Human Herpesvirus-6 in Human Lymphomas: Identification of Specific Sequences in Hodgkin’s Lymphomas by Polymerase Chain Reaction

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In search of a possible involvement of the human herpesvirus type 6 (HHV-6) in human Hodgkin’s and non-Hodgkin’s lymphomas, we studied the levels of anti-HHV-6 antibodies in the sera of 94 cases by an immunofluorescence assay, as well as the presence of HHV-6 sequences in the affected tissues of 66 cases by polymerase chain reaction, using one set of primer oligonucleotides. Our results showed higher anti-HHV-6 antibody titers in human lymphomas than in normal blood donors, but the difference is statistically significant only when normal donors are compared with Hodgkin’s lymphoma cases. HHV-6 sequences were detected in 3 of 25 Hodgkin’s lymphomas and 0 of the 41 cases of non-Hodgkin’s lymphomas studied. The three cases positive for HHV-6 sequences belong to the nodular sclerosis-lymphocyte depletion histologic subtype and share remarkable similarities in their clinical features. Furthermore, Southern blot analysis of total genomic DNA obtained from the neoplastic tissues of two of the three patients showed the same restriction fragment length polymorphism. Our results suggest that: (1) the high level of anti-HHV-6 antibodies in Hodgkin’s disease is due to an activation of the immune system not related to the presence of HHV-6 sequences in affected lymph nodes; (2) the presence of HHV-6 sequences in human lymphoid tissues is not a frequent event, rather it is in fact a very rare event in non-Hodgkin’s lymphomas, while in Hodgkin’s cases it is more frequent than previously reported on the basis of Southern blot analysis; and (3) the presence of HHV-6 sequences in Hodgkin’s lymphomas may have a relation with the clinical presentation of the disease.

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MATERIALS AND METHODS

Indirect Immunofluorescence Assay (IFA)

Replicating HSB-2 cells, treated with Polybrene (5 μg/mL; Sigma, St Louis, MO) for 2 hours, were infected with virus-rich culture supernatants from HHV-6/HSB-2 previously infected and dying cells. Five days later, large refractile cells, indicative of a...
successful infection, were prevailing in the culture. The cells were separated from cell debris on Ficoll (Sigma), applied to glass slides, and fixed with 50% acetone. The slides were incubated with a control-positive, human serum and developed using a fluoroscentated, goat antimouse IgG serum (Cappel-Organon Teknika, Veedijk, Belgium). IFA showed a characteristic, prevalently granular nuclear staining of acetone-fixed cells. Antibody titration was performed double blind by two operators. The assay was performed on 200 sera from normal blood donors and on 94 sera collected from human lymphoma cases at the time of diagnosis. The sera were stored at −20°C for no longer than 20 months.

Sources of Human Specimens and Clinical Histories

Genomic DNA was purified from 62 lymph node biopsies, from two fragments of surgically removed spleens, and from two cellular pellets derived from massive pleural effusions. All tissues were largely infiltrated by pathologic elements, while the cells from pleural effusions were more than 80% neoplastic. All the NHL cases were subjected to standard immunophenotyping procedures. All Hodgkin’s samples were from peripheral lymph nodes; in two cases the DNA extracted from peripheral blood mononuclear cells (PBMC), separated by Ficoll, was also examined. The clinical histories of the patients bearing the HHV-6 sequences were as follows.

Case 5. The patient, a woman born in 1960, was referred to the hospital in 1987 because of the presence of intermittent fever of unknown origin associated with left cervical lymphadenopathy. A standard chest radiography showed a bulky mediastinal involvement. The chest computed tomographic (CT) scanning confirmed the mediastinal mass, showing also a right hilar lymphadenopathy and the presence of small right pleural and pericardial effusions. The cervical lymph node biopsy showed a picture of nodular sclerosing Hodgkin’s disease (HD) of the subtype defined by Lukes as nodular sclerosis/lymphocyte depleted (NS-LD) and by Bennett et al as NS-LD with a prevailing “pleomorphic” basic pattern. The large pleomorphic Sternberg cells accounted for about 25% of the cellular elements. The patient, classified as stage II B with bulky mediastinal disease, was treated with associated chemotherapy (melphalanthamine, vincristine, procarbazine, doxorubicin, bleomycin, vinblastine, and dacarbazine) and radiation therapy. The therapy was well tolerated and the patient reached a complete remission that still holds.

Case 12. The patient, a woman born in 1958, first observed bilateral cervical lymphadenopathy in August 1986 but underwent medical examination only 4 months later, complaining of intermittent fever, weight loss, night sweats, fatigue, and initial wheeziness. At the time of admission to the hospital, generalized lymphadenopathy and hepatosplenomegaly were present, together with bilateral pleural effusions and ascites. The biopsy of an axillary lymph node showed a histopathologic picture consistent with nodular sclerosing HD of the subtype NS-LD according to Lukes and NS-LD with a prevailing “fibrohistiocytic” pattern according to Bennett. The lacunar cells accounted for about 25% of all cellular elements. The total body CT scanning showed mediastinal and retroperitoneal involvement of the disease, and the case was classified as stage II B with bulky mediastinal disease. A combined chemotherapeutic regimen (lomustine, vinblastine, procarbazine) and radiotherapy regimen was able to obtain only a partial reduction of the lymphomatous masses. The disease relapsed soon after the completion of the combined therapy and the patient died 15 months after the diagnosis, with extranodal pulmonary involvement and massive pleural and pericardial effusions, despite the intensive chemotherapy applied.

DNA Extraction and Southern Blot Analysis

The DNA was extracted from the solid tissue specimens with the technique described by Enrietto et al and from cellular pellets as reported elsewhere. To confirm the lineage origin of the affected cells in NHL cases, DNA samples obtained from each specimen were digested with EcoRI, BamHI, and HindIII restriction endonucleases and were hybridized to probes corresponding to Ig heavy and light chain genes and to the T-cell receptor β-chain (TCR-β) gene, as described elsewhere. The same enzymes and the same standard Southern procedure were used in a restriction pattern analysis of the DNAs of the Hodgkin’s samples positive for HHV-6 sequences. The cleaved DNAs were hybridized with the plasmid pZVH14, containing a 9-kb segment of the HHV-6 sequence, and pSL76, containing two copies of the EBV Bam W internal repeated sequences, and with Pr-1 (Oncogene Science Inc, Manhasset, NY), a commercially available genomic restriction fragment representing a portion of the human bcr gene.

Synthesis of Oligonucleotide Primers and Probes

Unmodified deoxyribonucleotides were synthesized on an automated solid-phase synthesizer (Applied Biosystems Inc, Foster City, CA, model 381A) by standard phosphoramidite chemistry. After several extraction cycles with ammonium hydroxide, the oligomers were ethanol precipitated, lyophilized, and dissolved directly in twice-distilled water.

The oligonucleotides used in the HHV-6 PCR assays have the following sequence: (1) 5'-CCCATTTACGATTTCCTGAC-3' as sense primer; (2) 5'-TTCAAGGGACGT-TATGTATTGAGCAGATGCG 3' as antisense primer; and (3) 5'-CCGTTAAAAATTTACACCTCCATTCATCTT 3' as internal probe.

For EBV PCR tests the sequences of primers (TC60 and TC51) and probe (TC62) were as specified by Saito et al.

PCR

One microgram of each DNA sample was included in a 100-μL reaction mixture containing 50 mmol/L KCl, 10 mmol/L Tris (pH 8.3), 2.5 mmol/L MgCl2, 225 μmol/L dATP, 225 μmol/L dCTP, 225 μmol/L dGTP, 225 μmol/L dTTP, 200 μg/mL of bovine serum albumin, 50 pmol of each primer, and 2 U of thermostable Taq DNA polymerase (Promega, Madison, WI). The sample denaturation was performed at 94°C for 1 minute, the annealing at 53°C for 1 minute, and the primer extension step was performed at 68°C for 2 minutes. Thirty cycles of amplification were performed with an automatic thermal cycler (MJ Research, Cambridge, MA).

At the end of the amplification procedure, one fifth of the reaction mixture was subjected to electrophoresis on a 2% agarose gel. The DNA was then transferred to a nylon membrane (NEN, Boston, MA) by electroblotting, fixed to the membrane at 80°C for
2 hours, and hybridized overnight at 65°C in 5X SSPE, pH 6.4, 0.6% sodium dodecyl sulfate (SDS), 0.2 mg/mL sonicated salmon sperm DNA, with the specific oligonucleotide probe end-labeled, by a standard procedure, with a γ^{32}P-ATP.\(^8\) At the end of the hybridization procedure the filters were washed in 2X SSPE, pH 6.4, 0.1% SDS for 15 minutes at room temperature and then in 5X SSPE, pH 6.4, 0.1% SDS for 1 hour at 60°C. The autoradiography of the membranes was performed for 2 and 7 days at ~80°C. To avoid false-positive results, all procedures were performed in strict adherence to the recommendations of Kwok and Higuchi.\(^7\)

The search for HHV-6 sequences was performed by a PCR procedure able to generate and amplify a segment of 186 nucleotides, part of the 9-kb HHV-6 sequence inserted in the pZVH14 plasmid.\(^9\) Dilution experiments, performed by diluting the pZVH14 in increasing amounts of normal human uninfected DNA, showed that, in our PCR conditions, it was possible to recognize one copy of the recombinant plasmid per 10,000 cell DNA equivalents. One positive (DNA from HHV-6-infected HSB-2 cells) and two negative (DNA from uninfected HSB-2) controls were used for each group of 10 samples examined. The test was discarded when one of the negative controls gave an even weak signal after a 7-day exposure.

The PCR procedure for EBV sequence detection was performed according to Saito et al.,\(^6\) resulting in the amplification of a 122-nucleotide segment of the reiterated viral DNA sequence Bam W.

RESULTS

HHV-6 Humoral Immune Response in Lymphoma Patients and Normal Individuals

Indirect immunofluorescence assay on HHV-6-infected cells was performed to detect serum antibodies against viral proteins in 200 sera from normal blood donors (male-to-female ratio 1:1) with a median age of 37 years. Data obtained with sera at 1:20 dilution were disregarded because of the aspecific immunofluorescence detected in control uninfected cells. At a serum dilution of 1:40, 54% of the normal blood donors were clearly positive for the presence of anti–HHV-6 antibodies. This percentage decreases to 26% and 6% at dilutions of 1:80 and 1:160, respectively, as presented graphically in the histograms of Fig 1. Ninety-four sera collected from human lymphoma cases, of which 31 were derived from HL (mean age 55 years) and 63 from NHL (mean age 63 years) cases, were studied using the same procedure and the same pool of reagents. Males and females in both groups were almost equally represented. The percentages of positive cases at 1:40, 1:80, and 1:160 dilutions are 80%, 68%, and 16%, respectively, in Hodgkin’s cases and 62%, 41%, and 19% in non-Hodgkin’s patients (Fig 1). Our results indicated a threefold increase in the number of patients with high antibody titers (1:160) against HHV-6 in the population with HL and NHL as compared with the normal population. These results are particularly meaningful in light of a known decrease of antibody titers against HHV-6 after age 40 in the normal population.\(^2\) However, when a severe discriminating criterion is used, the statistical analysis shows that only the differences between the values of normal subjects and Hodgkin’s patients are statistically significant ($X^2 = 16.0416$).

Presence of HHV-6 DNA Sequences in Affected Tissues of Lymphoma Patients

The presence of HHV-6 DNA sequences was investigated in the total DNA extracted, as described in Materials and Methods, from pathologic specimens derived from 66 cases of human lymphoma (Table 1). Forty-one were cases of NHL: 33 of the B lineage and eight of the T lineage. Twenty-five samples were obtained from HL cases. All 41 samples from NHL patients were negative for HHV-6 DNA sequences in two successive PCR tests. Three of 25 DNA samples extracted from lymph nodes of Hodgkin’s cases were clearly positive for the presence of HHV-6 sequences by PCR (cases 5, 12, and 14; Fig 2). When the standard Southern blot analysis was used with the pZVH14 plasmid as probe, the lymph nodes of patients 5 and 12 were positive. In these two cases, by comparison with the signals obtained with a probe representing a single copy gene (human bcr), the copy number of HHV-6 sequences was evaluated as 1 per 5 to 6 cell DNA equivalents. DNAs from PBMC and from a lymph node of patient 14, obtained at the time of diagnosis, were subjected to PCR analysis. HHV-6 sequences were detected in both DNA, but the lymph node DNA appeared to contain a higher copy number of viral sequences. The DNA of PBMC from patient 5, already in complete remission, gave a strong positive signal in a PCR.
test as well as in a standard Southern assay. Interestingly, no anti–HHV-6 antibodies were detected in the serum of patient 5 at 1:40 serum dilution. The serum of patient 14, obtained at the time of diagnosis, yielded a positive IFA test at dilution of 1:80. Both patient’s sera were negative for anti-HIV antibodies (enzyme-linked immunosorbent assay [ELISA] test) and weakly positive for anti-EBV IgG antibodies (ELISA tests). The serum of patient 12 was not available for analysis. The research of EBV sequences in the lymph node DNA of the three HHV-6-positive patients was negative by Southern blot, but positive in all cases by PCR, although the autoradiographic signals obtained were very faint (data not shown). These results suggest that, at least in the lymph nodes of patients 5 and 12, who were positive for HHV-6 sequences in Southern blot, the number of EBV sequences is remarkably lower than that of HHV-6 sequences. The same tests were negative when performed on the PBMC DNA of cases 5 and 14. These data are summarized in Table 2.

**Table 2. HHV-6 and EBV Sequences (PCR), Anti–HHV-6 (IFA), and Anti-EBV (ELISA) Antibodies in Hodgkin’s Patients Positive for HHV-6**

<table>
<thead>
<tr>
<th>Case No.</th>
<th>Sequences in Lymph Nodes</th>
<th>Sequences in PBMC</th>
<th>Serum Antibodies Against</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HHV-6</td>
<td>EBV</td>
<td>HHV-6</td>
</tr>
<tr>
<td>5</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>12</td>
<td>+</td>
<td>+</td>
<td>NT</td>
</tr>
<tr>
<td>14</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Abbreviation: NT, not tested.
*Tested in complete remission.

**Restriction Analysis of HHV-6 Sequences Found in Lymphoma Cases**

The lymph node DNAs of a control normal individual and of cases 5 and 12, as well as the PBMC DNA of case 5, were digested with EcoRI, HindIII, and BamHI restriction enzymes and hybridized with the pZVH14 plasmid. The negative control DNA did not show segments hybridizing with the probe used, whereas the restriction patterns obtained in the three positive samples, when compared with that obtained in the DNA of HSB-2-infected cells, showed some differences (Figs 3 and 4). The segments resulting from EcoRI digestion of infected HSB-2 DNA are all present in our cases. The HindIII digestion shows two bands, one of higher and one of smaller molecular weight compared with the 9-kb segment present in HSB-2 DNA, corresponding to the insert of the pZVH14 plasmid. The double digestion with EcoRI and HindIII (Fig 3) suggests that the S’ Hind site is lost in the cases examined, and a new Hind site is present in the pZVH14 sequence, possibly within the 5’ internal EcoRI fragment, as already reported.33 The digestion of infected HSB-2 DNA with BamHI (Figs 3 and 4) shows, as expected, four fragments: two short (0.9 and 1.1 kb) and two far longer. These latter segments are so similar in length that only a very short exposure time allows the distinction of two bands in the high molecular weight region of the gel. In the DNA of the patients, after BamHI digestion, the 0.9- and 1.1-kb fragments are absent, while the two longest, almost comigrating, fragments are only a little longer than those from infected HSB-2 cells. This behavior is consistent with the
loss of two of the three central BamHI sites. The double digestion with BamHI and HindIII (Fig 3) confirms that only the extreme 5' BamHI site is conserved, near the new 5' HindIII site. In conclusion, as shown by Southern blot analysis, the 9-kb viral sequence studied in two of the three positive Hodgkin's cases differs from that of the original HHV-6 isolate because of the absence of at least three restriction sites (one for HindIII and two for BamHI), with the formation of a new one (for HindIII), all in the 5' portion of the sequence examined (Fig 3).

DISCUSSION

The fact that about 50% of normal blood donors show the presence of IgG antibodies against HHV-6-related antigens at a 1:40 serum dilution is in agreement with the
widespread seroprevalence, ranging from 60% to 85%, reported in the normal population by several investigators examining the samples to serum dilutions as low as 1:10.13-15 A possible immunologic cross-reactivity between HHV-6 and other herpesviruses, mainly HCMV,19 has been ruled out by the large majority of investigators using indirect immunofluorescence techniques.13-15 Moreover, a nonspecific reaction able to influence the data obtained at a 1:40 dilution is unlikely. Uncertainties in interpreting immunofluorescence tests performed at low serum dilutions have already been reported in the literature.15,16 This ambiguity may be due to the fact that HHV-6 antibody reactivity, as shown by the ELISA test, is normally distributed from the weakest to the strongest responses without a discrete level of reactivity, as pointed out by Saxinger et al.13

In our series of 94 sera from human lymphomas, the percentage of tests positive for HHV-6 antibodies is higher than in normal blood donors as it was previously reported,13,25 but only the HL cases show a strong statistically significant difference (X² = 16.0416). Because the infection is a widespread phenomenon and the comparison is performed between values obtained at a 1:40 and higher serum dilutions, we conclude that the titer of anti-HHV-6 antibodies and not the frequency of infection is significantly higher in Hodgkin’s cases than in healthy subjects. A possible reason for these high antibody titers might be that the impairment of the cellular immune response, induced by the HD, allows the reactivation of a latent infection leading to higher antibody titers. A several-fold increase in HHV-6 antibodies has also been reported during EBV and HCMV infection.14,44 Thus, a possibly different mechanism for the raising of HHV-6 antibody titers in HD may act through a new infection by other viruses. We do not have a satisfactory explanation of this behavior; however, the serology data indicate that the immune system of Hodgkin’s patients is in some way activated against HHV-6 antigens. This activation is likely to be independent of the presence of HHV-6 sequences in lymph nodes, which seems to be a rather rare event, as judged by the results of the present and previous surveys.24-25 As reported above, HHV-6 is able to infect not only lymphocytes but also cells of other lineages. Then we may consider the possibility that a tissue different from the lymph node acts as a reservoir of the virus.

Previous studies, performed with Southern blot analysis, showed the presence of HHV-6 in only 5 of more than 150 cases of NHL, and in 0 of 37 cases of HD.24,25 In contrast, others described a high percentage of lymphoma cases bearing HHV-6 sequences.23 Despite the use of a sensitive technique, our findings suggest that the presence of HHV-6 sequences, as a rule, is not a frequent event in human lymphoid tissues, contrary to what is suggested elsewhere.25 This presence is in fact a very rare event in human NHLs, as previously reported, whereas in HD it is more frequent than previously suggested by the standard Southern blot technique. The high number of negative tests suggests that contamination was substantially absent from our PCR assays, although the sensitivity of the technique was high, as demonstrated by the dilution experiments reported in Materials and Methods. On the other hand, we must keep in mind that a positive PCR result does not either prove per se the presence of a complete viral genome or indicate the integrated or episomal form of the virus. It is also impossible to exclude that variant HHV-6, not hybridizing well to our primers, can be present in the examined tissues. Moreover, the positivity of a PCR test may be due to the presence of a very small amount of infected cells, which may not belong to the pathologic tissues in the sample examined. This warning holds particularly true in the case of HD in which only an in situ hybridization test, showing the presence of the infection in the Reed-Sternberg cells, would indicate a direct involvement of the neoplastic elements. However, in two of our cases the number of HHV-6 copies was high enough to allow an easy identification by standard Southern technique.

Our observations are suggestive of a possible role for HHV-6 in the pathogenesis of some cases of HD. The three young women found positive for HHV-6 sequences in our survey show some obvious similarities in their clinical pictures. All cases belong to the same histologic subgroup, NS-LD, generally accounting for a small proportion (about 5%) of the cases and associated with a poor prognosis. No patients belonging to other, more frequent, histologic subgroups were found infected. Two of the cases had a predominant bulky mediastinal involvement, while the third patient showed mediastinal involvement as part of a generalized disease. Mesothelial effusions (pleuropericardial in two cases and pleuroperitoneal in the third) were observed in the three patients, although at different times in the course of the disease. The number of cases is so small that several reasons may explain the coincidence of HHV-6 infection with these common clinical features. The histopathologic pattern, eg, frequently associated with poor prognosis, may involve a severe immune impairment, which in turn may favor exogenous or latent infections. However, one of our cases showed a 1:80 titer of anti–HHV-6 antibodies, indicating that HHV-6 infection is not necessarily associated with the absence of the immune response. Although a relationship between the presence of HHV-6 and a particular subset of patients is not solidly established, the possibility is raised, in our opinion, that HHV-6 infection may play a role in determining some basic clinical aspects of a limited number of cases. A better understanding of the relations between HHV-6 infection and HD requires the study of a larger number of cases and more information regarding the genomic structure of HHV-6, the possible integration of the virus in the host genome, the precise mechanisms of latency and reactivation of the infection, and the influence of a latent infection on the behavior of the immune system. Also, the results of a coinfection with other lymphotropic viruses must be carefully considered for their possible impact on clinical picture and prognosis. In fact, a latent EBV infection has been rather frequently reported in Hodgkin’s cases, although the meaning of this coexistence is still only matter of speculation.46-48 EBV sequences are present in the three cases reported here, which are also HHV-6 infected. Moreover,
cases of co-infection of HHV-6 with HIV and HTLV-I are reported, and the transactivation of HIV LTR by HHV-6 has been observed.

The restriction analysis performed on the HHV-6–positive cases showed several differences between the viral sequences present in the Hodgkin’s cases and the pZVH14 sequence of the original isolate. This is consistent with the reported polymorphism of HHV-6, but it has to be pointed out that the restriction pattern is identical in the two cases and partially similar to that already reported in a case of NHL infected with HHV-6. As the region analyzed is only 6% of the HHV-6 genome, the virus isolation and a more extensive restriction analysis are required to discuss the possible existence of a new strain of HHV-6.

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