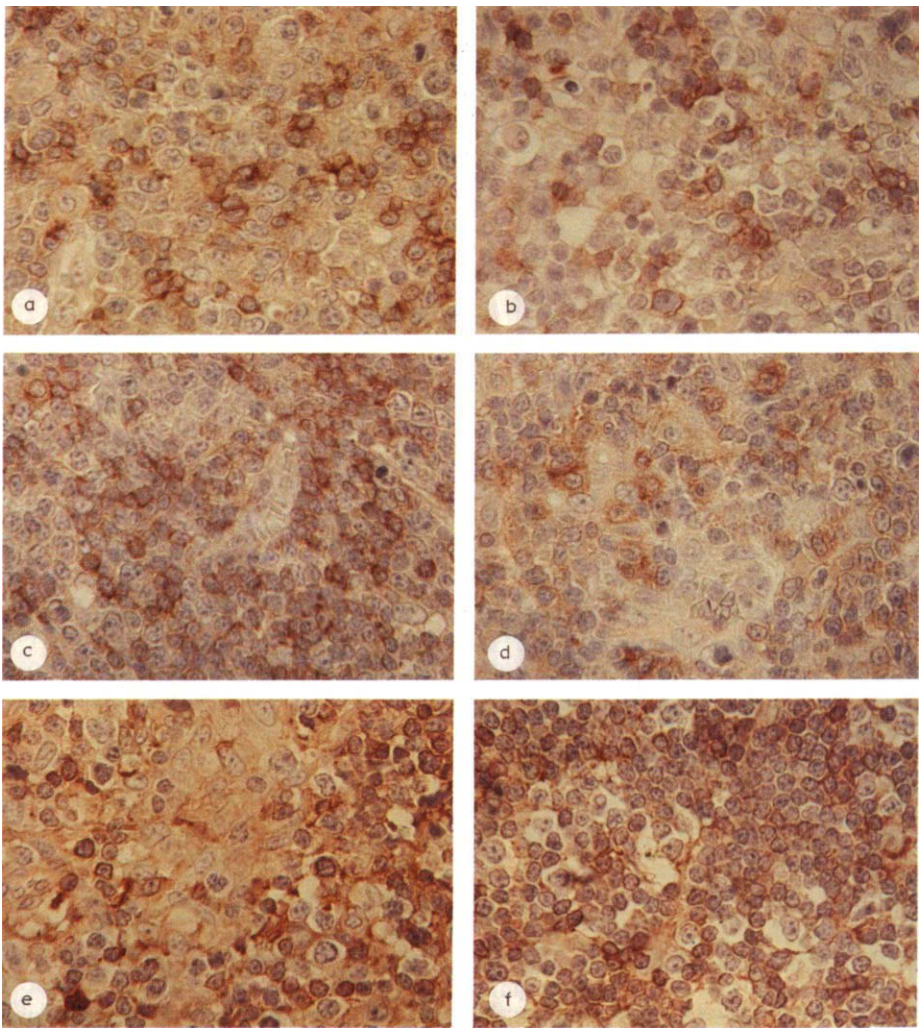


Fig. 15.2. Immunophenotyping of lymphoid cells in paraffin sections from a patient with APL. (a),(b) T lymphocytes (UCHL, MT1, PAP technique, magnification 600 ×); (c),(d) B lymphocytes (L26, KiB3, magnification 600 ×); (e),(f) immunoglobulin light chains in B lymphocytes (kappa, lambda, magnification 600 ×). (For printing purposes figure has been reduced.)

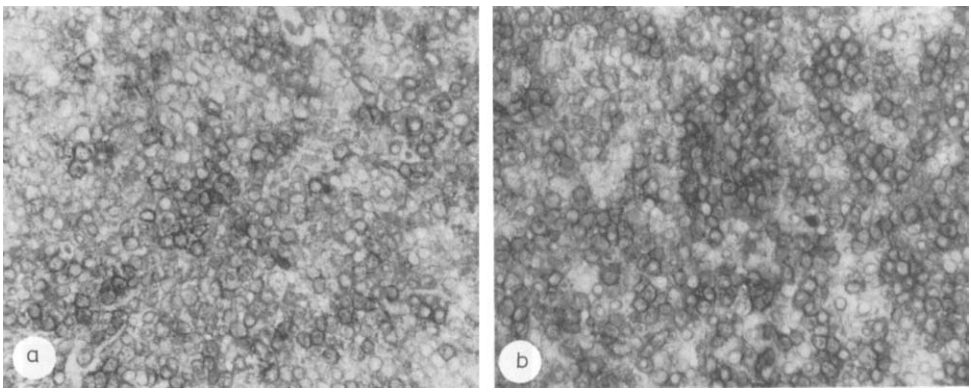


Fig. 15.3. Immunophenotyping of lymphoid cells in frozen section from patient with APL (from Fig. 15.2). (a) CD4 helper T cells; (b) CD8 suppressor T cells (PAP technique, magnification 375 ×). (For printing purposes figure has been reduced.)

TABLE 15.1.
Routine histologic diagnoses in APL

Massive lymphoid hyperplasia
Atypical hyperplasia simulating excessive mononucleosis
Hyperimmunization syndrome of unknown origin
Canale–Smith syndrome
Lymphoma in Sjögren's syndrome
Lymphoma in AIDS
Hodgkin's disease
Peripheral T cell lymphoma

malignant lymphomas, and (d) that they should be treated differently from conventional malignant lymphomas. Clinicopathologic examples for such cases of APL are summarized in Table 15.1.

The identification of APL in human pathology practically supports George Klein's concept of successive stages in tumor development (Klein, 1979, 1986). It has its counterpart in the various morphologically and immunophenotypically polyclonal cell proliferations preceding virus-induced animal lymphomas such as in the 'type B reticulum cell sarcoma' or in the Moloney- and Gross virus-induced lymphoblastic lymphomas in mice (Krueger et al., 1981; Krueger, 1989). Although it can theoretically be expected that forms of APL will also follow infections with lymphotropic RNA viruses such as HTLV-1 and HTLV-2, essentially all of today's identified cases had reactivated herpesvirus infections (EBV and/or HHV-6). Similarly, APL in HIV-1-positive patients is accompanied rather by the reactivated herpesviruses than follows HIV infection alone.

In the following we will discuss individual examples of APL associated with active HHV-6 infection.

15.3.1. Benign lymphocytosis and APL resembling Canale–Smith syndrome

Berthold et al. (1989) described a peculiar disease in 2 boys of a family presenting with persistent and recurrent lymphocytosis of 24.2 per µl to resp. 31.3 per µl, splenomegaly and recurrent indolent neck lymphadenopathy. Both came to medical attention for frequent upper respiratory tract infections, otitis media, and urinary tract infections suggesting some underlying immune deficiency. The patients' lymphocytes (PBL) consisted of lymphoblasts with basophilic cytoplasm

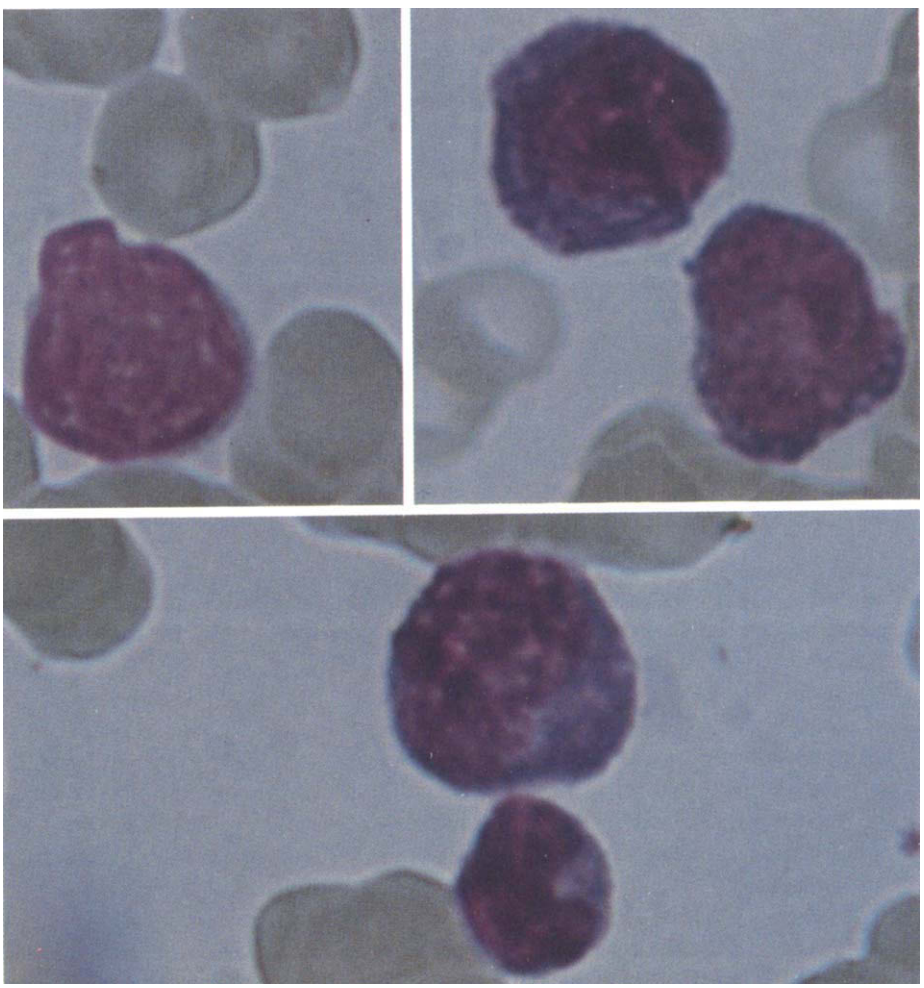


Fig. 15.4. Circulating atypical lymphoblasts with plasmacytoid cytoplasm in the peripheral blood of HHV-6 infected patient (Pappenheim, magnification 1450×). (For printing purposes figure has been reduced.)

(Fig. 15.4), and the peripheral blood smear reminded one of acute lymphocytic leukemia. Immunophenotyping of PBL exhibited polyclonality with the following average marker distribution: CD2 70%, CD3 38%, CD4 22%, CD8 30% (i.e., T4/T8 ratio 0.74), CD38 30%, CD57 0.3%, and CD19 12%. DNA flow cytometry revealed 11% aneuploid cells with DNA index 1.19. No atypical blasts were seen in the bone marrow; CALLA and TdT were negative.

An enlarged lymph node of one boy was investigated and showed histologically an over-all intact structure with pronounced follicular hyperplasia and paracortical expansion by a polymorphic lymphoid population with many blasts and occasional giant cells. Immunophenotyping of cells in this region yielded the following marker profile: CD2 50%, CD4 below 10%, CD8 30–50%, CD38 10%, CD57 below 10%, CD7 50%, CD19 10–30% focally distributed, and Tu1 70–80%. Atypical giant cells did not express CD30.

In situ hybridization of lymph node sections using the pZVH14 DNA probe of HHV-6 (Josephs et al., 1986) resulted in focal accumulations of positive cells. Numbers of HHV-6 DNA positive cells were clearly above those seen in latent infections ($175/10^5$ versus $1/10^5$). Productive HHV-6 infection was further proven by co-cultivation of the patient's PBL with HSB_2 cells which then underwent blastic transformation and replicated HHV-6. Southern blot analysis of lymph node tissue of one patient demonstrated JH immunoglobulin and TCRγ gene rearrangements (Fig. 15.5).

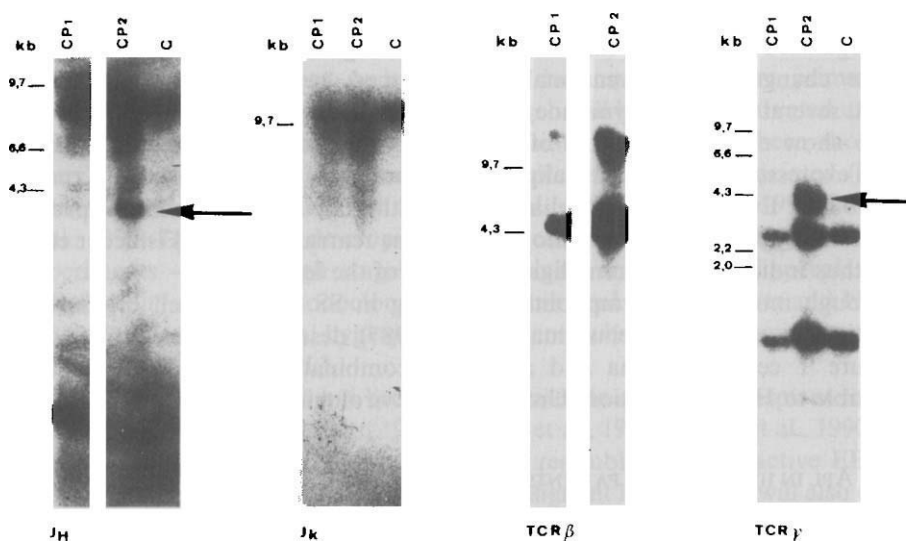


Fig. 15.5. Southern blot analysis of lymph node from a patient with APL and HHV-6 infection (CP2) and with nonspecific hyperplasia (CP1). As control (C) served DNA extracts from placenta of an HHV-6 negative person. Cloned probes: 3.1 kb EcoRI-HindIII fragment of JH, 1.8 kb SacI fragment of JK, 1.0 kb PstI fragment of TCRβ and 0.7 kb HindIII–EcoRI fragment of TCRγ (Krueger et al., 1989). Rearrangements were detected only in HHV-6-associated APL (CP2, arrows).

The described clinical course of the patients started soon after birth and lasted for 3 and 11 years with just symptomatic antibiotic treatment. The final clinical diagnosis was Canale–Smith syndrome as described in 1967 as chronic lymphadenopathy simulating malignant lymphoma (Canale and Smith, 1967). It is interesting to note that the boys' mother had a case history of 'acute lymphocytic leukemia' at the age of 3 years (in 1957) which was 'successfully treated'. She still had persistent lymphocytosis without other clinical signs of sickness. She and her 11-year-old son had IgG2 deficiency (0.28 and 0.21 μg/ml respectively) while the 3-year-old boy had elevated levels of IgG2 (2.70 μg/ml).

The data were interpreted as resulting from unusual lymphostimulation by persistently active HHV-6 infection probably on the basis of some immune deficiency (IgG2, NK cell). The patients and their mother are healthy and doing well at the time of this report (3 years after the initial report by Berthold et al.).

15.3.2. APL IN SJÖGREN'S SYNDROME

Sjögren's syndrome (SS) is an autoimmune disorder related to collagen-vascular diseases and complicated in a considerable number of cases by the development of malignant lymphomas (Talal et al., 1987). Its frequent association with HHV-6 infection is described by DeClerck and colleagues in Chapter 22, this volume. One of the three lymphoma cases with HHV-6 infection published by Josephs et al. (1988a) had a history of SS. In the same year, Jarrett et al. (1988) reported two more cases of HHV-6-associated malignant lymphomas, one of which had SS. Lymphoproliferation in Sjögren's syndrome generally progresses from orderly hyperplasia to disorganized hyperplasia (actually APL) to malignant lymphoma.

These changes are conventionally also named 'myoepithelial sialoadenitis' (MESA; several stages) or 'lymphoepithelial lesion' of the salivary gland. We were able to show elevated numbers of HHV-6 genome-carrying cells and HHV-6 antigen expression in APL and malignant lymphomas of patients with SS (Krueger et al., 1988b). Even lymphoepithelial lesions in the salivary gland without proven monoclonality may exhibit immunoglobulin gene rearrangements (Fishleder et al., 1987), thus indicating the premalignant nature of the lesion.

Although most of the lymphomas occurring in SS are of B cell origin, it is interesting to note that Schuurman et al. (1987) described a case of SS with immature T cell lymphoma and a marker combination CD4/CD38like cells susceptible to HHV-6 infection. Unfortunately, virological studies were not done.

15.3.3. APL IN HIV-1 POSITIVE PATIENTS WITH AIDS

Similar to EBV, HHV-6 reactivation was described repeatedly in HIV-1-infected patients (Ablashi et al., 1988; Krueger et al., 1988a). Although these 3 viruses were shown *in vitro* to co-infect cells and to transactivate their intracellular activities (Gendelman et al., 1986; Pagano et al., 1988; Ensoli et al., 1989; Lusso et al., 1989; Schonnebeck et al., 1991), careful clinical studies do not show apparent differences in the course of the HIV-induced disease whether herpesviruses are reactivated or

not (Lewin et al., 1990). Those HIV-1$^+$ cases, however, in which lymphoproliferative lesions develop, show regularly reactivated EBV, HHV-6 or frequently both (Krueger et al, 1988b; 1989). APL in AIDS patients frequently simulates malignant lymphomas, although they are still polyclonal by immunophenotyping, miss the characteristic DNA aneuploidy of malignant lymphomas and rarely show gene rearrangements (Auffermann et al., 1986; Krueger et al., 1989). Their histologic appearance is frequently even more bizarre than in the non-AIDS cases and hard to distinguish from a pleomorphic or unclassified malignant lymphoma.

HHV-6 IgG antibody titers vary from 1:40 to 1:1 280 with absent IgM and positive virus isolation from PBLs. The cellular HHV-6 DNA load in lymphoid tissues exceeds significantly the one in latent infections. Double-active infections with EBV and HHV-6 appear even more frequent in AIDS patients with APL (Krueger et al., 1989).

15.3.4. APL IN TRANSPLANT PATIENTS

The increased risk of allograft recipients to develop malignant lymphomas is a well-documented fact (Penn, 1978). Early mouse experiments suggested that a combination of persistent immunosuppression and immunostimulation may be responsible for progressive lymphoproliferation (Krueger et al., 1971). This had led to the formulation of the dysregulative versus the transformative theory of lymphoma development (Krueger, 1989). Lymphotropic viruses in mice as in man, whether RNA viruses or DNA viruses, are immunostimulants and immunosuppressants at the same time. They could thus induce as well progressive lymphoproliferation if they remain persistently active.

One would expect that such viruses in human patients are primarily the omnipresent lymphotropic herpesviruses, which will become reactivated under the condition of posttransplant immunosuppression. It has indeed been shown that many lymphomas arising in transplant recipients are EBV-associated (Forman et al., 1987; Nalesnik et al., 1988; Shapiro et al., 1988a; List et al., 1990; Randhawa et al., 1990). Lymphomas developing under these conditions – similar to the animal experiments – go through stages of hyperplasia and atypical hyperplasia with polyclonality and final monoclonality (Nalesnik et al., 1988; Locker and Nalesnik, 1989). Atypical polyclonal hyperplasia represents what we call atypical polyclonal lymphoproliferation (APL).

Reactivation of HHV-6 has been demonstrated to occur in transplant recipients just like EBV (Chou and Scott, 1990; Okuno et al., 1990; Wrzos et al., 1990). Since tissue reactions in active HHV-6 infections resemble those in active EBV, it is possible that HHV-6-associated APL and malignant lymphomas will also occur in transplant recipients, but this needs to be studied in more detail.

15.3.5. APL IN PATIENTS WITHOUT APPARENT IMMUNODEFICIENCY

There are sporadic cases of HHV-6-associated APL identified in patients without AIDS, allografting or other defined immunodeficiency disorders (Krueger et al.,

1989). Lymph nodes are biopsied usually in these patients for suspicion of a malignant lymphoma. Routine diagnostic measures, however, including immunophenotyping, reveal a polyclonal cell population although the lymph node structure appears widely or completely effaced. T cell receptor gene or immunoglobulin gene rearrangement studies usually result in germline structures, although occasional rearrangements do not suffice to identify a true malignancy. The clinical course is characterized by a several years' history of upper respiratory tract 'flu-like' infections or nonexudative oropharyngitis and recurrent lymphadenopathy finally with massive local or systemic lymph node enlargement. Lymph nodes are repeatedly biopsied, and pathologists unfamiliar with the problem (or unaware of the clinical course) will provide diagnoses such as massive lymphoid hyperplasia, giant lymph node hyperplasia (Castleman's disease), angioimmunoblastic lymphadenopathy, chronic proliferative Kikuchi's syndrome, Rosai–Dorfman's syndrome, lymphoplasmacytoid lymphoma, monocytoid B cell lymphoma, malignant lymphoma unclassified, Hodgkin's disease or T cell lymphoma pleomorphic type (Krueger et al., 1988b, 1989; Manak, unpublished data, 1990). Differential diagnosis from lymphomas with a pleomorphic appearance (e.g., pleomorphic T cell lymphoma or lymphogranulomatosis) can be extremely difficult, and should be attempted in those cases after clinicopathologic correlation and careful immunovirological studies. In doubtful cases, an initial therapeutic trial with interferon a2a and iv immunoglobulin appears warranted (Hoffmann et al., 1988; Shapiro et al., 1988b; Shapiro and Filipovich, 1989) before instituting classical antiproliferative chemotherapy. Examples of the characteristic histological appearance of such cases of APL are given in Figs. 15.1–15.3.

Although serologic antibody titers against HHV-6 are usually elevated (above 1:640), IgM antibodies may not be detectable. In a few cases, serology gives only borderline elevations, yet virus is readily recovered from the patients' PBL and tissues (Krueger et al., 1989). PBL in tissue culture show sustained growth and pleomorphism with pseudopodia and some spontaneous blastic transformation (Fig. 15.6). Lymphoid tissues contain increased numbers of HHV-6 genome carrying cells in focal distribution by *in situ* hybridization (Fig. 15.7), and HHV-6 antigen expression can be demonstrated by immunocytology and monoclonal antibodies in frozen sections and in tissue culture cells.

Three HHV-6 isolates from cases of sporadic APL were grown in HSB_2 cells. Initial infection of HSB_2 cells by supernatants from the patients' PBL harvested between 2 and 6 weeks resulted in expression of HHV-6 antigens (p41, p135 and gp180) in 70–80% of the cells by day 14 (Ablashi and Salahuddin, Chapter 7, this volume; Krueger, unpublished results). Large numbers of extracellular and intracellular virions were detected by electron microscopy (Fig. 15.8). High molecular weight DNA was extracted from HHV-6-infected HSB_2 cells and Southern blots were prepared after digestion with EcoRI, BamHI or HindIII and probing with the HHV-6 pZVH14 DNA fragment (Josephs et al., 1986; Ablashi and Salahuddin, Chapter 7, this volume). Restriction enzyme banding patterns of the APL HHV-6 isolates equaled the ones from the original NIH GS prototype virus with 1.2 and 2.1 kb bands for EcoRI, a 22 kb doublet for BamHI and a 2.7 kb band for HindIII.

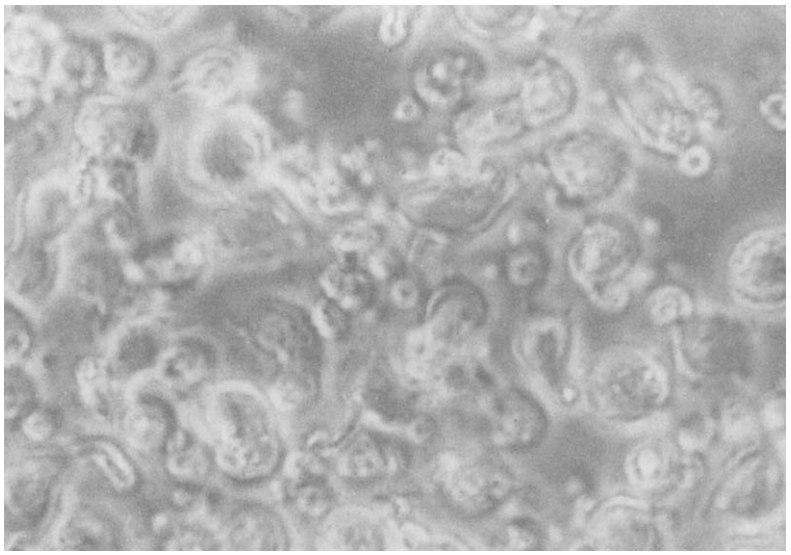


Fig. 15.6. Cell culture of peripheral blood lymphocytes from a patient with active HHV-6 infection (virus isolation by co-culture with HSB_2 cells) (Krueger et al., 1991b). Note marked polymorphism and spontaneous blast formation (phase contrast, magnification 375 ×).

They were distinct from banding patterns of HHV-6 isolates from patients with exanthem subitum and with chronic fatigue syndrome (Ablashi and Salahuddin, Chapter 7, this volume). However, a certain selection for GS-similar virus isolates by our tissue culture system using HSB_2 cells cannot be excluded at this time.

15.4. Malignant lymphomas

Original isolates of HHV-6 were obtained from patients with malignant lymphomas, AIDS and dermatopathic lymphadenopathy and with angioimmunoblastic lymphadenopathy (Salahuddin et al., 1986). The virus at that time was designated as human B lymphotropic virus (HBLV in contrast to the T lymphotropic viruses HTLV1–3), although one patient suffered from a cutaneous T cell lymphoma. Subsequently, HHV-6 was identified by Southern blotting, *in situ* hybridization and selective virus isolation in such diversified malignant lymphomas as nodular histiocytic lymphoma, small cleaved cell lymphoma, Burkitt's lymphoma, diffuse large cell lymphoma, pleomorphic T cell lymphoma, high grade germinal center cell lymphoma, immunoblastic lymphoma, malignant lymphoma small and large cleaved cell, lymphoplasmacytoid lymphoma, small cleaved cell lymphoma, sinusoidal B cell lymphoma, adult T cell leukemia, hairy cell leukemia, mycosis fungoides, and Hodgkin's lymphoma of various subtypes (Becker et al., 1988; Jarrett et al., 1988; Josephs et al., 1988a; Krueger, 1989; Krueger et al., 1989;

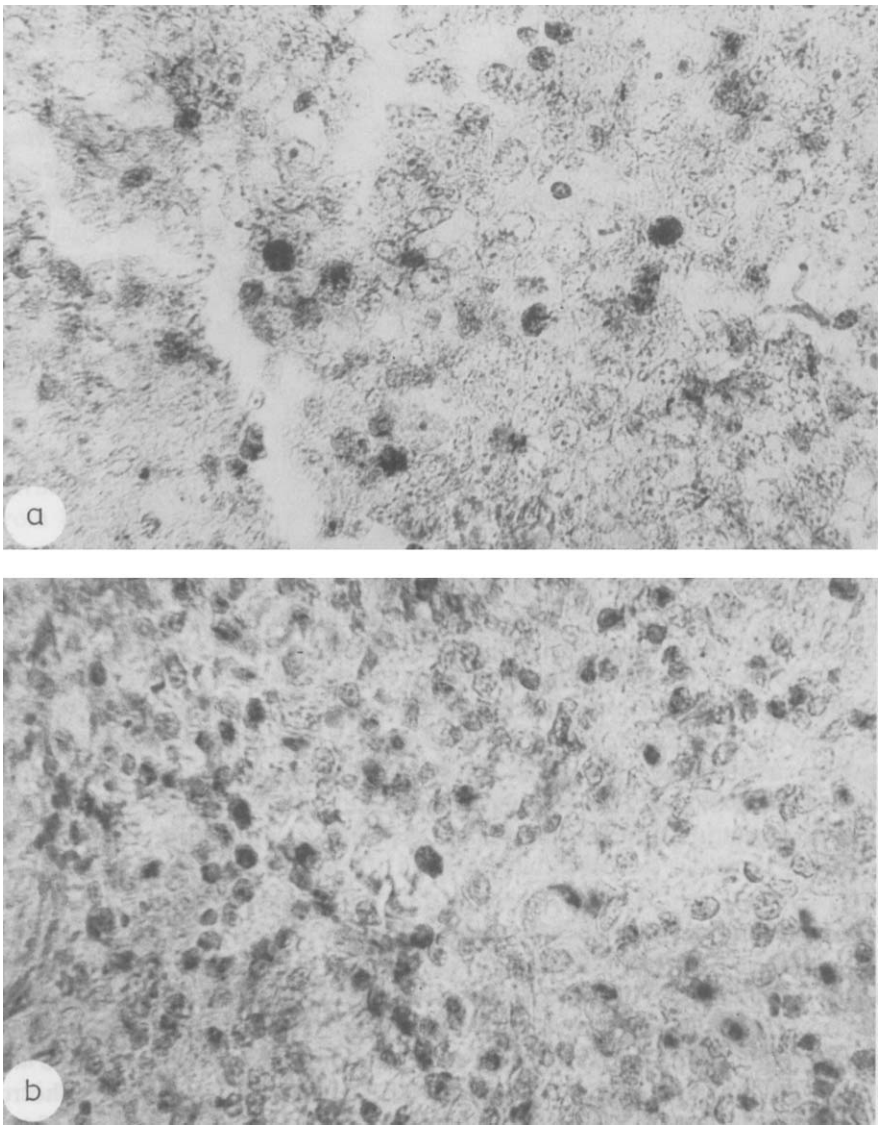


Fig. 15.7. *In situ* hybridization of lymph node section of patient with HHV-6$^+$ (a) APL and (b) lymphoma using the biotinylated pZVH14 HHV-6 DNA probe (S.F. Josephs, NCI) with NBT/BCIP visualization (Bethesda Res. Labs.). Note scattered DNA positive cells in both sections (magnification 375 ×).

Borisch-Chappius et al., 1989; Fox et al., 1990; Eizuru et al., 1991; Ellinger et al., Chapter 16, this volume). Comparative evaluation of data is complicated since different classification schemes for malignant lymphomas were applied by the individual research groups. Data are summarized, therefore, again in Table 15.2

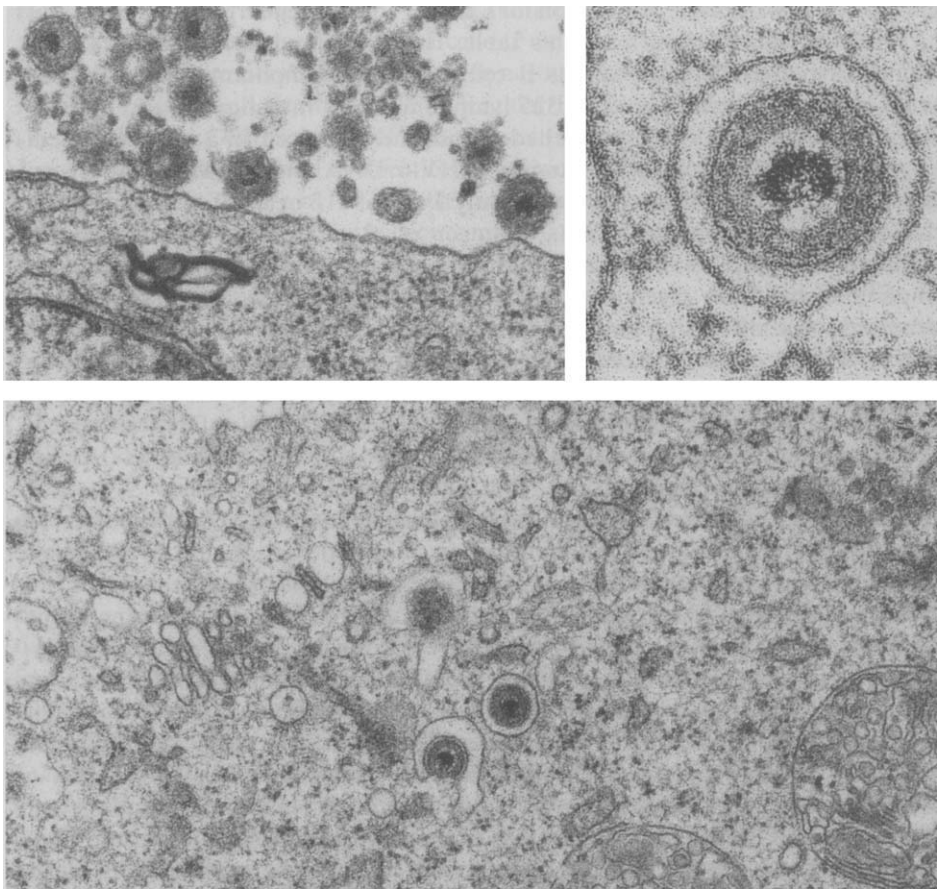


Fig. 15.8. Characteristic HHV-6 virus particles in tissue culture lymphoblasts (CD4/CD38$^+$ cells) budding into intracytoplasmic vesicles and in the extracellular space (magnification 46 000 ×, 60 000 × and 220 000 ×). (For printing purposes figure has been reduced.)

TABLE 15.2.
Histologic lymphoma entities with possible HHV-6 reactivation[a]

Hodgkin's disease
ML, small cleaved and large cell
ML, small cleaved cell diffuse
ML, lymphocytic with plasmacytoid cells
Monocytoid ('sinusoidal') B cell lymphoma
ML, T cell pleomorphic

[a] New working formulation a.o. entities.

using the New Working Formulation for Clinical Use (Rosenberg, 1982; Krueger et al., 1983). As can be seen from this table, Hodgkin's and non-Hodgkin's lymphomas carried HHV-6, as well as B cell and T cell lymphomas. To make the matter even more complicated, of 325 lymphoma cases investigated serologically for antibodies to HHV-6, only 21 had significantly elevated IgG antibody titers (Krueger et al., 1989). In other studies, HHV-6 DNA was shown by Southern blotting or by PCR in biopsy tissues of only 3 out of 112 or 2 out of 16 malignant lymphomas (Josephs et al., 1988a; Ellinger et al., Chapter 16, this volume). Buchbinder et al. (1988), however, found a significantly larger percentage of lymphomas with HHV-6 genome in his PCR studies. This phenomenon needs to be addressed when discussing pathogenesis (Section 15.5).

HHV-6 DNA extracted from malignant lymphomas resembled in its restriction enzyme patterns the prototype virus (NIH GS strain) (Jarrett et al., 1988; Josephs et al., 1988a) with some minor variations. Characteristic HHV-6 DNA sequences were detectable in EcoRI restriction patterns with pZVH14 probing at 1.2 and 2.1 kb with additional bands at 6.3 and 8.2 kb.

Similar to APL (Section 15.3), HHV-6 genome and antigen can be demonstrated in virus-positive malignant lymphomas by *in situ* hybridization and by immunohistology using specific monoclonal antibodies. Optimal for these techniques are frozen sections from freshly submitted and in liquid nitrogen shock-frozen tissue biopsies; paraffin embedded tissue sections are less reliable, and should be supplemented by frozen section studies when negative. Distribution of HHV-6 DNA positive cells in lymphoma tissue may be focal or diffuse without apparent significance with regard to the eventual malignancy of the proliferative process contrary to what was suggested before (Borisch-Chappius et al., 1990). HHV-6 DNA load appears to be generally higher in malignant lymphomas than in latent infection or in APL (Krueger et al., 1989; Eidt et al., 1991). Negative results do not exclude that virus is present. Studies for HHV-6 antigen expression or virus isolation, as described for APL cases, serve to additionally prove the presence of HHV-6. Virus isolation was accomplished in our laboratory from PBL, tissue biopsies and from bone marrow trephine biopsies (Krueger et al., 1989).

15.5. Pathogenetic considerations of HHV-6-induced lymphoproliferation and malignancy

HHV-6, like EBV, is an ubiquitous virus which causes upon infection lifelong equilibrium between HHV-6-infected cells and the host's defense system. Disease develops in a rather low percentage of infected individuals (Krueger et al., 1988a). Being a predominantly lytic virus, HHV-6 may cause acute diseases such as exanthem subitum, heterophil-negative infectious mononucleosis and Kikuchi's syndrome (Yamanishi et al., 1988; Bertram et al., 1989; Eizuru et al., 1989; Steeper et al., 1990; Hoffmann et al., 1991; Horwitz et al., Chapter 13, this volume). There is

increasing evidence that HHV-6 – again similar to EBV – may reside in salivary gland and bronchial gland epithelial cells (Fox et al., 1990; Krueger et al., 1990) which could be the site of viral persistence not necessitating persistent lymphocyte infection (Allday and Crawford, 1988). Regular HHV-6 shedding from this site, as suggested by frequent isolation of infectious virus from saliva (Harnett et al., 1990; Levy et al., 1990), may lead to infection of bystanders, yet not to disease as long as the virus is kept at bay by an effective immune system. This explains that viral spread in the human community occurs usually already during the first year of life without causing significant clinical symptoms. If the immune system will be incompetent to reduce viral spread and replication in newly recruited cells like in transplant recipients and AIDS patients, persistent activity in lymphocytes may become associated with disease just like in EBV infection (Krueger et al., 1983, 1988b; Purtilo et al., 1985; Allday and Crawford, 1987). Were HHV-6 only a lytic virus, extensive necrotic lymphoid lesions should follow (similar to extensive Kikuchi's syndrome). Razzaque and Josephs (1989) and Razzaque (1990) provided suggestive evidence for HHV-6 being able to transform cells as well. Thus malignant lymphomas are a possible consequence of persistent active HHV-6 infections.

There is, so far, no such clear-cut model of HHV-6-induced malignant lymphoma as there is for EBV and the Burkitt's lymphoma. Instead, lymphoproliferative lesions associated with reactivated HHV-6 are quite variable and are frequently of B cell nature although HHV-6 infects apparently T cells (Lusso et al., 1989). This all suggests that the effect of HHV-6 on lymphomagenesis is an indirect one rather than a direct malignant transformation of lymphocytes.

There appears to exist a conceptionally direct relationship between infectious mononucleosis (IM), Kikuchi–Fujimoro's syndrome (KFS), APL and malignant lymphoma (ML) (Krueger et al., 1988b): IM and KFS apparently represent the response of the immunocompetent individual to HHV-6-infected lymphoid cells at different levels of hyperactivity of the immune system and/or different infectious doses. APL and ML, instead, signal a progressive response secondary to persistent viral activity in immunologically incompetent persons. Thus with developing immune deficiency, lymphoproliferation progresses from benign reactive hyperplasia, atypical hyperplasia ('autonomous hyperplasia' as Lennert once called it (Lennert, 1967) or 'progressive hyperplasia' (Robb-Smith, 1975)) to malignant lymphoma. Locker and Nalesnik (1989) provided support for this concept by their investigation of EBV-associated posttransplant lymphomas identifying polymorphic nonclonal tumors, mixed clonal and nonclonal tumors, polymorphic clonal tumors and monomorphic clonal tumors.

Provided that EBV and HHV-6 genomes in lymphoid tissues indicate a pathogenetic relationship to the development of APL and ML, the low number of infected cells even in ML, as well as the variety of morphologic entities similarly associated with EBV and/or HHV-6 remain to be explained. As indicated before, both observations suggest an indirect rather than a direct effect of the viruses in lymphomagenesis such as by polyclonal B cell stimulation (Purtilo et al., 1985;

Spring et al., 1989) and by enlarging the pool of cells susceptible for (another) transforming and oncogenic event. This second oncogenic event may then determine the histologic entity of the ML.

Persistent activity of herpesviruses can explain pronounced non-malignant (or pre-malignant) lymphoproliferation (Dorfman and Warnke, 1974; Weiss et al., 1989). Herpesviruses with a specific lymphotropism such as EBV and HHV-6 may also contribute to immunological dysfunction thus enhancing immune deficiency (Purtilo et al., 1985; Krueger and Ramon, 1988) and favoring their own persistence. This suggested pathogenesis of EBV- and HHV-6-induced lymphomas is well in keeping with George Klein's concept of a multistep mechanism of lymphomagenesis (Klein, 1979, 1986) and it also supports our previous hypothesis of coincident persistent immunosuppression and immunostimulation conditioning the body for lymphoma development (Krueger et al., 1971; Krueger, 1972).

HHV-6 stimulates and infects T lymphocytes of apparently different degree of differentiation (CD38/CD4 cells). Davies et al. (1990) have theorized that T cell stimulation may be an important factor preceding lymphoma development through the production of various cytokines. They cite interleukin-2 (IL-2) as one example for a cytokine related to chronic T cell activation. IL-2 can contribute to cell proliferation by its stimulatory and mitogenic activity for various cells including lymphocytes, T lymphocytes and macrophages (Balkwill and Burke, 1989). It also activates natural killer cells (NK cells) and lymphokine-activated cells (LAK cells), thus exerting a certain control of the proliferation of transformed (atypical) cells and, in addition, IL-2 was shown to inhibit HHV-6 replication in susceptible cell populations (Roffman and Frenkel, 1990).

Contrarily, HHV-6 infection apparently stimulates the production of interleukin-1 (IL-1) and of tumor necrosis factor (TNF) (Flamand et al., 1991). IL-1 and TNF are quite pluripotential cytokines with diverse stimulatory activities for cell proliferation and function including of T and B lymphocytes (Dinarello, 1988).

Interleukin-6 (IL-6) production decreases upon HHV-6 infection (Flamand et al., 1991). On the other hand, we have shown that IL-6 producing and IL-6 receptor carrying cells of established Hodgkin cell culture lines express a p41 antigen cross-reactive (or identical ?) with a HHV-6 protein (Fig. 15.9). Upon HHV-6 infection of such cell lines, rapid transformation of lymphoid blasts occurs to form multinucleated Reed–Sternberg-type cells (Krueger et al., 1991d) (Fig. 15.10). Thus, although no conclusive conceptional connections can be made yet of such alterations in cytokine activities, HHV-6 persistence and progressive lymphoproliferation, their coincidence appears more than incidental and further study is warranted.

There are two more effects of HHV-6 infection on lymphoid cells that may carry some importance with regard to uncontrolled cell proliferation and thus should be mentioned here: changes in cell membrane receptor expression and function, and the potential promotion of double infections of cells. Double infections of lymphoid cells by HHV-6, EBV, HIV and measles were repeatedly shown (Suga et al., 1990). This mechanism may actually support the above-mentioned second oncogenic event, if the superinfecting virus has transforming capacities (such as EBV). In fact,

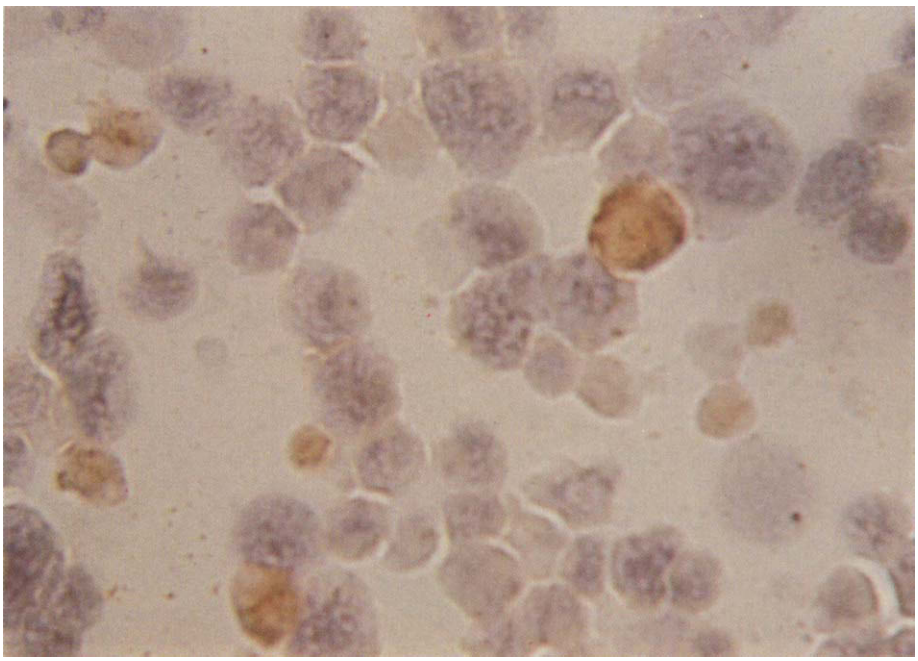
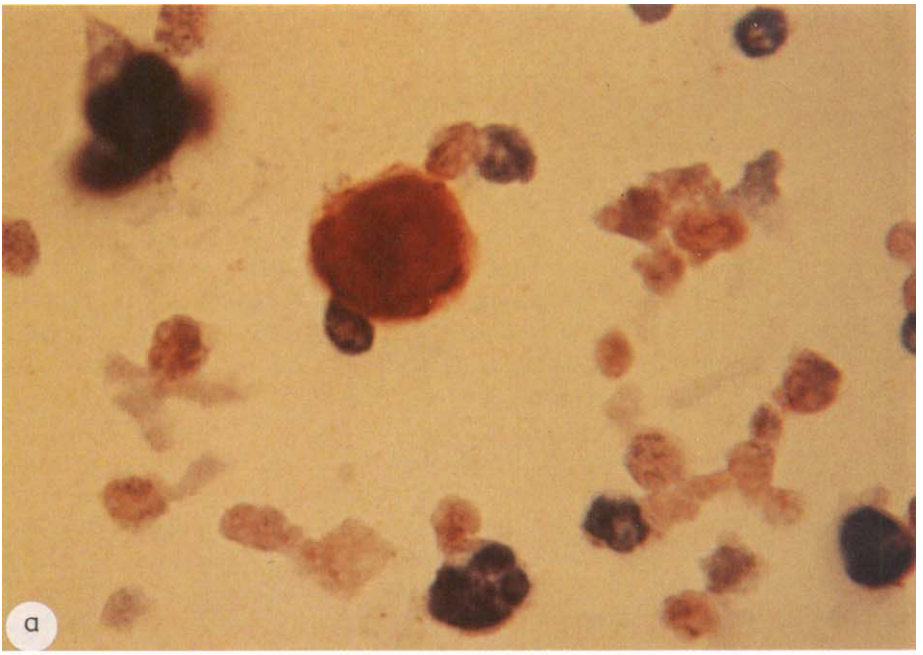


Fig. 15.9. Hodgkin cell line L-428 (Drexler et al., 1989) expressing spontaneously p41 (9A5D12) early antigen of HHV-6 (Balachandran et al., 1989; Krueger et al., 1991d). Note occasional cell showing a positive brown PAP reaction (magnification 1500 ×). (For printing purposes figure has been reduced.)

in a number of ML there is serological evidence for double reactivations of HHV-6 and EBV (Krueger et al., 1989). HHV-6 infection also will alter the expression of cell membrane receptors with probable consequences for cell function (Krueger et al., 1987, 1990). In certain instances, this again may favor superinfection of such HHV-6-infected cells (Krueger et al., 1991c; Schonnebeck et al., 1991). Alteration of cell membrane function and the reduction of the cell's own synthetic activities (Flamand et al., 1991) following HHV-6 infection will render it incompetent to fulfill its natural function. While individual cells will die shedding more infectious virus, the composite lymphoid tissue will become progressively incompetent, and the co-existence of such incompetence with persistent immune stimulation (e.g., by the virus) will cause 'pseudo-reparatory' cell proliferation as described before (Krueger, 1972, 1989).

15.6. Summary and conclusions

There exists a documented significant association of human herpesvirus-6 reactivation and persistent activity with certain lymphoproliferative disorders. It occurs preferentially in patients with some immunological incompetence and resembles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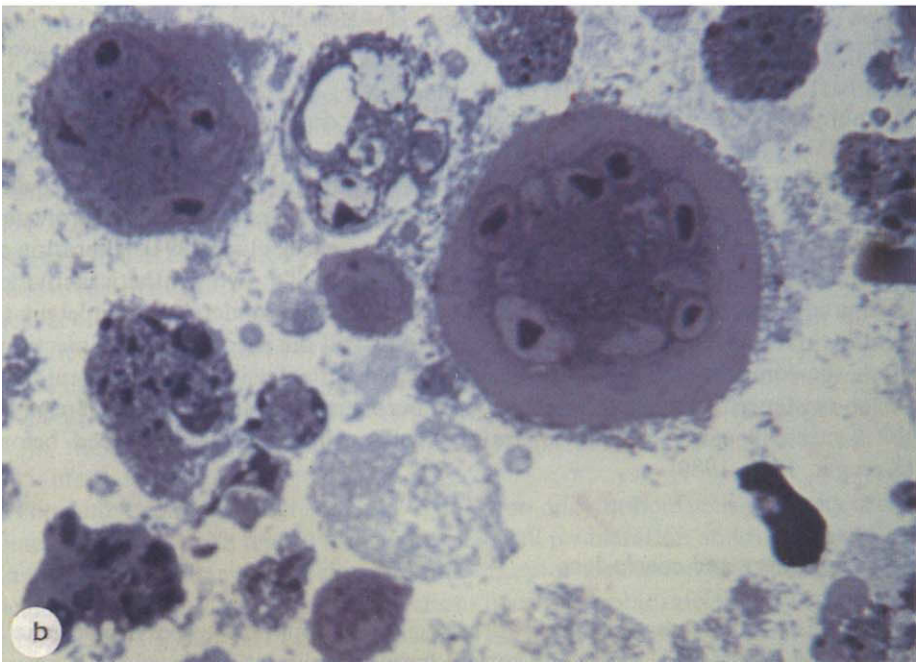
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similar reactions in Epstein–Barr virus infections. Lymphoproliferative patterns may progress from benign polyclonal hyperplasia such as in infectious mononucleosis to progressive polyclonal hyperplasia ('persistent infectious mononucleosis'), mixed polyclonal and monoclonal hyperplasia to monoclonal malignant lymphoma (Krueger et al., 1987, 1991e). Identification of the individual stage of virus associated lymphoproliferation is necessary for therapeutic reasons (Shapiro et al., 1988b; Shapiro and Filipovich, 1989; Krueger et al., 1989) and requires all of the currently available techniques in histology, immunophenotyping of cells and molecular biology (Locker and Nalesnik, 1989).

The infectious dose of the virus and the state of immunocompetence of the infected host determine the kind and the extent of lymphoid tissue changes. Progressive lymphoproliferation with the risk of developing malignant lymphomas occurs preferentially in immunodeficient individuals with persistent viral activity. Lymphoproliferation appears to be a direct effect of polyclonal cell stimulation by viral (glyco-)proteins, a reaction of host cells to viral-infected (and possibly transformed) cells, as well as a result of the production of various cytokines. Monoclonal malignant lymphomas probably arise on the basis of virus-associated imbalanced cell and cytokine responses when an additional oncogenic event will occur. In case of oncogenicity of HHV-6 (like EBV), such oncogenic event may arise from the virus itself.

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Fig. 15.10. Hodgkin cell line L-428 infected by HHV-6 (GS strain) showing a dual effect of rapid cell degeneration and of excessive giant cell formation resembling huge Reed–Sternberg cells. (a) PAP reaction with monoclonal antibody against HHV-6 p41 early antigen shows markedly increased expression of this antigen. (b) Semithin section shows pronounced giant cell formation (magnification 1500 ×). (For printing purposes figure has been reduced.)

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Ablashi/Krueger/Salahuddin (eds) Human Herpesvirus-6

CHAPTER 16

Detection of human herpesvirus type 6 (HHV-6) genomes in lymphoproliferative diseases

KLAUS ELLINGER[1], FRANK NEIPEL[1], STEPHAN SEIDL[2], BETTINA BORISCH-CHAPPUIS[2], KONRAD MÜLLER-HERMELINK[2], and BERNHARD FLECKENSTEIN[1]

[1] Institut für *Klinische und Molekulare Virologie, Universität Erlangen-Nürnberg, W-8520 Erlangen, Germany, and* [2] *Institut für Pathologie, Universität Würzburg, W-8700 Würzburg, Germany*

16.1. Introduction

Human herpesvirus type 6 (HHV-6), designated first as human B lymphotropic virus (HBLV), was originally isolated from American patients with various forms of lymphoproliferative disorders, such as AIDS-related lymphoma, angioimmunoblastic lymphadenopathy, cutaneous T cell lymphoma, immunoblastic lymphoma and acute lymphocytic leukemia (Salahuddin et al., 1986; Lopez et al., 1988). Other strains of the same virus were obtained from severely immunosuppressed individuals in Africa, from Ugandan (strain U1102) (Downing et al., 1987), Zairian (strain Z-29) (Lopez et al., 1988) and Gambian AIDS patients (Tedder et al., 1987), and from a man in South Africa with hairy cell leukemia who was infected with human T cell leukemia virus (Becker et al., 1988, 1989). A distinct, however related herpesvirus, tentatively termed HHV-7, was recovered from peripheral blood T4 lymphocytes of a healthy individual (Frenkel et al., 1990). HHV-6 replicates lytically in primary cultures of stimulated peripheral blood lymphocytes (Black et al., 1989) and macrophages (Levy et al., 1990a) and in a number of T cell-derived human tumor cell lines (Ablashi et al., 1987, 1988).

The genome of HHV-6 is linear ds-DNA of high complexity (Josephs et al., 1986). Mapping of the genome from HHV-6, isolate Z-29, revealed that the virion DNA was 171 kb; a long unique (U_L) region flanked by 12.5–13.5 kb terminal repetitions in tandem orientation (P. Pellett, personal communication). Although the overall genome structure is quite different, HHV-6 DNA shares significant nucleotide sequence homologies with human cytomegalovirus (HCMV), an

important pathogen that is generally taken as prototype of the β-herpesvirus subgroup (Efstathiou et al., 1988; Lawrence et al., 1990; Littler et al., 1990).

Also, serologic cross-reaction seemed to hint at a phylogenetic relationship between HHV-6 and HCMV (Larcher et al., 1988). Other groups, however, assumed that the dual antibody rises to HHV-6 and HCMV that are occasionally seen are probably not the consequence of shared antigenicity (Buchbinder et al., 1989; Chou and Scott, 1990; Irving et al., 1990). Although structural proteins of HHV-6 have been analyzed in a preliminary study (Balachandran et al., 1989), far more work will be required to identify the relevant polypeptides and their antigenic epitopes that need to be identified for improving serodiagnostic procedures; also, importance of strain variability and cross-reactivity for antibody detection needs to be evaluated.

The first serological studies quickly indicated that HHV-6 is widely spread in the world population (Krueger and Ablashi, 1987; Krueger et al., 1987; Linde et al., 1987; Saxinger et al., 1988; Larcher et al., 1989). Children are nearly always infected at the end of the second year of life. The incidence of detectable antibodies declines with increasing age (Briggs et al., 1988; Brown et al., 1988; Okuno et al., 1989); it means that nearly all humans become infected with HHV-6 during the first years of life; however, antibody titers gradually decline below detection level if measured by conventional methods such as indirect immunofluorescence on fixed HHV-6-infected culture cells. Very high titers of HHV-6 antibodies are sometimes found in leukemia and lymphoma patients; they are significantly higher than in primary infection of otherwise healthy individuals (Horvat et al., 1989; Clark et al., 1990). The common occurrence of HHV-6 in the healthy population also becomes evident from the high frequency of infectious HHV-6 in saliva (Pietroboni et al., 1988; Fox et al., 1990; Harnett et al., 1990; Levy et al., 1990b).

HHV-6 causes exanthema subitum (ES), a common mild disease of childhood. ES is always accompanied by primary infection with HHV-6 and seroconversion (Yamanishi et al., 1988; Ueda et al., 1989); the virus can be found in ES by isolation from peripheral lymphocytes (Yoshida et al., 1989) and by polymerase chain reaction (Kondo et al., 1990). HHV-6 was found in lymphatic tissues of patients with Sjögren's syndrome (Fox et al., 1989). It has been hypothesized that HHV-6 may accelerate progression of HIV disease. Simultaneous infection is frequent (Agut et al., 1988) and the same type of cells is infected by both viruses (Lusso et al., 1989). HIV expression can be stimulated by HHV-6 gene products at the level of transcription regulation (Ensoli et al., 1989). However, clinical observations seemed not to prove that destruction of T cells is potentiated by HHV-6 in AIDS patients (Fox et al., 1988). HHV-6 is capable of persisting in renal tissues (Asano et al., 1989). Increased antibody titers in the rejection crisis of renal transplant recipients could hint at a pathogenic role of the virus; this, however, could also be due to concomitant endogenous HHV-6-reactivation in immunosuppression (Morris et al., 1989; Okuno et al., 1990). A causal role has been attributed to HHV-6 in the generation of chronic fatigue syndrome (CFS), a disease entity that can be accompanied by myalgic encephalomyelitis and depression. Increased antibody titers were found in CFS, but causal links have not been proven (Krueger et al.,

1987; Kirchesch et al., 1988; Wakefield et al., 1988). HHV-6 has a pronounced tropism for T cell lymphocytes; the virus replicates in T lymphocytes and it is activated in disturbances of the cellular immune systems. However, its potential role in inflammatory lymph node lesions, lymphoproliferative disorders and/or lymphomas is far from being established (Biberfeld et al., 1988; Jarrett et al., 1988; Niederman et al., 1988; Eizuru et al., 1989). Krueger et al. (1988, 1989) found persistent active HHV-6 infection in parallel to the presence of Epstein–Barr virus in certain types of atypical polyclonal lymphoproliferation (APL) and malignant lymphoma; the same group described HHV-6 infection in monoclonal B cell proliferation (Berthold et al., 1989). We found a rising titer against HHV-6 in a patient with intestinal lymphadenopathy (Borisch-Chappuis et al., 1989), without knowing if HHV-6 had a pathogentic effect by lymphocyte growth stimulation or if it had a mere passenger role in the altered lymphatic tissues. We wanted, therefore, to investigate more closely the possible association between HHV-6 and lymphoproliferative syndromes. Based on a structural study of the viral genome, we established polymerase chain reaction (PCR) and *in situ* hybridization diagnostics to search for viral markers in a broad spectrum of lymphatic disorders.

16.2. Genome structure and taxonomy

To facilitate the search for viral DNA in biopsy materials, we cloned the entire genome of HHV-6, strain U1102 (Downing et al., 1987), as overlapping fragments in cosmid and plasmid vectors. Cleavage maps of the HHV-6 genome were derived for a number of restriction endonucleases, and collinearity to other herpesvirus genomes was determined by partial sequencing. The virus was grown in the lymphoid T cell line HSB_2 (Ablashi et al., 1988) or in cord blood lymphocytes that were stimulated by phytohemagglutinin (PHA) and interleukin-2 (IL-2). Virus particles were purified from culture fluids by sedimentation in sucrose gradients (Fleckenstein et al., 1978). Virion DNA was isolated by isopyknic banding in CsCl density gradients and was partially digested with restriction endonuclease Sau3A. Fragments of about 30 kb were cloned into the unique BamHI site of the cosmid vector pWE15 (Wahl et al., 1987). Genomic ends of HHV-6 were treated with mung bean nuclease prior to cleavage with either PstI or BamHI. These fragments of virion DNA molecules were cloned between the SmaI site and either the BamHI or PstI sites of a plasmid vector. More than 1000 cosmid and plasmid clones were screened by a quick lysis procedure and cleavage with restriction enzymes. We selected a series of 7 clones containing 160 kb of continuous sequence as overlapping fragments. Detailed cleavage maps were constructed for the restriction endonucleases BamHI, EcoRI, NotI, and SmaI by partial and double digestion and cross-hybridization with isolated ^{32}P-labeled fragments (Fig. 16.1). Two plasmid clones, 348–2 and 307–10, were identified to contain the 2 end fragments (Fig. 16.1). Both clones hybridized with the cosmids 220–21 and 228–14. Further mapping of the end fragments showed that the genome of HHV-6 strain U1102 is flanked by direct terminal repeats of 10.5 kb; the unique region between the repeats is 140 kb in

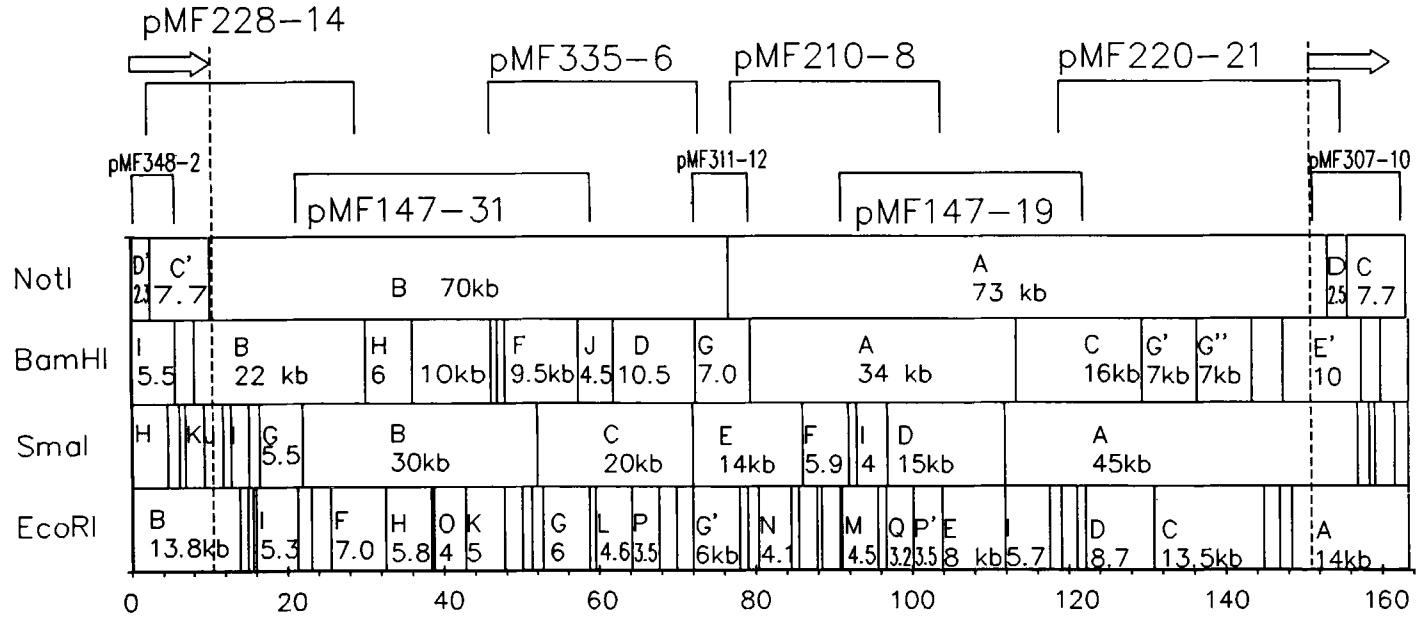

Fig. 16.1. Restriction maps of the HHV-6 strain U1102 genome and genomic position of overlapping cosmid and plasmid clones. The cleavage maps were derived by partial and double digestion mapping and cross-hybridization with ^{32}P-labeled restriction fragments. Arrows indicate sizes of the terminal repeats.

size (Fig. 16.1). To identify map positions of typically conserved herpesvirus genes, 59 short stretches of DNA (290–470 nt) randomly distributed over the genome were sequenced. The ends of cosmid clones and their EcoRI and PstI subclones were used as double-stranded templates for sequencing by the dideoxynucleotide chain termination (method described in detail by Albrecht and Fleckenstein (1990). Sequence data were analyzed by the Genetics Computer Group (GCG) (Madison, Wisconsin) sequence analysis package. Open reading frames (ORFs) of at least 50 amino acids were used for homology searches with the computer program FASTA (Lipman and Pearson, 1985). 12 out of the 59 sequences showed significant homologies to ORFs of HCMV AD169 (Fig. 16.2). Seven of these ORFs belong to blocks of genes that are typically conserved across all of the sequenced human herpesvirus genomes. The HHV-6 reading frames 1–3 were homologous to the HCMV genes UL31–UL33 (Chee et al., 1990). The gene UL32 of HCMV codes for the large structural phosphoprotein pp150 which is a constituent of the virion matrix (Jahn et al., 1987) and is, due to well-conserved and highly immunogenic epitopes, successfully used as antigen for serodiagnostics. The HHV-6 ORF2 shares sequences with the UL33 gene of HCMV that codes for a protein of the G-protein-coupled receptor family. The series of overlapping cosmid and plasmid clones of the HHV-6 strain U1102 will help in further gene mapping and localizing antigenic epitopes for serological diagnostics, and it will allow for the analysis of gene expression patterns. As indicated in Fig. 16.2, all of the 10 HHV-6 ORF sequences are in collinear arrangement relative to the U_L region of HCMV. In contrast, the corresponding genes of other herpesviruses, e.g., herpes simplex virus type 1 and Epstein–Barr virus, are differently arranged relative to the order of HCMV and HHV-6 genes.

Restriction mapping of HHV-6 strain U1102 has confirmed and extended what P. Pellett reported about the overall genome structure of the Z-29 strain. Virion DNA consists of a long unique segment that is flanked by terminal repeats in tandem orientation. A similar gross-structure was found before in the genome of channel catfish herpesvirus (Cébrian et al., 1983), however, not in any other herpesvirus characterized up to now. The genome of strain U1102, which had 161 kb, in our hands appeared significantly shorter than the DNA of HHV-6 isolate Z-29 with 170 kb. Based on nucleotide sequencing of a continuous 21 kb segment of HHV-6 DNA, Lawrence et al. (1990) concluded that HHV-6 is much more closely related to HCMV than to any of the other human herpesviruses. We have shown by sequencing numerous short stretches of DNA that the entire unique region of HHV-6 DNA is mostly collinear with the U_L region of HCMV. At least for the left end of the HHV-6 genome, this relationship extends beyond the gene blocks that were found conserved in all herpesviruses. In general, it is consistent with serological studies that found antigenic relationship between HHV-6 and HCMV and identified cross-reactive epitopes (Larcher et al., 1988; Littler et al., 1990). HCMV is generally taken as prototype of the β-herpesvirus subgroup (Roizman, 1982; Honess, 1984). Sequence homologies, collinear genome organization, and antigenic cross-reactivity appear to justify classification of HCMV and its close relatives in a β_1-herpesvirus subgroup, and designating HHV-6 as a β_2-herpesvirus.

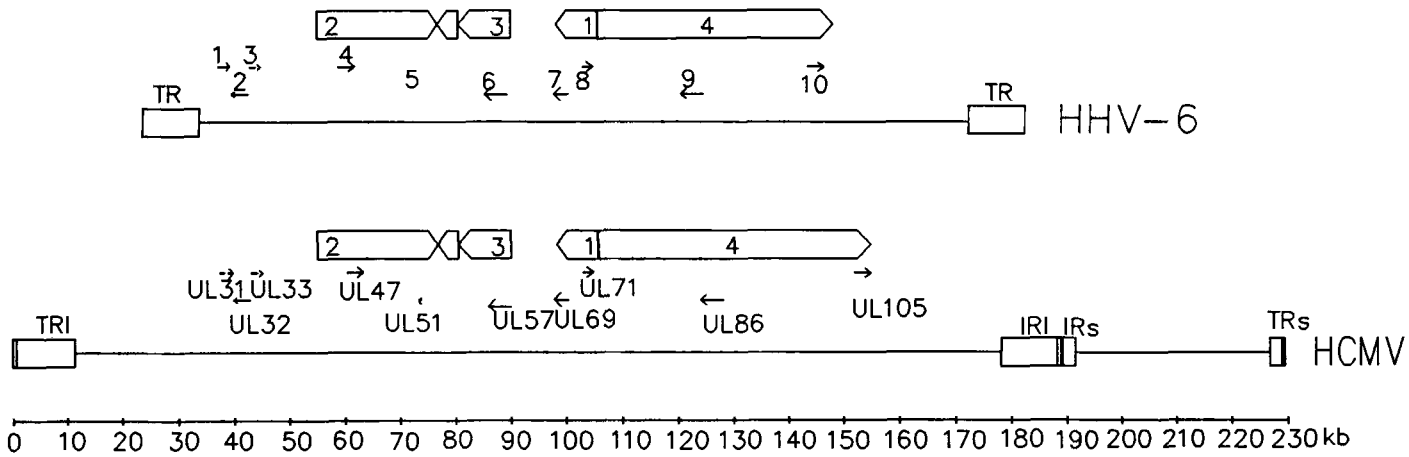

Fig. 16.2. Genomic organization of human herpesvirus type 6 (HHV-6) and human cytomegalovirus (HCMV). Repeat structures are indicated by boxes, conserved blocks of genes (1–4) are symbolized by hatched arrows. The figure was conceived following Gompels et al. (1988). All reading frames of HHV-6 (#1–#10) appear collinear in genomic position and orientation to the homolgous genes of HCMV (UL31–UL105) (Chee et al., 1990). TR represents terminal repeat of HHV-6; TRl terminal repeat large; TRs terminal repeat small; IRs internal repeat small; and IRl internal repeat large of HCMV.

16.3. PCR and *in situ* hybridization for virus detection in biopsies.

Polymerase chain reaction (PCR) was established in order to screen for HHV-6 DNA in biopsy materials from patients with various forms of lymphatic disorders. *In situ* hybridization was applied to localize viral DNA sequences in the PCR-positive biopsy tissues. A PstI-fragment of 1060 bp (pKE-M) from the EcoRI-I-fragment was sequenced and chosen to establish PCR. It falls into an ORF. Computer comparisons revealed no sequence homology of this ORF with HCMV or other human herpesviruses. Two primer pairs of 24 and 33 nucleotides, respectively, were used (Fig. 16.3), the last with a synthetic BamHI-site at the 5'-end to allow convenient cloning of amplified fragments (Fig. 16.3). Synthesis results in DNA products of 305 or 295 bp, respectively. Whole cellular DNA from fresh biopsy materials or from cell cultures was isolated by standard methods. Samples of 1–2 µg cellular DNA were used for PCR; the DNA was amplified in a total volume of 100 µl with 2.5 U Taq polymerase (Perkin Elmer), 200–400 µmol dNTP, and 1 µg of each primer. Amplification was performed in 40 cycles, denaturating for 30 s at 93°C, renaturating at 55°C for 40 s, and polymerizing for 40 s at 72°C. Aliquots of 10 µl were analyzed for HHV-6 DNA by agarose gel electrophoresis. The reaction was confirmed by Southern blot hybridization with a ^{32}P-labeled oligonucleotide (20 nt) that hybridizes within the amplified DNA region. Specifity of the primers for the PCR was evaluated by using DNA from cells infected with the 4 other human herpesviruses (HCMV, HSV-1, EBV, VZV); no cross-reaction occurred. Other strains of HHV-6 (Z-29, R104) always gave positive signals. Sensitivity of the reaction was monitored by amplification from dilutions of plasmid pKE-M DNA (Fig. 16.4).

In order to localize infected cells in PCR-positive tissues, biopsies were screened by *in situ* hybridization. Deparaffinized sections were mounted on glass slides and denaturated in 0.2 N HCl and probed with various ^{35}S-labeled plasmid clones. Hybridization was performed in 2 × SSC (SSC = 0.15 M sodium chloride, 0.015 M sodium citrate) and 50% formamide, followed by washing with 4 × SSC at 42°C, 2 × SSC at 37°C and 0.2 × SSC at 20°C. The slides were counterstained with hematoxylin and eosine (HE). Human cord blood lymphocytes (CBL) infected with HHV-6 were used as positive controls. Hybridization of infected cells with a HCMV-specific probe and with vector DNA was used as negative control.

16.4. HHV-6 DNA in tissue from lymphoproliferative diseases

Biopsy materials from 31 patients with disorders of the lymphatic system were screened for the presence of HHV-6 sequences. The probes were taken from 16 non-Hodgkin lymphomas, 6 tissues of Hodgkin's disease, 2 thymomas and 1 case of acute infectious mononucleosis (Table 16.1). Most of these biopsies were free of HHV-6 DNA. Only 2 out of 16 non-Hodgkin's lymphomas were positive by PCR. They remained, however, negative by *in situ* hybridization. HHV-6 DNA was found in 4 (out of 6) lymphoproliferative lesions that were difficult to classify. We

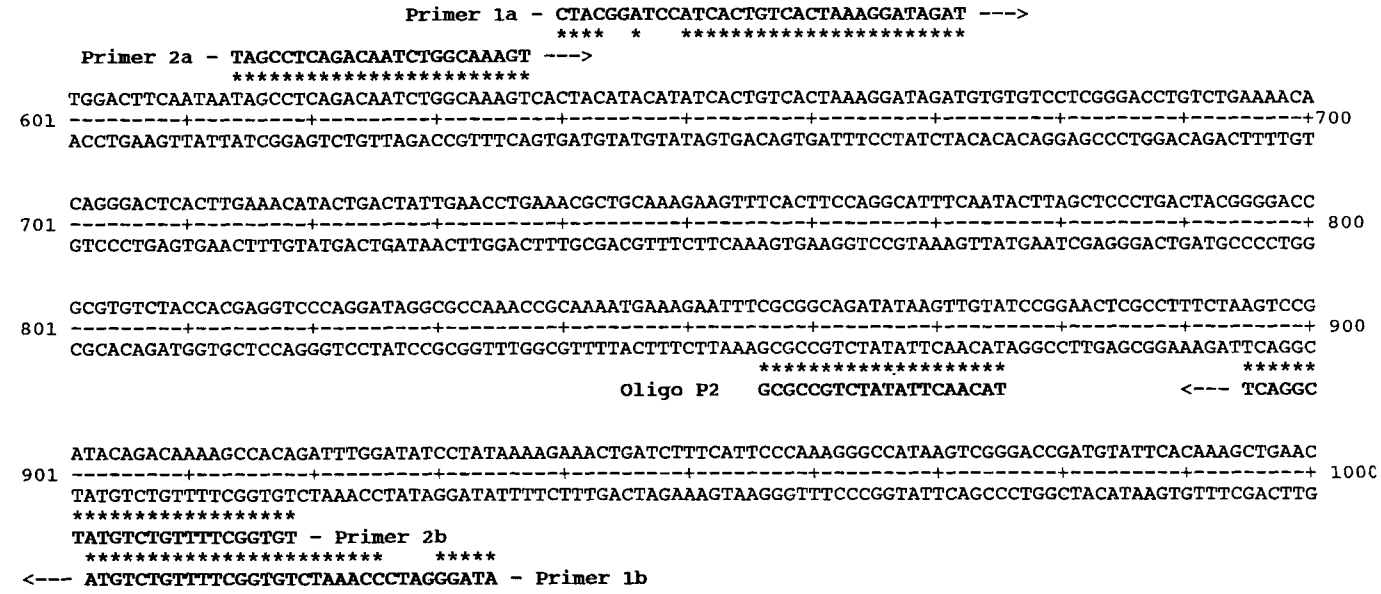

Fig. 16.3. Nucleotide sequences of the primers used for polymerase chain reaction and the oligonucleotide probe applied for the detection of amplification products C * indicating perfect matches). The genomic HHV-6 sequence represents part of the plasmid pKE-M containing 1.06 kb virion DNA (Map position EcoRI-fragment I; Fig. 16.1)

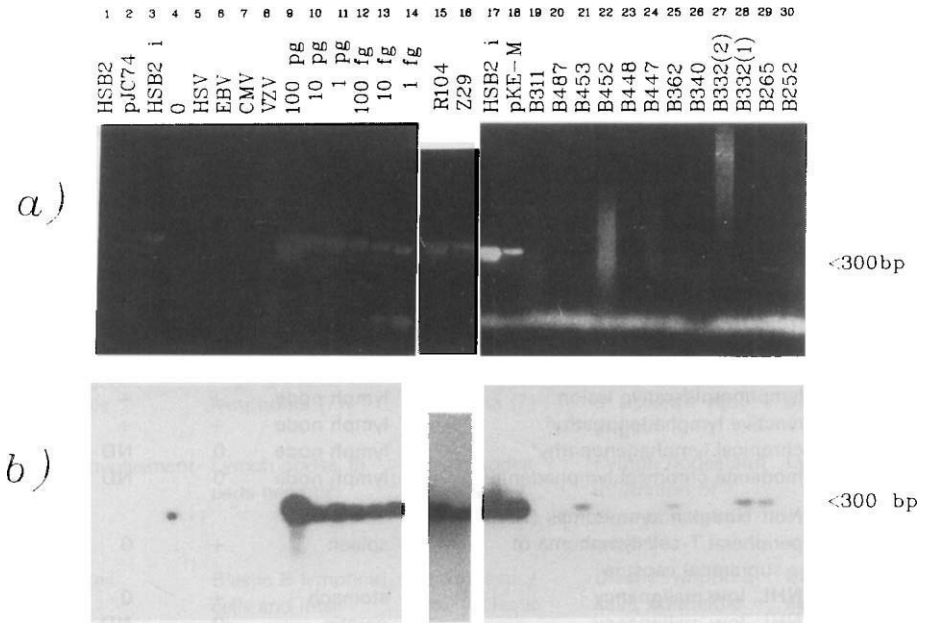

Fig. 16.4. Polymerase chain reaction for the detection of HHV-6 DNA sequences. Amplification products were separated by electrophoresis through 2.5% agarose and stained with ethidium bromide (panel a) or were visualized by Southern blot hybridization with a ^{32}P-end-labeled oligonucleotide (panel b). Lane 1: uninfected HSB_2 cells (100 ng DNA); lane 2: cosmid vector pJC74 (100 ng) as a negative control; lane 3: HSB_2 cells infected with HHV-6 strain U1102 (100 ng); lane 4: no DNA; lane 5: Vero cells infected with herpes simplex virus type 1 (100 ng); lane 6: Epstein–Barr virus carrying Raji cells (100 ng); lane 7: cytomegalovirus-infected human fibroblasts (100 ng); lane 8: varicella–zoster-virus-infected human fibroblasts (100 ng); lanes 9–14: decreasing amounts of the plasmid pKE-M containing 1 kb of HHV-6 DNA (see Fig. 16.3) (100 pg to 1 fg); lane 15: cord blood lymphocytes infected with HHV-6 strain R104 (100 ng); lane 16: cord blood lymphocytes infected with HHV-6 strain Z-29 (a gift from P. Pellett, 100 ng); lane 17: HSB_2 cells infected with HHV-6 strain U1102 (100 ng); lane 18: plasmid pKE-M (100 pg); lanes 19–30: biopsies as listed in Table 16.1.

want to describe those 4 cases of lymphoproliferative syndromes and their histopathology (Table 16.2).

A 72-year-old man (Ku) presented with a localized cervical lymph node swelling without fever or weight loss. Paraffin and cryostat sections of the lymph node biopsy could not clearly define differential diagnosis of high-grade non-Hodgkin's lymphoma of germinal center origin or reactive lymphoproliferation. One of the lymph nodes showed a subtotal necrosis (Fig. 16.5a). Histology revealed pleomorphic proliferation of blastic B lymphoid cells, starting in the germinal centers and infiltrating into the pulp (Fig. 16.5b); this normally indicates malignant lymphoma. The blastic cells, examined by immunohistochemistry, had typical pan-B cell markers such as L26 (Fig. 16.5c). Few blastic cells expressed T cell antigens such as CD2, CD4 and CD8. Southern blot with T cell receptor probes showed rearrangement, indicating oligo- or monoclonal T cell proliferation. No rearrangements of

TABLE 16.1.
Biopsy materials from lymphoproliferations and lymphoma tested for HHV-6 DNA by PCR and, in part, by *in situ* hybridization

Biopsy-number	Diagnosis	Organ	PCR	*In situ*
	Lymphoproliferations			
602	lymphoproliferative lesion (diff. diagnosis: B cell lymphoma, high malignancy)	lymph node	+	+
362	lymphoproliferative lesion; (diff. diagnosis: B cell lymphoma, high malignancy)	lymph node	+	+
265	lymphoproliferative lesion	lymph node	+	+
332	reactive lymphadenopathy[a]	lymph node	+	+
340	chronical lymphadenopathy[a]	lymph node	0	ND
449	moderate chronical lymphadenitis	lymph node	0	ND
	Non-Hodgkin Lymphomas (NHL)			
447	peripheral T-cell-lymphoma of suprarenal capsule	spleen	+	0
487	NHL, low malignancy	stomach	+	0
311	NHL, low malignancy	parotis	0	ND
310	NHL, low malignancy	parotis	0	ND
486	NHL, low malignancy	stomach	0	ND
252	NHL, low malignancy	stomach	0	ND
448	NHL, immunoblastic lymphoma, low malignancy	stomach	0	ND
488	NHL, high malignancy	stomach	0	ND
489	NHL, high malignancy	stomach	0	ND
492	NHL, high malignancy	stomach	0	ND
493	NHL, high malignancy	stomach	0	ND
341	NHL, high malignancy	parotis	0	ND
538	NHL, high malignancy		0	ND
556	peripheral T cell lymphoma		0	ND
490	Ki1-lymphoma, high malignancy	stomach	0	ND
537	Ki1-lymphoma, high malignancy	lymph node	0	ND
452	thymoma, mixed type	thymus	0	ND
453	thymoma, mixed type	thymus	0	ND
	Hodgkin's Disease (HD)			
376	HD, mixed type	lymph node	0	ND
377	HD, mixed type	lymph node	0	ND
379	HD, mixed type	lymph node	0	ND
378	HD, nodular sclerosis	lymph node	0	ND
484	HD, nodular sclerosis	lymph node	0	ND
483	HD, lymphocyte rich, nodular paragranuloma	lymph node	0	ND
539	infectious mononucleosis	lymph node	0	ND

[a] = With progressive transformation of germ centers.

TABLE 16.2.
Symptoms and pathology of HHV-6-associated lymphoproliferations

Patient	602 Ku.	362 Pi.	265 Ba.	332 Ha.
Sex	m	f	m	m
Age	72	70	57	19
Clinical diagnosis	Cervical lymph node swelling	Cervical lymph node swelling	Cervical lymph node swelling, generalized lymph-adenopathy	Enlarged lymph node of the neck
Outcome	Remission after chemotherapy	Remission after chemotherapy	Death of septic shock	Recovery
Histological diagnosis	High grade B cell lymphoma (?)	High grade B cell lymphoma (?)	Lympadenopathy of possible viral origin	Reactive process
Organ involvement	Lymph nodes, in parts necrotic	Lymph nodes	Lymph nodes and infiltration of parenchymatous organs	Lymph nodes
Cell types	Blastic B lymphoid cells and inter-follicular T cells	Centroblastic/ immunoblastic cells	Blastic lymphoid cells, epitheloid cells, monocytotic cells	Blastic B and T cells
Clonalit	Oligo- or mono-clonal T cells, polyclonal B cells	Monoclonal B cells	Polyclonal	Polyclonal
PCR	+	+	+[a]	+
In situ	+	+	+[a]	+
Serology	1:64	1:32	ND	ND

[a] Also positive with EBV DNA probe.

B cell receptors (JH, CK) were found. IgG antibodies against HHV-6 were detected in serum samples of the patient by indirect immunofluorescence at low titer. The patient received local radiotherapy. Enlarged lymph nodes disappeared completely within a short time and the patient has recovered completely up to now (8 months).

In the second case, a 70-year-old woman (Pi) with cervical lymph node swelling, biopsy material was classified first as a B cell type lymphoma of high grade malignancy. Histology revealed a diffuse proliferation of blastic lymphoid or immunoblastic cells in large parts of the lymph node tissue. Preserved follicles were seen in other areas. This case revealed clonality of B cells by JH-rearrangement. The patient received chemotherapy (3 cycles of COP-BLAM) and adjuvant radiotherapy. This led to complete remission and the patient has felt well up to now.

The third case (Ba) also did not allow easy classification. A 57-year-old man suffered from cervical lymph node enlargement in 1984 with the histology of lymphadenopathy that disappeared spontaneously. The patient was well for 2

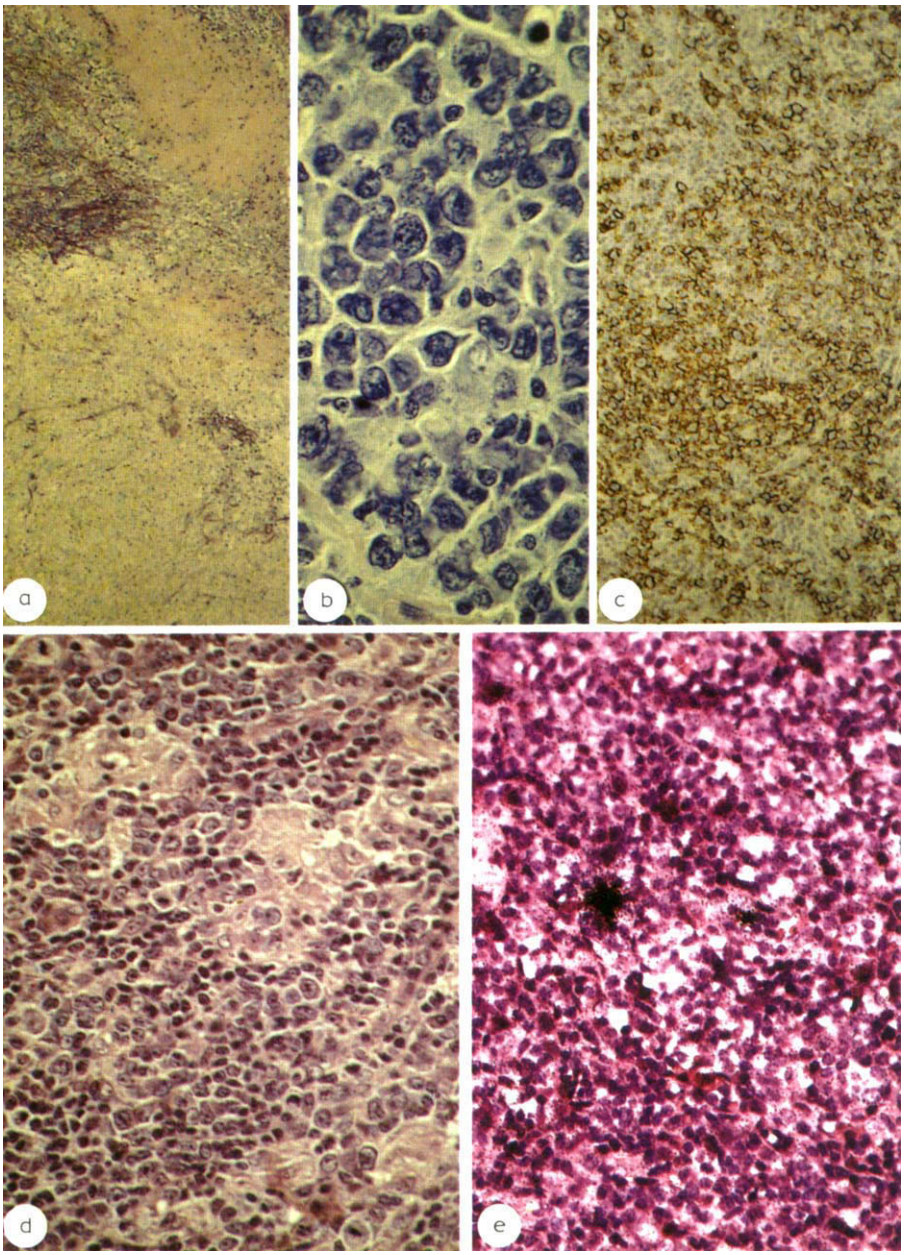

Fig. 16.5. (a)–(c) Histopathology of lymph node tissue from patient Ku. (d)–(e) Lymph node tissue from patient Ba. (a) Lymph node with subtotal necrosis (H&E). (b) Blastic B lymphoid cells infiltrating into the pulp (Giemsa). (c) Blastic cells were identified with a monoclonal antibody against the pan-B cell marker L26 and peroxidase-labeled polyclonal antibodies. (d) Morphology of angioimmunoblastic lymphadenopathy (H&E). (e) Proliferating lymphatic tissue after *in situ* hybridization with ^{32}P-labeled HHV-6 DNA (counterstained with H&E).

years until another lymph node swelling appeared. At this time, histology suggested angioimmunoblastic lymphadenopathy. Later on, further lymph node enlargement developed and more biopsies were taken. Histology was described as possibly virus-induced lymphoproliferation with small groups of epitheloid cells and small areas of necrosis. At the same time, lymphoid cells with a morphology typical for some virus infections were found in the peripheral blood. The general status worsened rapidly, and the patient died of septic-toxic shock 3 months later. Autopsy showed a moderate generalized lymphadenopathy and lymphoid infiltration of liver, spleen and lung, as seen in infectious mononucleosis. The proliferation appeared polyclonal. PCR for HHV-6 was positive, and at the same time *in situ* hybridizations showed EBV-DNA as well as HHV-6 DNA in the proliferating lymphatic tissue (Fig. 16.5e). Intensive labeling appeared above scattered elements that were mostly described as blastic lymphoid cells.

The fourth patient, a 19-year-old man (Ha), presented with a nuchal lymph node that remained enlarged. Biopsy showed a reactive process as can be indicative of viral infection; the cells were characterized as blastic B and T lymphoid elements. Marker studies indicated polyclonal proliferation. The patient recovered spontaneously without specific treatment.

Clinical features and pathology of all 4 cases that were positive by PCR and *in situ* hybridization are summarized in Table 16.2. Histology showed a clear-cut B cell lymphoma in 1 case, polymorphic blastic proliferations in 2 cases, nearly destroying the normal structure of the lymph node, and reactive lymphadenitis probably of viral origin in 1 case. Additional features were sometimes necrosis, epitheloid cell clusters and immature sinus histiocytosis. Oligoclonal or monoclonal growth was found in 2 cases as shown by B or T cell gene rearrangement studies. In part, differential diagnosis between malignant lymphoma and reactive lymphoproliferation could not be established by histology alone. All of these cases showed the same pattern of *in situ* hybridization, indicating that viral genomes were present in a few scattered cells at high multiplicity. The role of HHV-6 infection in the pathogenesis of these peculiar lymphoproliferative diseases is not clear to date. Larger series will be necessary to show if they typically correlate with active HHV-6 infection. Possibly, certain kinds of polyclonal lymphoproliferation can be accompanied by endogenous HHV-6 reactivation and replication of the virus in a small subset of cells. Alternatively, HHV-6 might contribute to certain types of virus-induced lesions that are mimicking or even developing into malignant lymphomas. Lymphoproliferation may be a cause or consequence of HHV-6 replication. Another type of disease condition may possibly be defined by the presence of the virus that is usually self-limited and may represent an acute viral induced lymphadenitis.

16.5. Conclusions

Shortly after HHV-6 had been detected some 4 years ago, generally applicable procedures for virus isolation and serodiagnostics have been established, helping to

quickly understand epidemiology and some patterns of virus transmission. The virus ubiquitously occurs, infecting most children prior to the end of the second year. Seroconversion profiles and detection of the virus in blood and saliva clearly showed that exanthema subitum, a benign disease of childhood is causally linked to primary infections with HHV-6. Numerous questions, however, have remained open concerning pathogenicity and the potential role of HHV-6, persistence and endogenous reactivation. So far, the evidence has remained tenuous linking HHV-6 with chronic fatigue syndrome, Sjögren's disease and rejection crisis after renal transplantation. On the other hand, the unambiguous PCR-detection of HHV-6 DNA in biopsy samples from certain forms of lymphoproliferative syndromes calls for further elucidation of causal relationships. PCR may help to provide new diagnostic markers to differentiate those processes from malignant lymphomas (Hodgkin's or other types). It will not prove, however, if persisting or reactivated HHV-6 contributes to growth stimulation or if the virus is merely reactivated under certain forms of immunosuppression. Establishing cell lines from HHV-6-positive lymphoproliferative diseases and precise definition of virus-carrying cells by double labeling methods (immunohistochemistry and *in situ* hybridization) may help to identify the targets of the virus, if it causes lymphoproliferation and, possibly, clonal amplification. Moreover, it will be necessary to identify genes that are relevant for regulation of latency and persistence in lymphoid elements and, possibly, for dysregulated cell growth.

So far, first gene mapping of HHV-6 has only resulted because of computer comparisons with known genes of 4 other better-characterized human herpesviruses. The HHV-6 genome is similar in overall structure to the DNA of channel catfish herpesvirus (CCH) that, according to all other criteria, is entirely unrelated; CCH probably belongs to the α-herpesviruses. On the other hand, HHV-6 shares a cell tropism with gamma-herpesviruses; replication in culture seems restricted to primary lymphocyte culture or to tumor-derived T cell lines. Apparently, this cell tropism is not a taxonomic criterion. HHV-6 is much more closely related to cytomegalovirus, the human β-herpesvirus prototype; this is clear by sequence analyses of individual genes and from the striking collinearity in homologous reading frames. It seems to justify classification of HHV-6 as the first known member of a new β_2-herpesvirus subgroup.

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CHAPTER 17

Detection of HHV-6 using polymerase chain reaction amplification

RUTH JARRETT

Leukaemia Research Fund Virus Centre, Department of Veterinary Pathology, University of Glasgow, Bearsden, Glasgow G61 1QH, U.K.

17.1. Introduction

Over the last few years, the polymerase chain reaction (PCR) has been used increasingly for diagnosis as well as for studying the biology of viruses. As a means of detection of viral infection, PCR has many advantages over conventional assays. First, the technique directly detects the viral genome rather than the host's immune response to the virus. It avoids the technical difficulties and time involved in tissue culturing. Secondly, many different sample types, from body fluids to archival, paraffin-embedded specimens, can be analyzed. Thirdly, the reaction is extremely sensitive especially when compared to more traditional means of detecting viral nucleic acid. The main disadvantage of PCR is that low level contamination of samples, especially from carry-over of reaction products, can easily lead to false positive results unless extreme care is taken.

Several groups have used PCR to investigate the epidemiology and biology of herpesviruses, including HHV-6. For the detection of HHV-6, these studies can broadly be divided into 2 groups. First, those which have focused on the use of PCR to detect persistent and/or latent HHV-6 infection in healthy individuals. Secondly, those which have looked for an association between the virus and disease in various pathological specimens. Another application of PCR in the investigation of HHV-6 as well as other new herpesviruses is the use of conserved sequences among related viruses to amplify, clone and sequence related genes in these newer viruses. Among herpesviruses, this has been most widely used in the cloning of the polymerase gene.

The primer and probe sequences which have been used to detect HHV-6 are listed in Table 17.1. The choice of primer sequences has been limited until very

TABLE 17.1.

Oligos 5'	Sequence 3'	Product (bp)	Source Ref/strain
5'primer	TTCAGGGAC CGT TAT GTC ATT GAG CAT GTC G	123	Buchbinder et al. (1988)/GS[a]
3'primer	CCC ATT TAC GAT TTC CTG CAC CAC CTC TCT GC		
Probe	CTT GTC CGT AGA TGG AGT GGC		
5'primer	CCT TCT TAG CCA CTG CAC CC	500	Qavi et al. (1989)/GS[a]
3'primer	CTT GTC CGT AGA TGG AGT GGC		
Probe	PZVH14		
5'primer	GTG TTT CCA TTG TAC TGA AAC CGG T	776	Kido et al. (1990), Kondo et al. (1990)/ Hashimoto strain
3'primer	TAA ACA TCA ATG CGT TGC ATA CAG T		
Probe	SAL 1 CLONE OF HASHIMOTO STRAIN		
5'primer (H6-6)	AAG CTT GCA CAA TGC CAA AAA ACA G	223	Gopal et al. (1990)/U1102
3'primer (H6-7)	CTC GAG TAT GCC GAG ACC CCT AAT C		
Probe (H6-6/7)	AAC TGT CTG ACT GGC AAA AAC TTT T		
5'primer (LVC9)	TCT CAC AGC CCA GGA CAA TGG ATT ATA TAT	161	Jarrett et al. (1990)/GS[a]
3'primer (LVC10)	TGA GAT CAT TCT CCC GTT CTT GAG GG		
5'nested (LVC46)	TCC ACT ACT TAA AAC CGA	90	
3'nested (LVC47)	TGA TGA AGT ATG TGA TAG		
Probe	CTT CCA TCG AGG CCT CAT CTA TCA CAT ACT		

[a] These sequences were all derived from the plasmid pZVH14 published by Josephs et al. (1986).

recently by the availability of HHV-6 sequence data (Chapter 8, this volume). In all assays, attempts have been made to show that the primers are specific for HHV-6 and do not amplify other known herpesviruses. Sample types and reaction conditions have varied, therefore the results of different studies are not necessarily directly comparable. The use of PCR has yielded some consistent results which have widened our knowledge of the biology of HHV-6.

17.2. Detection of HHV-6 in healthy individuals

Serological studies have suggested that infection by HHV-6 is both widespread and common. The majority of individuals are seropositive by the age of 1 year, therefore infection occurs at a very young age (Chapter 2, this volume). These data suggest that the virus is transmitted efficiently and that there is a large reservoir of viral infection. Consistent with other herpesviruses, it seemed likely that following primary infection, HHV-6 persisted in a latent form in the host and was intermittently shed in body fluids. Both of these characteristics have been supported by information acquired using PCR amplification and detection.

HHV-6 was first isolated from the peripheral blood of patients with lymphoproliferative and immunosuppressive disorders (Salahuddin et al., 1986). The virus can infect a variety of cell types *in vitro*, but is most easily grown in T cell lines (Downing et al., 1987; Lusso et al., 1988) thus suggesting that the virus may be tropic for lymphocytes or perhaps monocytes *in vivo*. Three groups of workers have looked for HHV-6 genomes in the peripheral blood of normal, healthy individuals. Gopal et al. (1990) detected HHV-6 sequences in 49% of their samples. DNA extracted from peripheral blood mononuclear cells (PBMCs) was used as template, and 40 cycles of amplification were performed using standard reaction conditions. Dilution studies showed that DNA from the equivalent of 10^4–10^5 cells was required for specific amplification of HHV-6. Jarrett et al. (1990) could detect HHV-6 sequences in PBMCs from the majority of healthy individuals. This was, however, critically dependent on the number of cells assayed and the PCR conditions. Using 5×10^4 cells per reaction and 40 cycles of amplification, only 2 of the 10 samples were positive. When the cell numbers were increased to 5×10^5 cells per reaction, and a second round of amplification, using nested primers, was added (Table 17.1), 70% of the samples were positive. A titration experiment performed on 1 volunteer suggested that between 5×10^4 and 5×10^5 cells were required to reliably detect HHV-6.

Kondo et al. (1990) assayed samples from the peripheral blood of healthy individuals as well. These samples, however, were controls in a study designed to look for HHV-6 in exanthem subitum patients (see below). Using 100 ng of DNA (equivalent to the content of 1.5×10^4 cells) per reaction and 30 cycles of amplification, they did not detect HHV-6 DNA sequences in any of their healthy seropositive controls. The primers used did detect at least 8 different isolates, making it unlikely that the inability to detect HHV-6 was due to sequence variability. The lack of detection of the virus in these cases most likely reflects the small amount of template used in the reaction.

Although the virus has frequently been isolated from PBMC obtained from patients with HIV and patients with lymphoproliferative diseases (Salahuddin et al., 1986; Downing et al., 1987), it has very rarely been isolated from PBMC of healthy individuals (Lopez et al., 1988). This suggests that the virus detectable by PCR in healthy persons is in a latent state. These PCR studies suggest that HHV-6 genomes, although present in the PBMCs of the majority of healthy individuals, are probably in very few circulating cells. Sufficient DNA must be present and a sensitive assay used, in order to detect viral nucleic acid by PCR.

Gopal et al. (1990) and Jarrett et al. (1990) also used PCR to investigate saliva samples of healthy individuals for the presence of HHV-6. Gopal et al. used DNA extracted from cells recovered from saline gargles, while Jarrett et al. used crude saliva samples. In the former study, 63% of samples were positive, and in the latter, 90%. In both series, individuals were identified who were seronegative but had PCR-detectable HHV-6 in saliva. This may simply reflect the cut-off values used in serological experiments, but interestingly, this combination of results was not found in assays for Epstein–Barr virus (EBV) (Gopal et al., 1990). A third study reported the detection of HHV-6 in throat swabs by PCR. Overall, they reported only 1 of 30 adults, 2 of 9 adults with common cold, 2 of 10 infants with exanthem subitum and 4 of 39 febrile children to have detectable HHV-6 DNA by this method (Kido et al., 1990). The conditions and primers used for PCR are the same as those used by Kondo et al. (1990) and discussed below. The lower level of detection by this method could be due to sampling methods. The small amount of starting material on a throat swab may not provide enough template for adequate amplification.

Attempts to directly isolate HHV-6 in saliva by tissue culture have resulted in high rates of detection as well (Pietroboni et al., 1988; Harnett et al., 1990; Levy et al., 1990). In 2 of these studies (Pietroboni et al., 1988; Levy et al., 1990) the virus was isolated by co-cultivation of saliva with adult PBMCs. While is it possible that the HHV-6 might have been reactivated from the PBMCs used for culture, in at least 1 of the studies, the PBMC cultured without saliva failed to yield any virus (Levy et al., 1990).

In addition to these studies, Fox et al. (1990), using a combined approach of *in situ* hybridization and immunohistochemistry, have shown that HHV-6 is present in salivary glands. All of 9 submandibular glands and 1 of 4 parotid glands were positive for both HHV-6 genomes and for HHV-6 proteins. Using a similar approach, Krueger et al. (1990) detected HHV-6 in labial salivary glands and in transbronchial specimens.

Overall, the results suggest that HHV-6 establishes a persistent infection in salivary glands and is shed intermittently in saliva. It would seem likely that saliva is a vehicle for transmission of this virus.

17.3. Detection of HHV-6 in disease

PCR has been used to assay for HHV-6 sequences in diverse pathological conditions. Buchbinder et al. (1988), who were the first to use HHV-6 PCR, assayed PBMC samples from patients with AIDS. Using 1 μg of genomic DNA as template and 25 cycles of amplification, they detected HHV-6 sequences in 52/63 (83%) of

samples. This study was performed prior to the full appreciation of carry-over problems, however, internal negative controls were included in the analysis. Gopal et al. (1990) also analyzed DNA from PBMC of asymptomatic and symptomatic HIV-infected persons. They detected HHV-6 genomes in 29% of the former and 6% of the latter; both less than healthy controls. However, the average number of lymphocytes assayed was lower in the HIV-positive patients. In the same study, the frequency of EBV detection was greater in the HIV-positive compared to the HIV-negative samples. A number of possibilities exist for this finding including a lower number of appropriate lymphocytes in the samples from HIV-positive individuals due to the cytolytic effects of HIV infection alone or in combination with HHV-6. It, therefore, remains possible that the HHV-6 viral load is altered following HIV infection and accurate quantification of both starting material and PCR products will be required to clarify this.

Buchbinder et al. (1988) assayed DNA samples from lymph nodes obtained from patients with lymphoproliferative disorders and reactive hyperplasias. 20 out of 23 (87%) samples were positive. This includes 1 sample which had been shown (Josephs et al., 1988) to contain HHV-6 DNA by Southern blot analysis. *In situ* hybridization was carried out on the latter sample, and only 1 in 1000 cells was found to be positive for HHV-6 mRNA, indicating that the tumor cells were not all expressing HHV-6 (Buchbinder et al., 1988). Krueger et al. (1989), using *in situ* hybridization, have also demonstrated HHV-6-infected cells in lymph node biopsies from a large number of patients with lymphoproliferative disease. HHV-6 was not detected in any malignant cells, thus, if HHV-6 plays any role in the pathogenesis of these conditions, it is likely to be indirect. This highlights the problem of interpreting the results when a technique as sensitive as PCR is used to assay samples which are likely to contain latently infected cells.

More recently Torelli et al. (1991), using PCR amplification, showed that HHV-6 DNA was present in 3 of 25 lymph nodes from patients with Hodgkin's disease and none of 41 non-Hodgkin's lymphomas. All positive cases for HHV-6 sequences were nodular-sclerosing/lymphocyte depleted histologic subtype. 2 of the 3 PCR-positive lymphomas were also positive by Southern blot analysis, suggesting a greater viral burden and perhaps of greater significance than PCR positivity alone.

HHV-6 has been shown to be a causative agent of exanthem subitum, a febrile disease of infancy (Yamanishi et al., 1988). The virus can be isolated from 67% of patients with clinically diagnosed exanthem subitum (Kondo et al., 1990). Using PCR, Kondo et al. were able to detect HHV-6 in PBMCs from 7 out of 8 patients during the acute phase of the disease. The single negative patient, in contrast to the other patients, had no detectable antibody to HHV-6 during the convalescent phase of the disease. The PCR in this study utilized 100 ng of template DNA and 30 cycles of amplification. As mentioned above, all of the healthy controls were negative under these conditions. PCR performed on convalescent samples gave positive results, and although accurate quantification was not carried out, the viral load appeared to have decreased. A more recent report by the same group found that 70% of patients with exanthem subitum and neurological symptoms had PCR-detectable HHV-6 in the cerebral spinal fluid (CSF). Rates of detection in the CSF in children without neurological symptoms or their healthy counterparts are

not known and therefore the significance of such a finding remains in question. These studies do provide further evidence for a causal association between HHV-6 and exanthem subitum, and demonstrates that PCR could be a useful method of detecting HHV-6 infection during the acute phase of the disease especially when the reaction conditions are designed to limit the level of detection. However, the high incidence of presumably latent infection in the population limits the use of PCR to diagnose acute infection.

Qavi et al. (1989) used PCR to look for HHV-6 genomes in retinal tissue of HIV^+ patients. HHV-6 sequences were detected by PCR in 4 eyes from 3 patients. Sections from paraffin-embedded material were used in these experiments. This facilitated a comparison of the involved areas of retina and choroid with surrounding normal areas. Detection of HHV-6 was restricted to pathologic areas. In specimens from 3 of the patients, CMV was also detected, and in 2 patients, HIV was also present. In the second eye from one of these patients, only HHV-6 was detected. The precise role of HHV-6 in AIDS retinitis requires further study.

Using quantitative PCR, Cone et al. (1991) have recently demonstrated a higher HHV-6 viral burden in the lung tissue of bone marrow transplant (BMT) patients with pneumonia when compared to lung tissue from healthy controls. 9 of 11 control lungs did have detectable HHV-6; however, the mean concentration was 261 HHV-6 genomes/10^6 cells compared to 89 903 genomes/10^6 cells in the BMT patients. Although this clearly indicates a marked increase in virus load the role the virus plays in the pneumonia (verses secondary reactivation) is unclear.

17.4. Use of PCR for mapping genes of HHV-6

Available sequence analysis of the HHV-6 genome indicates areas of conserved sequences among all herpesviruses with the closest similarity to CMV (Lawrence et al., 1990; Josephs et al., 1991). This homology should allow the use of PCR to clone and sequence genes from HHV-6 using primers derived from known conserved sequences within a group of related herpesviruses. Teo et al. (1991) recently reported the successful amplification of a 510 bp fragment of the HHV-6 genome (Ugandan isolate U1102) using degenerate oligonucleotide primers derived from 3 highly conserved regions of 5 human herpesvirus DNA polymerases. This PCR product was subsequently used as a probe to screen a recombinant λ-library containing overlapping clones of the genome of the Ugandan U1102 isolate of HHV-6. The polymerase gene was successfully isolated and sequenced. A similar preliminary approach had been reported by Wolinsky et al. (1990). Such an approach should prove useful for identifying other genes with homologous regions among the herpesviruses.

17.5. Conclusions

The above studies highlight both the advantages and disadvantages of using PCR for the detection of herpesviruses. The sensitivity of the assay has proved extremely

useful in the detection of low levels of persistent viral infection and resulted in some important information concerning the biology of the virus. PCR amplification has shown viral genomes to be present in the peripheral blood of the majority of healthy individuals and has confirmed the presence of viral DNA in saliva. These data in conjunction with information derived from tissue culture suggest that the majority of healthy individuals carries the virus in a latent state in a small number of peripheral blood cells and shed it in the saliva. The experiments performed using PBMC samples emphasize an important aspect of PCR methodology – although the technique is extremely sensitive, the original sample must be sufficiently large to contain an amplifiable template.

The ability to detect HHV-6 in samples from healthy individuals limits the usefulness of PCR in diagnosing acute infection or when attempting to associate the virus with disease. These problems may, in part, be overcome by accurate quantification of PCR products. Particular difficulties, however, arise when examining samples from patients with immunosuppressive or lymphoproliferative diseases, who may have increased viral loads. Despite these problems, Kondo et al. (1990) have convincingly demonstrated the association between HHV-6 and exanthem subitum using relatively low level amplification PCR. Reverse-transcriptase polymerase chain reaction (RT/PCR) amplification, which allows amplification and detection of specific viral mRNA, should shed more light on the role of HHV-6 in specific disease processes as well as the biology of the virus in the normal host. This will be possible when specific viral mRNAs have been mapped. In addition, future studies combining PCR and RT/PCR with cell separation techniques should further delineate the sites of viral latency and replication in the host.

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CHAPTER 18

'Post-viral' chronic fatigue syndrome

ANTHONY L. KOMAROFF[1], SUDHIR GUPTA[2], and IRVING E. SALIT[3]

[1] *Division of General Medicine and Primary Care, Brigham and Women's Hospital, Harvard Medical School, Boston, MA 02115, U.S.A.,* [2] *Division of Basic and Clinical Immunology, University of California, Medical Sciences 1, C264A, Irvine, CA 92717, U.S.A., and* [3] *Department of Microbiology and Division of Infectious Diseases, University of Toronto, Toronto General Hospital, Toronto, Ontario M5S 1A8, Canada*

18.1. Introduction: fatigue in general medical practice

Every human being experiences fatigue. Furthermore, nearly everyone occasionally experiences an 'unnatural' degree of fatigue, not clearly explained by the mental, emotional and physical stresses of the previous days, but such fatigue usually is transient.

Chronic fatigue is a common problem in general medical practice (Allan, 1944; Morrison, 1980; Katerndahl, 1983; Nelson et al., 1987), accounting for 10–15 million office visits per year in U.S.A. Depression and anxiety with somatization (psychologic or social distress expressed in patients as bodily complaints) also is common in general medical practice, and one of the more common complaints among somatizing patients is fatigue (Stoeckle et al., 1964; Hoeper et al., 1979; Reifler et al., 1979; Nielsen and Williams, 1980; Barsky, 1981; Kessler et al., 1985).

Primary psychiatric disorders, particularly depression and anxiety, are the cause of most cases of chronic fatigue in a general medical practice. On occasion various 'organic' conditions also can produce fatigue – occult malignancies, anemia, thyroid disorders, multiple sclerosis, connective tissue disorders including systemic lupus erythematosis, etc. On these points most physicians would agree. Even when one of these well-recognized organic illness co-exists with fatigue, the relationship between the fatigue and the illness may not always be clear. While the diagnosis of malignancy is usually unambiguous (i.e., it is supported by tissue evidence), the connective tissue disorders and multiple sclerosis can present in a less than full-blown fashion, and thereby be impossible to diagnose definitively. Also, while the diagnosis of anemia and thyroid disease can be clear, it is not always clear whether these organic illnesses explain a patient's fatigue. The diagnosis of these organic

illnesses is not always clear, with different physicians making different diagnoses in the same patient. Again, on these points most physicians probably would agree. At this point, the controversy begins.

For over 100 years, the medical literature has described an illness characterized by a chronic debilitating fatigue, usually beginning suddenly with a 'flu-like' illness. The illness has been given a variety of names, but the descriptions of the illness are sufficiently similar to suggest the possibility that they are all part of the same syndrome. The pathogenesis of this illness (or these illnesses) has not been elucidated. Indeed, no reasonably specific and sensitive diagnostic test has been developed. For that reason, some clinicians question whether there really is an underlying organic disorder. In the following pages, we will describe briefly each of these conditions.

18.2. Fatigue syndromes: several labels, same disease?

Neurasthenia (or neurocirculatory asthenia) was first described in the mid-19th century (Paul, 1987). Typically an affliction of young adults, usually women, the illness is characterized by chronic malaise and often starts with an acute infectious illness. In the early 20th century, the illness was ascribed to 'weakness' of the nervous system and cardiovascular system, but no characteristic objective deficits were identified. For that reason, the illness slipped from favor, and has been rarely mentioned in the medical literature of the past 40 years. Despite the commonly-expressed opinion that the fast-paced life of modern industrial society is responsible for 'neurasthenia', the condition is seen even more frequently in agrarian societies such as mainland China (Kleinman, 1982).

Chronic mononucleosis (Komaroff, 1987; Straus, 1988) was first described 40 years ago. This illness starts with classical acute infectious mononucleosis, as characterized by clinical, hematologic and serologic features (Isaacs, 1948). However, instead of recovering, these patients remain ill for years. Some of them have serologic evidence of persistently active Epstein–Barr virus (EBV) infection, although in our experience there are some patients who remain chronically ill but whose EBV serologic studies become unremarkable.

Severe chronic active EBV infection (Edson et al., 1983; Schooley et al., 1986) is a very unusual chronic illness that often, but not always, follows acute infectious mononucleosis, like chronic mononucleosis. Indeed, it may be best thought of as a subset of chronic mononucleosis. These patients all have strikingly abnormal serologic studies (EBV–VCA-IgG greater than or equal to 1:5120, or early antigen-Ab greater than or equal to 1:320; sometimes without the expected antibodies to EBV nuclear antigens). Unlike most patients with chronic mononucleosis, many of these patients also have evidence of major organ involvement, such as recurrent interstitial pneumonia, persistent non-A, non-B hepatitis, splenomegaly and adenopathy, pancytopenia or selective cytopenia. Again, the parsimonious explanation is that these patients have an illness related to EBV infection, in which immunologic containment of EBV is impaired.

'Chronic Epstein–Barr virus infection' syndrome. Since 1985, an illness has been described in association with modest evidence of reactivated EBV infection. However, unlike chronic mononucleosis or severe chronic active EBV infection, this illness did not follow an episode of classic acute infectious mononucleosis, nor was the serologic evidence of reactivated EBV infection very striking. Because it had been felt that EBV reactivated only infrequently following primary infection, in healthy individuals, this led some to conclude that the syndrome might be caused by reactivation of EBV. Subsequent research has indicated, however, that reactivation of EBV (associated with modest elevations of EBV–VCA-IgG and anti-early antigens) is not uncommon in healthy individuals (Horwitz et al., 1985). Furthermore, one of us (I.E.S.) has looked for EBV excretion in these patients, using a sensitive EBV DNA probe. There was no increase in the usual expected levels of pharyngeal EBV excretion compared to controls. We have also observed this illness to occur after other definite (non-EBV) infections (Salit, 1985). For these reasons, an etiologic association of EBV with this illness was challenged, and the illness was renamed 'chronic fatigue syndrome' (CFS) as part of producing a case definition, an effort led by the Centers for Disease Control. CFS is described in detail later.

Myalgic encephalomyelitis (ME) is a very similar chronic fatiguing illness, variably epidemic neuromyasthenia, Akureyri disease or Icelandic disease (Sigurdsson et al., 1950; Sigurdsson and Gudmundsson, 1956; Poskanzer et al., 1957; Shelokov et al., 1957; The medical staff of the Royal Free Hospital, 1957; Acheson, 1959; Henderson and Shelokov, 1959). Most often, this illness strikes in mini-epidemics, affecting hundreds of individuals living in small towns, or large numbers of coworkers in a large institution. Typically the illness is heralded by acute respiratory infection symptoms, followed by months or years of profound fatigue, muscular weakness and twitching, muscular pain (especially in the neck, shoulder girdle, low back and thighs), pharyngitis, nausea, vomiting, abdominal cramps, swelling in the fingers and feet, cognitive problems, emotional instability, depression, insomnia, paresthesias, and a tendency to transpose words. Not infrequently, these patients note that their symptoms worsen in damp weather, or in the premenstrual period. Physical examination often is entirely unremarkable, but a substantial number have been reported to suffer from low-grade fevers, adenopathy (especially in the posterior cervical chain), splenomegaly, and nystagmus. Past outbreaks have led to disability and work loss lasting many months or years. The few long-term follow-up studies which have been done suggest gradual improvement in the following years, although many patients continue to experience mild but similar episodes of illness. No particular viral agent has been definitively associated with these syndromes.

Fibromyalgia syndrome, also termed *fibrositis*, was first described in the 19th century and is now considered to be a very common cause of chronic musculoskeletal pain and fatigue (Goldenberg, 1987). The illness has been described and investigated primarily by rheumatologists. Up to 5% of patients at a general medical clinic and 12% of new patients seen by rheumatologists may have fibromyalgia (Yunus et al., 1981; Wolfe et al., 1984; Goldenberg, 1987). Indeed,

some rheumatologists believe that primary fibromyalgia is the most common rheumatologic condition seen in their practice, particularly in women under the age of 50 (Yunus et al., 1981; Wolfe et al., 1984; Dinerman et al., 1986; Felson and Goldenberg, 1986; Goldenberg, 1987). From 4–20% of new patients seen in ambulatory rheumatology clinics are diagnosed as having fibromyalgia; it has been estimated that 3–6 million people in the United States suffer from fibromyalgia (Yunus et al., 1981; Campbell et al., 1983; Wolfe and Cathey, 1983).

Muscular pain, most commonly of the axial skeleton (Yunus et al., 1981; Wolfe et al., 1984; Dinerman et al., 1986; Felson and Goldenberg, 1986; Goldenberg, 1987) is the hallmark of fibromyalgia. The chronic pain is accompanied by morning stiffness and increased tenderness at specific sites known as 'tender points'. The disorder is frequently accompanied by poor sleep, headaches, irritable bowel syndrome, and major affective disorders. Patients with fibromyalgia also complain of fatigue. Indeed, Yunus has stated that "one may question the diagnosis of primary fibromyalgia in the absence of tiredness" (Yunus et al., 1981). Between 9–21% of patients are partially or totally work-disabled (Cathey et al., 1986; Wolfe and Cathey, 1990).

The presence of tender points on physical examination have been shown to differentiate patients with fibromyalgia from normals and from patients with other chronic rheumatic disorders (Yunus et al., 1981; Wolfe et al., 1985; Goldenberg, 1987). However, such diagnostic criteria have not been tested in patients with other poorly understood chronic pain disorders such as chronic idiopathic low back pain.

In fibromyalgia there is a disturbance in stage 4 or deep sleep, termed alpha wave intrusion into delta sleep (Moldofsky et al., 1975). Moldofsky and coworkers produced this sleep disturbance in normal controls studied in a sleep laboratory; these normal subjects then developed symptoms and tender points which are consistent with fibromyalgia (Moldofsky and Scarisbrick, 1976). More recent investigations have focused on the tissue changes in fibromyalgia. Such studies have described excessive cold sensitivity (Dinerman et al., 1986) and neurogenic hyperactivity (Littlejohn et al., 1987), type II muscle fiber atrophy (Kalyan-Raman et al., 1984), as well as alterations in muscle metabolism (Bengtsson et al., 1986; Lund et al., 1986; Bonfede et al., 1987). Preliminary reports have described abnormalities in T cell subpopulations and natural killer (NK) cell activity, as well as detectable cytokine levels in subsets of patients with fibromyalgia (Peter and Wallace, 1988; Russell et al., 1988). Furthermore, a subset of patients with fibromyalgia is reported to have a positive antinuclear factor and immunoglobulin deposition at the dermal-epidermal junction (Caro et al., 1986; Dinerman et al., 1986).

One of us (A.L.K.) and colleagues have found close parallels between fibromyalgia and chronic fatigue syndrome. Although this had not been previously reported, we found that patients with fibromyalgia often had symptoms thought typical of CFS (but not fibromyalgia): the sudden onset of their syndrome with an 'infectious-like' illness, chronic fevers, sore throat, cough, and adenopathy (Buchwald et al., 1987). In a second study, when examining patients with CFS, we found tender

points with a frequency approaching that seen in fibromyalgia, and much more often than in healthy control subjects (Goldenberg et al., 1990). One of us (I.E.S.) also noted that on polysomnography the characteristic sleep disorder of fibrositis was present in 14/14 subjects who met the criteria for CFS (Whelton et al., 1988).

18.3. Chronic fatigue syndrome

As stated earlier, the illness now called chronic fatigue syndrome (CFS) was defined in a case definition developed by the Centers for Disease Control, and summarized in Table 18.1 (Holmes et al., 1988). The definition relies entirely on a combination of symptoms and signs (not laboratory data), and on the exclusion of chronic active 'physical' or psychiatric illnesses that can produce chronic fatigue. It is not yet clear whether the current case definition accomplishes the objectives of any case definition: the identification of a group of individuals with a common and characteristic pathological abnormality and/or a common and characteristic prognosis. Indeed, we have found that those who do not fully meet the case definition are otherwise indistinguishable from those who do (Komaroff and Geiger, 1989).

Elsewhere, we have summarized in some detail the symptoms and signs of CFS (Salit, 1985; Komaroff and Buchwald, 1991). CFS is characterized by varying degrees of chronic fatigue, fever, pharyngitis, myalgias, headache, arthralgias, paresthesias, depression, and cognitive deficits. Typically, the chronic illness begins abruptly with an acute 'infectious-like' syndrome that includes respiratory and/or gastrointestinal symptoms, with associated fever, myalgias, and arthralgias. While the full syndrome has been described only in recent years (Ballow et al., 1982; Tobi et al., 1982; Edson et al., 1983; Hamblin et al., 1983; DuBois et al., 1984; Jones et al., 1985; Salit, 1985; Straus et al., 1985), earlier reports which may have been reporting the same phenomenon exist in the literature. A similar syndrome may follow definite infection with a variety of infectious agents (Benjamin and Hoyt, 1945; Cluff et al., 1959; Imboden et al., 1959; Lawton et al., 1970; Rosene et al., 1982; Salit, 1985).

The onset of the syndrome typically seems to be in late adolescence or young adulthood (Ballow et al., 1982; Tobi et al., 1982; Edson et al., 1983; Hamblin et al., 1983; DuBois et al., 1984; Jones et al., 1985; Straus et al., 1985), although it may also occur in childhood (Jones et al., 1985). By definition, patients with this syndrome have been evaluated for a variety of chronic infectious, rheumatologic, endocrinologic and malignant diseases, and no chronic disease is apparent (Tobi et al., 1982; DuBois et al., 1984; Jones et al., 1985; Straus et al., 1985). The diagnosis has been made about twice as often in women as in men (Tobi et al., 1982; Hamblin et al., 1983; DuBois et al., 1984; Jones et al., 1985; Straus et al., 1985). For most patients, the illness takes the form of a chronic, recurring 'flu-like' illness (DuBois et al., 1984; Jones et al., 1985; Straus et al., 1985). Virtually all patients perceive themselves to be impaired in some way. Some patients are completely disabled by the fatigue, muscular weakness and pain.

Table 18.1.
Working case definition of chronic fatigue syndrome[a]

A case of chronic fatigue syndrome must fulfill major criteria 1 and 2, and the following minor criteria: 6 or more of the 11 symptom criteria and 2 or more of the 3 physical criteria; or 8 or more of the 11 symptom criteria.

Major criteria

(1) New onset of persistent or relapsing, debilitating fatigue or easy fatigability in a person who has no previous history of similar symptoms, that does not resolve with bedrest, and that is severe enough to produce or impair average daily activity below 50% of the patient's premorbid activity level, for a period of at least 6 months.

(2) Other clinical conditions that may produce similar symptoms must be excluded by thorough evaluation, based on history, physical examination, and appropriate laboratory findings. These conditions include: malignancy; autoimmune disease; localized infection (such as occult abscess); chronic or subacute bacterial disease (such as endocarditis, Lyme disease, or tuberculosis), fungal disease (such as histoplasmosis, blastomycosis, or coccidioidomycosis), and parasitic disease (such as toxoplasmosis, amebiasis, giardiasis, or helminthic infestation); disease related to human immunodeficiency virus (HIV) infection; chronic psychiatric disease, either newly diagnosed by history (such as endogenous depression, hysterical personality disorder, anxiety neurosis, schizophrenia), or chronic use of major tranquilizers, lithium, or antidepressive medications; chronic inflammatory disease (such as sarcoidosis, Wegener granulomatosis, or chronic hepatitis); neuromuscular disease (such as multiple sclerosis or myasthenia gravis); endocrine disease (such as hypothyroidism, Addison disease, Cushing syndrome, or diabetes mellitus); drug dependency or abuse (such as alcohol, controlled prescription drugs, or illicit drugs); side effects of a chronic medication or other toxic agent (such as a chemical solvent, pesticide, or heavy metal); or other known or defined chronic pulmonary, cardiac, gastrointestinal, hepatic, renal, or hematologic disease.

Minor criteria

Symptom criteria

To fulfill a symptom criterion, a symptom must have begun at or after the time of onset of increased fatigability, and must have persisted or recurred over a period of at least 6 months (individual symptoms may or may not have occurred simultaneously). Symptoms include:

(1) Mild fever – oral temperature between 37.5 and 38.6°C, if measured by the patient – or chills. (Note: oral temperatures of greater than 38.6°C are less compatible with chronic fatigue syndrome and should prompt studies for other causes of illness.)
(2) Sore throat.
(3) Painful lymph nodes in the anterior or posterior cervical or axillary distribution.
(4) Unexplained generalized muscle weakness.
(5) Muscle discomfort or myalgia.
(6) Prolonged (24 hours or greater) generalized fatigue after levels of exercise that would have been easily tolerated in the patient's premorbid state.
(7) Generalized headaches (of a type, severity, or pattern that is different from headaches the patient may have had in the premorbid state).
(8) Migratory arthralgia without joint swelling or redness.
(9) Neuropsychologic complaints (one or more of the following: photophobia; transient visual scotomata; forgetfulness; excessive irritability; confusion; difficulty thinking; inability to concentrate; depression).
(10) Sleep disturbance (hypersomnia or insomnia).

TABLE 18.1. (continued)

(11) Description of the main symptom complex as initially developing over a few hours to a few days (this is not a true symptom, but may be considered as equivalent to the above symptoms in meeting the requirements of the case definition).

Physical examination criteria

Physical criteria must be documented by a physician on at least 2 occasions, at least 1 month apart.

(1) Low-grade fever – oral temperature between 37.6 and 38.6°C, or rectal temperature between 37.8 and 38.8°C. (See note under symptom criterion 1.)
(2) Nonexudative pharyngitis.
(3) Palpable or tender anterior or posterior cervical or axillary lymph nodes. (Note: lymph nodes greater than 2 cm in diameter suggest other causes. Further evaluation is warranted.)

[a] From Holmes et al. (1988).

18.3.1. Symptoms

The principal symptoms of CFS are summarized in Table 18.2. Two particularly remarkable findings are worth highlighting: chronic post-exertional malaise and recurrent, often drenching night sweats. The post-exertional malaise is characterized not only by symptoms that could simply represent deconditioning – pain and weakness of the involved muscles – but also by exacerbation of 'systemic' symptoms – e.g., fatigue, fevers, pharyngitis, adenopathy, and impaired cognition.

In the experience of one of us (A.L.K.), patients with CFS state that these symptoms and others were typically not a chronic problem in the years before the onset of their illness, but became common after the illness began. As an example, here are the frequencies of several common chronic symptoms after the illness began versus before the illness began: arthralgias (76% versus 6%); morning stiffness (62% versus 3%); distractibility (82% versus 4%); forgetfulness (71% versus 2%); dizziness (61% versus 4%); paresthesias (52% versus 2%); sleep disorder (90% versus 7%); irritability (68% versus 4%); depression (66% versus 7%). All of these symptoms are surely nonspecific, and several are thought to be concomitant symptoms of depression. However, the fact that these symptoms typically started abruptly in the context of an acute 'infectious'-type illness suggests that the symptoms are not likely to be due exclusively to a psychiatric disorder.

A few of the patients that one of us (A.L.K.) has been following have had transient acute neurologic events: primary seizures (7%); acute, profound ataxia (6%); focal weakness (5%); transient blindness (4%); and unilateral paresthesias (not in a dermatomal distribution). The clinical and laboratory findings in these relatively few patients with dramatic neurologic events are very similar to those of the larger group of patients with chronic fatigue, except for the neurologic events themselves. These acute and transient neurologic events also are similar to the findings occasionally reported in outbreaks of ME.

TABLE 18.2.
Frequency of chronic symptoms and signs[a]

Symptom/sign	Frequency (%)
Fatigue	75–100
Low-grade fever (by self-report)	60–95
Low-grade fever (at time of examination)	15–30
Myalgias	30–95
Depression (following onset of CFS)	70–85
Headaches	35–85
Pharyngitis	50–70
Impaired cognition	50–70
Sleep disorder	15–70
Anxiety (following onset of CFS)	50–70
Adenopathy	40–60
Nausea	50–60
Arthralgias	40–60
Diarrhea	30–40
Cough	30–40
Odd skin sensations	30–40
Rash	30–40
Weight loss	20–30
Weight gain	50–70
Low basal body temperature (95.0–97.6°F) (recorded in the office)	10–20

[a] Adapted from the experience of one of us (A.L.K.), plus others (DuBois et al., 1984; Jones et al., 1985; Straus et al., 1985).

18.3.2. Past medical history

A high frequency of atopic or allergic illness (in approximately 50%), as was first highlighted by Jones and his colleagues (Olson et al., 1986a,b), and confirmed by Straus et al. (1988), is one of the striking findings in the past medical history of patients with CFS. In some cases, subjects develop new allergies after the onset of CFS.

18.3.3. Physical examination

Uncontrolled experience suggests that relatively few findings on physical examination may be indicative of CFS; this remains to be determined from controlled studies with blinded observers. As listed in Table 18.2, unusual and abnormal findings are observed in 15–50% of the patients: fevers; unusually low basal body temperature (below 97° F); posterior cervical adenopathy; and abnormal tests of balance (Romberg and tandem gait).

18.3.4. Standard laboratory testing

Elsewhere we have summarized knowledge about laboratory test findings in CFS (Buchwald and Komaroff, 1991). In the laboratory of one of us (A.L.K.), *hemato-*

logic testing has revealed results outside the normal range in 15–50% of the patients: leukocytosis; leukopenia; relative lymphocytosis; atypical lymphocytosis; monocytosis; elevated sedimentation rate; and unusually low sedimentation rates. These results have not yet been formally compared to results in a control group of healthy patients.

In the laboratory of one of us, standard serum *chemistry* testing is remarkable only for modestly elevated transaminases on one or more occasions in a quarter of the patients we have seen. None of these patients has had serologic evidence of active infection with hepatitis A, B or C virus. One of us (S.G.) has observed an unusually high frequency of fasting blood sugar levels at the lower limit of normal.

18.3.5. Immunologic testing

The data on immunological analysis are conflicting. We and others (DuBois et al., 1984; Jones et al., 1985; Straus et al., 1985; Tosato et al., 1985; Olson et al., 1986a; Caligiuri et al., 1987; Murdoch, 1988; Peter and Wallace, 1988; Wallace and Margolin, 1988; Cheney et al., 1989; Klimas et al., 1990; Gupta and Vayuvegula, 1991) have found evidence of subtle and diffuse dysfunction; partial hypogammaglobulinemia (25–80%); partial hypergammaglobulinemia (10–20%); increased numbers of B cells; low titers of autoantibodies, particularly antinuclear antibodies (15–35%), with one of us (I.E.S.) finding antibodies to single-stranded DNA in an ELISA assay to be frequently elevated (anti-double-stranded DNA being undetected); increased numbers of the subset of B cells ($CD20^+$, $CD5^+$) that are dedicated to the production of autoantibodies; low levels of circulating immune complexes (30–50%); elevated ratios of helper/suppressor T cells (20–35%); reduced EBV-specific cytotoxic T cell activity; reduced lymphoproliferative responses after stimulation with various mitogens and antigens; increased numbers of activated cytotoxic T cells; reduced *in vitro* synthesis of interleukin-2 and interferon by cultured lymphocytes; increased IgE-positive cells; reduced numbers and deficient functional activity of natural killer cells; anergy or hypoergy by skin testing; and elevated levels of various cytokines. Some investigators have found increased levels of circulating interferon, whereas others have not. Straus demonstrated a significant increase in levels of leukocyte 2′,5′-oligoadenylate synthetase activity, an enzyme induced during acute viral infections (Straus et al., 1985), and one of us (I.E.S.) has confirmed that finding – although the levels are much lower than are observed in patients with AIDS. In approximately half of the few patients who have had lumbar punctures, in the patients studied by one of us (A.L.K.) there has been pleocytosis, predominantly lymphocytic, without other abnormalities. One of us (S.G.) has examined cerebrospinal fluid from 3 advanced cases of CFS. No evidence for HHV-6 or HTLV-I antibodies was observed.

18.3.6. Neurologic evaluation

Formal neuropsychological tests of cognition performed by Bastien, Albert and their colleagues on patients referred by one of us (A.L.K.) suggest that one-third to one-half of our patients have cognitive impairment – particularly impairment of

concentration and attention (unpublished data). It is the judgment of the neuropsychologists that the pattern of test performance suggests an 'organic' deficit, rather than cognitive dysfunction secondary to a mood disorder. Contrary evidence has recently been published (Altay et al., 1990). In that study, patients with CFS were found to have subjective complaints of cognitive impairment which could not be confirmed using standard neuropsychological instruments. CFS subjects performed as well as controls on these tests, suggesting that psychological factors were playing an important role.

Because of the cognitive and neurologic complaints, and because of the similarity of some of these symptoms to symptoms experienced by patients with multiple sclerosis, we obtained magnetic resonance images (MRI) of the brain. In 79% of 144 patients, vs. 21% of matched healthy control subjects, areas of high signal were seen in the white matter (Buchwald et al., 1992); it remains uncertain if this result is generalizable to other groups of patients. One of us (I.E.S.) has performed single-photon emission computed tomography (SPECT) on 60 CFS patients and 14 control subjects. The most striking finding was the diminished blood flow to the basal ganglia in 60% of CFS patients. These findings were similar to those observed in patients with major depression (M. Ichise, I.E. Salit, et al., unpublished observations). Another of us, with a much smaller experience, has found frequent temporal lobe perfusion abnormalities (A.L.K., unpublished observations); others have reported similar findings (I. Mena, unpublished observations).

18.4. Viruses and chronic fatigue syndrome

Infectious agents have been suspected as triggering agents with each of the chronic fatigue syndromes we have discussed. The speculation has centered most often on viruses, although, as observed by one of us (I.E.S.), nonviral infectious agents may also be able to trigger a similar post-infectious malaise (Rosene et al., 1982; Salit, 1985). Indeed, noninfectious events such as major surgery and severe allergic reactions can be associated with the onset of CFS.

18.4.1. Human herpesvirus-6

In our judgment, HHV-6 is an interesting candidate to play a pathogenetic role in some cases of CFS; it is a lymphotropic and gliotropic virus (Josephs et al., 1986; Salahuddin et al., 1986; Ablashi et al., 1987; Komaroff, 1990), and CFS is characterized by immunologic and neurologic symptoms. Studies conducted by one of us (A.L.K.) in association with Ablashi and Saxinger, as well as studies by others, have indicated a serologic association of this virus with both chronic fatigue syndrome and fibromyalgia (Ablashi et al., 1988; Komaroff et al., 1988), although some studies have not found such an association.

Sero-epidemiologic studies suggest that infection with HHV-6 is ubiquitous, and occurs in most people at a young age (Saxinger et al., 1988). Moreover, as with the other herpesviruses, infection with HHV-6 is lifelong. Therefore, the finding of a serologic association is not itself strong evidence of an etiologic role for HHV-6 in CFS. Stronger evidence of an association between HHV-6 and CFS comes from primary cell culture studies looking for active replication of the virus. We found that in 2 patients, sisters who became ill at about the same time, one of whom had a dramatic lymphoproliferative syndrome, active HHV-6 infection was present (Buchwald et al., 1990).

More recently, we have found active replication of HHV-6 in 70% of over 100 patients vs. only 20% of matched healthy controls (Buchwald et al., 1992). Given that primary infection with HHV-6 occurs early in life in most individuals, the finding of active HHV-6 in adults with CFS almost surely represents secondary reactivation of the virus, rather than primary infection. This secondary reactivation of HHV-6 may merely be an epiphenomenon reflecting immune dysregulation. Alternatively, even if secondary, reactivated HHV-6 might play a central role in the production of CFS symptoms, as will be discussed later.

18.4.2. Enterovirus

For years, myalgic encephalomyelitis was thought to be produced by a less virulent strain of poliovirus. Recently, Mowbray has reopened the possibility that enteroviral infection may indeed be associated with some cases of CFS, by demonstrating circulating enteroviral antigen more often in patients than in control subjects (Yousef et al., 1988).

18.4.3. Epstein–Barr virus

As discussed earlier, Epstein–Barr virus also has been the subject of investigation in chronic fatiguing illnesses. A triggering role for EBV seems plausible and even likely in chronic mononucleosis and in severe active chronic EBV infection. In patients with CFS, fibromyalgia and ME, however, there is no evidence that EBV plays a central role in the illnesses. In most patients with CFS, the EBV serologic results in most patients probably represent *secondary* evidence of some immunologic perturbation, rather than a primary pathogenetic role for EBV.

18.4.4. Retrovirus

Several investigators, including one of us (A.L.K.), have looked for evidence of infection with the known human retroviruses in hundreds of patients with CFS. In a few cases, serologic evidence of infection with human T lymphotropic virus type I or II (HTLV-I, -II) has been identified; the etiologic role of HTLV-I or -II in these patients remains obscure. In the great majority of patients we and most others have

tested, however, there has been no evidence of infection with the known human retroviruses. In a few patients without serologic evidence of infection with HTLV-I, HTLV-II or HIV, we have looked for evidence of reverse transcriptase in primary lymphocyte cultures from patients with CFS, to see if we could find evidence of a novel retrovirus, but without success (Buchwald et al., 1992).

Recently, DeFreitas and colleagues have reported evidence that a novel retrovirus related to HTLV-II may be present in a large fraction of patients with CFS; this possibility has been suggested from polymerase chain reaction and *in situ* cytohybridization assays, although no viral cytopathic effect, no evidence of reverse transcriptase and no electron microscopic evidence of a retrovirus have yet been uncovered (DeFreitas et al., 1991). This preliminary work, if it indeed leads to the identification of a novel retrovirus in a large fraction of patients with CFS, would be an important step forward.

18.5. CFS and psychological illness

Much confusion has resulted from a failure to distinguish chronic fatigue, a common problem for which patients seek medical attention, from chronic fatigue syndrome. As stated at the outset, most patients seeking medical care for chronic fatigue probably are suffering from depression and/or anxiety (and the related condition called somatization). Moreover, most patients seeking medical care for chronic fatigue probably do not have CFS (Kroenke et al., 1988; Manu et al., 1988).

It can be very difficult to study antecedent or subsequent psychiatric disorders in patients who may have an underlying organic illness. One reason is because the affective disorders, not being measurable by an objective laboratory test, are formally defined by subjective findings – symptoms expressed by the patient and the appearance of the patient to the skilled observer. Moreover, many of the symptoms taken as characteristic of depression, anxiety and somatization – e.g., fatigue, cognitive impairment, sleep disturbance, nausea – also are seen with a variety of organic illnesses. Therefore, subjects may meet the criteria for major depression but may not always have a depressive state because the symptoms of CFS may be similar.

The evidence presented earlier of objective immunologic and virologic findings that distinguish patients with CFS from healthy control subjects suggests that CFS is probably not simply a primary psychiatric disorder. However, that suggestion must be confirmed by doing similar studies on patients with primary psychiatric disorders, particularly major depression, to make sure that the findings seen in CFS patients are not just previously unrecognized biologic concomitants of depression. (Of course, if that were to be found, it could change profoundly our concepts of what psychiatric disorders are.)

One piece of evidence that CFS and major depression are not the same thing is the work of Demitrack and colleagues who have found abnormalities of the hypothalamic-pituitary-adrenal axis that are entirely different from those seen in

patients with major affective disorder (Demitrack et al., 1991). Furthermore, the sleep disorders seen in CFS by one of us (I.E.S.) and the sleep disorders seen in major depression are profoundly different.

At the same time, we think that psychiatric disorders play a role in CFS. Every study of the question, including the work of two of us (A.L.K. and I.E.S.) and our colleagues, indicates that the majority of patients with CFS *become* depressed and anxious following the (usually sudden) onset of their disorder (Taerk et al., 1987; Kruesi et al.,1989; Wessely and Powell, 1989; Gold et al., 1990; Hickie et al., 1990). For many patients, the depression and anxiety become the most debilitating parts of their illness.

The more difficult question is whether patients who develop CFS have had evidence of a psychiatric disorder in the years *before the onset* of CFS, and/or had an active psychiatric disorder *at the onset* of the CFS. Addressing this question requires retrospective interrogation, a difficult process. Nevertheless, most observers believe that retrospective interrogation using formal instruments such as the diagnostic interview schedule (DIS) can elicit reliable information.

Several studies have evaluated the presence of psychiatric disorders in the years before the onset of CFS, although none of these studies has carefully evaluated whether there was an active psychiatric disorder at the time of onset of CFS (Taerk et al., 1987; Kruesi et al., 1989; Wessely and Powell, 1989; Gold et al., 1990; Hickie et al., 1990). It also should be noted that the published studies were largely organized and conducted before the development of a formal case definition of CFS; thus not all of the patients may meet CFS criteria, and the studies may not be comparable with one another.

These caveats aside, by and large the studies find a somewhat higher past history of psychiatric disorders in patients with CFS than in the population at large: the average across all studies is around 30% (range, 20–50%) of CFS patients versus 5–10% in the population at large (Robins et al., 1984; Taerk et al., 1987; Kruesi et al., 1989; Wessely et al., 1989; Gold et al., 1990; Hickie et al., 1990). The studies vary greatly with regards to the reported relative frequencies of the different disorders – depression, anxiety and somatization. However, these findings must be incorporated into any hypothesis about the pathogenesis of CFS.

One of us (I.E.S. and colleagues) has also noted an extremely high prevalence of major stress factors in the 6 months prior to the onset of CFS: 90% of the patients had such stressful events, whereas only 5% of controls had similar events. Common stressful events included: major changes on the job; difficulties with a relationship; financial problems; and moving a home. Another of us (A.L.K.), in a study of about 290 patients, has found that 50% of patients describe themselves as being under great stress at the time of the onset of CFS (unpublished data).

If a background of psychiatric disease, particularly depression, is present more often in patients with CFS, what might that mean? It could indicate that CFS is just depression expressed with predominantly somatic symptoms. Or it could indicate that CFS is triggered by an organic illness, such as an infection, that is followed and perhaps supplanted by the reemergence of an underlying depression. Or it could

indicate that the biologic underpinnings of depression somehow render one vulnerable to the 'organic' abnormalities (e.g., the immunologic and virologic findings) seen in CFS.

We are more inclined to favor the last hypothesis, although it is not mutually exclusive with the 2 other hypotheses. We are more inclined to view affective disorders as biologically determined disorders of neurochemistry, disorders that can affect immune function and that, in turn, can be perturbed by the immune system. According to this model, 'mind' and 'body' are not separate and discrete, but inevitably linked: biological forces that increase the likelihood of affective disorder also may increase vulnerability to disorders of immunity. In patients with CFS, who have a current and/or past affective disorder, and who also have evidence of immune dysfunction and active viral infection, it may never be possible to determine whether the affective disorder, the immune dysfunction or the viral infection came first. Rather, the practical question is what form of management will be most effective: psychotherapy; pharmacotherapy of the affective disorder; 'immune modulating' pharmacotherapy; anti-microbial therapy; or some combination of these. There are no good studies of these issues, at this time.

18.6. A model for the pathogenesis of CFS

Our pervasive ignorance about the pathogenesis of CFS makes possible the elaboration of many models, but provides strong support for none of them. Our current view of this illness is reflected in Fig. 18.1. We think the disease is due to both physical and psychological factors. And, as with any illness, the degree of disability seen in CFS must be due, in part, to physical and to psychological factors.

We currently view CFS as centrally an immunologic disturbance, one that allows reactivation of latent and ineradicable infectious agents, particularly viruses. The reactivation of these viruses may only be an epiphenomenon. However, we feel it is more likely that, once secondarily reactivated, these viruses contribute to the morbidity of CFS – directly, by damaging certain tissues (e.g., the pharyngeal

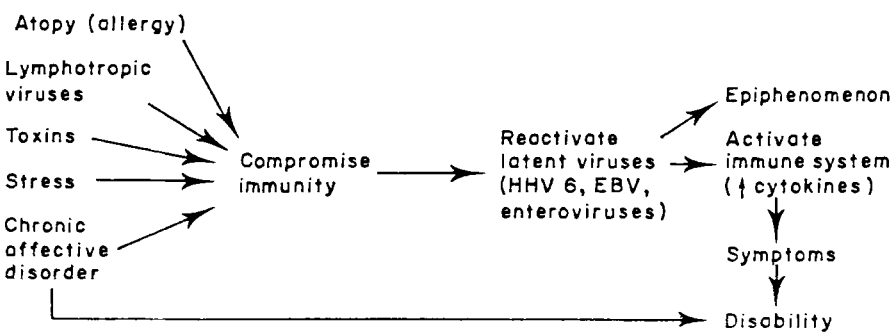

Fig. 18.1. Current favorite model.

mucosa), and indirectly, by eliciting an on-going immunologic response. In particular, the elaboration of various cytokines (e.g., interferon-alpha, interleukin-6, tumor necrosis factor and interleukin-2) as part of this on-going immunologic war may produce many of the symptoms of CFS – the fatigue, myalgias, fevers, sleep disorder, adenopathy, and even the disorders of mood and cognition. This is suggested by the finding of increased levels of various cytokines in CFS and related conditions (Peter and Wallace, 1988; Wallace and Margolin, 1988; Cheney et al., 1989), and the experience with infusing cytokines made by recombinant DNA techniques for various therapeutic purposes (Erstoff and Kirkwood, 1984; Quesada et al., 1986; Belldegrun et al., 1987; Denicoff et al., 1987; Muss et al., 1987; Rosenberg et al., 1987; Ettinghausen et al., 1988).

What triggers the immune dysfunction in the first place? It is likely that many factors could do so: atopic disorders; exogenous lymphotropic infectious agents; environmental toxins; stress; and, as argued earlier, the biology of an underlying affective disorder. This illness seems likely to have a multifactorial etiology, like most illnesses. While the discovery of a single explanation, such as a novel infectious agent or a specific inherited immunological defect, might simplify the search for solutions to this illness, we are dubious that such a simple answer will emerge from the growing amount of research conducted by laboratories around the world.

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Ablashi/Krueger/Salahuddin (eds) Human Herpesvirus-6

CHAPTER 19

Human herpesvirus-6 and HIV-1 infection

MARK H. KAPLAN

Department of Medicine, Division of Infectious Diseases, North Shore University Hospital/ Cornell University Medical College, Manhasset, NY 11030, U.S.A.

19.1. Introduction

Since the discovery of HHV-6, attempts have been made to determine what role it plays during the progressive destruction of the immune system by the HIV-1 virus.

Human herpesvirus-6 was initially isolated from 2 patients with AIDS. One homosexual patient had HIV-1 infection complicated by Kaposi's sarcoma and lymphoma (Josephs et al., 1986; Salahuddin et al., 1986). A second patient, an intravenous drug abuser, had HHV-6 isolated from peripheral blood mononuclear cells. After 3 episodes of pneumocystis pneumonia, he developed severe granulomatous dermatitis with associated dermatopathic lymphadenopathy. This second patient also had concomitant HTLV II infection. Several other isolates have been obtained from HIV-infected patients, including one from a Ugandan patient with HIV-1 (Downing et al., 1987) now called strain 1102, one from a 19-year-old girl from the Ivory Coast with HTLV-I-associated acute T cell leukemia complicating HIV-2 infection (Agut et al., 1988), a third from a Gambian HIV-2 AIDS patient (Tedder et al., 1987) and a fourth from a 58-year-old Scandinavian man with AIDS (Becker et al., 1989). Virus has also been cultivated from 10 HIV seropositive patients tested in Atlanta, Georgia (Lopez et al., 1988). One isolate called Z-29 has been characterized. The saliva of HIV-infected patients in San Francisco has yielded an isolate called HHV-6_{SF} (Levy et al., 1990a).

Initial studies showed that HHV-6 is tropic for the $CD4^+$ cell and could be lytic to this cell (Lusso et al., 1988). The infected $CD4^+$ population was shown to be a fairly immature phenotype bearing the $CD7^+$ antigen, the earliest known T lymphocytic marker, as well as the pan-T marker CD5, and the sheep erythrocyte marker CD2. Often, infected cells expressed cytoplasmic $CD3^+$, rather than surface

CD3 antigen, and both $CD4^+$ and $CD8^+$ antigens were co-expressed (Lusso et al., 1990). The ability of HHV-6 to produce a lytic infection in the CD4 cell population suggested that it might act synergistically with HIV-1 to destroy CD4 cells and degrade the immune system (Lusso et al., 1989).

19.2. Dual infections with HHV-6 and HIV-1

Initially, Lusso et al. (1989) showed that HHV-6 and HIV-1 could infect the same cell. Budding HIV-1 virions were present in cells that also showed both unenveloped intracytoplasmic HHV-6 and mature extracellular HHV-6 virions. The expression of viral messenger RNA of HIV-1 was accelerated in the course of co-infection with HHV-6. In addition, these authors noted a more rapid induction of cytopathicity in the course of dual infection. Finally, they were able to show that the viral LTR of HIV-1 transfected into MOLT-3 and CEM cells could be transactivated by infection with HHV-6. Similar observations were made by Horvat et al. (1989). The human T cell line, HuT-78 was transfected with a plasmid containing the HIV LTR region ligated upstream of the chloramphenicol acetyltransferase gene (CAT). The transfected cells were then infected with HHV-6, either by co-cultivation with infected HuT-78 and HSB_2 cells, or by cell free virus at 5×10^4. Cells were extracted 2 days after infection and assayed for CAT activity. CAT activity significantly increased by co-cultivation with HHV-6-infected cells. Cell-free virus infection had less of an effect on CAT activity. These experiments suggested that HHV-6 transactivates the LTR of HIV-1. The ability of HHV-6 to transactivate the HIV-1 LTR suggests that it can convert a latent HIV viral infection to an acute infection acting synergistically to enhance the viral activity of HIV-1.

The HHV-6 virus was also shown to infect the peripheral mononuclear cells of the chimpanzee (*Pan troglodytes*) in cells that expressed both the $CD4^+$ and $CD8^+$ phenotype (Lusso et al., 1990). T cell activation antigens were expressed on HHV-6-infected cells. As in human cells, HHV-6-infected cells could be dually infected with HIV-1. The cytopathic effect produced by HHV-6 in chimpanzee cells was accelerated by HIV-1.

Somewhat conflicting results have been reported by Carrigan et al. (1990) who showed that at relatively low levels of infectivity of both HHV-6 and HIV-1, HHV-6 suppressed HIV-1 expression. They used 4 different strains of HHV-6, including the KF strain of HHV-6 isolated from the peripheral blood of a patient with chronic lymphocytopenia and legionella pneumonia, the Ba strain isolated from the bone marrow of an autologous bone marrow transplant patient, and the Z-29 and GS strain originally isolated by Salahuddin et al. (1986). They were unable to show that HHV-6 enhanced replication of HIV-1. Instead, HIV-1 replication was consistently almost completely suppressed by HHV-6 when they monitored p24 antigen production. Multinucleate giant cell induction by HIV-1 was also suppressed in dual infection. In contrast, HHV-6 expression in infected cells was not changed by dual infection with HIV-1. HHV-6 induced cytopathic effect (CPE)

progressed in a similar fashion in the HIV-1 co-infected cells as it did in uninfected cells. They showed that the suppression of HIV-1 was not a result of induction of interferon, nor was it a result of HHV-6 destruction of the $CD4^+$ cell. This suppressive effect of HHV-6 on HIV-1 replication was observed with all 4 strains of HHV-6 utilized, and for different strains of HIV-1. It was felt that intracellular changes induced by HHV-6 might act to inhibit the production of HIV-1 in dual infection. At high input moi of $HHV\text{-}6_{KF}$, ganciclovir treatment of co-infected cultures inhibited suppression of HIV-1 by HHV-6. This suggested that the inhibitory effect of HHV-6 on HIV-1 may be mediated by an immediate-early, or early gene product of HHV-6 which is suppressed by ganciclovir.

Similar findings have been reported by Levy et al. (1990b), using strain $HHV\text{-}6_{SF}$ isolated from saliva of an HIV-1 infected individual and HIV-SF33 to infect lymphocytes harvested from HHV-6 and HIV-1 antibody-negative individuals. When these cells were co-infected with HIV-SF33 and $HHV\text{-}6_{SF}$, marked reduction of reverse transcriptase activity was observed, in contrast to that produced by HIV-SF33 alone. Different strains of HIV-1, with different cytopathic properties, were similarly inhibited by HHV-6. Significant numbers of $CD4^+$ cells survived dual infection indicative of inhibition of HIV-1 replication by HHV-6. The experiments of Carrigan et al. (1990) and Levy et al. (1990b) are at odds with those of Lusso et al. (1990). Carrigan thinks these discrepancies can be accounted for by the different levels of infectivity of HHV-6 used by Lusso, who used high levels of virus to perform experiments. At high levels of infectivity, there may have been a more synchronous infection of the 2 viruses resulting in different interaction. Carrigan speculates that this inhibitory effect of HHV-6 may be beneficial to patients with HIV-1. Late in AIDS, when HHV-6 may be activated, it may act on the $CD4^+$ cell to slow down the rate of replication of HIV-1 acting in a beneficial way to slow the progression of HIV-1-induced immune destruction. Clearly more work is necessary to understand the interaction between HHV-6 and HIV-1.

19.3. Detection of HHV-6 in patients with HIV-1 infection

Attempts have been made on a small scale to detect HHV-6 during the course of HIV-1 infection. Lopez et al. (1988) showed that HIV-1 cultures contaminated with HHV-6 showed a sudden drop in reverse transcriptase activity. These cultures showed herpesvirus particles when examined by electron microscopy. They were able to isolate 10 strains of HHV-6 viruses and characterized the Z-29 strain. Buchbinder et al. (1988) have shown that HHV-6 is easily detected in peripheral blood of HIV-1 patients using the method of polymerase chain reaction amplification and *in situ* hybridization to detect the virus. Specific HHV-6 primers were synthesized using sequences present in the pZVH14 probe developed by Josephs et al. (1986). The primers were a 32 mer CCCATTTACGATTTCCTGCA-CCACCTCCTCGC and a 31 mer CGACATGCTCAATGACATAACGG-TCCCTGAA which were 123 base pairs apart. A probe 31 base pairs long was used to detect an amplified product of 186 base pairs. It had a sequence of

CCGTAAAAAATTTACACCTCCATTTCATCTT. 52 out of 63 (83%) of patients with AIDS were found to have amplification of the HHV-6 specific sequence. 9 (14%) had no signal, and 2 had an equivocal signal. 20 tumors out of 23 from non-AIDS patients showed amplification of signal. The extreme sensitivity of this method made it hard to define what role, if any, HHV-6 has in HIV infection. Polymerase chain amplification will not only amplify actively replicating virions, but it will also amplify unexpressed virus which may be lying dormant in some lymphocyte population. Methods of quantitative PCR were not tried out in this study. Nonetheless, it does indicate that HHV-6 circulates in the peripheral blood of HIV-1-infected patients.

Unlike cytomegalovirus, which produces retinitis, esophagitis and colitis late in HIV-1, no discernible or distinct clinical syndrome has yet been ascribed to HHV-6 in the AIDS patient.

19.4. Serological studies of HHV-6 in HIV-1 infected patients

Serological studies have been equally disappointing in helping to define the role of HHV-6 in HIV-1 infection. Initial serologic studies yielded conflicting results because virus was difficult to grow in cord blood lymphocytes, making serologic testing inconsistent from laboratory to laboratory. Initial studies by Spira et al. (1990) showed that serologic titers in homosexual patients with HIV-1 infection had no bearing on the extent of disease or the outcome of illness. The percentage of positive titers was no different in HIV-1 positive patients with lymphadenopathy (65.9%) and HIV-1 seronegative controls (65.9%). Titers ranged to 1:320. In contrast, patients with AIDS had a significantly lower percentage of seropositivity (43.6%) versus early LAD and versus seronegative patients. In patients with lymphadenopathy, those who progressed to AIDS did not differ in titer from those who had stationary disease. In serial samples collected over long periods of time, most titers to HHV-6 were stationary or decreased by one or two dilutions. Similar studies in intravenous drug users showed no difference in the titer of HHV-6 in both HIV-1 seropositive IVDA's and HIV negative IVDA's (Huemer et al., 1989).

With the adaptation of virus to HSB_2 cell lines and to other continuous cell lines, more consistent results have been obtained. These results indicate that many HIV-1-infected patients have antibody to HHV-6. No clear association between the level of antibody and the extent of HIV infection has been seen. We have been able to show that HHV-6 antibody titers stay relatively stable during HIV-1 infection. In 3 HIV-1 seroconverters, antibody to HHV-6 did not change. However, 1 patient who developed a mononucleosis-like illness with seroconversion to HIV-1, also developed very high antibody titers to HHV-6 to 1:80000 with a positive HHV-6 IgM response. Following seroconversion, this patient went on to develop persistent generalized lymphadenopathy. This case suggests that the mononucleosis-like illness described with HIV-1 infection may in part be due to HHV-6 infection. We also found that titers to HHV-6 are generally stable throughout the course of persistent lymphadenopathy, and even during collapse of

the immune system with the development of AIDS defining illnesses. However, in a small group of AIDS patients, antibody to HHV-6 suddenly abruptly rose after initiation of azidothymidine (AZT) therapy. One of these patients developed a flare up of acute lupus erythematosis after AZT was initiated. A second patient with severe Reiter's syndrome complicating HIV-1 infection got abruptly better just after initiation of AZT. In these patients titers rose from 1:20 to 1:5120, and 1:40 to 1:640, respectively. In 2 other patients no discernible other illness complicated their abrupt change in HHV-6 titers after AZT was initiated. Their titers rose from 1:320 to 1:20480 and 1:20 to 1:320, respectively. These same patients did not show any concomitant changes in EBV serology at the time of sudden rise in titer to HHV-6, nor was there any change in their seroreactivity to p24 gag protein during serological change to HHV-6. In these patients, the change in IgG antibody was not accompanied by a concomitant change in IgM antibody to HHV-6, suggesting that this response was an amnestic rise in titer triggered by AZT. In 1 patient in whom sera was available only after initiation of AZT, titers fell from 1:80000 to 1:640 over 1.5 year. In patients who showed serologic rises after AZT, concomitant changes could be seen in viral specific proteins precipitated by their serum, using the method of radioimmunoprecipitation (RIPA). A characteristic

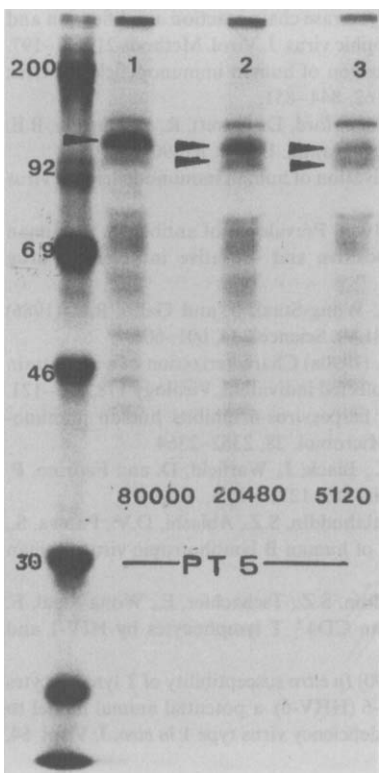

Fig. 19.1. A characteristic RIPA pattern.

RIPA pattern is shown in Fig. 19.1. We are not able to explain this interesting titer change to HHV-6 following initiation of AZT in HIV-1 infection. If HIV in fact restricts the replication of HHV-6 as suggested by Levy et al. (1990b), then perhaps control of HIV-1 by AZT may allow more productive HHV-6 infection which could boost titers to HHV-6.

Clearly much more work has to be done to understand what role, if any, HHV-6 plays in HIV-1 infection. This ubiquitous virus must have some important effect on the pathogenesis of AIDS. The mechanism of inhibition of the HIV-1 virus by HHV-6, as suggested by Levy and others, may be very important in our strategies to control this devastating viral infection.

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Ablashi/Krueger/Salahuddin (eds) Human Herpesvirus-6

CHAPTER 20

HIV-1 and HHV-6 infections of human retina and cornea

HAMIDA B. QAVI[1], MARY T. GREEN[1,2], and GARY K. SEGALL[3]

[1] *Division of Molecular Virology,* [2] *Department of Ophthalmology, and* [3] *Department of Pathology, Baylor College of Medicine, Houston, TX 77030, U.S.A*

20.1. Introduction

More than 110 000 Americans have died and more than one million are believed to be infected with the human immunodeficiency virus (HIV-1). About 270 000 individuals are projected to have developed acquired immunodeficiency syndrome (AIDS) in the United States by the end of 1991. It has been predicted that the disease will strike deep into mainstream America and will devastate Asia before the turn of the century. Although thousands of those affected are young adults and teenagers, perhaps some of the saddest cases are seen in infants and very young children who are victims of this deadly disease. The CDC reported 2 734 cases of pediatric AIDS as of November 30, 1990. Since the leukocytes and antibodies from children are deficient in providing antiviral activity, the disease progresses more rapidly in children than in adults.

Eye infection, a common complication of AIDS, strikes as many as 98% of all people infected with HIV-1 (AIDS Weekly, July 1, 1991, p 17). 50–80% of individuals with HIV-1 infection experience significant visual loss prior to death, further complicating an already devastating disease process. The spectrum of ophthalmic lesions associated with AIDS ranges from asymptomatic cotton-wool spots to blinding retinal necrosis. The cotton-wool spots, which are nerve fiber layer infarcts, are thought to be associated with a microangiopathy which ultimately leads to increased permeability and ischemic changes in the retina (Holland et al., 1983; Rosenberg et al., 1983; Freeman et al., 1984; Palestine et al., 1984; Pepose et al., 1984, 1987a). With the exception of eyelid and conjunctival involvement by Kaposi's sarcoma, most ocular pathology has been found to involve the retina, retinal pigment epithelium, choroid, and uvea (Schumann and Friedmen,

1983; Cole et al., 1984; Pepose et al., 1985a; Brody et al., 1991). Although a herpesvirus, cytomegalovirus (CMV) is most commonly involved in AIDS-associated retinitis (50–70% of the cases), a wide variety of opportunistic agents including Epstein–Barr virus (EBV), varicella zoster virus (VZV), herpes simplex virus type 1 (HSV-1), human herpesvirus type 6 (HHV-6), *Candida*, *Cryptococcus*, and *Pneumocystis carinii* have been reported (Neuwirth et al., 1982; Brooks et al., 1984; Humphry et al., 1986; Sandor et al., 1986; Levine et al., 1989; Qavi et al., 1989, 1991; Gabriel et al., 1990; Kawe et al., 1990). An abnormal deposition of immune complexes has been shown in the walls of retinal vessels in patients with AIDS-associated retinitis. Even in the presence of documented CMV retinitis, these deposits contain no known viral antigen.

Human immunodeficiency virus type 1 (HIV-1) has been identified in retinal tissue of 30–60% of the AIDS patients analyzed (Pomerantz et al., 1987; Cantrill et al., 1988; Qavi et al., 1991a). More than 50% (7 of 12) of the HIV-1 positive retinal tissues were also positive for HHV-6 DNA sequences or transcriptional activity in one of the studies (Qavi et al., 1991a). HHV-6 is a recently described human herpesvirus which has been isolated from peripheral blood leukocytes of patients with AIDS and other immunoproliferative disorders in the United States, Europe and Africa (Josephs et al., 1986; Salahuddin et al., 1986a; Downing et al., 1987; Krueger and Ablashi 1987; Lusso et al., 1987; Efstathiou et al., 1988). It is the only herpesvirus that shares cell tropism with HIV-1. Of further potential importance, it has been shown to act as a co-factor resulting in increased expression of HIV-1 antigens and more rapid depletion and destruction of $CD4^+$ T lymphocytes (Ensoli et al., 1989; Gallo et al., 1989; Lusso et al., 1989; Krueger et al., 1990a,b; Schonnebeck et al., 1991). Because the etiology of AIDS-associated retinitis remains unknown, it is tempting to speculate that HIV-1 alone or HIV-1 in association with HHV-6 may set the stage for invasion of retinal vascular endothelium by opportunistic agents such as CMV. There is an urgent need to clarify the role of HIV-1 and HHV-6 in the development of AIDS-associated retinitis.

Transmission of HIV-1, with subsequent development of AIDS via contaminated blood products, is well established. Because of the increasing incidence of HIV-1 seropositivity, and the undetermined window of negative serology prior to conversion following infection with HIV-1, there is increasing concern that the virus may be transmitted by organ transplantation. Kidney, heart, liver, and hip joint recipients from a single donor have seroconverted. HIV-1 has been isolated from tear film (Fujikawa et al., 1985a, 1986), conjunctiva (Fujikawa et al., 1985b), and corneas (Salahuddin, 1986b; Ablashi et al., 1987; Doro et al., 1987; Cantrill et al., 1988; Qavi et al., 1991b) from patients with AIDS and from patients with asymptomatic HIV-1 infection. Our group has reported the isolation of HIV-1 and HHV-6 from corneas of asymptomatic HIV-1-positive donors (Qavi et al., 1991b), suggesting that corneal tissue may be capable of supporting HIV-1 and HHV-6 infection. Recently, a low grade iritis with associated stellate corneal endothelial deposits was reported in 81% of AIDS patients with CMV retinitis (Brody et al., 1991). Although HIV-1 is a neurotropic pathogen, and in that respect similar to rabies and Jakob–Creutzfeldt disease, which have been transmitted by

corneal transplantation, there is currently no clinical or serological documentation of transmission of HIV-1 by that means (Pepose et al., 1985b, 1987b). Even so, the growing frequency of HIV-1 infection, and the frequency of corneal transplant in the United States (approximately 35000 per year) emphasize the importance of determining whether HIV-1 can be transmitted through donor corneal buttons.

20.2. Human immunodeficiency virus type 1 and infection of the retina

The retina consists of numerous layers of different cell types, and is considered to be an extension of the brain. The central nervous system is one of the principal tissues infected by HIV-1 (Shaw et al., 1985; Gabuzda et al., 1986; Koenig et al., 1986; Wiley et al., 1986; Pumarola-Sune et al., 1987). These observations and the established neurotropism of HIV-1 led to the hypothesis that HIV-1 might also infect retinal tissue. Pomerantz et al. (1987) were the first to document the presence of HIV-1 antigens (using immunohistochemical techniques) and to isolate HIV-1 from retinal tissues of 2 AIDS patients. Cantrill et al. (1988) reported similar observations on 2 additional patients.

Recently, we have cultured 26 retinas from AIDS patients and 4 from asymptomatic HIV-1 carriers. 4 of the 26 retinas from the 16 AIDS patients and none from asymptomatic HIV-1 carriers were culture positive for HIV-1 (Table 20.1). Using the polymerase chain reaction technique (PCR), HIV-1 DNA sequences were found in 15 of 29 retinas from AIDS patients and in 1 of the 4 retinas from the asymptomatic HIV-1 carriers (Table 20.1 and Qavi et al., 1989, 1991a). To determine whether the DNA sequences detected were restricted to lesioned areas, the template DNAs extracted from areas of retinal involvement and normal areas were subjected to PCR amplification. The results showed that HIV-1 DNA sequences were localized in areas of retinitis only (Qavi et al., 1989). An area of necrosis in the peripapillary region of 1 retina from an AIDS patient stained positive for HIV-1 using immunoperoxide. In another case, giant cells present in several different retinal layers stained positive for HIV-1. Because of the necrotic nature of the lesions, the cells type(s) involved could not be determined (Qavi et al.,

TABLE 20.1.
Analyses of retinal tissue

Donor status	Retinas analyzed	Serum antibody		DNA sequences, PCR		Viral culture	
		HIV-1	HHV-6	HIV-1	HHV-6	HIV-1	HHV-6
Asymptomatic (HIV^+)	4 (2 pr)	2/2	2/2	1/4	0/4	0/4	0/4
AIDS	26 (13 pr)	13/13	13/13	12/26	7/26	4[a]/26	0/26
Normal (HIV^-)	4 (2 pr)	0/2	0/2	0/2	0/2	0/2	0/2

[a] 2 of 4 HIV-1 culture positive retinas were positive for HIV-1 and HHV-6 transcriptional activity. 1 of the 2 retinas with HIV-1 and HHV-6 transcriptional activity also contained CMV antigens and DNA sequences.

1989). However, Pomerantz et al. (1987) demonstrated HIV-1 antigens in endothelial and neuroretinal cells using immunoperoxidase staining. Some of the retinal lesions were subjected to *in situ* hybridization using ^{35}S-labeled RNA HIV-1 probes for detection of transcriptional activity. 2 of the lesions were positive when screened by confocal microscopy suggesting active HIV-1 infection (Fig. 20.1).

Schumann et al. (1987) documented the presence of a single particle resembling a HIV-1 virion in retinal tissue from an AIDS patient. In our study, 50 sections from each of 4 sample blocks containing lesioned retina from 4 HIV-1 culture positive retinas were screened by electron microscopy (EM) for the presence of intact HIV-1 particles. No HIV-1 particles were detected in retinal sections. However, intact HIV-1 particles and cytoplasmic tubuloreticular inclusions were readily visible in peripheral blood monocytes (PBMC) used for culture of the retinal tissue (Fig. 20.2). Cytoplasmic tubuloreticular inclusions are characteristic of HIV-1 infections in PBMC and have been observed in lymph nodes and lymphocytes of AIDS patients. (Onerheim et al., 1984; Daskal et al., 1985; Kostianovsky and Grimley, 1985; Yoffe et al., 1989; Font et al., 1990).

20.3. Human herpesviruses and infection of the retina

Several herpes group viruses (CMV, HSV-1, VZV, HHV-6) have been implicated in the development of AIDS-associated retinitis (Cole et al., 1984; Pepose et al., 1984, 1985a; Qavi et al., 1989, 1991a).

The most common opportunistic agent associated with AIDS retinitis is CMV (Holland et al., 1983; Rosenberg et al., 1983; Freeman et al., 1984; Palestine et al., 1984; Pepose et al., 1984, 1985a, 1987a). CMV retinitis is much more frequent in AIDS than in other immunosuppressed patients (Henderley et al., 1987). The prognosis for eyes with CMV retinitis in AIDS patients is poor (Humphry et al., 1987). Skolnik et al. (1989) isolated CMV from retinal tissues of AIDS patients and confirmed its presence by immunohistochemical staining. Others have documented the presence of CMV antigens, DNA sequences, and intact viral particles in lesion-containing areas of retina from AIDS patients (Holland et al., 1983; Pepose et al., 1984, 1985a, 1987a; Qavi et al., 1989, 1991a; Schmitt-Graff et al., 1990).

VZV and HSV involvement in retinitis is much less frequent than CMV (Pepose et al., 1984, 1987a). These agents have been implicated in acute retinal necrosis syndrome (ARN) which is a sudden onset, fulminant form of retinitis often resulting in severe visual deficit. ARN occurs in normal, in non-HIV-1-infected immunocompromised individuals and in AIDS patients (Culbertson et al., 1986; Freeman et al., 1986; Pepose, 1986). VSV infections are more severe in young immunocompromised patients (Cole et al., 1984; Sandor et al., 1986; Kestelyn et al., 1987; Jabs et al., 1989). VZV antigens and DNA sequences have been detected in the retina of a pediatric AIDS patient utilizing immunohistochemical staining and *in situ* hybridization (Pepose, personal communication).

HHV-6 is a neurotropic virus and has been detected in brain lymphomas from AIDS patients (Albrecht et al., unpublished observations). Reactivation of latent

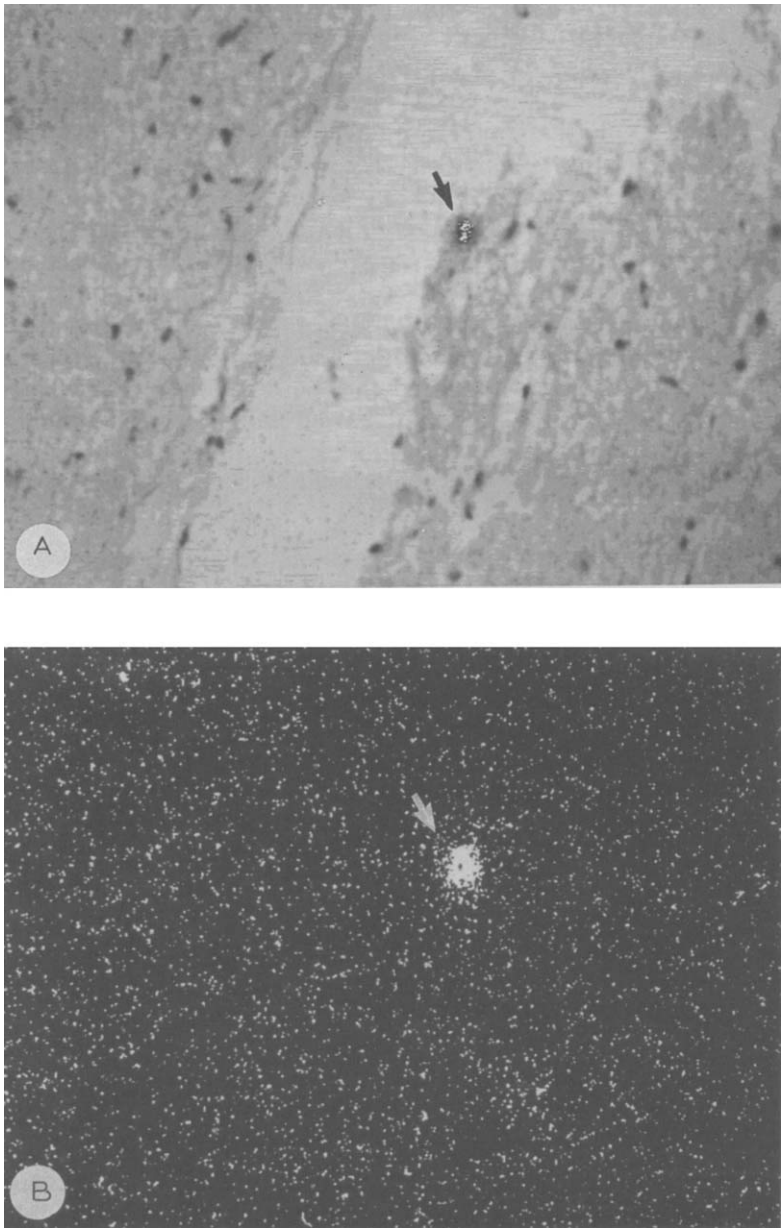
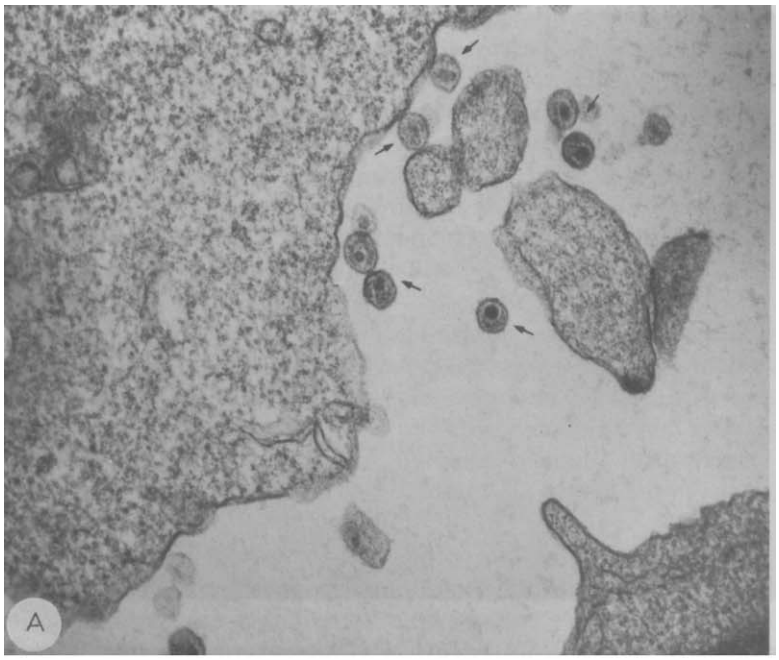


Fig. 20.1. *In situ* hybridization showing HIV-1 transcriptional activity in the retinal sections. (A) Retinal section form an AIDS patient (arrow shows label in the retinal cells, *highlighted*). (B) Same section as in (A), confocal image (arrow shows label in retinal cells).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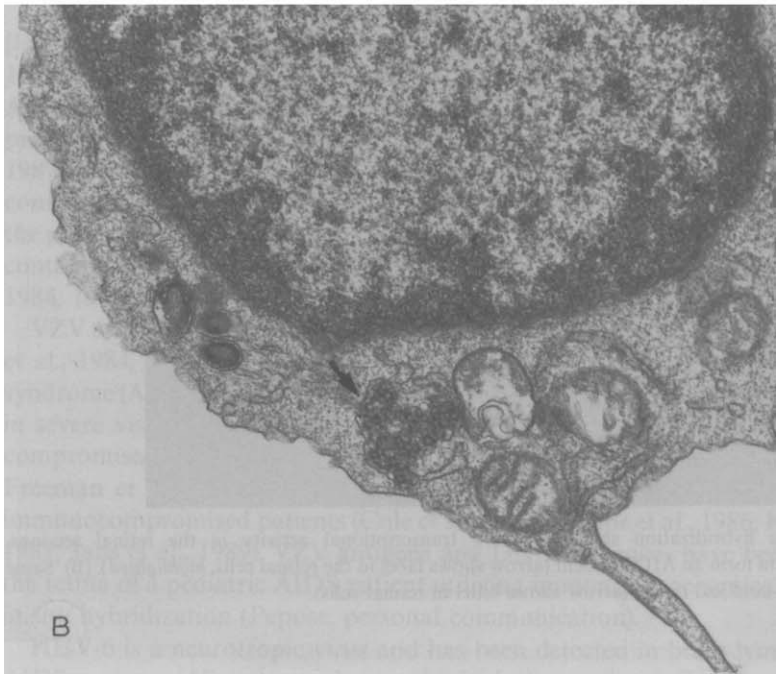

B

HHV-6 is also not uncommon in normal and immunocompromised patients (Russler et al., 1991). Because of our interest in determining the etiology of AIDS-associated retinitis, we have screened retinal tissues from patients with AIDS and asymptomatic HIV-1 infection for the presence of HHV-6. Initially we detected HHV-6 DNA sequences in several retinal lesions from AIDS patients using PCR and dot blot hybridization techniques (Qavi et al., 1989, 1991a). Recently, we detected HHV-6 DNA sequences in 7 of 26 retinas from 16 AIDS patients by PCR (Table 20.1). Using ^{35}S-labeled RNA probes, HHV-6 transcriptional activity was demonstrated in 2 of the 7 retinas containing HHV-6 DNA sequences (Table 20.1, Fig. 20.3).

Although not specifically identified, herpesvirus-type particles (spherical shape with icosahedral symmetry and 150 nm diameter) have been shown to be present in retinal tissue from AIDS patients by several investigators (Pepose et al., 1985a; Schmitt-Graff et al., 1990; Qavi et al., 1991a).

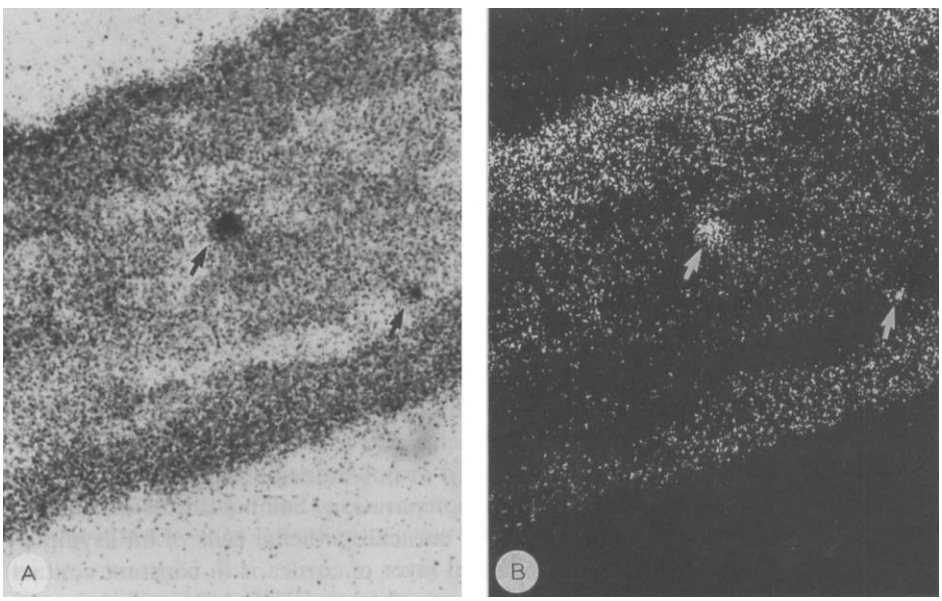


Fig. 20.3 *In situ* hybridization showing HHV-6 transcriptional activity in retinal sections. (A) Retinal section from an AIDS patient (arrows show label in retinal cells); (B) same section as in (A), confocal image (arrows show label in retinal cells).

Fig. 20.2. Electron micrograph of PBMC from positive retinal culture. (A) HIV-1 virions (arrows), magnification 54 600 ×. (B) Cytoplasmic tubuloreticular inclusions (arrow), magnification 29 400 ×. (For printing purposes figure has been reduced.)

20.4. Co-infection of the retina with HIV-1, HHV-6 and CMV

Co-infection of the human retina with HIV-1 and herpesvirus may have implications with regard to the pathogenic mechanism(s) of AIDS-associated retinitis. It is tempting to speculate that an interaction between 2 viruses may result in activation of one or both of the viruses. Using tissue culture and immunohistochemical staining, Skolnik et al., (1989) demonstrated simultaneous infection, with HIV-1 and CMV, of 2 of 13 retinal specimens from AIDS patients. We have also documented the presence of HIV-1 and CMV antigens and HHV-6 DNA sequences in a single retinal lesion and dual infection of several retinal lesions from AIDS patients (Qavi et al., 1989). In a recent study, we detected HHV-6 and HIV-1 DNA sequences in 7 of 26 retinas from AIDS patients. 4 of those 7 retinas were culture positive for HIV-1. 2 of the HIV-1 culture positive retinas which showed HIV-1 and HHV-6 transcriptional activity also contained CMV DNA sequences and antigens (Table 20.1). Rummelt et al. (1991) documented the presence of HIV-1, CMV, and HSV-1 antigens in a single retinal lesion from an AIDS patient. Using immunoperoxidase staining, Schmitt-Graff et al. (1990) reported HIV-1 and CMV antigens in several retinal lesions from a single AIDS patient.

20.5. Human immunodeficiency virus type 1 and infection of the cornea

Although avascularity protects the cornea from blood-borne infection to a certain extent, this structure is vulnerable to infection from both external and internal sources. HSV-1 infection is the leading cause of corneal blindness in this country (O'Day, 1984). Likewise, VZV is capable of causing devastating corneal disease (Liesegang, 1985; Uchida, 1985). Although much is known about HSV and VZV infection of the cornea, little information is available with regard to HIV-1 infection of the cornea. HIV-1 has been isolated from corneas of AIDS patients by several investigators (Salahuddin et al., 1986b; Ablashi et al., 1987; Doro et al., 1987; Cantrill et al., 1988). Ablashi et al. (1987) demonstrated HIV-1 antigen in cultured epithelial cells from corneas of AIDS patients and asymptomatic HIV-1 carriers. Endothelial cells were either weakly stained or not stained at all. No staining was observed in the stroma. Using a touch preparation, Salahuddin et al. (1986b) detected HIV-1 p24 antigens in 2–3% of corneal epithelial cells of an asymptomatic carrier of HIV-1. Since the epithelial layer of cornea is in constant contact with tear film, it could be questioned whether the HIV-1 identified in the preceeding studies was secondary to tear film contamination.

In a recent study we analyzed 35 pairs of corneas from asymptomatic carriers of HIV-1 and 8 pairs from AIDS patients for the presence of HIV-1 viral transcripts, DNA sequences, and intact and infectious virus particles. Serum from all donors was positive for HIV-1. 3 corneas from asymptomatic carriers of HIV-1 and 3 corneas from AIDS patients were culture positive for HIV-1 (Table 20.2) (Qavi et al., 1991b). All 6 culture positive corneas also contained HIV-1 DNA sequences. HIV-1 transcriptional activity in the form of positive label was seen in stromal

TABLE 20.2.
Analyses of corneal tissue

Donor status	Corneas analyzed	Serum antibody HIV-1	Serum antibody HHV-6	DNA sequences, PCR HIV-1	DNA sequences, PCR HHV-6	Viral culture HIV-1	Viral culture HHV-6
Asymptomatic (HIV^+)	70 (35 pr)	35/35	23/30	3/70	3/70	3[a]/70	1/70
AIDS	16 (8 pr)	8/8	6/6	0/16	0/16	3/16	0/16
Normal (HIV^-)	4 (2 pr)	0/2	0/2	0/4	0/4	0/4	0/4

4 of the 6 HIV-1 culture positive corneas showed HIV-1 transcriptional activity in corneal kerotocytes.

[a] 1 of the transcriptional active corneas was also culture positive for HHV-6.

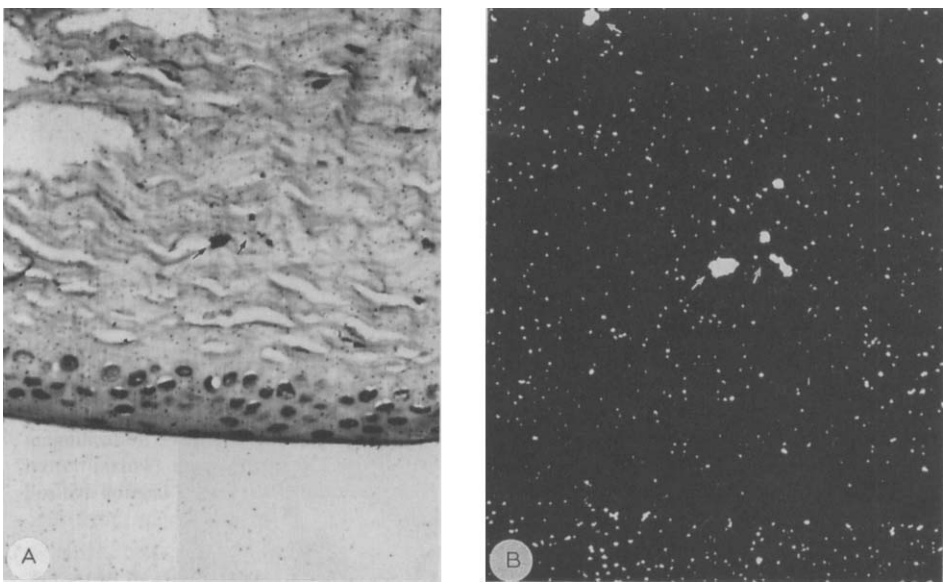
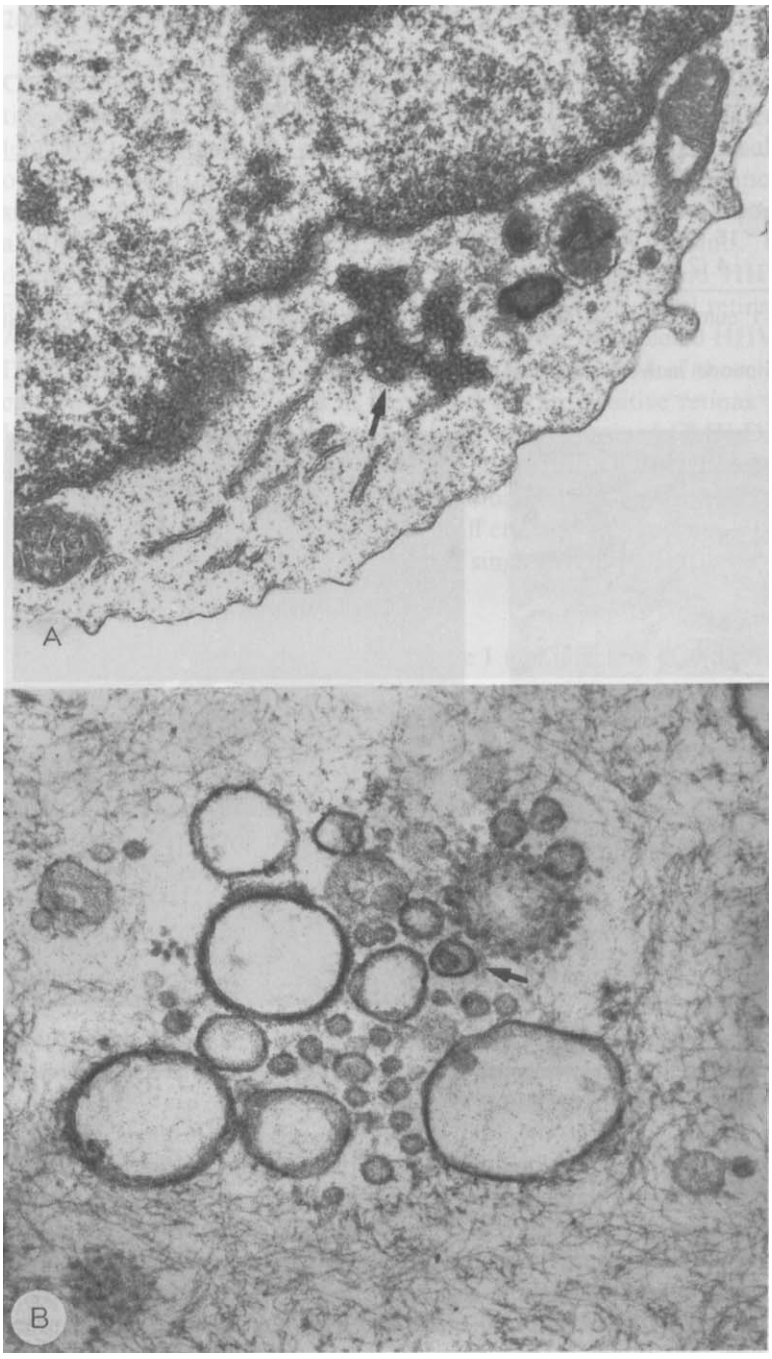


Fig. 20.4. *In situ* hybridization showing HIV-1 transcriptional activity in corneal sections (Qavi et al., 1991b). (A) Corneal section from an asymptomatic carrier of HIV-1 (arrows show label in keratocytes); (B) same section as in (A), confocal image (arrows show label in keratocytes).

keratocytes of 2 of the 3 HIV-1 culture positive corneas from each group (Table 20.2, Fig. 20.4) (Qavi et al., 1991b). These findings coupled with the fact that the 8 mm central corneal buttons were extensively washed prior to co-cultivation argue against tear film contamination.

Corneal tissue and PBMC harvested at day 10 from the positive culture were examined by EM. No intact HIV-1 particles were visible in corneal sections, however, cytoplasmic tubuloreticular inclusions were present in the PBMC (Fig. 20.5). No HIV-1 particles or cytoplasmic tubuloreticular inclusions were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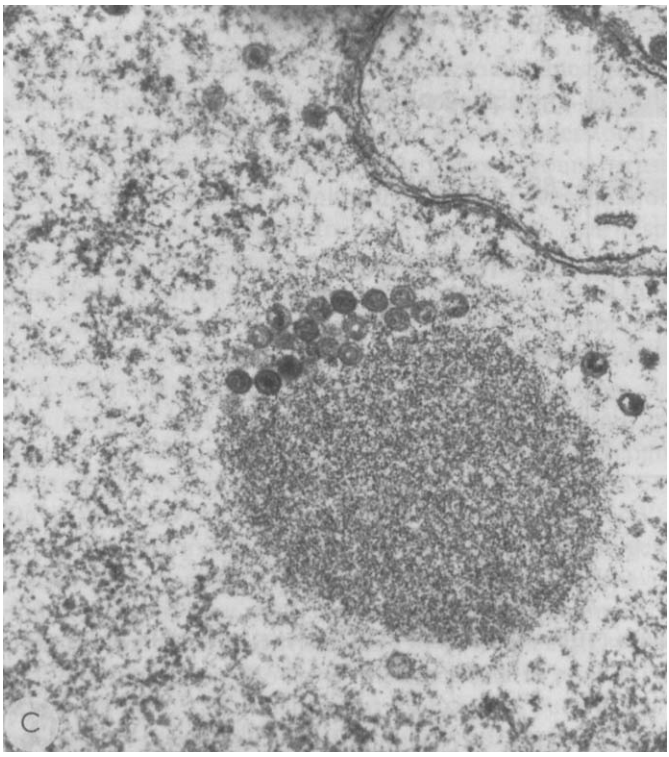

Fig. 20.5. Electron micrographs of corneal tissues and corneal cultures (Qavi et al., 1991b). (A) Cytoplasmic tubuloreticular inclusion in PBMC from HIV-1 positive corneal culture (arrow), magnification 29 400×. (B) HIV-1 virion in an epithelial cell of an HIV-1 culture positive corneal button (arrow), magnification 72 800×. (C) Herpes group virus particles in PBMC from an HIV-1 positive corneal culture, magnification 39 900×. (For printing purposes figure has been reduced.)

detected in similarly treated normal corneas or PBMC from negative cultures. One of the culture positive corneas revealed the presence of a single viral particle (in a corneal epithelial cell) which appeared to have a similar shape and size as the HIV-1 virion in T lymphocytes (Fig. 20.5) (Haseltine and Wong-Staal, 1988).

20.6. Co-infections of the cornea with HIV-1 and HHV-6

In our study HHV-6 was isolated from 1 of the 70 corneas from asymptomatic HIV-1 carriers. HHV-6 DNA sequences were demonstrated in all 3 of the HIV-1 culture positive corneas. The single HHV-6 and HIV-1 culture positive cornea showed HIV-1 transcriptional activity. This was thought to be located in corneal keratocytes; however, migrating cells cannot be ruled out (Table 20.2, Fig. 20.4) (Qavi et al., 1991b). Although no herpesviruses were noted in corneal sections by

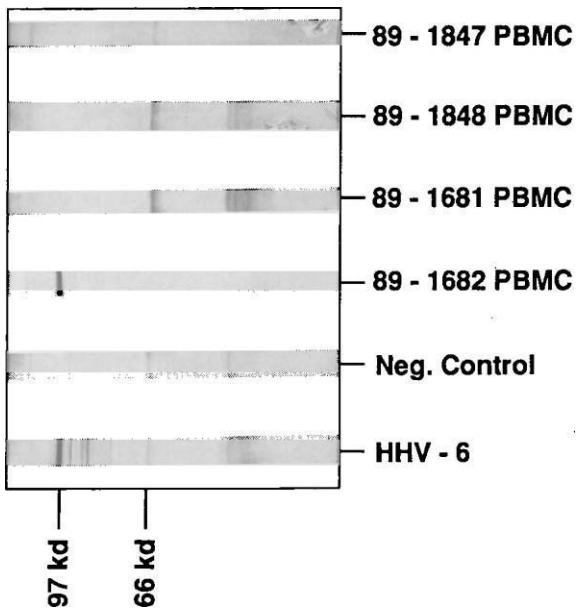

Fig. 20.6. Western blot analysis of HHV-6 antigens in PBMC from an HIV-1 positive corneal culture (Qavi et al., 1991b).

EM, herpesviruses were present in the nucleus as well as in the cytoplasm of the PBMC from the positive corneal cultures (Fig. 20.5), (Qavi et al., 1991b).

Antigen studies of formalin-fixed, paraffin-embedded corneal sections using monoclonal antisera to HIV-1 and polyclonal antisera to HHV-6 were negative. PBMC harvested from 1 of the HIV-1 positive corneal cultures on day 10 were extracted and subjected to Western blot analysis using antisera to HIV-1, HHV-6, CMV, HSV-1, and HSV-2. With anti-HIV-1, a weak band was visible in the vicinity of the p24 (data not shown). When the blots were treated with anti-HHV-6, a strong band was identified which did not react with antisera to any of the other herpesviruses (Fig. 20.6). Antisera to HSV-1 gave a positive reaction, antisera to CMV and HSV-2 gave a negative reaction. To rule out the possibility that HIV-1 and HHV-6 were contaminants from the PBMC used for co-cultivation, similar Western blot analyses were performed on 3 other negative corneal cultures in which the same batch of PBMC was used. These samples gave negative reactions with HIV-1, HHV-6 and HSV-1 antibodies. These observations confirm that HIV-1 and HHV-6 are capable of infecting non-CD4 expressing cells as has been documented earlier (Dalgleish et al., 1984; Klatzmann et al., 1984; Xi et al., 1990). Although preliminary, this data suggests that HIV-1 and HHV-6 may be capable of infecting corneal tissue.

20.7. Significance and conclusions

Although AIDS-associated retinitis leads to blindness prior to death in 70–80% of patients, significantly adding to the morbidity of this tragic syndrome (Pepose et al., 1985a), the pathobiology is not clearly understood.

While several opportunistic agents (herpes groups viruses) have routinely been observed in this form of retinitis, the common denominator of the process has not been determined. Whether the pathology arises as a response to infection with HIV-1 or another herpes group virus, or as a result of combined infection (helper virus effect) is currently unknown. It has been suggested that HIV-1 may directly or indirectly damage retinal tissue and interact with opportunistic pathogens, thus leading to the variety of ocular abnormalities observed with AIDS (Schmitt-Graffe et al., 1990). Interestingly, HIV-1 has been isolated from and demonstrated within both lesioned and nonlesioned retinal tissue (Skolnik et al., 1989). This data suggests that HIV-1 alone may not cause clinically recognized retinal lesions. In our study, HIV-1 and HHV-6 were confined to the lesion containing areas of retina (Qavi et al., 1989, 1991a). The diversity of responses to HIV-1 infection suggest that multiple co-factors are likely to be involved in the pathogenesis of the disease. One of these co-factors may be herpes group viruses. Whenever dual infection occurs viral interaction may result. Several human herpesviruses such as HSV-1 (Mosca et al., 1987a,b), CMV (Davis et al., 1987; Nelson et al., 1989), and EBV (Kenney et al., 1988) have been shown to trans-activate *in vitro* HIV-1 LTR directed gene expression by a "tat" independent mechanism, but none of these viruses have the capability of invading $CD4^+$ T lymphocytes. Therefore their mechanism of activation of HIV-1 is unclear. HHV-6, on the other hand, has been shown to have the capability of infecting $CD4^+$ T lymphocytes and is the only herpes group virus which appears to share cell tropism with HIV-1. Several lines of evidence indicate that HHV-6 and HIV-1 can productively co-infect $CD4^+$ lymphocytes leading to accelerated cytopathic effect as compared to HIV-1 infection alone (Ensoli et al., 1989; Gallo et al., 1989; Lusso et al., 1989;. Krueger et al., 1990a,b; Schonnebeck et al., 1991) It has also been shown that HHV-6 infection induces nuclear factors that specifically bind to the enhancer region of the HIV-1 LTR (Ensoli et al., 1989). Since replication of HHV-6 itself is cytopathic to $CD4^+$ T lymphocytes, it might accelerate the rate of the disease process. Our data support this hypothesis. 2 of the 4 HIV-1 culture positive retinas which were also positive for both HIV-1 and HHV-6 transcriptional activity showed a rise in HIV-1 p24 and reverse transcriptase (RT) activity as early as day 4. The remaining 2 retinas which contained only the HHV-6 DNA sequences showed an increase in HIV-1 p24 and RT activity much later. This suggests that activation of HIV-1 (at least *in vitro*) may require active co-infection with HHV-6. The finding of HIV-1 and HHV-6 in combination with CMV in the retinas of AIDS patients may have etiological implications concerning AIDS associated retinitis. Infection with HIV-1 or HHV-6, alone or in combination, may predispose retinal tissue to infection by other agents, such as CMV. The fact that CMV retinitis is a late manifestation of AIDS and a poor prognostic sign further supports this hypothesis. A suitable animal model is

urgently needed to firmly establish the relationship of HIV-1, HHV-6 and CMV to AIDS-associated retinitis.

Another line of support of this hypothesis is the corneal data. Interestingly, one HIV-1 culture positive cornea from an asymptomatic carrier of HIV-1 which was also culture positive for HHV-6 showed a rise in HIV-1 p24 and RT activity at day 10, whereas the other 2 corneas from asymptomatic HIV-1 carriers which of contained only HHV-6 DNA sequences exhibited increases in HIV-1 p24 and RT activity much later. These observations again suggest that HHV-6 may be associated with activation of HIV-1. HHV-6 is usually recovered in immature T lymphocytes (CCRB-HSB_2 cells), but 1 cornea from an asymptomatic carrier of HIV-1 was culture positive for both HIV-1 and HHV-6 only in PBMC cells (Qavi et al., 1991b). The reason for lack of recovery of HHV-6 from CCRB-HSB_2 cells is uncertain. One possibility is that HIV-1 may have activated the growth of HHV-6 in the PBMC culture. CCRB-HSB_2 cells do not support HIV-1 growth and hence there was no activation of HHV-6 in such a culture. This observation suggests a bidirectional activation (activation of HHV-6 by HIV-1). Similar bidirectional activations have been shown between HIV-1 and CMV by Skolnik et al., (1988). Finally, 4 of the 6 HIV-1 culture positive corneas were positive for the HIV-1 transcriptional activity, showing for the first time the presence of an active HIV-1 infection in corneal buttons. Although preliminary, our results indicate that HIV-1 and HHV-6 may be capable of invading corneal tissues lending credence to the need for a vigorous serologic screening of corneal donors.

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Ablashi/Krueger/Salahuddin (eds) Human Herpesvirus-6

CHAPTER 21

Human herpesvirus-6 and bone marrow transplantation

DONALD R. CARRIGAN

Department of Pathology and the Bone Marrow Transplant Program, Medical College of Wisconsin, Milwaukee, WI 53226, U.S.A.

21.1. Introduction

Marrow transplantation is being increasingly used for the treatment of a variety of diseases, including aplastic anemia, leukemia, and breast cancer. Viral infections are an important cause of morbidity and mortality in marrow transplant patients with interstitial pneumonitis being the most serious clinical manifestation. Cytomegalovirus (CMV) accounts for the majority of these infections, and untreated CMV interstitial pneumonitis has a mortality rate of approximately 90% (Bortin and Rimm, 1986). The mortality of interstitial pneumonitis for which no specific etiology is identified, i.e., idiopathic interstitial pneumonitis (IIP), is less than that of CMV pneumonitis, but it is still substantial with a case fatality rate of 50–60% (Thomas, 1983). That some cases of IIP are of viral origin is suggested by the similar histopathological changes seen in IIP and in cases of proven viral pneumonitis. Identification of viruses capable of causing IIP is important since infection with some of them may have high case fatality rates and yet be treatable with currently available antiviral agents (Bortin et al., 1989).

Another important way in which viral infections can manifest in marrow transplant patients is invasion of the grafted marrow with a concomitant decrease or delay in hematopoietic reconstitution. CMV is the pathogen most clearly demonstrated to be capable of causing this complication (Bolger et al., 1986) although other viruses such as parvovirus B19 (Kurtzman et al., 1987) and Epstein–Barr virus (EBV, Baranski et al., 1988) have been theoretically implicated as well. Mechanisms involved in the marrow suppression are likely to be complex, but probably include both destruction and induced dysfunction of stem cells and disruption of the biochemical micro-environment necessary for stem cell

proliferation and differentiation (Young et al., 1984; Simmons et al., 1990). The most common signs of such infections are poor graft function with leukopenia, anemia and thrombocytopenia, and exacerbation or prolongation of the immunosuppressed state. Clinically, hemorrhagic complications and increased susceptibility to opportunistic infections, both of which can be life-threatening, are frequently seen.

We have identified a newly discovered herpesvirus, human herpesvirus-6 (HHV-6) (Salahuddin et al., 1986), as a cause of severe interstitial pneumonitis, either directly or in concert with other respiratory pathogens, in marrow transplant patients (Carrigan et al., 1991). Also, a series of clinical and experimental observations have implicated HHV-6 marrow infection as an important cause of poor graft function in marrow transplant recipients (Carrigan et al., 1991; Knox and Carrigan, 1992). HHV-6 is a beta-herpesvirus closely related to CMV with respect to both genome homology and biological properties (Russler et al., 1989; Lawrence et al., 1990). In cell culture, the primary target of HHV-6 appears to be the CD4 positive T lymphocyte (Lusso et al., 1988; Takahashi et al., 1989), although a number of other cell types have been reported to be infectable (Ablashi et al., 1988). Work with human tissues suggests that HHV-6 can replicate within lymphocytes (Eizuru et al., 1989), salivary gland epithelial cells (Fox et al., 1990; Krueger et al., 1990), and renal tubular epithelial cells (Okuno et al., 1990) and macrophages (Carrigan et al., 1991). Estimates of the seroprevalence of HHV-6 in normal populations range from 60 to 90% (Brown et al., 1988; Krueger et al., 1988; Okuno et al., 1989; Yoshikawa et al., 1989). Limited data (Asano et al., 1989) and analogy with the other herpesviruses (Jordan, 1983) suggest that latent infections with HHV-6 occur.

Viral isolation and serological studies have confirmed HHV-6 as the cause of exanthum subitum, a mild illness of young children (Yamanishi et al., 1988; Ueda et al., 1989). HHV-6 can be intermittently isolated from the saliva of most normal, seropositive individuals (Pietroboni et al., 1988b; Levy et al., 1990a) similar to the situation with EBV (Gerber et al., 1972). HHV-6 has been isolated from the saliva and peripheral blood of patients with the acquired immunodeficiency syndrome (AIDS) (Salahuddin et al., 1986; Pietroboni et al., 1988b) and from the peripheral blood mononuclear cells (PBMC) of patients with a variety of lymphoproliferative diseases including both lymphomas (Becker et al., 1989) and leukemia (Salahuddin et al., 1986; Becker et al., 1988). It has also been isolated from the PBMC of a child with fatal hemophagocytic syndrome (Huang et al., 1990), an infant with fatal hepatitis (Asano et al., 1990), from the PBMC of 2 patients during episodes of renal allograft rejection (Okuno et al., 1990) and from a kidney biopsy taken from a renal transplant patient (Asano et al., 1989). In normal individuals primary infections with HHV-6 can cause a mononucleosis syndrome (Steeper et al., 1990) as well as a mild, self-limited febrile illness (Niederman et al., 1988). Transient febrile episodes occasionally occur in renal transplant patients with HHV-6 infections (Morris et al., 1989). Using viral isolation and serologic methods, the virus has been implicated as being involved in lymphomas and sarcoidosis (Biberfeld et al., 1988) and in the chronic fatigue syndrome (Krueger et al., 1987; Buchwald et al., 1990). It has been proposed that HHV-6 may synergize with the human immunodeficiency virus

(HIV) in causing AIDS (Gallo, 1988), although it is unclear whether the virus enhances (Lusso et al., 1989) or suppresses the replication of HIV (Pietroboni et al., 1988a; Carrigan et al., 1990; Levy et al., 1990b).

In spite of these efforts, the pathogenic potential of HHV-6 remains largely undefined, especially with respect to its potential to cause serious disease in immunocompromised patients. As noted above, CMV, a virus closely related to HHV-6, is a common and dangerous pathogen in immunocompromised patients (Griffiths and Grundy, 1988). Also, EBV, another herpesvirus similar to HHV-6 with respect to its seroprevalence and apparent mode of spread (Gerber et al., 1972; Levy et al., 1990a), causes serious and occasionally fatal disease in both immunocompromised and normal individuals (Miller, 1990). Therefore, it would not be surprising to discover that HHV-6 is capable of causing life-threatening infections under the proper circumstances. Further, the lytic nature of HHV-6 infection of T lymphocytes and other leukocytes suggests that the virus has the potential to induce or exacerbate an immunocompromised state in its host. This chapter summarizes the results of a series of studies performed in our laboratory concerning the role of HHV-6 as a pathogen in marrow transplant patients.

21.2. Prospective estimation of HHV-6 infection rate in marrow transplant patients

In collaboration with the Marrow Transplant Unit of the Medical College of Wisconsin under the direction of Dr. Robert Ash and the laboratory of Dr. Michael Dunne at the Children's Hospital of Wisconsin, our laboratory is conducting a prospective study of HHV-6 infections in adult marrow transplant patients. Full results are not yet available. However, an interim summary of data concerning the serological assessment of the patients is shown in Table 21.1. Biweekly serum specimens were obtained from the patients and analyzed for the presence of HHV-6 specific IgM antibodies and for the titer of HHV-6 specific IgG antibodies. The enzyme immunoassay (EIA) procedure used was similar to that described by Chou and Scott (1990) and utilized the GS strain of HHV-6 ($HHV\text{-}6_{GS}$) grown in the HSB_2 cell line as the source of viral antigen. This virus and the HSB_2 cells were provided by Dr. Dharam Ablashi of the National Cancer Institute. IgM antibodies were detected by a mu-chain specific, enzyme labeled antiserum combined with IgG depletion of the serum specimen by means of immunoprecipitation with human IgG-specific antibodies (Whittaker Bioproducts; Walkersville, Maryland). Since the patients were receiving prophylactic intravenous human IgG (IVIG) during these studies, at least a ten-fold increase in IgG titer was required for the alteration to be considered indicative of an active HHV-6 infection. The IVIG protocol produced serum titers of HHV-6 IgG of between 1:50 and 1:500 in previously seronegative patients. As shown in the table, approximately 60% (8/13) of the patients were seropositive for HHV-6 prior to their transplant, a proportion similar to that described by others for adults (Brown et al., 1988; Krueger et al., 1988; Okuno et al., 1989; Yoshikawa et al., 1989). It should be noted, however, that

TABLE 21.1.
Prospective serological study of HHV-6 infections in marrow transplant patients

Patient	Days after BMT	HHV-6 IgM detected	Ten-fold increase in IgG titer
JS(?)[a]	180	+(102)[b]	+(102)
NB(+)	160	+(91)	+(105, 153)
CC(−)	158	−	+(53)
GC(+)	148	+(102)	+(148)
MB(+)	123	+(39)	+(102)
TC(+)	115	−	−
DG(+)	99	+(99)	+(92)
SS(?)	95	+(59)	−
CT(−)	95	−	−
MN(−)	88	−	−
CA(+)	88	−	−
MS(?)	76	+(47)	−
LS(?)	71	−	−
TM(?)	68	+(54)	−
JE(−)	68	−	+(47)
JH(?)	66	−	−
CK(+)	59	−	−
KF(−)	56	+(40)	+(40)
BL(+)	32	−	−

[a] HHV-6 serostatus prior to transplant: (+) HHV-6 IgG detected; (−) HHV-6 IgG not detected; (?) serum not available or not determined.
[b] Numbers refer to the day after transplant on which antibodies were detected.

the sensitivity of the EIA used may have been inadequate to detect low levels of HHV-6-specific IgG in some seropositive patients. Current estimates of the HHV-6 reactivation rate in the seropositive patients are 50% (4/8) using HHV-6 IgM positivity, changes in IgG titer or both measures. This must be considered a minimal estimate since several patients have been followed for less than 2 months after their transplants. Also, patient BL developed mental status changes and focal CNS disease contemporaneously with repeated isolation of HHV-6 from his peripheral blood. No serological manifestations of this infection have yet appeared although the follow-up period has been short (2 weeks). The mean time of HHV-6 reactivation in the patients was approximately 80 days after marrow transplantation with a range of 39–102 days. These figures are similar to the corresponding times for CMV reactivation in marrow transplant patients (Apperley and Goldman, 1988). 60% (3/5) of the patients who appeared to be seronegative prior to transplantation have later shown evidence of active HHV-6 infection, although only one became HHV-6 IgM positive. In this patient primary infection with the virus appears to have occurred since she showed an IgG titer rise from less than 1:500 1 week after her transplant, while receiving IVIG, to greater than 1:5000 during her fifth week after transplant. Also, the appearance of the HHV-6-reactive IgM antibody was accompanied by high fever, fatigue and leukopenia that were not attributable to another cause.

The range of clinical consequences of the HHV-6 infections that were detected and the results of concurrent viral isolation studies are being evaluated. However, 3 patients (NB, GC and BL) developed serious illnesses that correlated with their HHV-6 infections in the absence of any other detected pathogen, including CMV. HHV-6 was isolated from the peripheral blood of 2 of the patients (NB and BL) at the time of illness onset. The significance of these isolations is supported by the observations that multiple subsequent viral isolation attempts from these patients during asymptomatic periods, and 20 of 21 isolation attempts using samples of peripheral blood from 15 other marrow transplant patients were negative during the same time period. Manifestations of the HHV-6 infections seen in these 3 patients included malaise, leukopenia, fever, lymphadenopathy, mental status changes, focal central nervous system (CNS) disease and interstitial pneumonitis in different combinations. All had illnesses of sufficient severity to warrant treatment with foscarnet or ganciclovir, and both NB and GC responded positively to treatment, dramatically so in the case of GC. Thus far, BL has not shown a positive effect of ganciclovir treatment for his HHV-6-associated CNS disease, but the time of treatment has been short at this writing. NB continues to have poor graft function which corresponds to continued activity of her HHV-6 infection (fluctuations in her serum HHV-6 IgG titer). A more complete description of these serological and virological studies will be forthcoming, but it can now be concluded that HHV-6 reactivations are common in seropositive patients after marrow transplantation, post-transplant HHV-6 primary infections occur and serious peripheral and CNS clinical manifestations of these infections are possible.

21.3. Examples of HHV-6 infections in marrow transplant patients

The full range of the clinical manifestations of HHV-6 infections remains unknown, but our experience to date can best be summarized in the descriptions of the 2 marrow transplant patients in which severe HHV-6 infections were first documented (Carrigan et al., 1991). They illustrate the abilities of the virus to infect lung tissue and cause interstitial pneumonitis and to infect marrow cells and interfere with their differentiation and response to cellular growth factors. Summaries of their courses are shown in Fig. 21.1.

21.3.1. Patient 1

This was a 19-year-old CMV seronegative and HHV-6 seropositive man with testicular carcinoma who received an autologous BMT. One week following a planned second autologous BMT he developed fever and progressive bilateral interstitial infiltrates and required mechanical ventilatory support. Over the next 2 weeks the pulmonary infiltrates slowly resolved, and the patient was removed from the ventilator. However, 3 weeks later fever and interstitial infiltrates recurred. Progressive respiratory failure then occurred over the next 2 months leading to his death. Marrow function was poor throughout his course with his peripheral

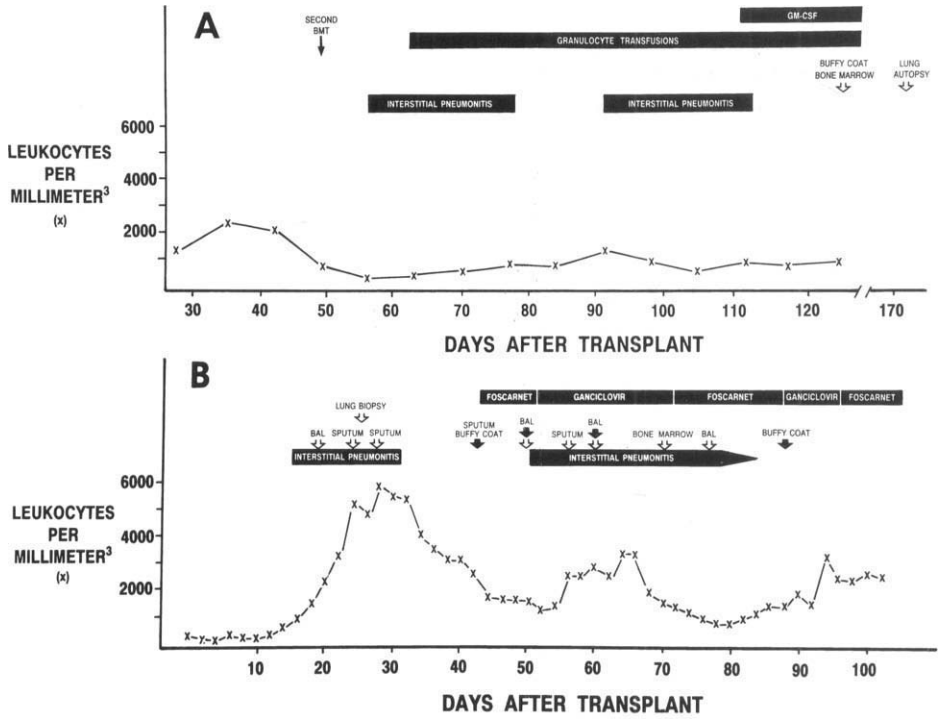

Fig. 21.1. Summaries of the post marrow transplantation courses of patients with HHV-6 interstitial pneumonitis. Downward pointing arrows designate times of viral isolation or immunohistochemical detection of virally infected cells in the indicated specimen. ⇩ denotes HHV-6, while ⬇ denotes CMV. (A) 19-year-old male patient after autologous marrow transplant for testicular carcinoma. (B) 32-year-old female patient after allogeneic marrow transplant for myelodysplasia.

leukocyte count rarely exceeding 1000 cells per mm^3. HHV-6 (designated the $HHV\text{-}6_{BA}$ strain) was isolated from samples of blood and marrow obtained immediately before his death. Immunohistochemical staining of lung tissue samples obtained at autopsy showed widespread HHV-6 infection in close association with tissue necrosis, inflammation and fibrosis. An example of the HHV-6-infected cells observed in lung tissue obtained at autopsy is shown in Fig. 21.2. The infected cells appeared to be macrophages and lymphocytes with no evidence of epithelial cell involvement. No other respiratory pathogen was identified at autopsy or at any other time throughout his course. CMV was never isolated from any specimen during his course, no CMV-infected cells were detected by immunohistochemical staining of lung tissue obtained at autopsy using procedures described in detail previously from this laboratory (Toorkey and Carrigan, 1989), and CMV DNA was not detected in lung tissue by means of the polymerase chain reaction (PCR) technique. While the etiology of this patient's initial episode of interstitial pneumonitis remains unproven, the pneumonitis present at the time of autopsy was probably due to the HHV-6 infection since it fulfilled accepted criteria for the

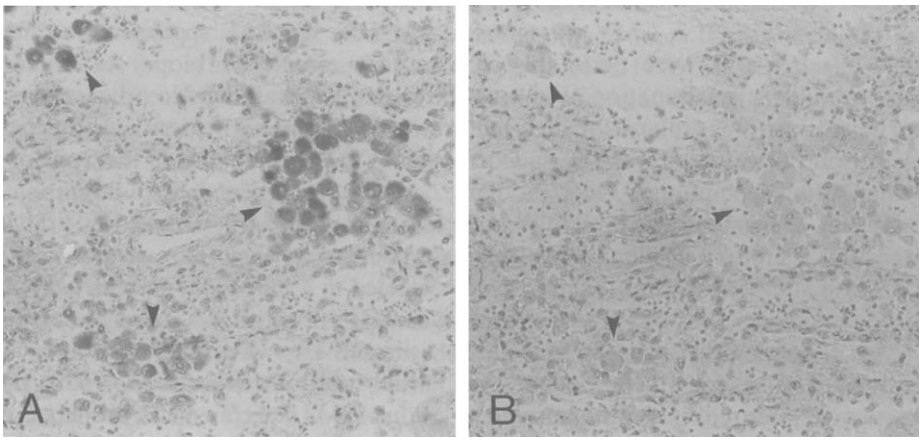

Fig. 21.2. Immunohistochemical detection of HHV-6 infected cells in lung tissues from marrow transplant patient with HHV-6 interstitial pneumonitis. Details of the avidin-biotin based immunohistochemical staining procedures used in this laboratory for the detection of HHV-6-infected cells in sections of fixed and paraffin embedded tissues have been described in detail previously (Russler et al., 1991). Briefly, a rabbit hyperimmune serum raised against HSB_2 cells infected with the GS strain of HHV-6 was used after being made HHV-6 specific by extensive absorption with lysates of uninfected HSB_2 cells. A matched control was prepared by subjecting a sample of the same original hyperimmune serum to an identical absorption procedure with HHV-6_{GS}-infected HSB_2 cells. This control serum showed no residual reactivity with HHV-6_{GS}-infected HSB_2 cells. The HHV-6-specific and control sera were used at dilutions giving identical IgG concentrations (nuclear fast red counterstain). (A) A section of lung tissue stained with the HHV-6-specific antiserum. Arrowheads indicate clusters of HHV-6-infected cells. (B) An immediately adjacent serial section of tissue after staining with the control serum. Arrowheads indicate the same cell clusters as in (A).

diagnosis of viral pneumonitis (Emanuel et al., 1986; Cordonnier et al., 1987; Paradis et al., 1988). Isolation of HHV-6 from the patient's marrow suggests that the poor performance of his marrow graft may have been due to that infection since herpesviruses are known to infect and inhibit the function of marrow cells (Bolger et al., 1986; Simmons et al., 1990; Knox and Carrigan, 1992). Experimental evidence for HHV-6-associated suppression of marrow cell differentiation and proliferation is presented in a later section.

21.3.2. Patient 2

This was a 32-year-old CMV and HHV-6 seropositive woman with myelodysplastic syndrome who underwent allogeneic marrow transplantation. Two weeks after transplant, the patient developed fever and bilateral interstitial infiltrates. A bronchoalveolar lavage (BAL) specimen was positive for HHV-6 by viral isolation in PBMC cultures and by immunohistochemical staining. Numerous HHV-6-infected cells, primarily intra-alveolar macrophages as shown by morphology and positive staining for alpha-1 chymotrypsin, were detected in a lung biopsy sample

using an immunohistochemical staining procedure identical to the one described in the legend of Fig. 21.2. CMV was not isolated from the BAL specimen, and no CMV-infected cells were detectable in the BAL sample or biopsy tissue by histopathological examination or immunohistochemical staining. No other respiratory pathogen was isolated or detected in any of the patient's specimens during this period. Therefore, it was concluded that HHV-6 was the cause of this episode of interstitial pneumonitis. Over the next 2 weeks the patient had radiographic resolution of her lung abnormalities but continued to have persistent low-grade fevers and a gradual decline in her leukocyte count.

42 days after transplantation, CMV was detected in a buffy coat specimen. Due to the possibility of simultaneous infection with HHV-6 and CMV, treatment with foscarnet, an agent with broad anti-herpesvirus activity, was begun. However, 8 days into the treatment, the patient's interstitial infiltrates reappeared and progressive dyspnea required mechanical ventilation. A BAL specimen was positive for HHV-6 by isolation of the virus in a PBMC culture, and numerous HHV-6-infected cells were detected in the BAL specimen by immunohistochemical staining. CMV was also detected in the same lavage fluid by a rapid shell vial procedure. However, no CMV was isolated in cell culture and no CMV-infected cells were detected in the specimen by immunohistochemical staining, suggesting that the amount of CMV present was low. Foscarnet and ganciclovir therapies were then alternately applied in a regimen designed to minimize ganciclovir-associated marrow suppression while maintaining continuous anti-herpesvirus treatment. Fever persisted, but there was no further deterioration in respiratory status. A progressive pancytopenia was noted on about the sixty-eighth day, and numerous HHV-6-infected cells were immunohistochemically detected in a sample of marrow. No CMV was isolated from this marrow specimen, and no CMV-infected cells were detected by immunohistochemical staining. The patient's respiratory status slowly improved, and she was weaned from the ventilator. She remained severely leukopenic (< 2000 cells per mm^3) with an absolute lymphocytopenia (< 50 lymphocytes per mm^3) for the remainder of her course. She died several months later of a disseminated adenovirus type 1 infection.

We believe that the initial episode of interstitial pneumonitis in this patient was due to HHV-6 since no other pathogen, including CMV, was detected in any of the patient's specimens during this period despite exhaustive isolation attempts and the virological parameters fulfilled criteria used to diagnose viral pneumonitis (Emanuel et al., 1986; Cordonnier et al., 1987; Paradis et al., 1988). Co-infection with HHV-6 and CMV probably caused the later periods of interstitial pneumonitis; a synergistic effect of the 2 viruses on the pneumonitis is likely but cannot be proven. A role for CMV in the poor function of this patient's marrow graft cannot be ruled out, but HHV-6 was the only pathogen whose presence in the marrow was demonstrated. Thus the course of this patient confirms the ability of HHV-6 to serve as a pulmonary pathogen and lends support to the possible role of HHV-6 as a cause of marrow dysfunction. Finally, both of these marrow transplant patients developed HHV-6 pneumonitis (probable in the first patient and definite in the

second) within a few weeks after their transplants. This time corresponds to a period of rapid proliferation of marrow derived cells in many tissues, including the lungs. This fact, when combined with the tropism of HHV-6 for actively proliferating cells (Frenkel et al., 1990), suggests that this period after transplantation may be one of selective vulnerability of the patients to HHV-6 infection and disease.

21.4. Retrospective identification of lung infections with HHV-6 in marrow transplant patients with pneumonitis

Identification of 2 marrow transplant patients with HHV-6-associated interstitial pneumonitis within 1 year suggested that such infections may be relatively common. In order to test this possibility archival lung tissues from 9 marrow transplant patients known to have died of pneumonitis were immunohistochemically examined for the presence of HHV-6-infected cells. Cases were chosen with no knowledge of the diagnosed etiology of the pneumonitis. One block of tissue was examined for each patient. The serostatus of the patients with respect to HHV-6 was unknown. The staining procedure used was identical to the one described previously (Russler et al., 1991) and in the legend of Fig. 21.2. Results are shown in Table 21.2. HHV-6-infected cells were identified in the lungs of 3 of the 9 patients examined. In all 3 the infected cells were intra-alveolar macrophages and lymphocytes scattered throughout the tissue, especially in proximity to areas of inflammation and necrosis. Morphology and distribution of the antigen-positive cells were similar to those seen in the 2 patients described above. Concentrations of positively staining cells varied widely, but in all 3 focal areas were seen that contained high numbers of HHV-6-infected cells. In both cases with a co-existent CMV pneumonitis (A122-85 and A173-86) no staining of CMV-infected cells with the HHV-6 specific antiserum was observed, and at least as many HHV-6-infected cells were present as cells infected with CMV. However, the relative contributions of HHV-6

TABLE 21.2.
Presence of HHV-6-infected cells in lung tissues from BMT patients with pneumonitis

Autopsy number	Age/sex of patient	Time from BMT to onset of fatal pneumonitis	Autopsy diagnosis of pneumonitis etiology	HHV-6 staining
A152-84	28y/M	3 months	idiopathic	–
A122-85	30y/M	1 month	CMV	+
A7-86	28y/F	2 months	idiopathic	–
A44-86	7y/F	6 weeks	pseudomonas	–
A134-86	31y/M	5 months	idiopathic	–
A173-86	24y/F	5 months	CMV and adenovirus	+
A96-87	30y/M	5 months	bacteria	–
A102-87	31y/F	7 months	bacteria	–
A35-88	30y/M	6 months	aspergillus	+

and the other pathogens could not be determined. Finally, since only 1 tissue block was analyzed for each case, this rate of HHV-6 positivity (approximately 30%) must be considered a minimal estimate of the true lung infection rate in such patients.

Therefore, it appears that HHV-6 can infect macrophages and lymphocytes within the lung tissues of marrow transplant patients and, in some cases, this infection is associated with an interstitial pneumonitis. Also, lung infections with HHV-6 appear to be common since cells infected with the virus were detected in relatively high numbers in tissues of a sizable proportion of patients dying with pneumonitis. It can be postulated that HHV-6 can synergize with other pathogens (e.g., CMV, adenovirus or aspergillus) in causing pneumonitis in marrow transplant patients by killing or causing dysfunction of macrophages and lymphocytes in the infected tissues.

21.5. *In vitro* infection of macrophages by HHV-6

The prominent macrophage infection with HHV-6 in the lungs of the marrow transplant patients just described made it of interest to explore and document the ability of macrophages to support the replication of HHV-6 *in vitro*, which has been previously noted in passing by other investigators (Levy et al., 1990a). PBMC were obtained from a HHV-6 seronegative donor and stimulated for 3 days with 10 μg/ml of PHA. The cells were then cultured for 21 days with weekly changes of the culture medium. At the end of this period, nonadherent cells were removed from the cultures by vigorous washing, and the adherent cells were infected with either the HHV-6 strain obtained from the marrow of the first patient described above ($HHV\text{-}6_{BA}$) or another HHV-6 strain isolated in this laboratory ($HHV\text{-}6_{KF}$) (Russler et al., 1991). The adherent cells obtained by this procedure were over 95% macrophages as determined by a-naphthyl acetate esterase staining (Sigma Diagnostics; St. Louis, Missouri). The majority of the macrophages infected with either of the 2 strains of virus developed a progressive cytopathic effect (CPE) that destroyed essentially all of the cells by the tenth day after infection. An example of this CPE is shown in Fig. 21.3. These findings were consistent with the widespread infection of macrophages in the lungs of the patients and supported the idea that HHV-6 infection of macrophages may be important in the induction of lung disease by the virus.

21.6. Suppression of marrow stem cell differentiation by HHV-6

Our experience to date suggests that suppression of marrow function may be one of the most common, and potentially most serious, effects of HHV-6 infections in marrow transplant patients. Both of the patients described in detail above, an immunologically normal individual with HHV-6/legionella pneumonia described

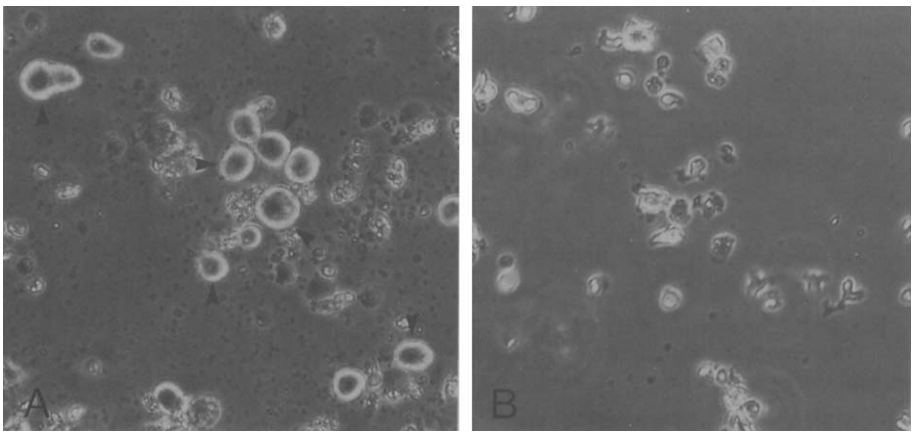

Fig. 21.3. Viral cytopathic effects in macrophages infected with HHV-6. Plastic adherent cells in cultures of PHA-stimulated PBMC were washed free of nonadherent cells and infected with HHV-6_{KF}. Greater than 95% of the adherent cells were macrophages as shown by a-naphthyl acetate esterase staining. (A) HHV-6_{KF}-infected macrophages on the eighth day after infection. Arrowheads indicate some of the cells showing typical HHV-6 CPE. (B) A control, uninfected macrophage culture at the same time. Photographs were taken of living cultures using phase contrast microscopy.

previously by this laboratory (Russler et al., 1991), and the symptomatic patients represented in Table 21.1 all showed evidence of various degrees of marrow dysfunction in concert with their HHV-6 infections. Direct infection by HHV-6 of marrow cells was documented in both of the patients described in detail above (Fig. 21.1). Precedents for marrow suppression by viruses, and especially herpesviruses, are common (Bolger et al., 1986; Baranski et al., 1988; Simmons et al., 1990), and suppression of hematopoietic colony formation by CMV has been described (Rakusan et al., 1989; Sing and Ruscetti, 1990). Therefore, a standardized methylcellulose-based colony formation assay (Human Bone Marrow Stem Cell Proliferation Kit; GIBCO BRL; Gaithersburg, Maryland) (Fauser and Messner, 1979) was used to determine whether HHV-6 infection can interfere with differentiation of several types of marrow stem cells (Knox and Carrigan, 1992). Briefly, purified marrow mononuclear cells from a normal adult donor were infected with either HHV-6_{KF} or HHV-6_{GS} and subjected to a marrow stem cell proliferation assay. The assay medium contained 5 units per milliliter of erythropoietin, and results were determined 2 weeks after initiation of the cultures. 'Mock' viral preparations, i.e., materials prepared from uninfected cells handled in parallel to those used for viral stocks, were included as controls. 250 000 marrow cells were used per culture, and all cultures were run in triplicate. Results of one such experiment are summarized in Table 21.3. The stem cells evaluated were CFU-GM (precursor of the granulocyte/macrophage cell lineage), BFU-E (erythroid blast unit representing the precursor of erythroid differentiation), and CFU-GEMM (multipotential precursor of the erythroid, myeloid and megakaryocyte cell

TABLE 21.3.
Effect of HHV-6 infection on differentiation of hematopoietic stem cells in normal marrow[a]

	Granulocyte/macrophage (CFU-GM)	Erythroid (BFU-E)	Multipotential (CFU-GEMM)
Control medium	21 +/− 1[b]	84 +/− 18	4 +/− 1
HHV-6_{KF}	16 +/− 2	17 +/− 18	1 +/− 1
Mock HHV-6_{KF}	18 +/− 3	118 +/− 16	7 +/− 1
HHV-6_{GS}	10 +/− 4	37 +/− 8	0.3 +/− 0.5
Mock HHV-6_{GS}	10 +/− 3	93 +/− 19	4 +/− 1

[a] Experimental details presented in text.
[b] Mean number of respective colonies in thirty-two 10× microscopic fields +/− one standard deviation. Assay performed in triplicate.

lineages). Infection of the marrow cells with either HHV-6_{KF} or HHV-6_{GS} had little or no effect on CFU-GM differentiation. In contrast, a sizable suppression of BFU-E differentiation was seen with both viral strains (86% and 60% suppression with HHV-6_{KF} and HHV-6_{GS}, respectively). Although the numbers of cells involved in the assay were smaller, similar suppression of CFU-GEMM differentiation by both viruses was observed. The magnitude of the inhibition can be estimated at 80–90% for both virus strains, although the accuracy of these estimates is suspect due to the small numbers. The mechanism or mechanisms involved in these effects are unknown, but the potential for HHV-6 to infect and adversely effect the differentiation of marrow stem cells is clear.

21.7. Inhibition of marrow stem cell responses to growth factors by HHV-6 infection

In the few marrow transplant patients with HHV-6-associated marrow suppression that have been studied to date, weak or transient responses to growth factors such as granulocyte/macrophage-colony stimulating factor (GM-CSF) have been seen (e.g., patient number one described above, Fig. 21.1a). This observation, combined with the ability of HHV-6 infection to suppress the differentiation of marrow stem cells suggested that the virus may be directly interfering with the growth factor response. This possibility has now been tested. Purified marrow mononuclear cells were infected with either HHV-6_{KF} or HHV-6_{GS} as described for the colony formation assay above. 'Mock' viral materials were once again used as controls. One million marrow cells were used per culture, and all cultures were run in duplicate. Cells were cultured for 1 week in the presence of either 10 ng/ml of human recombinant DNA derived GM-CSF (Sandoz Pharmaceuticals) or 1 ng/ml of human recombinant DNA derived interleukin-3 (IL-3) (Intergen Company), a growth factor having a stimulatory effect on marrow stem cells (Ogawa, 1989). At the end of this period, the nonadherent cells in the flasks were removed, the cultures

were thoroughly washed and the remaining adherent cells were stained to identify macrophages using an a-naphthyl acetate esterase staining procedure (Sigma Diagnostics). The darkly staining esterase positive macrophages were then counted, and the numbers present in the different cultures were compared. Numbers of nonadherent cells present in the different cultures were determined to assure culture viability and to assess viral cytopathogenicity. Photographic examples of the infected and uninfected GM-CSF treated macrophage cultures are shown in Fig. 21.4, and the quantitative results are summarized in Table 21.4.

Addition of human GM-CSF or IL-3 to the marrow cells dramatically increased the number of macrophages in the cultures, consistent with the known biological effects of these growth factors. However, both HHV-6_{KF} and HHV-6_{GS} suppressed the GM-CSF and IL-3 induced macrophage outgrowth from the marrow stem cells by more than 99%. This effect of the virus on macrophage production was not due simply to general cell destruction by the virus since the number of viable nonadherent cells in all of the different cultures were approximately equal. The mechanism or mechanisms of this macrophage suppression is unclear. However, it is likely that HHV-6 infection either prevented the growth factor induced differentiation of macrophages from the marrow stem cells or the virus infected and destroyed newly formed macrophages soon after, or during, their differentiation. The lack of measurable effect of either HHV-6_{KF} or HHV-6_{GS} on CFU-GM

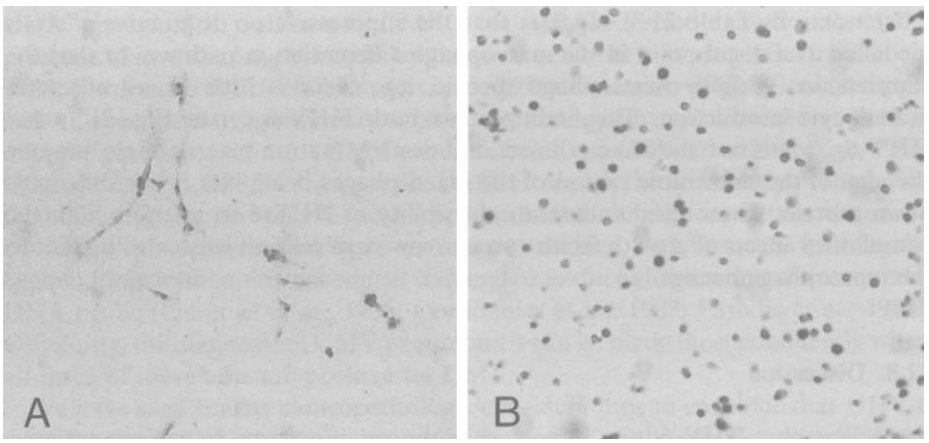

Fig. 21.4. Effect of HHV-6-infection of macrophage differentiation from marrow stem cells treated with GM-CSF. Purified marrow mononuclear cells were infected with HHV-6_{KF} and cultured in the presence or absence of 10 ng/ml of recombinant DNA-derived human GM-CSF. On the seventh day after infection the nonadherent cells present were removed, and the adherent cells remaining were stained for nonspecific esterase to identify macrophages. Quantitative results are given in Table 21.3. (A) The HHV-6_{KF}-infected culture. Few esterase positive cells are present in small foci. (B) The uninfected control culture treated with a 'mock' virus preparation. Note the large number of esterase positive cells evenly distributed across the culture.

TABLE 21.4.
Effect of HHV-6 infection of GM-CSF and interleukin-3 (IL-3) stimulated macrophage differentiation from normal marrow stem cells[a]

Sample	Plain medium	Medium + GM-CSF	Medium + IL-3
Control Medium			
macrophages	3[b]	914	447
nonadherent cells/ml	4.7×10^5	5.1×10^5	4.6×10^5
HHV-6_{KF}			
macrophages	2	4	1
nonadherent cells/ml	3.9×10^5	5.4×10^5	5.3×10^5
Mock HHV-6_{KF}			
macrophages	10	715	303
nonadherent cells/ml	4.9×10^5	5.6×10^5	5.2×10^5
HHV-6_{GS}			
macrophages	1	2	2
nonadherent cells/ml	4.4×10^5	3.5×10^5	3.9×10^5
Mock HHV-6_{GS}			
macrophages	4	983	340
nonadherent cells/ml	4.1×10^5	5.4×10^5	4.4×10^5

[a] Experimental details given in text.
[b] Number of esterase positive macrophages per sixteen 20× microscopic fields. Mean of duplicate cultures.

differentiation (Table 21.3) suggests that the suppressive or destructive effect is mediated at a distal point in the macrophage differentiation pathway or that the suppression is highly macrophage specific, e.g., there is little or no effect on granulocyte production. The findings that both HHV-6_{KF} (see Fig. 21.3) and HHV-6_{GS} (data not shown) can infect and destroy mature macrophages support the idea of the vulnerable period of the macrophages being late in the differentiation process. These results illustrate the ability of HHV-6 to interfere with the stimulatory effects of growth factors on marrow stem cells, at least with respect to the macrophage lineage.

21.8. Discussion

We believe that HHV-6 is an important pathogen in marrow transplant patients, both autologous and allogeneic. The 2 most common serious clinical manifestations of HHV-6 infection in these patients appear to be interstitial pneumonitis and poor marrow function, and each of these carries with it a significant degree of morbidity and mortality. Reasons for the failure of previous studies to identify the virus as a factor in the outcomes of these patients center around the difficulties involved in diagnosing HHV-6 infections. Like cells infected with EBV, HHV-6-infected cells in tissues do not develop intranuclear or cytoplasmic inclusion bodies

as do those infected with CMV. Thus detection of the virus by means of routine histopathological examination is difficult or impossible. Routine viral isolation procedures cannot reliably detect the presence of HHV-6 in patient specimens due to its failure to replicate in most human or other cell lines, although an abortive infection of human fibroblasts with some strains of HHV-6 can occasionally be detected. Isolation of the virus requires the use of mitogen-stimulated peripheral T lymphocytes, and these cells are rarely used routinely outside of laboratories involved with the isolation or study of HIV. HHV-6 serology is relatively well defined, but the high seroprevalence in normal adults makes exact clinical correlations difficult, especially in the setting of marrow transplantation where infections with a variety of agents are common and simultaneous involvement of several pathogens is not infrequent. Finally, detection of HHV-6-infected cells in patient specimens requires the use of specialized immunological and molecular biologic reagents and procedures that are not in common use. Therefore, most HHV-6 infections in marrow transplant patients probably go unrecognized or are misdiagnosed as being caused by other infectious agents. At the present time we are exploring the idea that some illnesses attributed to CMV in marrow transplant patients may, in fact, be due to HHV-6 infections or to HHV-6/CMV co-infections. Examples of such co-infections can be found in patient 2 above and in 2 of the patients shown in Table 21.1.

The HHV-6/CMV co-infections just mentioned raise the important topic of causation with respect to disease in marrow transplant and other patients. This deserves special comment since the determination of disease etiology, especially for interstitial pneumonitis, is frequently complex. For example, in the diagnosis of CMV pneumonitis isolation of CMV from a BAL-derived sample reflects the presence of invasive lung infection by the virus in 60–70% of cases (Emanuel et al., 1986; Cordonnier et al., 1987; Paradis et al., 1988). Detection of CMV-infected cells in BAL specimens by immunohistochemical staining or by cytological analysis has a higher predictive value for CMV pneumonitis of over 80% (Emanuel et al., 1986; Cordonnier et al., 1987; Paradis et al., 1988). However, the laboratory finding with the highest predictive value for CMV pneumonitis is the demonstration of numerous CMV-infected cells in biopsy- or autopsy-derived lung tissue by histological examination, immunohistochemical staining, or *in situ* hybridization with a specific DNA probe (Emanuel et al., 1986; Cordonnier et al., 1987; Paradis et al., 1988). Obviously, the diagnosis of CMV pneumonitis can be made most confidently when all three of these tests are positive for CMV.

We have used similar clinicopathologic considerations to conclude that HHV-6 caused episodes of interstitial pneumonitis in the 2 patients discussed in detail above. This conclusion should be considered carefully since it cannot be completely excluded that HHV-6 was either a copathogen with some undetected agent or that it was not involved directly in the disease process. In summary, the evidence implicating HHV-6 as the cause of the pneumonitis present at autopsy in the first patient rests upon the following observations: (1) active HHV-6 infection of the patient was demonstrated late in his course by isolation of the virus from his blood and marrow, (2) no other virus or other respiratory pathogen was isolated from

any respiratory specimen at any time during his course or at autopsy, and (3) large numbers of HHV-6-infected cells were detected throughout his lungs at autopsy. The postulated causative role of HHV-6 in the initial episode of pneumonitis in the second patient is supported by the following results: (1) HHV-6 was repeatedly isolated from sputum and BAL samples, (2) numerous HHV-6-infected cells were detected in several BAL and sputum specimens by immunohistochemical staining, (3) no other respiratory pathogen, including CMV, was detected in any respiratory or other specimen obtained during this period in spite of rigorous testing, and (4) numerous HHV-6-infected cells, but no cells infected with CMV, were detected by immunohistochemical staining of a biopsy derived sample of lung tissue. The etiology of the later episodes of pneumonitis seen in this patient was less clear due to the intermittent presence of low levels of CMV in several respiratory and buffy coat specimens during the later periods of her course. An accumulation of experience and more extensive experimental work will be necessary before a causative role for HHV-6 in interstitial pneumonitis can be unequivocally proven.

However, in this context it is important that HHV-6 infections are potentially immunosuppressive because of the viral tropism for macrophages and CD4 positive lymphocytes. Thus, in many instances HHV-6 may play an indirect role in lung, and other organ, infections by suppressing local immunological responses and, in that way, synergizing with other pathogens. Also, the absence of epithelial cell involvement in the HHV-6 lung infections seen to date suggests that the pathogenic mechanism of HHV-6 pneumonitis may be more dependent upon inflammation than parenchymal cell destruction. Widespread HHV-6 infection of macrophages could lead to the dysregulated production of physiologically active cytokines such as interleukin-1 (IL-1) and tumor necrosis factor (TNF) which would potentiate any inflammatory process present and possibly lead to an immunopathologic pneumonitis. Abnormal production of IL-1 and other cytokines by CMV-infected macrophages has been described (Dudding et al., 1989; Lewis et al., 1990; Moses and Garnett, 1990), and an immunopathological mechanism has been proposed for CMV pneumonitis (Zaia and Forman, 1987). The prompt response of a pneumonitis associated with a HHV-6/legionella co-infection in an immunologically normal adult to the administration of high dose corticosteroids supports an immunopathological component for HHV-6 lung infections (Russler et al., 1991).

Conclusions regarding the role of HHV-6 infections as the direct cause of graft dysfunction in marrow transplant patients must be drawn carefully for many of the same reasons just discussed for pneumonitis. However, the effects of HHV-6 infection on marrow stem cell differentiation (Knox and Carrigan, 1992) and on the induction of macrophage differentiation by growth factors *in vitro* support a role for the virus as a marrow pathogen. The cellular and molecular mechanisms involved in these effects are unclear, but 3 basic possibilities have to be considered. First, HHV-6 may infect a small population of marrow cells leading to the production of cytokines such as the interferons that have strong suppressive effects on cell proliferation and differentiation (Clemens and McNurlan, 1985; Rossi, 1985). Evidence for the existence of this mechanism has already been obtained (Knox and Carrigan, 1992). Second, the virus may productively infect and destroy

the differentiated cells as they develop from stem cells. The abilities of HHV-6_{KF} and HHV-6_{BA} to infect and destroy mature macrophages (Fig. 21.3) could be reflective of this mechanism. Third, HHV-6 may abortively infect marrow stem cells with only partial expression of its genome, e.g., only immediate-early gene products, which could modify the differentiation and proliferation of the infected cells without killing them. Such abortive infection of peripheral blood leukocytes by CMV has been described (Rice et al., 1984). Further, CMV infection has been shown to alter the expression of cell genes and gene products that are likely to influence cell differentiation such as proto-oncogenes (Boldogh et al., 1990) and major histocompatibility complex proteins (Browne et al., 1990). Similar interactions may be involved in the suppression of marrow cell proliferation and differentiation by HHV-6.

The importance of the observation that HHV-6 infection can suppress marrow function extends beyond marrow transplantation since HHV-6 has been isolated on numerous occasions from leukemia and lymphoma patients (Salahuddin et al., 1986; Becker et al., 1988, 1989), and growth factors such as GM-CSF are being increasingly used to ameliorate marrow suppression associated with high dose chemotherapy in such patients. Thus, the possibility of HHV-6 infections being associated with poor patient responses to growth factor therapy should be evaluated in both marrow transplant and other patient populations.

Finally, HHV-6 infections are currently treatable since the virus has a spectrum of antiviral drug sensitivities similar to that of CMV (Meyers, 1988; Russler et al., 1989). However, use of antiviral agents must be carefully considered with respect to the probable role of the virus in the illness, since causation can often be difficult to establish. As noted previously, in our experience, isolation of the virus in cell culture (using mitogen stimulated T lymphocytes from adult HHV-6 seronegative donors as indicator cells) from peripheral blood, bronchoalveolar lavage samples, or bone marrow is infrequent and often correlates with clinical manifestations. However, the study of HHV-6 pathogenesis in marrow transplant and other immunocompromised patients is just beginning, and conclusions are difficult to draw with confidence. Descriptions of HHV-6-associated diseases will become clearer as the methods for the detection or diagnosis of HHV-6 infections become more refined and as experience is gained in dealing with this new human pathogen.

21.9. Summary

We have identified human herpesvirus-6 (HHV-6) as a cause of severe interstitial pneumonitis, either directly or in concert with other respiratory pathogens, in marrow transplant patients. Also, a series of clinical and experimental observations have implicated HHV-6 marrow infection as an important cause of poor graft function in marrow transplant recipients. Prospective serological and virological evaluation of marrow transplant patients has shown that HHV-6 reactivations are common in seropositive patients after marrow transplantation, that post-transplant HHV-6 primary infections occur and that these infections frequently have peripheral and CNS clinical manifestations. Immunohistochemical analysis has

demonstrated that HHV-6 can infect macrophages and lymphocytes within the lung tissues of marrow transplant patients and this infection is often associated with an interstitial pneumonitis. Also, lung infections with HHV-6 are not rare since cells infected with the virus were detected in relatively high numbers in tissues of a sizable proportion of patients dying with pneumonitis of a variety of diagnosed causes. Colony formation assays with normal marrow stem cells revealed that HHV-6 infection suppressed the differentiation and/or proliferation of both erythroid precursors and multipotential stem cells. Also, HHV-6 infection virtually eliminated the response of marrow stem cells to 2 growth factors (GM-CSF and interleukin-3) currently used in marrow transplant patients to speed or enhance marrow engraftment and recovery. In summary, we believe that HHV-6 is an important pathogen in marrow transplant patients, and the 2 most common serious clinical manifestations of HHV-6 infection in these patients appear to be interstitial pneumonitis and poor marrow function.

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Ablashi/Krueger/Salahuddin (eds) Human Herpesvirus-6

CHAPTER 22

Human herpesvirus-6 in Sjögren's syndrome

L.S. DE CLERCK[1], N. BOURGEOIS[2], G.R.F. KRUEGER[3] and W.J. STEVENS[1]

[1] *Department of Immunology, Allergy and Rheumatology, University of Antwerp, 2610 Antwerp, Belgium;* [2] *Department of Pathology, University of Antwerp, 2520 Edegem, Belgium;* [3] *Department of Pathology and Laboratory Medicine, University of Texas Medical Center, Medical Science Building, Houston, TX 77030, U.S.A.*

22.1. Introduction

Sjögren's syndrome (SS) is a chronic disease characterized by keratoconjunctivitis sicca (dry eyes) and xerostomia (dry mouth). Moreover, features of autoimmune disease are prominent: lymphocytic infiltration in exocrine glands and augmented B cell activity with production of autoantibodies (rheumatoid factors and antinuclear antibodies, especially SS-A (Ro) and SS-B (La)).

The disease occurs isolated ('primary SS'), or in association with another autoimmune disorder, most commonly rheumatoid arthritis, systemic lupus erythematosus or scleroderma ('secondary SS'). The clinical spectrum of SS is broad: from a limited exocrinopathy to systemic disease with thyroiditis, atrophic gastritis, primary biliary cirrhosis, vasculitis and involvement of kidneys, lungs, central and peripheral nervous systems.

Furthermore, SS is at the cross-roads of autoimmune disease and lymphoid malignancy, especially B cell lymphomas and Waldenström's disease: the risk of lymphoma development in SS patients is more than 40 times greater than in matched normal control subjects (Kassan et al., 1978).

22.2. Histopathology of Sjögren's syndrome

A common feature of all organs affected by Sjögren's syndrome is a progressive lymphocytic infiltration. The pathologic findings can include:

- focal lymphocytic adenitis of salivary and lacrimal glands;
- primary biliary cirrhosis, sclerosing cholangitis, pancreatitis and atrophic gastritis;

- interstitial nephritis;
- lymphocytic interstitial pneumonitis;
- peripheral vasculitis;
- progression to pseudolymphoma or B cell lymphoma.

The histopathological characteristics of labial salivary glands in Sjögren's syndrome are those of a primary lymphocytic infiltration in otherwise generally normal-appearing glands and include:

- focal aggregates of at least 50 lymphocytes and plasma cells adjacent to and replacing normal-appearing acini;
- consistent presence of these foci in all or most of the glands in the specimen, but with variability in the number of foci per gland. Larger foci often resemble germinal centers.

22.2.1. Diagnostic specificity of focal sialadenitis in labial salivary glands

Because focal sialadenitis in accessory salivary glands is a hallmark of Sjögren's syndrome, and because xerostomia is difficult to assess and is not specific to Sjögren's syndrome, focal sialadenitis in a labial salivary gland biopsy specimen was introduced as the diagnostic criterion for the salivary component of Sjögren's syndrome (Daniels et al., 1975).

Questions about the disease specificity of focal sialadenitis in labial salivary gland biopsy specimens have arisen. Indeed, focal sialadenitis has been described in minor glands taken from areas where the overlying mucosa shows chronic inflammation or in glands showing diffuse acinar atrophy. Furthermore, foci have been found in postmortem studies on patients not suffering from SS. Hence the need to obtain biopsy specimens from clinically normal-appearing mucosa to avoid false-positive biopsies.

In a study comparing the specificity of various methods of salivary gland function to the diagnosis of Sjögren's syndrome, it was found that focal sialadenitis correlated more strongly with keratoconjunctivitis sicca, or with keratoconjunctivitis sicca and another connective tissue disease, than did either reduced parotid flow rate or the presence of xerostomia symptoms (Daniels, 1984).

Moreover, some authors suggest that quantitative histomorphometric investigation of labial salivary gland tissue will give a substantial reduction in the number of false-positive results (9% false positives when only the focus score is used) (De Wilde et al., 1986).

22.2.2. Immunohistology

There is general agreement that the predominant cell in the infiltrates of the minor salivary gland is of the T helper-inducer phenotype (55–75% of the lymphocytes in the infiltrates are CD3-positive T lymphocytes, of which 45–55% are CD4-positive and 10–20% CD8-positive) (Moutsopoulos et al., 1986).

20–35% of the infiltrating lymphocytes are B cells (CD22-positive). The presence of B cell clusters within large T cell foci is similar to germinal centers in lymph nodes (Adamson et al., 1983), suggesting that salivary gland lesions in Sjögren's syndrome evolve by initial infiltration of activated T cells with the subsequent development of germinal centers that produce Ig and autoantibodies (rheumatoid factors, antinuclear antibodies such as anti-SS-A(Ro) and anti-SS-B(La)).

A recent study demonstrated that the activated cells in the salivary gland lesions, although few in total number, are B cell in origin (Segerberg-Konttinen et al., 1987). CD5-positive B cells, recently found to be increased in blood and salivary glands of SS patients, may play a fundamental role in the development of B cell hyperreactivity and autoantibody production in SS (Youinou et al., 1988). Indeed, these CD5-positive B cells are responsible for autoantibody production, at least in animal models and possibly also in humans.

Plasma cells, and not pre-B cells, are the principal cell type staining positive for intracytoplasmic immunoglobulins in minor salivary glands in patients with Sjögren's syndrome. The majority of plasma cells infiltrating the minor salivary glands containing IgG in contrast to controls which contained IgA (Lane et al., 1983; Matthews et al., 1985). Some authors even proposed a new diagnostic criterium for Sjögren's syndrome based on the reversed ratio of IgG- and IgA-containing plasma cells with a specificity of more than 95% and a sensitivity of 100% (De Wilde et al., 1989). Natural killer cells (CD57- and CD16-positive cells) are absent in the salivary glands of Sjögren patients, while these cells can be found in the peripheral blood of these patients (Fox et al., 1985).

Using the monoclonal antibody CD11 for monocytes/macrophages and granulocytes, it was observed that less than 5% of the cells in the round cell infiltrates were stained.

It was shown that more than 90% of the cells in the infiltrates were stained positive for HLA class II (DR) antigens, suggesting that the majority of T cells are activated in the salivary gland lesions (Moutsopoulos et al., 1986). In addition, anti-HLA-DR antibodies intensively stained epithelial cells (ducts and acini), particularly in areas close to the round cell infiltrates. There is some evidence that local production of gamma-interferon plays a role in this induction (Fox et al., 1986a). HLA class II expression was not observed in normal salivary gland tissue.

The expression of DR may render salivary gland epithelium capable of functioning as antigen presenting cells, leading to further T cell activation. However, in a recent study it was shown that only a minority of the DR-positive inflammatory cells expressed the T cell activation antigen TAC (IL-2-receptor) or were thymidine-incorporating blasts (Segerberg-Konttinen et al., 1987). In any case, T lymphocyte activation and blast transformation seemed to be more extensive in glandular tissue in SS than in normal control glands.

In patients with Sjögren's syndrome, the whole spectrum of lymphoproliferation ranging from benign lymphoepithelial lesions (so-called myoepithelial sialadenitis) to malignant lymphoma may be observed. At first, no proliferation areas are found in the reactive lymphocytic salivary gland infiltrations. Later, small proliferation

areas with polyclonal cytoplasmatic immunoglobulin-producing cells occur. Finally, in these areas monoclonality occurs, with possible transition into a B cell lymphoma (Bridges and England, 1989; Schmidt et al., 1989). In this context, it is of interest that oligoclonal rearrangements of kappa and lambda genes have been described in salivary gland lymphocytes of SS patients (Freimark et al., 1989). Escape from the immune regulation of one of these clones may lead to development of a malignant lymphoma. The inciting event in this plot might be Epstein–Barr virus or another herpesvirus.

22.3. Etiology of Sjögren's syndrome

22.3.1. Epstein–Barr virus (EBV)

The etiology of Sjögren's syndrome is unknown. The potential role of EBV in this disorder has recently been reviewed (Fox, 1988). EBV is an ubiquitous agent and can be isolated from saliva in 15–20% of normal individuals who have had prior EBV infection. Infection of B lymphocytes may occur through their contact with infected salivary glands in which the virus can persist (Wolf et al., 1984). A recent study using *in situ* hybridization showed that EBV DNA was present in some SS labial salivary glands in the epithelial cells of acini and ducts (Schuurman et al., 1989).

It has been proposed that the initiating event in primary SS is infection with EBV (Whittingham et al., 1987). It has been shown that patients with SS have higher titers of serum anti-EBV capsid antigen antibodies, have higher frequency of oropharyngeal excretion of EBV and have higher frequencies of EBV-transformed B cells secreting autoantibodies than controls (Yamaoka et al., 1988). Moreover, anti-SSB antibodies (a marker for primary SS) react with a ribonucleoprotein (the SSB autoantigen), to which bind not only all cellular RNAs transcribed by RNA polymerase III, but also the viral RNAs EBER 1 (Epstein–Barr virus encoded RNA 1) and EBER 2. It is proposed that during EBV infection, there are multiple copies of the EBERs available to bind to the SSB ribonucleoprotein and when infection occurs in subjects who have an impaired T cell mediated response to EBV, and who are genetically predisposed to autoimmunity, there is loss of immunological tolerance to SSB with the production of anti-SSB. Thus the inflammatory process in exocrine glands might be due to chronic EBV infection and induction of autoimmunity. Clinical evidence for this hypothesis could be the onset of SS after EBV infection (Steinberg, 1984; Wittingham et al., 1987).

Another possibility is that viral reactivation in salivary glands could lead to the expression of class II histocompatibility molecules by epithelial cells with subsequent capability of these cells to present EBV antigenic determinants to sensitized T cells, leading to destruction of the epithelium in predisposed patients (Fox, 1988). Since it is known that SS is strongly associated with HLA-DR3, it is suggested that EBV or a related herpesvirus plays a role in the perpetuation of local immune responses within the salivary gland of genetically predisposed HLA-DR3-positive individuals (Fox, 1988).

22.3.2. Human immunodeficiency virus (HIV)

It should be noticed that similar lymph node lesions are described in AIDS and Sjögren's syndrome (Diebold, 1984) and that HIV has been isolated from salivary glands in AIDS patients (Lecatsas et al., 1985). Occasionally, infiltration of salivary lymph nodes can give rise to salivary gland enlargement in AIDS (Ryan et al., 1985). Cases of acquired immune deficiency syndrome (AIDS) in patients presenting with signs and symptoms of SS and systemic lupus have been described (De Clerck et al., 1988, Calabrese, 1989). In contrast with SS or SLE patients, these patients generally have depressed CD4/CD8 ratios (De Clerck et al., 1988) and the diagnosis AIDS should always be excluded in patients presenting with a clinical picture compatible with autoimmune diseases such as SLE and Sjögren's syndrome, especially when the CD4/CD8 ratio is markedly depressed. Furthermore, it should be noted that not only false-positive HIV tests (ELISA, immunofluorescence and sometimes Western blot) but also false-negative testing (Western blot and virus isolation) can be misleading in the differential diagnosis between AIDS and systemic diseases.

22.3.3. Hhv-6

It has been reported that latent HHV-6, similar to EBV or other herpesviruses, may become reactivated in patients with Sjögren's syndrome (Ablashi et al., 1988). HHV-6 has been isolated from saliva (Levy et al., 1990). Moreover, a recent study using *in situ* hybridization and immunohistochemical staining showed evidence for HHV-6 replication in a high proportion of salivary glands from adults (Fox et al., 1990). In order to study the role of HHV-6, we examined labial salivary gland tissue of primary SS patients.

22.4. Materials and methods

Serum samples were obtained from patients with primary SS and controls for HHV-6 serology. Paired labial salivary gland tissue was available from most of these patients and controls.

Patients and controls: the diagnostic work-up of patients and controls included a detailed history taking with special attention to signs and symptoms of connective tissue diseases and a physical examination performed by a rheumatologist. Xerostomia was diagnosed by diminished excretion of technetium 99^m on salivary scintigraphy and by the Saxon test (Kohler and Winter, 1985). A quantitative rose bengal test according to Van Bijsterveld (1969) objectivated xerophtalmia. Immunological evaluation included determination of rheumatoid factor by Waaler–Rose and latex fixation test, antinuclear antibodies (ANA) by immunofluorescence using Hep-2 cells and extractable nuclear antigens by double immunodiffusion. Furthermore, a labial salivary gland biopsy was performed according to Daniels (1984): a focus score of > 1 was considered diagnostic for focal sialadenitis.

Furthermore, a morphometric analysis was performed using a semiautomatic analysis system (MOP-Videoplan, Kontron, Munich, Germany) in order to calculate the percentage infiltrated area at the largest cross section of the salivary gland. According to these investigations, the patients were classified as primary SS when they fulfilled the criteria as proposed by Fox et al. (1986b) or controls when no objective abnormality was found.

HHV-6 SEROLOGY: inactivated and filtered patient sera were titred using HHV-6 (GS line) infected HSB_2 cells according to standardized techniques (Krueger et al., 1991a). For IgM antibody determination, sera were preabsorbed with RF absorbens (Behring Werke, Marburg, Germany).

IMMUNOHISTOCHEMICAL STAINING: the immunophenotype of inflammatory cells was studied on frozen sections with monoclonal antibodies from Dako (Glostrup, Denmark) for CD1, CD3, CD4, CD5, CD8, CD11c, CD15, CD22, CD23, CD25, CD38, CD68, CD71 and L26 using the peroxidase antiperoxidase bridge technique. CD21 was obtained from Becton Dickinson (Mountain View, CA, USA). For HLA DR the monoclonal antibody LN3 from Biotest (Dreieich, Germany) was employed. Also a PAS and a Giemsa stain were done.

DETECTION OF HHV-6 ANTIGENS: HHV-6 p41 antigen (9A5D12); (Balachandran et al., 1989) was demonstrated in frozen sections from lip biopsies by routine immune peroxidase sandwich technique. The monoclonal antibody against p41 (kindly provided by Balachandran, University of Kansas, U.S.A.) is specific for HHV-6 and does not cross-react with proteins of other herpesviruses. The antigen is demonstrated in HHV-6 infected cells early during the course of viral replication (Ablashi et al., 1991; Krueger et al., 1991a).

IN SITU HYBRIDIZATION: For *in situ* hybridization studies on sections of lip biopsies, the HHV-6 DNA probe pZVH14 (kindly provided by Josephs, National Cancer Institute, U.S.A.) was biotinylated by nick translation. Specific binding was visualized after routine hybridization methods by the NBT/BCIP method (Seyda et al., 1989).

22.5. Results

SEROLOGY: According to our current experience, 6 out of the 11 patients with SS had serological evidence for active HHV-6 infection: 2 with IgM antibody titers of 1:20 or above, and the others with IgG titers of 1:640 or above. 2 additional cases had IgA antibodies of 1:40, the implication of which is not clear at this time. Contrarily, 2 of the 9 control cases without SS had IgM anti-HHV-6 antibody titers of 1:20, none had IgA.

IMMUNOHISTOCHEMICAL STAINING: In SS patients the labial salivary glands displayed a lymphocytic-plasmacytic inflammation consisting of focal and diffuse components of variable size. The lymphocytic part was characterized by an immunophenotype of peripheral T lymphocytes: CD3 CD5 with predominance of CD4 (3/4) (Fig. 22.2A) over CD8 (1/4). Many of these cells were immunoreactive for CD38, few for CD71, but negative for CD1 and HLA DR. Immunoreactivity for

CD38 was conspicuous in the interstitial infiltrate where histiocytes and plasma cells were numerous (Fig. 22.2B). CD38 cells, however, were always located in the diffuse infiltrates, not in the focal areas where B lymphocytes predominated. Smaller groups of CD22 L26 lymphocytes were partially positive for CD23. These groups were incorporated into the larger inflammatory foci. CD21 was found as a membranous immunoreactivity on a lymphocytic aggregate, the smaller part of a larger group of B lymphocytes (Fig. 22.2C). A follicular germinal center was sporadically present. The plasma cells were disseminated at the borders of the lymphocytic foci but were often more numerous in the interstitial part of the inflammatory infiltrates. Granulocytes were absent but CD11c CD68 mononuclear-histiocytic cells (Fig. 22.2D) were present in between the acini even in the absence of lymphocytes or plasma cells. In some cases such cells displayed a weak immunoreactivity for CD15 and CD1. Also a faint natural peroxidase activity was seen. Mast cells, conspicuous in the Giemsa stain, showed the same distribution as the histiocytes and occurred also in fibrotic and lipomatous fields.

In general the labial salivary glands of the control patients with sicca symptoms were devoid of extensive inflammatory infiltrates except for some plasma cells, histiocytes and mast cells. Finally in the majority of these minor salivary glands obtained from SS and control patients the lobules displayed mild, moderate or severe dilatation of the intralobular ducts and also of some extralobular ducts. The lumina contained mucoid secretion. These features probably contributed to the symptoms or dyschylia from which the patients complained.

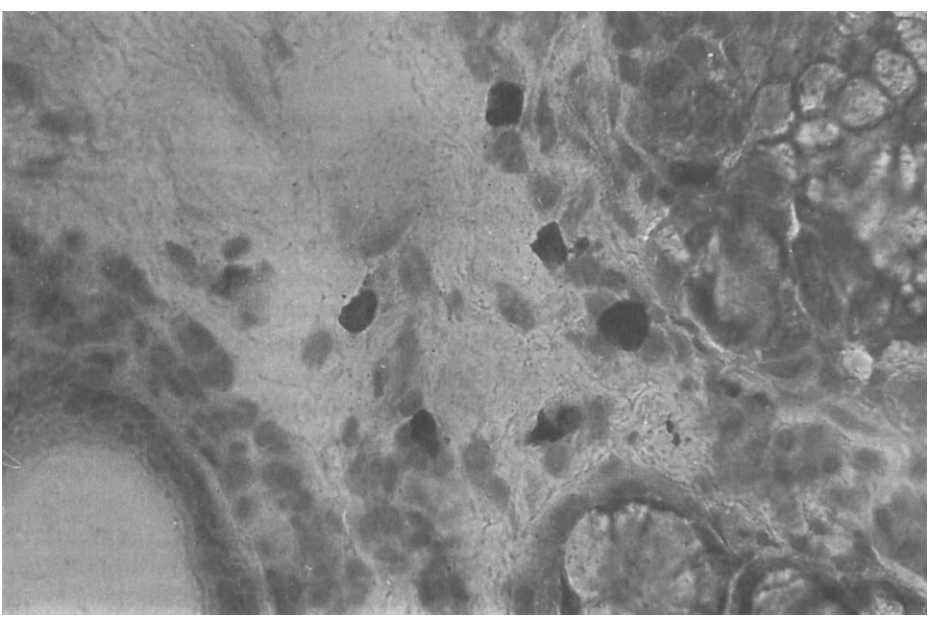

Fig. 22.1. Large dark stained cells in the interstitium immunoreactive for HHV-6 (magnification 300 ×).

TABLE 22.1.
Inflammatory infiltrate (focus score and % infiltrated area), titers of anti-HHV-6 antibodies in serum and immunohistochemical staining in salivary glands of SS patients (SS) and controls (CTRL) (ND denotes not done)

Nr.	Age	Sex	Diagnosis	Focus score/ % infiltr. area	Serum anti-HHV-6			HHV-6 saliv. gland
					IgG	IgM	IgA	
1	27	F	SS	2.5/2.7	160	0	40	+ + +
2	62	F	SS	4.0/4.6	1280	0	0	+ + +
3	65	F	SS	5.5/20.2	320	0	40	(+)
4	64	F	SS	3.0/10.3	40	0	0	(+)
5	64	F	SS	5.0/46.0	320	0	0	(+)
6	59	F	SS	4.0/N.D.	80	0	0	+ + +
7	43	F	SS	4.0/ < 0.1	640	20	0	+ +
8	53	F	SS	3.5/N.D.	1280	0	0	+ + + +
9	26	F	SS	9.0/4.6	2560	0	0	N.D.
10	44	F	SS	6.0/1.7	160	0	0	N.D.
11	36	F	SS	3.0/10.8	2560	80	40	N.D.
12	50	F	CTRL	0.0/ < 0.1	320	0	0	N.D.
13	68	F	CTRL	0.0/ < 0.1	40	0	40	+ + + +
14	70	F	CTRL	0.0/ < 0.1	80	0	0	+ +
15	76	F	CTRL	0.0/ < 0.1	40	0	0	+ + + +
16	63	F	CTRL	0.0/ < 0.1	320	20	0	+ +
17	54	M	CTRL	0.0/ < 0.1	40	20	0	+ +
18	74	F	CTRL	0.0/N.D.	N.D.	N.D.	N.D.	(+)
19	52	F	CTRL	0.5/ < 0.1	0	0	0	N.D.
20	54	F	CTRL	1.0/ < 0.1	80	0	0	N.D.

In situ hybridization with the HHV-6 pZVH 14 probe was weakly positive in only 2 of the SS patients (results not shown). However, strong staining of the cytoplasm was seen with the HHV-6 monoclonal antibody 9A5D12 in SS patients as well as in controls (Fig. 22.1, Table 22.1). Positive cells were found primarily in the interstitial stroma, especially in the lymphoid infiltration. Although there was no clear difference in the number of antigen-positive mononuclear round cells in the interstitium of SS and control patients, the percent infiltrated area varied significantly. Therefore, the over-all HHV-6 antigen load appeared higher in salivary glands of SS patients (i.e., HHV-6-positive cells × infiltrated area) than of normal controls. Some salivary glands of SS patients showed a positive reaction of brush border or secretory product (Fig. 22.2). There was no clear-cut positive reaction of glandular cells.

22.6. Discussion

SS is a common autoimmune disorder, possessing unique features that make it suitable for research in pathogenesis of both autoimmunity and malignancy. In the

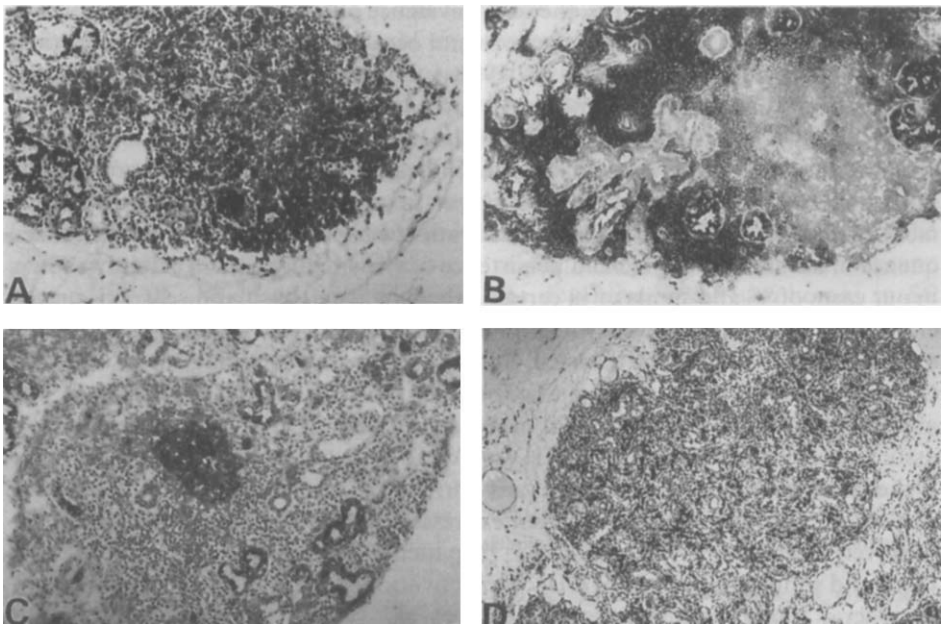

Fig. 22.2. (A) Small dark stained cells $CD4^+$ replacing acini (magnification 120×); (B) pale stained lymphoid focus devoid of immunoreactivity for CD38 which is strongly expressed in the interstitial infiltrate (magnification 75×); (C) compact group of $CD21^+$ lymphocytes part of an extensive lymphocytic infiltrate (magnification 120×); (D) lobule with numerous interstitial polymorphous stained cells immunoreactive for CD38 (magnification 120×). (For printing purposes figure has been reduced.)

same line, herpesviruses such as EBV and HHV-6 are possible candidates in the etiopathogenesis of autoimmune and malignant diseases. However, it remains difficult to conceive how exactly trigger events and autoimmunity in SS can lead to specific damage of salivary and lacrimal glands, to extraglandular systemic and neoplastic manifestations.

There is no compelling evidence to implicate one of the herpesviruses (e.g., EBV, CMV or HHV-6) as direct causative agents in human autoimmune disease. However, since the etiopathogenesis of diseases such as rheumatoid arthritis and SS is probably multifactorial including genetic and environmental factors, certain viruses might trigger immunopathological events leading to B cell hyperactivity with production of autoantibodies, eventually malignant transformation and cellular immune dysregulation.

The initial isolation of HHV-6 from patients with lymphoproliferative disorders suggested that this virus might have an etiological role in these diseases (Salahuddin et al., 1986). Since SS patients have a more than 40-fold risk of developing malignant lymphomas, we and others have studied the prevalence of antibodies to HHV-6 and the presence of HHV-6 in salivary gland tissue of SS patients and controls.

Similar to Epstein–Barr virus, the seroprevalence of HHV-6 is high in the general population with 60–80% latent infections and occasional spontaneous reactivation in healthy individuals (Krueger et al., 1991a). Consequently, HHV-6 can be easily isolated from saliva which is probably a source for viral spreading (Harnett et al., 1990). Fox et al. (1990) and Krueger et al. (1990) provided evidence for HHV-6 presence in salivary and bronchial glands in healthy individuals. Viral presence in salivary glands and occasional activation *per se* could hardly explain the development of disease. If disease follows reactivation, it appears to be rather a consequence of an unusual degree and persistence of virus replication as actually shown in our cases of SS and similarly in certain other collagen-vascular diseases (Krueger et al., 1991b). However, even when an association between virus replication and autoimmune disease can be revealed, this is not yet proof of etiological association. Occurrence of IgM (and IgA) antibodies, IgG titers against HHV-6 above 1:640 and increased HHV-6 antigen load in diseased tissues of our SS patients might be associated with reactiviation of HHV-6. Viral replication appears to occur at a higher level in inflamed salivary glands correlating with the degree of the mononuclear cell infiltrate. HHV-6-antigen expression in salivary gland epithelial cells, however, is apparently not increased. In patients with systemic lupus erythematosus and similar indicators of HHV-6 reactivation, virus was easily cultured from peripheral blood lymphocytes (Krueger et al., 1991b). Conditions leading to excessive herpesvirus reactivation and persistence include continuous abnormal stimulation of virus genome-carrying cells, defective immune control of viral spread, and defective host control of intracellular viral reproduction (Krueger et al., 1991c).

Although we are unable to offer any conclusive explanation yet for the pathogenic role of HHV-6 in SS, this virus infects cells of the immune system and may thus contribute to immune dysregulation in these patients. Polyclonal B cell stimulation, T cell lysis and alteration of cell membrane receptor expression are among known effects of HHV-6 infection of the immune system (Krueger and Ramon, 1988; Ablashi et al., 1991; Schonnebeck et al., 1991). Whether HHV-6, like EBV, can induce tissue lesions by molecular mimicry still needs investigation. Other mechanisms shown by HHV-6 which may influence the inflammatory response in infected tissues are stimulation of cytokine production such as interleukin-1 and tumor necrosis factor (Flamand et al., 1991).

Furthermore, HHV-6 DNA sequences have been found in non-Hodgkin lymphomas and atypical polyclonal lymphoproliferation of patients with SS (Jarrett et al., 1988; Josephs et al., 1988; Krueger et al., 1989). However, since antibodies to HHV-6 are common and the virus is frequently found in salivary gland tissue of normal controls, its specific role in the pathogenesis of SS and the development of lymphomas in these patients remains to be proven.

22.7. Summary

Sjögren's syndrome (SS) is an autoimmune disease characterized by a lymphocytic infiltration of exocrine glands, various extraglandular manifestations including

vasculitis and evolution into a B cell neoplasia. Significant progress has recently been made in the understanding of the immunopathogenesis of this disease. SS patients have a generalized B cell hyperreactivity that may become more restricted in time to turn out oligoclonal and later monoclonal. Moreover, SS is associated with defective T cell functions and focal lymphocytic infiltration of affected organs. Current data on the histopathology of SS are reviewed and the etiopathogenic role of Epstein–Barr virus (EBV), human immunodeficiency virus (HIV) and especially human herpesvirus-6 (HHV-6) are discussed. Although HHV-6 is probably not the primary etiologic agent of the disease, it might have a role in the autoimmune cascade in SS and could be a trigger in the development of lymphomas in SS patients.

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CHAPTER 23

HHV-6: response to antiviral agents

MARSHALL V. WILLIAMS

Department of Medical Microbiology and Immunology, The Ohio State University and Comprehensive Cancer Center, Columbus, OH 43210, U.S.A.

23.1. Introduction

Human herpesvirus-6 (HHV-6) was originally isolated from patients with acquired immunodeficiency syndrome (AIDS) (Salahuddin et al., 1986) and subsequently from patients with a variety of lymphoproliferative disorders (Downing et al., 1987; Krueger et al., 1987; Tedder et al., 1987; Ablashi et al., 1988a; Becker et al., 1989), as well as from normal individuals (Pietroboni et al., 1988). Recent studies have demonstrated that HHV-6 is the etiological agent of exanthema subitem a benign self-limiting disease of children characterized by a skin rash and high fever (Takahashi et al., 1988; Ueda et al., 1989; Yamanishi et al., 1988; Suga et al., 1990). While serological studies have associated HHV-6 with several disorders (Krueger et al., 1987; Biberfield et al., 1988; Kirchesch et al., 1988; Krueger et al., 1988; Bertram et al., 1989; Steeper et al., 1990), the importance of these associations are unknown since seroepidemiological studies have demonstrated that HHV-6 infections are widespread in the human population and that seroconversion occurs early in life (Briggs et al., 1988; Brown et al., 1988; Linde et al., 1988; Saxinger et al., 1988; Okuno et al., 1989). While virtually nothing is known regarding the latent state of HHV-6, the virus has been identified in lymph nodes (Yamanishi et al., 1989) and in peripheral blood monocytes from transplant patients (Asano et al., 1989; Wrzos et al., 1990). It has also been suggested that HHV-6 may play a role in the activation of human immunodeficiency virus (HIV-1) (Ensoli et al., 1989; Horvat et al., 1989; Lusso et al., 1989). Because of its possible association with a variety of lymphoproliferative disorders, it is important to identify agents that may be useful in treating infections caused by HHV-6. Furthermore, the identification of agents that inhibit HHV-6 replication could be useful in studies directed towards

determining features associated with HHV-6 replication both at the molecular and cellular level.

In the past few years, there has been an increase in the search for novel chemotherapeutic agents that can be employed for antiviral chemotherapy and/or chemoprophylaxis. This is due in part to progress in the areas of molecular virology and biochemistry that have allowed investigators to distinguish specific virus functions from host cellular functions. This has resulted in the development of several agents that inhibit viral replication without being overly toxic to the host.

In this chapter, we will review the information that has been developed thus far concerning the response of HHV-6 to antiviral agents, examine problems that are associated with determining the sensitivity of HHV-6 to these agents and finally look at approaches that should be taken in the future for these types of studies.

At the present time, there have been very few studies directed towards determining the response of HHV-6 to antiviral agents (Agut et al., 1988, 1989b; Steicher et al., 1988; Kikuta et al., 1989a; Russler et al., 1989; Shiraki et al., 1989; DiLuca et al., 1990). These studies have focused on antiviral agents that have previously been demonstrated to be effective in treating infections caused by various herpesviruses and HIV-1. These compounds are phosphonoacetic acid, phosphonoformic acid, azidothymidine, acyclovir and ganciclovir. The structures of these compounds are shown in Figure 23.1.

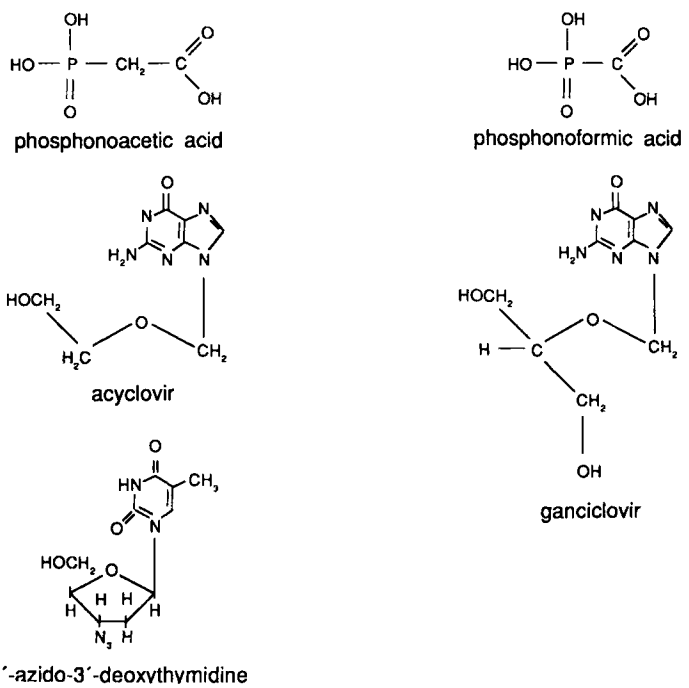

Fig. 23.1. Structure of antiviral agents tested against HHV-6.

23.2. Pyrophosphate analogs

Phosphonoacetic acid (PAA) and phosphonoformic acid (PFA, Foscarnet) are pyrophosphate analogs that have been reported to inhibit the replication of several viruses including members of the herpesvirus group (Summers and Klein, 1976; Ablashi et al., 1977; May et al., 1977; Boezi, 1979; Svennerholm et al., 1979; DeClercq et al., 1980; Oberg, 1983), visna (Sundquist and Larner, 1979), Friend leukemia virus (Varnier et al., 1982) and HIV-1 (Sandstrom et al., 1985; Sarin et al., 1985; Vrang and Oberg, 1986). PAA and PFA are selective inhibitors of viral replication due to their ability to inhibit virus-specific DNA polymerases and reverse transcriptases at concentrations that have no significant effect on the activities of cellular DNA polymerases (Sabourin et al., 1978; Eriksson et al., 1982). PAA and PFA do not require cellular activation to be effective. They act as noncompetitive inhibitors of virus-encoded DNA polymerases and reverse transcriptases by binding directly to the enzyme at the pyrophosphate binding site (Leinbach et al., 1976; Eriksson et al., 1982).

HHV-6 is similar to other herpesviruses in that it induces a virus-specific DNA polymerase in infected cells (Bapat et al., 1989; Shiraki et al., 1989; Williams et al., 1989). Studies with the partially purified HHV-6 DNA polymerase have demonstrated that both PAA and PFA inhibit the activity of the enzyme with IC_{50} (concentration of the compound required to inhibit 50% of the enzyme activity) values of 2.5–3 μM for PAA (Bapat et al., 1989; Shiraki et al., 1989) and 0.4 μM for PFA (Bapat et al., 1989) (Table 23.1). These values are within the range that has been reported previously for the DNA polymerases from other herpesviruses (PAA: 1–5 μM; PFA: 0.3–22 μM (Huang, 1975; Mao et al., 1975; Miller and Rapp, 1977; Miller et al., 1977; Reno et al., 1978; Ostrander and Cheng, 1980; Cheng et al., 1981; Datta and Hood, 1981; Eriksson et al., 1982) and in the case of PFA for the reverse transcriptases encoded for by feline immunodeficiency virus and HIV-1 (Ki of 0.18 and 0.72 μM respectively) (North et al., 1990). In addition to inhibiting the DNA polymerase activity, both PAA and PFA inhibit the replication of HHV-6 in tissue culture (Table 23.1) (Steicher et al., 1988; Agut et al., 1989a; DiLuca et al., 1990) at

TABLE 23.1.
Effect of pyrophosphate analogs on replication of HHV-6

Agent	Virus Strain	Cells	Concentrations tested	Effect	Reference
PAA[a]	MA	CBMC	71.4 μM	> 99% reduction of IF^+ cells	Shiraki et al. (1989)
	Z-29	PBMC	714 μM; 1.2 mM	66–88% reduction in viral DNA synthesis; > 99% reduction of IF^+ cells	DiLuca et al. (1990)
PFA	GS	HSB_2	66 μM	> 99% reduction of IF^+ cells	Steicher et al. (1988)
	SIE	PBMC	59 μM	90% reduction of IF^+ cells	Agut et al. (1989b)

[a] PAA: phosphonoacetic acid; PFA; phosphonoformic acid; CBMC: cord blood mononuclear cells; PBMC: peripheral blood mononuclear cells.

concentrations which are similar to those reported for other herpesviruses (Overby et al., 1974; Boezi, 1979; Oberg, 1983). Thus, it may be possible to use either PAA or PFA for treating infections caused by HHV-6.

Studies to determine the clinical usefulness of these pyrophosphate analogs have focused on PFA since it is less toxic and generally more effective in inhibiting the replication of herpesviruses (Oberg, 1983). Both PAA and PFA have been demonstrated to be effective as topical agents for the treatment of herpes infections in both animal models (Shipkowitz et al., 1973; Alenius et al., 1978, 1982; Kern et al., 1978, 1981) and humans (Dolin, 1985). PFA has also been reported to be effective in controlling HCMV infections in transplant patients (Klintmalm et al., 1985). It has been reported that PFA is effective either alone or in combination with other agents in inhibiting the replication of HIV-1 *in vitro* (Sandstrom et al., 1985; Sarin et al., 1985; Hartshorn et al., 1986). Recent studies have demonstrated that HHV-6 infects $CD4^+$ cells that are infected with HIV-1 (Agut et al., 1988; Lusso et al., 1988; Takahashi et al., 1989), that HHV-6 can transactivate the HIV-1 promoter (Horvat et al., 1989; Lusso et al., 1989) and that this may lead to the enhanced expression of HIV-1 (Ensoli et al., 1989) leading to the more rapid development of cytopathic effects in infected cells (Lusso et al., 1989). Thus, the use of an agent such as PFA that could simultaneously inhibit the replication of both HHV-6 and HIV-1 may be useful in treating patients with AIDS or ARC and clinical trials are presently underway to determine the effacement of PFA in such patients (Sarin, 1988).

A limitation of PAA and PFA is that they have no effect on latent virus genomes. Studies with HCMV and VZV have demonstrated that while these agents inhibit virus replication, the virus genome persists in cells initially infected and that the genome is reactivated following removal of PAA or PFA (Wahren and Oberg, 1980; Walz-Cicconi et al., 1980; Wahren et al., 1985). At the present time, virtually nothing is known concerning the latency of HHV-6, but since seroepidemiological studies have demonstrated that individuals are infected with HHV-6 early in life, it is probable that HHV-6 is maintained in some cells in a latent state. Thus, while PAA and PFA may inhibit the replication of the HHV-6 in active infections, it probably would not have any effect on the latent virus genome. However, further studies are necessary to determine what effect that these pyrophosphate analogs may have on the latent HHV-6 genome.

23.3. Nucleoside analogs

Numerous nucleoside analogs have been synthesized and examined for their ability to inhibit the replication of herpesviruses (for reviews, see Prusoff and Ward, 1976; Dolin, 1985; Flowers et al., 1988) and HIV-1 (for reviews, see Kim et al., 1987; Furman and Berry, 1988; Sarin, 1988; Yarchoan et al., 1989). Studies to determine the response of HHV-6 to nucleoside analogs have been limited and they have focused primarily on those agents that are being used clinically to treat infections caused by HIV-1 and members of the herpesvirus group.

23.3.1. Azidothymidine

3′-azido-3′-deoxythymidine (AZT, zidovudine) is a nucleoside analog that is presently being used for treatment of patients with AIDS (Mitsuya et al., 1985; Yarchoan et al., 1986; Fischel et al., 1987; Richmond et al., 1987). AZT is phosphorylated to its 5′-triphosphate derivative by cellular enzymes in the thymidine-salvage pathway (Furman et al., 1986). The triphosphate, which has low affinity for cellular DNA polymerase (Furman et al., 1986), acts as a competitive inhibitor of the HIV-1 reverse transcriptase (Furman et al., 1986; Cheng et al., 1987; North et al., 1990). The IC_{50} value of AZT for HIV-1 has been reported to be 0.05 μM (Furman and Berry, 1988). Lin et al. (1988) examined the effect of AZT on the replication of EBV, HSV-1, HSV-2, VZV and HCMV and while AZT inhibited the replication of EBV (EC_{50}, 3 μM), it had no effect on the replication of the other herpesviruses. Agut et al. (1989a) reported that AZT (1–8 μM) did not have any effect on the replication of HHV-6 (strain SIE) in peripheral blood mononuclear cells and thus they concluded that AZT would not be useful for treating infections associated with HHV-6. No further studies have been conducted with this agent.

23.3.2. Acyclovir and ganciclovir

Acyclovir (ACV, 9-(2-hydroxyethoxymethyl)guanine) and ganciclovir (GCV, 9-(1,3-dihydroxy-2-propoxymethyl)guanine) are purine nucleosides that are highly potent and specific inhibitors of some herpesviruses (Elion et al., 1977; Elion, 1982; Cheng et al., 1983b; Field et al., 1983). Both ACV and GCV exert their antiviral effect by being converted to 5′-triphosphates and then acting as substrates for the viral-specific DNA polymerases but not for the cellular DNA polymerases (Cheng et al., 1983a,b; Furman et al., 1986). Incorporation of ACV-triphosphate into the DNA causes chain termination (Furman et al., 1980) and the terminated DNA template can also inhibit DNA polymerase through noncompetitive binding (Derse et al., 1981). The major difference in ACV and GCV is the manner in which they are phosphorylated to their triphosphate derivative. ACV is a poor substrate for cellular thymidine kinase (TK), but it is an efficient substrate for virus specific TKs (Elion et al., 1977; Field et al., 1983). Conversely, GCV can be efficiently phosphorylated to the 5′-monophosphate by both cellular and virus specific TKs (Field et al., 1983). The 5′-monophosphates of both ACV and GCV are phosphorylated to 5′-triphosphates by cellular enzymes, but again, the 5′-monophosphate derivative of GCV is more efficiently phosphorylated than ACV (Miller and Miller, 1980; Field et al., 1983). Thus, ACV is effective against HSV (Cheng and Ostrander, 1976), VZV (Cheng et al., 1979) and EBV (Roubal and Klein, 1981) which encode for a viral specific TK, while GCV is more effective against CMV (Crumpacker et al., 1979; Cheng et al., 1983a; Erice et al., 1987) which lack a specific virus TK.

At the present time, the most controversial aspect relating to the response of HHV-6 to antiviral agents is whether ACV will be useful in treating infections caused by the virus. Some investigators have concluded that ACV can be used to treat HHV-6 infections (Kikuta et al., 1989a; Russler et al., 1989) while others have

reached the opposite conclusion (Steicher et al., 1988; Agut et al., 1989a,b, DiLuca et al., 1990).

The IC_{50} value of ACV for various strains of HHV-6 has been reported to be in the range of 18–100 μM (Agut et al., 1988, 1989b; Kikuta et al., 1989a; Russler et al., 1989) (Table 23.2). However, the IC_{50} may be underestimated since other studies reported that concentrations of ACV up to 300 μM had no significant effect on the replication of HHV-6 (Steicher et al., 1988; DiLuca et al., 1990). The most comprehensive study on the effects of ACV on HHV-6 replication have demonstrated that only high concentrations (400 μM) of ACV have any effect on virus spread and on infectious virus production; lower concentrations have no effect or just delay virus spread and infectious virus production (DiLuca et al., 1990). Previous studies have demonstrated that the IC_{50} value of ACV for sensitive strains of HSV, VZV and EBV is in the range of 0.1–6.75 μM (Schaeffer et al., 1978; Crumpacker et al., 1979; DeClercq et al., 1980, Cole and Balfour, 1986; Erlich et al., 1988). Conversely, the IC_{50} of ACV for resistant strains of HSV and CMV is reported to be in the range of 27–300 μM (Crumpacker et al., 1979; Ingrand et al., 1984; Tocci et al., 1984; Erlich et al., 1988). Based upon IC_{50} values, it appears that HHV-6 responds to ACV in a manner that is similar to that of ACV-resistant HSV mutants and HCMV. This apparent resistance to ACV is supported by studies that have demonstrated that ACV triphosphate is not as good as a substrate for the HHV-6 DNA polymerase when compared to other herpesvirus DNA polymerases (Bapat et al., 1989) and that HHV-6 is similar to HCMV in that it does not induce a virus-specific TK in infected cells (Williams et al., 1989; DiLuca et al., 1990). This is also consistent with the studies demonstrating that HHV-6 is related

TABLE 23.2.
Effect of nucleoside analogs on replication of HHV-6

Agent	Virus strain	Cells	Concentrations tested (μM)	Effect	References
ACV[a]	GS	HSB_2	200, 320	80% inhibition of IF^+ cells	Steicher et al. (1988)
	SIE	PBMC	100	> 99% inhibition of CPE	Agut et al. (1988)
	SIE	PBMC	6–200	IC_{50}: 30 μM; IC_{90}: 82 μM[a,b]	Agut et al. (1989b)
	KF	PBMC	16–120	IC_{50}: 100 μM	Russler et al. (1989)
	PL	CBMC	0.4–444	> 99% inhibition at 444 μM	Kikuta et al. (1989a)
	229	PBMC	20, 100, 400	0, 60 and 85% inhibition of viral DNA replication	DiLuca et al. (1990)
GCV	GS	HSB_2	320, 600	No effect	Steicher et al. (1988)
	SIE	PBMC	1–20	IC_{50}: 2 μM; IC_{90}: 5.6 μM[b]	Agut et al. (1989b)
	KF	PBMC	1–11	IC_{50}: 2 μM	Russler et al. (1989)
AZT	SIE	PBMC	8	No effect	Agut et al. (1989b)

[a] ACV: acyclovir; GCV: ganciclovir; AZT: azidothymidine; CBMC: cord blood mononuclear cells; PBMC: peripheral blood mononuclear cells; IC_{50}: concentration of agent required to cause 50% inhibition; IC_{90}: concentration required to cause 90% inhibition.

[b] Represent the average of 3 different methods used to quantitate IC_{50} and IC_{90}.

to HCMV (Efstathiou et al., 1988; Lawrence et al., 1990). Thus, since it is probably not possible to obtain significant plasma levels of ACV (Bridgen et al., 1981) that would inhibit the replication of HHV-6, it is possible to conclude that ACV will not be useful for the treatment of infections caused by HHV-6.

Conflicting data has also been reported concerning the response of HHV-6 to GCV. Agut et al. (1989b) reported that GCV is a potent inhibitor of HHV-6 replication with 10 μM completely inhibiting virus replication and an IC_{50} value of 1.1 μM. This value is similar to those (0.50–5.9) reported for other herpesviruses including HCMV (Field et al., 1983; Erice et al., 1988). Conversely, Steicher et al. (1988) reported that concentrations of GCV as high as 150 μg/ml (600 μM) had no effect on HHV-6 replication. It is not known whether these differences reflect differences in the strains of virus or in the experimental protocols that were used in the studies. However, it is apparent that further studies are necessary before any conclusions can be reached regarding the use of GCV for treatment of infections caused by HHV-6.

23.3.3. Mercurithio-deoxyuridine analogs

It has been suggested that the herpesvirus encoded deoxyuridine triphosphate nucleotidohydrolase (dUTPase) (Caradonna and Cheng, 1981; Williams, 1984;

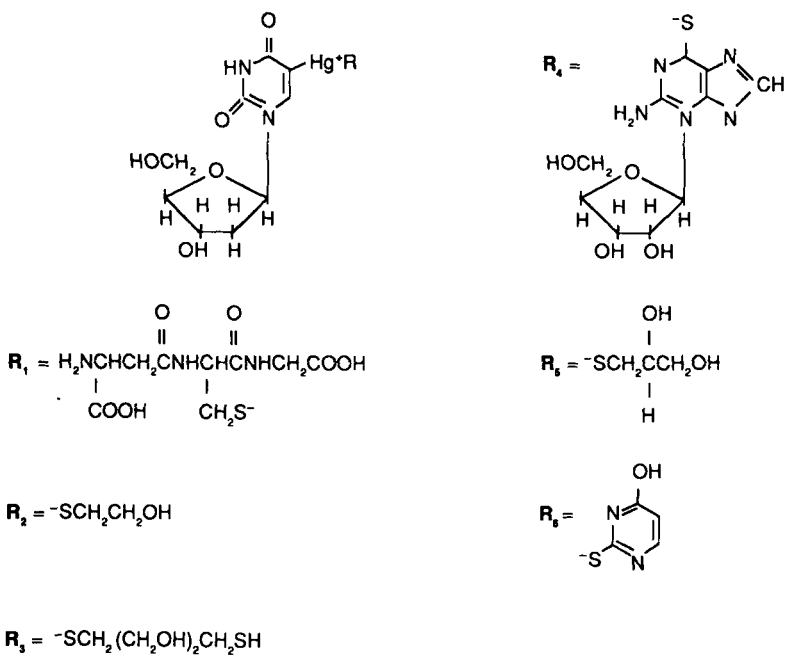

Fig. 23.2. Structure of various 5'-mercurithio-2-deoxyuridine (HgdUrd) analogs. R_1: glutathione–HgdUrd; R_2: 2-mercaptoethanol–HgdUrd; R_3: dithiothreitol–HgdUrd; R_4: 6-mercaptoguanosine–HgdUrd; R_5: α-thioglycerol–HgdUrd; R_6: 2-thiouracil–HgdUrd.

TABLE 23.3.
Effect of analogs of 5′-mercuric-2-deoxyuridine (HgdUrd) on the replication of HHV-6[a]

Compound	Effect
dUrd	nontoxic, no effect on CPE
HgdUrd	nontoxic, no effect on CPE
glutathione–HgdUrd	nontoxic, no effect on CPE
dithiothreitol–HgdUrd	toxic
β-mercaptoethanol–HgdUrd	toxic
α-thioglycerol–HgdUrd	nontoxic, no effect on CPE
2-thiouracil–HgdUrd	nontoxic, no effect on CPE
6-mercaptoguanosine–HgdUrd	nontoxic, decreased CPE

[a] Assays were performed as described previously using HSB_2 cells and HHV-6 (strain GS) (Steicher et al., 1988). The concentration of the test compound was 20 μM.

Williams and Parris, 1987) may be a potential target site for the development of antiviral agents for use in treating infections caused by herpesviruses (Williams 1988). We have demonstrated that some thio-derivatives of 5′-mercuric-2-deoxyuridine-5′-triphosphate (HgdUTP) act as irreversible-active-site directed inhibitors of eukaryotic dUTPases and that some of these inhibitors selectively inhibit the HSV-1 and HSV-2 specific dUTPases (Williams, 1986). We have also demonstrated that the glutathione, mercaptoethanol, mercaptoguanosine and thioglycerol derivatives of 5-mercuric-2-deoxyuridine (HgdUrd) (Fig. 23.2) inhibit the replication of HSV-1 and/or HSV-2 *in vitro* and that these compounds are relatively noncytotoxic (Holliday and Williams, manuscript submitted for publication). Furthermore, we have used the selectivity of the mercaptoguanosine derivative to demonstrate the presence of an EBV specific dUTPase (Williams et al., 1985).

While we have not been able to demonstrate the induction of dUTPase activity in cells infected with HHV-6 (Williams et al., 1989), our initial studies with the thio-derivatives of HgdUrd indicate that the mercaptoguanosine derivative has no cytotoxic effect on HSB_2 cells and that it decreases the CPE induced by HHV-6 in HSV-2 cells (Table 23.3) (Steicher and Williams, unpublished results). However, further studies are needed to determine the effect of these mercurithio-derivatives of dUrd on HHV-6 replication and to determine whether HHV-6 encodes for a specific dUTPase.

23.4. Problems

23.4.1. Quantitation of infectious virus

The major problem associated with determining the response of HHV-6 to antiviral agents is in the lack of a rapid and sensitive method for quantitating the

effect of the agent on HHV-6 replication. HHV-6 is similar to EBV in that a plaque assay does not exist for the quantitation of the virus. Because of this, end-point dilution assays have been used to quantitate infectious HHV-6 in a sample based on the $TCID_{50}$ (amount of virus required to infect 50% of the cells). End-point dilution assays are generally not suitable for determining the effect of antiviral agents on virus replication because they lack accuracy. Furthermore, end-point dilution assays are time consuming and they require relatively large numbers of cells. Therefore, in order to circumvent the problems associated with end-point dilution assays, several methods (Table 23.4) have been used to quantitate the effect of antiviral agents on the replication of HHV-6. While these methods have been useful, all have a specific limitation in that they do not quantitate the production of infectious virus but rather measure some effect of the virus such as a change in cell morphology (cytopathic effect) and/or the production of some virus-specific protein or viral DNA, thus inferring that antiviral agent decreases virus production. However, such assumptions can be misleading. For example, several studies have demonstrated that a specific antiviral agent decreases the number of cells in culture that exhibit specific CPE (pleiomorphic, balloon-like cells). However, CPE is dependent to some degree upon the cell type used in the study and there is no qualitative relationship between CPE and the amount of infectious virus that is produced. Other studies have used immunofluorescence (IF) or Western blotting techniques to demonstrate that a specific antiviral agent decreases the replication of HHV-6. Potential problems associated with IF techniques are that there are differences in the sera and/or monoclonal antibodies (mAb) used. Perhaps more importantly is that while IF techniques can demonstrate that an antiviral agent

TABLE 23.4.
Methods used for quantitation of response of HHV-6 to antiviral agents

Method	Reference
Cytopathic effect	Russler et al. (1989) Agut et al. (1988, 1989a) Roffman and Frenkel (1990) DiLuca et al. (1990)
End-point dilution	DiLuca et al. (1990)
Western blot – human serum	Russler et al. (1989)
DNA hybridization	
p2H14 plasmid	Kikuta et al. (1989a)
cloned ClaI 3.9 Kb fragment	Agut et al. (1989b)
Anticomplement immunofluorescence – human serum	Shiraki et al. (1989)
Indirect immunofluorescence – human serum	Steicher et al. (1988) Agut et al. (1989b)
Monoclonal antibody to cytoplasmic protein from HHV-6 infected cells	Kikuta et al. (1989a)
Monoclonal antibody 9A5D12	Roffman and Frenkel (1990) DiLuca et al. (1990)

reduces the number of cells infected by HHV-6, this reduction in IF^+ cells may reflect a decrease in the ability of the virus to infect adjacent cells when in the presence of the antiviral agent rather than an inhibition of viral replication. Furthermore, there have not been any attempts to correlate a decrease in IF positive cells with a decrease in the production of infectious virus. Thus, as pointed out by Kikuta et al., (1989a), no definitive method has been found for quantitating HHV-6 and such a system must be developed before definite conclusions can be reached regarding the effects of some agents of HHV-6 replication.

Harada et al. (1985) reported the development of a focus-forming assay using chemically attached cells for use in quantitating HIV-1. Asada et al. (1989) have used this technique to evaluate the effect of neutralizing antibodies on HHV-6. Quantitation of infectious HHV-6 is determined in this assay by focus formation using chemically attached MT-4 cells (T-cell line). Foci of infected cells are identified using indirect immunofluorescent-antibody staining. Their results with this assay have demonstrated that there is a linear relation between focus formation and virus number. Furthermore, this assay is relatively rapid, is more reproducible and accurate than end-point dilution assays and uses a homogenous cell type rather than a heterogenous cell population. The importance of this latter feature will be discussed later. Thus, it should be possible to use this assay to accurately determine the effect of antiviral agents on the replication of HHV-6 and on the production of infectious virus by quantitating the reduction in focus-formation following exposure of HHV-6 to an antiviral agent. A similar assay based upon the reduction of focus formation has been used to assess the effects of antiviral agents on the replication of HCMV (Wahren and Oberg, 1980).

23.4.2. Multiplicity of infection (MOI)

Harmenberg et al. (1980) demonstrated that the sensitivity of a virus to a given antiviral agent is dependent to some degree upon the moi. In studies concerning the response of HHV-6 to antiviral agents, the moi has ranged from 0.001 to 0.1. Such variation in the moi probably effects the response of HHV-6 to an agent but none of these studies with HHV-6 have addressed this potential problem.

23.4.3. Cell culture conditions

In addition to the problems associated with quantitation of infectious HHV-6, the type of cell used for the study may have an effect on the results concerning the sensitivity of HHV-6 to antiviral agents. In natural infections, HHV-6 exhibits T cell tropism (Downing et al., 1987; Ablashi et al., 1988a; Agut et al., 1988; Lopez et al., 1988; Lusso et al., 1988, 1989; Takahashi et al., 1989) and HHV-6 has also been reported to infect a variety of cell lines (Downing et al., 1987; Tedder et al., 1987; Ablashi et al., 1988a,b; Asada et al., 1989). Most studies concerning the sensitivity of HHV-6 to antiviral agents have been performed using peripheral

blood mononuclear cells (PBMC) (Agut et al., 1989a,b; Russler et al., 1989; DiLuca et al., 1990) or human cord blood mononuclear cells (CBMC) (Kikuta et al., 1989a; Shiraki et al., 1989). While the use of these cells probably resembles an *in vivo* situation with regards to the interaction of the virus with susceptible cells, primary mononuclear cell cultures represent a highly heterogenous population of cells and the constituents in this population will vary between individuals, thus making the results variable not only between individual experiments, but also between different laboratories. Furthermore, different culture conditions have been used to grow these cells. Cells have been grown in media that has been supplemented with various amounts of the mitogen phytohemagglutinin (PHA, 0–5 μg/ml) and the polypeptide interleukin-2 (IL-2, 0–10 units/ml). Culture conditions are important in that they could affect cell-virus interactions and this, in turn, could affect the response of HHV-6 to a particular antiviral agent (DeClercq et al., 1980; Harmenberg et al., 1980). Recent studies have demonstrated that HHV-6 may be similar to HIV-1 (Gowda et al., 1989) in that T cell activation is required for efficient replication of the virus (Frenkel et al., 1990). Additional studies have demonstrated that while IL-2 may be required for the efficient replication of HHV-6 (Black et al., 1989; Frenkel et al., 1990), high levels of IL-2 can delay and/or inhibit the replication of the virus (Frenkel et al., 1990; Roffman and Frenkel, 1990). It is apparent from these studies that complex interactions occur at both the cellular and molecular levels that allow for the efficient replication of HHV-6 and at the present time, we do not understand these interactions. Thus, before such heterogenous populations of cells are used for studies concerning the sensitivity of HHV-6 to various antiviral agents, standard culture conditions should be established and even then caution should be used in the interpretation of the results.

In view of the potential problems associated with using PBMC and/or CBMC, it may be more appropriate to use tissue culture cell lines for studies concerning the effect of antiviral agents on HHV-6 replication. Tissue culture cell lines are routinely used to assess the chemotherapeutic efficiency of antiviral agents against many viruses including other herpesviruses and such systems are advantageous in that they contain a single cell type. However, it has also been demonstrated that the concentration of an agent required to inhibit the replication of herpes simplex virus is dependent upon the cell line employed (Harmenberg et al., 1980; DeClercq, 1982). This suggests that the host cell may have a role in determining the chemotherapeutic efficiency of a given antiviral agent. HHV-6 can infect a variety of tissue culture cell lines but at different efficiencies (Downing et al., 1987; Tedder et al., 1987, Ablashi et al., 1988a,b; Asada et al., 1989). At the present time, the significance of the differences in replication efficiency in these cell lines is unknown, but it further demonstrates that the host cell may have a role in determining the response of a virus to an antiviral agent. However, at the present time, there has only been one study to determine the effect of antiviral agents on the replication of tissue culture cells (Steicher et al., 1988) and, as pointed out previously (DeClercq, 1982), generalizations concerning the action of antiviral agent should not be done based on studies that have employed only one cell line.

23.4.4. Strain variation

HHV-6 has been isolated from normal individuals (Pietroboni et al., 1988) as well as from patients with a variety of diseases (Salahuddin et al., 1986; Downing et al., 1987; Krueger et al., 1987; Tedder et al., 1987; Ablashi et al., 1988a; Yamanishi et al., 1988; Becker et al., 1989). Only a few isolates of HHV-6 have been examined for their sensitivity to antiviral agents (Steicher et al., 1988; Agut et al., 1989b; Kikuta et al., 1989a; Russler et al., 1989; Shiraki et al., 1989; DiLuca et al., 1990; Roffman and Frenkel, 1990) and recent studies have demonstrated that there is genetic pleomorphism within some of these isolates (Jarrett et al., 1987; Josephs et al., 1988). Similar studies have demonstrated that genetic pleomorphism exists between different isolates of EBV (Bornkamm et al., 1980). While there is virtually no information known regarding the biological implications of this genomic variation, we have demonstrated that antigenic differences exist between the alkaline deoxyribonucleases (DNase) that are encoded by different strains of EBV (Williams et al., 1988) and such changes in the structure and/or function of a viral specific protein could change the response of the organism to an antiviral agent. Similarly, DeClercq et al. (1980) demonstrated that various strains (both laboratory and clinical isolates) of HSV-1 and HSV-2 differed in their response to several nucleoside analogs which are used as antiviral agents. While strain variation could be responsible for some of the differences in sensitivities of the various isolates of HHV-6 to antiviral agents, at this time, it is not possible to make any firm conclusion about the biological importance of these strain variations because of the limited studies.

23.5. Conclusions

It is apparent that while some progress has been made, there is a considerable amount of research that needs to be done to determine the response of HHV-6 to antiviral agents. A limited number of studies have been performed thus far and they have focused on a small number of compounds. Since HHV-6 seems to be related to HCMV, further studies at both the molecular and cellular levels need to be performed with ganciclovir to determine whether this agent can effect the replication of HHV-6. Furthermore, other agents, particularly those that do not need to be phosphorylated by a virus-specific thymidine kinase, need to be examined for their effect on viral replication.

A more pressing problem may be in the development of a rapid and sensitive assay that can be used for the quantitation of infectious virus. Also, the procedures that are used to determine the response of HHV-6 to various antiviral agents should be standardized. This is particularly important since differences in protocols, i.e., virus strain, multiplicities of infection, cell type and culture conditions affect the response of HHV-6 to antiviral agents, thus making the interpretation of results from different laboratories more difficult.

Finally, as we gain a better understanding of the biochemical mechanisms involved with HHV-6 replication, cellular interactions and with its interaction with

HIV-1, we may be able to rationally develop antiviral agents that will be useful in treating infections caused by HHV-6.

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