Background and Objectives. The prevalence and pathogenic role of human herpesvirus 6 (HHV-6) in various benign and malignant hematologic diseases remain largely unknown. The aim of this study was to search for a possible involvement of HHV-6 in the pathogenesis of hematologic diseases.

Design and Methods. The presence of HHV-6 DNA sequences was examined by polymerase chain reaction (PCR) in bone marrow mononuclear cells from 241 patients with benign and malignant hematologic diseases in China. Platelet-associated immunoglobulin (PAIg) of 66 idiopathic thrombocytic purpura (ITP) patients was measured by competitive enzyme-linked immunosorbent assay. The presence of HHV-6 DNA in sera from 31 ITP patients was examined by PCR. Paired serum samples from 19 ITP patients were analyzed for anti-HHV-6 IgG titers using an indirect immunofluorescence assay.

Results. HHV-6 DNA was detected in 41% and 37.5% of ITP and acute leukemia patients respectively, but in only 6.7% of patients with iron deficiency anemia. HHV-6 positivity for ITP patients with excessive PAIgG was significantly higher than in patients with a normal level of PAIgG. HHV-6 DNA was not detected in any of the serum samples from ITP patients. None of the 19 cases of ITP showed a significant increase in anti-HHV-6 antibody titers during the convalescent phase compared with the onset phase.

Interpretation and Conclusions. Our results indicate that HHV-6 infection might be associated with excessive PAIgG in some cases of ITP, and that the virus persists in a latent state. The pathogenic role of HHV-6 in ITP needs to be confirmed by further investigations.

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Key words: human herpesvirus 6, idiopathic thrombocytic purpura, acute leukemia, platelet-associated immunoglobulin

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in ITP patients, the presence of HHV-6 DNA in sera was examined by PCR, and paired serum samples were analyzed for anti-HHV-6 IgG titers using an indirect immunofluorescence assay (IFA).

Design and Methods

Patients

Two hundred and forty-one patients with hematologic diseases originating from our hospital and 44 healthy donors were included in this study. The patient series included 15 patients with iron deficiency anemia (IDA), 19 with aplastic anemia (AA), 105 with ITP, 25 with myelodysplastic syndromes (MDS), 48 with acute leukemia (AL) and 29 with chronic myeloid leukemia (CML). ITP was diagnosed on the basis of the findings of thrombocytopenia or shortened platelet survival, presence of normal or increased numbers of megakaryocytes in bone marrow, normal spleen size and no other known cause of thrombocytopenia.17 Diagnosis of the other diseases was based on morphologic, cytochemical and immunophenotypic criteria.18-24 The patients’ characteristics, including their age and sex, are summarized in Table 1. Of the 44 healthy donors, twenty were females and 24 were males, with a median age of 32 years.

All the bone marrow samples, the blood samples for measurement of PAIg and the serum samples for PCR were obtained at diagnosis. Informed consent was obtained from all the patients.

PCR detection of HHV-6 sequence

Bone marrow and peripheral blood were collected in heparin; mononuclear cells were isolated by Ficoll-Hypaque gradient separation. All samples were stored frozen in liquid nitrogen until DNA extraction. DNA was extracted using the DNA isolation kit for mammalian blood (Boehringer Mannheim, Germany) according to the manufacturer’s instructions. One microgram of DNA was used as the template for PCR.

DNA extraction from serum samples (20 µL) was performed using a commercially available kit, Ready PCR™ serum-virus DNA purification system (Sino-American Biotech Co., China). Two microliters of the extract was used in PCR.

PCR was performed in a Gene-Amp PCR System 2400 thermocycler (Perkin Elmer, Foster City, CA, USA), using the set of primers described by Aubin et al.25 which detect a conserved region for both variants A and B of HHV-6. The primers (A: 5'—GAT CCG ACG CCT ACA AAC AC-3', C: 5'-CGG TGT CAC ACA GCA TGA ACT CTC-3') amplify a fragment of 830bp. The total reaction volume was 50 µL containing 10 mM Tris-HCl pH8.3, 1.5 mM MgCl2, 50 mM NaCl, 0.01% (wt/vol) gelatin, 100 µM of each deoxynucleotide triphosphate (dNTP), 20 pmol of each primer, and 1.5 U of Taq polymerase (Golden World Biological Co., Beijng, China). The 30 cycles consisted of 1 minute at 94°C, 50 seconds at 55°C, 90 seconds at 72°C preceded by 5 minutes of denaturation at 94°C. After the last cycle, the extension step was extended to 10 minutes at 72°C. Ten microliters of PCR product was electrophoresed on a 1.5% agarose gel containing 0.5 µg/mL ethidium bromide. The results were observed under UV illumination.

pZVH14, the plasmid containing a 9kb segment of the HHV-6 sequence,26 was used as a template for PCR to evaluate the lower detection limit of the PCR test. Serial dilution experiments showed that, in our PCR conditions, it was possible to recognize amplified products obtained from ten copies of the recombinant plasmid.

Table 1. Prevalence of HHV-6 genomes in BM/MNC from patients with hematologic diseases.

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>HHV-6 positivity (%)</th>
<th>Male</th>
<th>Female</th>
<th>p value</th>
<th>Adult (&gt;14 years)</th>
<th>Children (≤14 years)</th>
<th>p value</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>ITP</td>
<td></td>
<td>38.1 (16/42)</td>
<td>42.9 (27/63)</td>
<td>&gt;0.05</td>
<td>47.9 (34/71)</td>
<td>26.5 (9/34)</td>
<td>&lt;0.05</td>
<td>41.0 (43/105)*</td>
</tr>
<tr>
<td>acute</td>
<td></td>
<td>36.0 (9/25)</td>
<td>34.4 (11/32)</td>
<td>&gt;0.05</td>
<td>38.2 (13/34)</td>
<td>30.4 (7/23)</td>
<td>&gt;0.05</td>
<td>35.1 (20/57)†</td>
</tr>
<tr>
<td>chronic</td>
<td></td>
<td>41.2 (7/17)</td>
<td>51.6 (16/31)</td>
<td>&gt;0.05</td>
<td>56.8 (21/37)</td>
<td>18.2 (2/11)</td>
<td>&lt;0.05</td>
<td>47.9 (23/48)‡</td>
</tr>
<tr>
<td>AL</td>
<td></td>
<td>35.7 (10/28)</td>
<td>40.0 (8/20)</td>
<td>&gt;0.05</td>
<td>36.8 (14/38)</td>
<td>40.0 (4/10)</td>
<td>=0.278</td>
<td>37.5 (18/48)‡</td>
</tr>
<tr>
<td>AML</td>
<td></td>
<td>31.6 (6/19)</td>
<td>54.5 (6/11)</td>
<td>=0.145</td>
<td>40.7 (11/27)</td>
<td>33.3 (1/3)</td>
<td>=0.452</td>
<td>40.0 (12/30)§</td>
</tr>
<tr>
<td>ALL</td>
<td></td>
<td>44.4 (4/9)</td>
<td>22.2 (2/9)</td>
<td>=0.244</td>
<td>27.3 (3/11)</td>
<td>42.9 (3/7)</td>
<td>=0.311</td>
<td>33.3 (6/18)</td>
</tr>
<tr>
<td>MDS</td>
<td></td>
<td>18.8 (3/16)</td>
<td>44.4 (4/9)</td>
<td>=0.147</td>
<td>30.4 (7/23)</td>
<td>0 (0/2)</td>
<td>=0.510</td>
<td>28.0 (7/25)</td>
</tr>
<tr>
<td>RA</td>
<td></td>
<td>11.1 (1/9)</td>
<td>33.3 (1/3)</td>
<td>=0.409</td>
<td>16.7 (12/72)</td>
<td>0 (0/1)</td>
<td>=0.846</td>
<td>16.7 (2/12)</td>
</tr>
<tr>
<td>RAEB</td>
<td></td>
<td>25.0 (1/4)</td>
<td>50.0 (1/2)</td>
<td>=0.533</td>
<td>40.0 (2/5)</td>
<td>0 (0/1)</td>
<td>=0.667</td>
<td>33.3 (2/6)</td>
</tr>
<tr>
<td>RAEBT</td>
<td></td>
<td>33.3 (1/3)</td>
<td>50.0 (2/4)</td>
<td>=0.514</td>
<td>42.9 (3/7)</td>
<td>—</td>
<td>—</td>
<td>42.9 (3/7)</td>
</tr>
<tr>
<td>CML</td>
<td></td>
<td>23.8 (5/21)</td>
<td>40.0 (2/8)</td>
<td>=0.365</td>
<td>24.1 (7/29)</td>
<td>—</td>
<td>—</td>
<td>24.1 (7/29)</td>
</tr>
<tr>
<td>chronic phase</td>
<td></td>
<td>27.3 (3/11)</td>
<td>25.0 (1/4)</td>
<td>=0.484</td>
<td>26.7 (4/15)</td>
<td>—</td>
<td>—</td>
<td>26.7 (4/15)</td>
</tr>
<tr>
<td>acute phase</td>
<td></td>
<td>66.7 (2/3)</td>
<td>0 (0/1)</td>
<td>=0.500</td>
<td>50.0 (2/4)</td>
<td>—</td>
<td>—</td>
<td>50.0 (2/4)</td>
</tr>
<tr>
<td>blast crisis</td>
<td></td>
<td>0 (0/7)</td>
<td>33.3 (1/3)</td>
<td>=0.300</td>
<td>10.0 (1/10)</td>
<td>—</td>
<td>—</td>
<td>10.0 (1/10)</td>
</tr>
<tr>
<td>AA</td>
<td></td>
<td>18.2 (2/11)</td>
<td>12.5 (1/8)</td>
<td>=0.454</td>
<td>14.3 (2/14)</td>
<td>20.0 (1/5)</td>
<td>=0.470</td>
<td>15.8 (3/19)</td>
</tr>
<tr>
<td>IDA</td>
<td></td>
<td>0 (0/1)</td>
<td>7.1 (1/14)</td>
<td>=0.933</td>
<td>7.1 (1/14)</td>
<td>0 (0/1)</td>
<td>=0.933</td>
<td>6.7 (1/15)</td>
</tr>
</tbody>
</table>

Comparisons of HHV-6 positivity were made between male and female patients, adult and childhood patients, and also between the IDA group and all the other groups. Significantly higher than IDA: * p<0.01; † p=0.024; ‡ p<0.005; § p=0.018. Significance for each pair was derived from the χ² test or Fisher’s exact test.

For each group of 10 samples examined, DNA from HHV-6-infected T-cell line HSB-2 and uninfected HSB-2 cells were used as positive and negative controls, respectively. To determine possible contamination, a control without DNA was also included. For serum samples, additional controls were used: serum DNA extracts from a renal transplant patient from whom HHV-6 had been isolated, and those of a seronegative patient were used as positive and negative control, respectively. Every DNA sample was checked for the presence of PCR inhibitors that might have survived the extraction and precipitation processes by analyzing a separate PCR mix that contained an aliquot of the sample plus 10³ copies of pZVH14.

Platelet preparation

Platelets were prepared from EDTA anticoagulated blood by differential centrifugation, then washed three times with 0.01M PBS containing 10 mM EDTA. Platelets were resuspended at a count of 50,000/µL in 900 µL PBS-Tween (0.05% Tween 20 instead of EDTA) containing leupeptin (100 µg/mL) and solubilized by adding 100 µL 10% Triton-X-100. The solubilized platelets were centrifuged at 5,000g for 10 minutes. One hundred microliters of supernatant were used in the ELISA assay.

Competitive ELISA for PAIg

The competitive ELISA was performed according to the method described by Blumberg et al. with minor modifications. Briefly, microtiter wells (Sigma Chemical Co., St. Louis, MS, USA) were coated overnight at 4°C with 100 µg/mL of purified human IgG (IgM or IgA) (Sigma) at a concentration of 1 µg/mL, then blocked with blocking solution containing 3% BSA. The soluble IgG (IgM or IgA) standard curve dilutions were added to individual wells coated with IgG (IgM or IgA) in triplicate, as were 100 µL of platelet supernatant. One hundred microliters of horseradish peroxidase (HRP)-conjugated affinity-purified antihuman IgG (IgM or IgA) (Sigma) were added to each well and incubated at 37°C for 90 minutes. After four washes with PBS-Tween, 200 µL of substrate (0.015% H₂O₂, 0.4 mg/mL OPD in 0.1 mol/L citrate buffer, pH 5.0) were added to each well, and incubated at 37°C for 20 minutes. After stopping the reaction with 50 µL of 2N H₂SO₄, the color was read on an automatic microtiter plate reader at 492 nm. The IgG (IgM or IgA) content (µg/mL) in each sample could be calculated from the standard curve. Normal range for PAIg: PAIgG: 0–108 ng/10⁷ platelets; PAIgM: 0–40 ng/10⁷ platelets; PAIgA: 0–22 ng/10⁷ platelets.

Serology by IFA

Serum samples obtained from 19 ITP patients were analyzed for anti-HHV-6 IgG according to the method described by Salahuddin et al. Briefly, HHV-6-infected or uninfected HSB-2 cells were deposited on slides, and fixed in cold acetone for 10 minutes. Patients’ sera were added to the slides, incubated at 37°C for 30 minutes, and stained with FITC-conjugated, goat anti-human IgG (Sigma) for 30 minutes. IgG titers ≥ 1:10 were considered positive.

Statistical analysis

HHV-6 DNA positivity for different groups was compared using the chi-squared test or Fisher’s exact test. The significance level was set at p<0.05.

Results

Detection of HHV-6 sequences in BMMC from patients with hematologic diseases and in PBMC from healthy donors

HHV-6 DNA was detected in 79 (32.8%) of 241 hematologic disease samples. The positivity for different disease groups ranged from 6.7% to 41.0% (Table 1, Figure 1). HHV-6 DNA positivity in ITP patients was the highest among all the patient groups and was significantly higher than that for IDA (Table 1). An evaluation of HHV-6 DNA positivity by sex and subtype of patients with ITP showed that HHV-6 positivity in adult patients was significantly higher than in childhood patients (Table 1). When the adult and childhood patients were further divided by subtype, a statistically significant difference was found between adult and childhood patients with chronic disease (Table 1).

Analysis of BMMC from AL patients showed the presence of HHV-6 in 18 (37.5%) of 48 samples. The difference between the positivity in AML and IDA patients was not statistically significant (Table 1). No significant difference of HHV-6 positivity was found between the positivity in AML and IDA patients was statistically significant (Table 1). When the adult and childhood patients were further divided by subtype, a statistically significant difference was found between adult and childhood patients with chronic disease (Table 1).

HHV-6 DNA positivity in MDS, CML and AA patients was higher than that in IDA patients, but the differences were not statistically significant. When types of MDS were evaluated, an increase of HHV-6 DNA positivity with progression of MDS was observed (Table 1). HHV-6 DNA was detected in PBMC from 3 (6.8%) of 44 healthy donors.

Correlation of HHV-6 infection with PAIg production in ITP patients

To determine whether HHV-6 infection is correlated with excessive PAIg production, PAIgG, PAIgM, PAIgA...
HHV-6 in hematologic diseases

Table 2. Comparison of HHV-6 DNA positivity in ITP patients divided according to PAIg level.

<table>
<thead>
<tr>
<th>PAIg level</th>
<th>HHV-6 positivity (%)</th>
<th>PAIgG</th>
<th>PAIgM</th>
<th>PAIgA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>25.8 (8/31)</td>
<td>40.9 (18/44)</td>
<td>46.7 (21/45)</td>
<td></td>
</tr>
<tr>
<td>Abnormal</td>
<td>68.6 (24/35)</td>
<td>63.6 (14/22)</td>
<td>52.4 (11/21)</td>
<td></td>
</tr>
<tr>
<td>p value</td>
<td>&lt;0.005</td>
<td>&gt;0.05</td>
<td>&gt;0.05</td>
<td></td>
</tr>
</tbody>
</table>

The 66 ITP patients were grouped according to normal and abnormal levels of PAIg; comparisons of HHV-6 positivity were made between the normal and abnormal groups. Significance for each pair was derived from χ² test statistic analysis.

and PAIgA were measured in 66 patients with ITP by competitive ELISA. Of these, 35 (53%) had an abnormal PAIgG, 22 (33.3%) an abnormal PAIgM, and 21 (31.8%) an abnormal PAIgA. The abnormal rates of PAIgG, PAIgM and PAIgA in the different ITP groups divided by sex, age and subtype were similar (data not shown).

In these 66 patients, HHV-6 DNA positivity in patients with a normal level of PAIgG was statistically significantly lower than in patients with an abnormal level of PAIgG (Table 2). An evaluation by sex, age and subtype showed significantly higher HHV-6 positivity in female, adult, acute and chronic patients with a normal level of PAIgG (Table 3). An evaluation by sex, age and subtype showed significantly higher HHV-6 positivity in female, adult, acute and chronic patients with a normal level of PAIgG vs patients with an abnormal level of PAIgG. Significance for each pair was derived from the χ² test statistic analysis.

Table 3. Comparisons of HHV-6 DNA positivity for ITP patients with a normal level of PAIgG vs patients with an abnormal level of PAIgG.

<table>
<thead>
<tr>
<th>PAIgG Level</th>
<th>Normal</th>
<th>Abnormal</th>
<th>Male</th>
<th>Female</th>
<th>Adults</th>
<th>Children</th>
<th>Acute</th>
<th>Chronic</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>30.8 (4/13)</td>
<td>75.0 (6/8)</td>
<td>73.9 (17/23)</td>
<td>14.3 (1/7)</td>
<td>29.2 (7/24)</td>
<td>23.5 (4/17)</td>
<td>28.6 (4/14)</td>
<td></td>
</tr>
<tr>
<td>p value</td>
<td>&lt;0.005</td>
<td>&lt;0.005</td>
<td>&lt;0.005</td>
<td>=0.016</td>
<td>=0.015</td>
<td>=0.003</td>
<td>=0.007</td>
<td></td>
</tr>
</tbody>
</table>

The 66 ITP patients were divided into different groups according to sex, age and subtype; comparisons of HHV-6 positivity were made between patients with a normal level of PAIgG vs patients with an abnormal level of PAIgG. Significance for each pair was derived from the χ² test or Fisher’s exact test.

Discussion
To our knowledge, this study is the first description of the distribution of HHV-6 in bone marrow from patients with hematologic diseases. Since normal bone marrow samples were not available and IDA is a benign anemia with defined pathogenesis, HHV-6 positivity in IDA can, to some extent, represent the presence of HHV-6 in BMNC from normal people. Therefore, IDA was used as the control of hematologic diseases. A recent report demonstrated that HHV-6 latently infected bone marrow progenitors in 2 healthy subjects.28 In this study, HHV-6 DNA sequences were detected in 32.8% (79/241) of BMNC from patients with hematologic diseases, with the lowest rate of positivity in patients with IDA (6.7%). These findings suggest that besides infecting peripheral blood, HHV-6 may also persist in some bone marrow cells. In this study, HHV-6 DNA positivity in healthy blood donors was 6.8% (3/44). HHV-6 DNA prevalences in healthy blood donors in different reports are extremely variable. Because it has been suggested that the amount of DNA template used in PCR is an important factor influencing the detection of HHV-6 DNA,29,30 we compared the rate of positivity in this study with rates published in the literature which also used 1 µg DNA as template, and found that they ranged between 17-50%.29,31 Kadakia et al.32 found that some PCR negative samples converted to positive when another primer set was used. Thus the discrepancy of HHV-6 DNA prevalence might be explained by the different primer sets used. The positivity of 17% reported by Luca et al.,31 whose outer primers were the same as ours, was also relatively low in comparison with other reported prevalences. In addition, a possible role of regional variations in the positivity cannot be ruled out.

In the present study, the highest HHV-6 DNA positivity was found in ITP patients (41.0%). HHV-6 positivity in adult patients was significantly higher than that in childhood patients and the positivity in the adult patients was mostly confined to those adults with chronic disease. Since the samples used in this

ic disease, and one was a child with chronic disease. Nine were HHV-6 DNA positive in BMNC, ten were negative. Compared with during the onset phase, two patients had a 2 fold increase in antibody titers during the convalescent phase; six patients had 2 to 4 fold decreases; the titer remained unchanged in 11.
study were mainly bone marrow, and normal bone marrow controls from healthy people especially healthy children are too difficult to obtain, we tried to compare the findings for ITP with the results of other hematologic diseases. Childhood cases of AL, MDS, AA and IDA were available; HHV-6 DNA positivity was not found to be significantly higher in adult patients than in childhood patients in any of the 4 groups, suggesting that the association between HHV-6 and adult ITP patients might be specific. It has long been accepted that the etiology of ITP appears to be an autoimmune destruction of platelets. In order to investigate the association between HHV-6 and production of PAIg, PAIgG, PAIgM and PAIgA were measured in ITP patients. HHV-6 DNA positivity in patients of most groups divided by sex, age and subtype with abnormal levels of PAIgG were significantly higher than those with normal levels of PAIgG, suggesting a potential association between HHV-6 and excessive PAIgG. ITP-associated exanthem subitum and a case of exacerbation of ITP induced by primary HHV-6 infection were reported.\(^{33,34}\) However, a latent infection has been suggested that HHV-6 can be reactivated in immunocompromised hosts.\(^{10,11}\) Patients with acute leukemia are often immunosuppressed. Possibly, the high positivity found in our study reflects reactivation of the virus. Whether reactivated HHV-6 plays any pathogenic role in leukemia patients needs further investigation.

**Contributions and Acknowledgments**

XTM was responsible for the conception of the study, interpretation of the data and drafting the manuscript. XTM, DM and GL carried out the PCRs of all samples. GFM performed the serum antibody tests. LXJ and RCY were responsible for competitive ELISA measurements. YHS and KFW conceived the study, followed all phases of it and revised the paper. All authors contributed to the interpretation of the results. The first and the last authors had the main roles in performing this study, the order of the other authors was decided on the basis of the contributions they gave to the experiments.

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**Disclosures**

Conflict of interest: none.

Redundant publications: no substantial overlapping with previous papers.

**Manuscript processing**

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**Potential Implications for clinical practice**

- This study on the relationship between latent HHV-6 infection and ITP may provide new clues for elucidating the etiology of some cases of idiopathic thrombocytopenic purpura.

**References**


