Involvement of Human Herpesvirus-6 Variant B in Classic Hodgkin’s Lymphoma via DR7 Oncoprotein

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Abstract

Purpose: Hodgkin’s lymphoma (HL) is associated with the presence of EBV in Reed-Sternberg (RS) cells in ~40% of cases. Here, we studied the presence of human herpesvirus type 6 (HHV-6) variant B in RS cells of HL patients and correlated results with clinical parameters. We then examined the implication of HHV-6 DR7B protein in cell deregulation.

Experimental Design: HHV-6 DR7B protein was produced in a Semliki Forest virus system. Polyclonal antibodies were then generated and used for immunochemical HHV-6 localization in HL biopsies. Binding between DR7B and p53 was studied using a double-hybrid system. Transactivation of NFkB was observed after transient transfection using reporter gene assays. We looked for Id2 factor expression after stable transfection of the BJAB cell line by reverse transcription-PCR and Western blot analysis.

Results: HHV-6 was more common in nodular sclerosis subtype HL. and DR7B oncoprotein was detected in RS cells for 73.7% of EBV-negative patients. Colocalization of EBV and HHV-6 was observed in RS cells of doubly infected patients. DR7B protein bound to human p53 protein. p105-p50/p65 mRNA expression and activation of the NFkB complex were increased when DR7B was expressed. Stable expression of DR7B exhibited a strong and uniform expression of Id2. A slightly higher percentage of remission was observed in patients with RS cells testing positive for DR7B than in those testing negative.

Conclusions: Collectively, these data provide evidence for the implication of a novel agent, HHV-6, in cases of nodular sclerosis HL. 

Hodgkin’s lymphoma (HL) is one of the most frequent lymphomas in the Western world. Four subtypes of classic HL (cHL) have been identified: (a) the nodular sclerosis (NS) subtype, which is the most frequent; (b) the mixed cellularity subtype; (c) the lymphocyte-rich subtype; and (d) the lymphocyte-depleted subtype. HL (1) is characterized histologically by the pathognomonic expansion of Hodgkin and Reed-Sternberg (H/RS) cells (2) associated with an inflammatory infiltrate consisting of neutrophilic and eosinophilic granulocytes, histiocytes, T lymphocytes, and plasma cells (3, 4). RS cells are typical binucleated or multinucleated giant CD30+ cells. Interestingly, Bargou et al. (5) have described abundant constitutive nuclear NFkB activity in cultured H/RS cells, this factor being normally observed only for limited time intervals after stimulation with diverse inducers. These findings tended to identify NFkB as an important component for the understanding of HL pathogenesis.

On another hand, RS cells were shown to carry clonally rearranged IgV genes due to somatic mutations (6). The key to this extensive reprogramming was notably attributed to the B-cell–determining transcription factor E2A. This helix-loop-helix transcription factor plays important roles in promoting cell differentiation and in suppressing cell growth. E2A is notably inhibited in RS cells by the deregulated expression of its inhibitor of differentiation and DNA-binding Id2. Indeed, Id2 is strongly and uniformly expressed in RS cells from all cases of cHL (7).

Since its initial description, HL has been considered as an infectious disease (8) on the basis of sociogeographic and epidemiologic data. EBV is a herpesvirus described as linked to 25% to 40% of HL cases. EBV-associated HL is mostly encountered in infants, in elderly people, and in immunocompromised patients. EBV is frequently associated with the mixed cellularity subtype (9). EBV exerts its effects principally through its latent membrane protein-1 (LMP1) expressed in RS cells and is able to transform rodent fibroblasts in vitro and to render them tumorigenic in nude mice (10, 11).
Human herpesvirus type 6 (HHV-6) is a ubiquitous HHV initially isolated from six patients with various lymphoproliferative disorders (12, 13). The biological and immunologic properties and host cell tropism of this principally T lymphotropic virus have led to the identification of two different related variants: HHV-6A and HHV-6B (14). Almost all individuals over the age of 2 years are infected with HHV-6 (15). After primary infection, the virus remains latent in the host peripheral blood mononuclear cells and vascular endothelial cells (16) until reactivation occurs. Interestingly, HHV-6 association with different cancers (17) and angioimmunoblastic T-cell lymphomas was recently shown (18–20). About HL, several serologic studies have reported significantly higher prevalence of anti–HHV-6 lgG and higher anti–HHV-6 titers in patients compared with healthy controls (21–23). HHV-6 DNA sequences have been identified in lymph nodes from patients with HL (24–29), and the B variant was considerably more prevalent than the A variant (30). HHV-6 also shows transforming, transactivating, and oncogenic properties. Indeed, a 3.9-kbp Sall-L fragment was shown to transform human epidermal keratinocytes RHEK-1 and both primary and established rodent cells (31, 32). The transforming activity of the Sall-L fragment was localized to the DR7 gene (33), and cells expressing DR7 protein were tumorigenic when injected into nude mice, whereas cells expressing truncated DR7 protein were not. For the HHV-6 variant A, this oncogenic potential was related notably to the capacity of DR7 to bind and inactivate the human tumor suppressor protein p53 (34).

In this report, we looked for the presence of HHV-6 variant B and for the expression of DR7 protein in RS cells from HL patients’ lymph nodes, and then we studied the respective localization of both oncogenic viruses, HHV-6 and EBV, in doubly infected patients. Our goal was then to analyze the role of the DR7B oncogenic protein in cell deregulation during this lymphoproliferative disorder. Therefore, we tested the ability of DR7B to bind to human p53, to induce NFκB transactivation, and finally to overexpress Id2.

**Materials and Methods**

**Case selection**

Lymph node biopsies from 48 patients treated for cHL at Limoges University Hospital (France) from 1998 to 2003 were retrospectively included in this study. These patients belong to 68 patients previously found positive for HHV-6B DNA in their lymph nodes (≥10 HHV-6 copies/μg), as quantified by real-time PCR, among 86 patients tested (30). For the remaining 20 others, there was no further sample present. The study was approved by the Institutional Review Board. HL was initially diagnosed based on histologic analyses of lymph node biopsies, according to WHO guidelines for neoplastic diseases of lymphoid tissues (35). All patients underwent baseline examinations, and clinical parameters were recorded after diagnosis and during treatment about Ann Arbor stage, relapse, remission, and death in particular.

Among these 48 biopsies, 38 were negative for EBV detection in the classic immunohistochemical LMP1 (CS1-4, Dako) method. These 38 patients had a mean age of 34 years (range, 14-89 years) and a sex ratio of 0.9; 29 were hospitalized in the Clinical Haematology Department and 9 in the Pediatrics Department (≤18 years). All but two adult patients (one with mixed cellularity cHL and the other with lymphocyte-rich cHL) suffered from NS subtype cHL. The 10 other biopsies were HHV-6 and EBV positive. The mean age of these 10 patients was 58 years (range, 8-81 years) and the sex ratio was 2.3; 9 patients were hospitalized in the Department of Clinical Haematology and the last one in the Department of Pedi atrics. All except one patient (mixed cellularity) belonged to the NS subtype cHL.

**Cell lines and virus-infected cell cultures**

The MT4, Molt-3, JJHAN, and BHK-21 cell lines [obtained from the American Type Culture Collection (ATCC)]; the HHV-6- and EBV-negative BJAB B cell line (provided by I. Joab, U542-INSERM Université Paris 11, Paris, France); and the EBV-positive Raji, P3HR1, and Namalwa cell lines [provided by J. Icart, Unité de Physiopathologie Cellulaire et Moléculaire UPR2163 CNRS, Toulouse, France] were all grown in RPMI 1640 (Invitrogen) supplemented with 10% FCS (Invitrogen). HEp-2 cell line (ATCC) was grown in DMEM (Invitrogen) supplemented with 10% FCS. All cell lines were propagated at 37°C with 5% CO2. HHV-6 variant A strain U1102 and HHV-6 variant B strain Z29 (provided by H. Agut, Laboratoire de Virologie, CERVI, Groupe Hospitalier Pitié-Salpêtrière, Paris, France) and HHV-6 variant B strain HST (provided by B.M. Marcille-Imbert, EA4271, Laboratoire de Virologie, CHU, Nantes, France) were cultured in RPMI 1640 supplemented with

**Translational Relevance**

In this study, we showed the presence of human herpesvirus type 6 (HHV-6) and the expression of the viral DR7B oncprotein in Reed-Sternberg (RS) cells from Hodgkin’s lymphoma (HL) patients and particularly from EBV-negative HL. DR7B influenced proliferation by binding to p53 and by increasing the NFκB complex. DR7B expression also induced over-expression of Id2, the inhibitor of differentiation, and DNA binding of the transcription factor E2A. In our mind, these findings are of great interest because, for the first time, another virus other than EBV was shown to be implicated in HL. Interestingly, in this study, HHV-6 was found mostly in patients with nodular sclerosis HL in contrast to EBV. This study can be of consequence for clinicians because a slightly higher percentage of remission was seen in patients harboring DR7B in the RS cells.
10% FCS, 1 IU/ml of anti-IFN serum (Sigma-Aldrich), and 2.5 μg/ml polybrene (Sigma-Aldrich). They were respectively propagated in JJHAN, Molt-3, and MT4 T-cell lines.

**DR7 variant B protein production and antibody generation**

The DR7B gene was amplified using primers DR7-BamF (‘5′-ACCTTAAAGGATCCCGCCAGCAGGAGGCCG-3′) and DR7-BamR (‘5′-GCAGCCGGATCCACTAGTACCGCGCGCCG-3′). The DNA template used (close to the HHV-6 B HST strain) originated from a patient in this study and was selected because its DR7B sequence was representative of that most frequently found in our patients. A poly-His tag was previously added to this template. A Kozak sequence was included in the forward primer to facilitate expression. The amplified product was inserted into the BamHI site of the modified pSFV1 vector, the central element of the Semliki Forest virus (SFV) system carrying the nonstructural viral genes. Sequencing confirmed the presence of the insert in the appropriate orientation. The vector was linearized and transcription was carried out using a strong promoter and SP6 RNA polymerase. BHK-21 cells were transfected by electroporation (350 V, 750 μF) with the purified RNA obtained. RNA directed its own replication in these cells, resulting in the production of large amounts of the protein of interest and inhibition of host cell protein synthesis. Transfected cells were maintained in growth medium for 16 hours to allow for DR7B protein production. An anti-His antibody was used to test for the presence of DR7B protein in cells by immunofluorescence staining and Western blotting. A New Zealand female rabbit was inoculated with the dialyzed protein fraction purified by high-performance liquid chromatography and then diluted with complete Freund’s adjuvant for the first injection or with incomplete Freund’s adjuvant for the second injection 21 days later. Sera were collected from this rabbit before and after immunization and checked by immunofluorescence staining, first on a viral culture of HHV-6B strain HST and second on the HHV-6A strain U1102. It was also checked by Western blotting against a protein extract from a viral culture and against DR7B protein used for immunization. Serum was tested for specificity by testing on mock cell lines (BHK-21, MT4, and JJHAN cell lines). It was also tested against EBV-positive cell lines (Raji, P3HR1, and Namalwa) to verify the absence of labeling.

**Immunohistochemistry**

Anti-DR7B antibodies were incubated with Bouin-fixed, paraffin-embedded samples from the 48 patients. After dewaxing in toluene, these sections were pretreated with Dako buffer and endogenous biotin was blocked (Dako). Immunohistochemistry was then done with the LSAB2 system (Dako). Peroxidase staining was carried out with diaminobenzidine as the substrate, and sections were counterstained with hematoxylin. The same protocol was used for staining with anti-gp116/54/64 antibodies (Advanced Biotechnologies).

For samples testing positive with anti-DR7B antibodies, double immunohistochemical labeling was done with anti-DR7B and monoclonal anti-CD30 antibodies (Serotec). Alexa Fluor 488–conjugated goat anti-rabbit IgG and Alexa Fluor 594–conjugated goat anti-mouse IgG secondary antibodies (Invitrogen) were used, respectively.

For the 10 patients positive for HHV-6 and EBV, double immunohistochemical labeling was done with anti-DR7B and anti-LMP1 antibody. Paraffin sections were treated as previously described and then stained with mouse anti-LMP1 antibody (Dako) followed by Alexa Fluor 594–conjugated anti-mouse IgG as secondary antibody. The second labeling was then done with rabbit DR7B antibody followed by Alexa Fluor 488–conjugated goat anti-rabbit IgG. Observation was made by confocal fluorescent microscopy.

**Two-hybrid system**

Two-hybrid system was carried out using the CheckMate Mammalian Two-Hybrid System (Promega). The cDNA from human p53 (provided by C. Tomasetto, Institute of Genetics and of Molecular and Cellular Biology, Illkirch, France), derived from plasmid php53B (ATCC 57255), was inserted into pBIND mammalian vector after the yeast GAL4 DNA-binding domain sequence to generate a fusion protein. The DR7 gene from HHV-6B strain HST or from HHV-6A strain U1102 was cloned (DR7/EcoRI, 5′-TACGTCAATTCTAGGAGGGGCCCG-3′; DR7/Sall, 5′-CCGCGGCTCACTAATCCAGTACGCCG-3′) into pACT mammalian vector following the herpes simplex virus VP16 activation domain sequence to generate a fusion protein with this sequence. Sequencing checked orientation of each insert. The pGS5luc vector contains GAL4-binding sites upstream from a TATA box, which is upstream from the firefly luciferase reporter gene. Interaction between proteins p53 and DR7 results in the association of a transcriptional activation domain with a DNA-binding domain, which may promote the assembly of RNA polymerase II complexes at the TATA box and increase transcription of the firefly luciferase reporter gene. The pBIND vector, which expresses in addition the Renilla reniformis luciferase under control of the SV40 promoter, was used to normalize the efficiency of transfection and luciferase gene expression.

Briefly, HEp-2 cells cultured in six-well plates at a density of 106 per well during 24 hours at 37°C were transfected at ~70% confluence with 3 μg of each plasmid (pBIND, pACT, and pGS5luc) mixed with 27 μl liposome FuGENE 6 Transfection Reagent (Roche) in serum-free Dulbecco’s medium. Two milliliters of Dulbecco’s medium supplemented with 5% FCS were added 6 hours after beginning transfection. After 24 hours, cells were lysed and the amount of firefly luciferase was quantified using the Dual-Luciferase Reporter Assay System (Promega). Values were normalized to Renilla luciferase activity. For all assays, negative controls (each plasmid alone or each initial vector alone) were done. Each experiment was done in triplicate.

**Transient transfection of HEp-2 cells for NFκB transactivation study**

Fragments corresponding to the promoter region of the p105-p50 and p65 human NFκB gene were amplified from...
human genomic DNA by PCR using the following primers: p50/KpnI, 5′-ACAAAGGTACCGCTGGGTTTTTGTGTGTGG-3′; p50/BglII, 5′-GAGTTAGATCTACCCGGGTGGCGAGTCC-3′; p65/KpnI, 5′-GTTTAGGTACCGGATCCATGCAAGGTTCAATG-3′; and p65/HindIII, 5′-TAATAGAAGCTTCATGGCGGGGTCCCG-3′. After being purified and sequenced, p105-p50 or p65 amplified fragments were cloned into the pGL3 Enhancer Vector (Promega) upstream from the firefly luciferase reporter to generate p50PROMO-pGL3ev and p65PROMO-pGL3ev plasmids. Sequencing checked orientation of each insert. The DR7B gene of HHV-6 was amplified from the HST strain and then cloned directly into the transient mammalian expression vector pCI (Promega) to generate DR7-pCI. It was transfected with p50PROMO-pGL3ev or p65PROMO-pGL3ev in HEp-2 cells using FuGENE 6. pBIND vector was used to normalize transfection efficiency. Negative controls (each plasmid alone, or pCI or pGL3 initial vectors alone) were done, and the assay was done in triplicate.

**Transient transfection of HEp-2 cells for NFκB activity**

pNFκB-luc vector (Clontech) is a reporter vector especially designed for monitoring activation of the NFκB signal transduction pathway. It contains the firefly luciferase gene as a reporter located after multiple copies of the NFκB consensus sequence. After endogenous NFκB proteins bind to the κ enhancer element, transcription is induced and the reporter gene is activated. pTAL-luc vector (Clontech) containing the TATA-like promoter region from the herpesvirus thymidine kinase promoter, but not the NFκB consensus sequence, was used as a negative control to normalize the effect of the minimal HSV1 promoter present in the pNFκB-luc vector. DR7-pCI was cotransfected with pNFκB-luc or pTAL-luc vector into HEp-2 cells using FuGENE 6. Luciferase activity was determined after 24 hours. Negative controls were done and the assay was repeated three times.

**Stable transfection of DR7B gene into Burkitt’s lymphoma B-cell line BJAB**

Amplification of DR7B gene was done from a template originating from a representative patient with the primers DR7/ClaI-S (5′-TGTAGATCGATACATGAGGGCG-3′) and DR7/ClaI-AS (5′-CCGGGGATCGATCTACCCGGG-3′), and the amplified product was cloned into the Clal restriction site of the pTRIN76 vector (36). In this plasmid, elements of the tetracycline regulatable system are in opposite directions and the selection gene (NeoR) is downstream from an IRES sequence. Electroporation of

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**Fig. 1.** RS cells labeled with antibody against DR7B. A, RS cells are labeled with the rabbit anti-DR7B primary antibody and horseradish peroxidase–conjugated goat anti-rabbit IgG+A secondary antibody. Magnification, ×400. B, the same labeling reveals DR7B-positive mummified cells. Magnification, ×1,000. C, a RS cell is labeled with the rabbit anti-DR7B primary antibody and Alexa Fluor 488–conjugated goat anti-rabbit IgG secondary antibody. D, the same cell is labeled by mouse anti-CD30 primary antibody and Alexa Fluor 594–conjugated goat anti-mouse IgG secondary antibody. E, double labeling (DR7B/CD30) of the same cell.
Table 1. Characteristics of the 38 HL patients positive for HHV-6B and negative for EBV: general population, DR7B, and gp116/54/64 labeling

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>General population (N = 38)</th>
<th>DR7B+ in RS cells (n = 28)</th>
<th>DR7B− in RS cells (n = 10)</th>
<th>gp complex+ in RS cells (n = 15)</th>
<th>gp complex− in RS cells (n = 23)</th>
<th>DR7B+ in infiltrating cells (n = 17)</th>
<th>DR7B− in infiltrating cells (n = 21)</th>
</tr>
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<tbody>
<tr>
<td>Mean age ± SD in years</td>
<td>34.0 ± 17.0 (14-89)</td>
<td>35.6 ± 18.1 (14-89)</td>
<td>29.5 ± 13.1 (16-51)</td>
<td>36.1 ± 19.5 (14-89)</td>
<td>32.6 ± 15.5 (15-73)</td>
<td>25.8 ± 12.7 (14-56)</td>
<td>40.6 ± 17.4 (15-89)</td>
</tr>
<tr>
<td>Sex ratio</td>
<td>0.9</td>
<td>1.0</td>
<td>0.7</td>
<td>0.9</td>
<td>0.9</td>
<td>0.9</td>
<td>0.9</td>
</tr>
<tr>
<td>Stages I-II (%)</td>
<td>20/35 (57.1%)</td>
<td>13/25 (52.0%)</td>
<td>7/10 (30.8%)</td>
<td>4/13 (30.8%)</td>
<td>16/22 (72.7%)</td>
<td>9/16 (56.2%)</td>
<td>11/19 (57.9%)</td>
</tr>
<tr>
<td>Stages III-IV (%)</td>
<td>15/35 (42.9%)</td>
<td>12/25 (48.0%)</td>
<td>3/10 (30.0%)</td>
<td>9/13 (69.2%)</td>
<td>6/22 (27.3%)</td>
<td>7/16 (43.7%)</td>
<td>8/19 (52.6%)</td>
</tr>
<tr>
<td>Remission, OS (%)</td>
<td>27/37 (73.0%)</td>
<td>21/27 (77.8%)</td>
<td>6/10 (60.0%)</td>
<td>11/15 (73.3%)</td>
<td>16/22 (72.7%)</td>
<td>12/17 (70.6%)</td>
<td>15/20 (75.0%)</td>
</tr>
<tr>
<td>Median amounts of HHV-6 in copies/μg DNA</td>
<td>164</td>
<td>165</td>
<td>147</td>
<td>181</td>
<td>116</td>
<td>181</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviation: OS, overall survival.

10⁷ cells with 10 μg plasmid DNA (pTRIN76-DR7B or pTRIN76 alone) was carried out at a capacitance of 960 μF and a voltage of 270 V. After 48 hours of incubation, clones with stably integrated pTRIN76 or pTRIN76-DR7B were selected by culturing in the presence of 400 μg/mL G418 (Dako). Inducible DR7B expression was revealed with the same medium supplemented with doxycycline (0 and 1 μg/mL). Medium was changed after 24 hours to preserve doxycycline repression.

Real-time reverse transcription-PCR

DR7B and Id2 expressions were determined by real-time PCR, and ratios were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression. Total RNA was extracted from cultured cells by a single-step guanidium thiocyanate-phenol chloroform method using Trizol reagent (Life Technologies) according to the manufacturer's instructions. Any contaminant DNA was eliminated by the use of DNase I RNase-free (Roche) before reverse transcription (37). After priming with hexanucleotide mix (Roche), first-strand cDNA synthesis was carried out on 200 ng DNase I-treated RNA with Moloney murine leukemia virus reverse transcriptase (Invitrogen). PCRs were then done on 100 ng cDNA with the following primers and Taqman probes: DR7B, 5′-GACCGCCTTACTGTTTCTG-3′ (forward) and 5′-CCGAATACGTCCAATGTCCT-3′ (reverse); Id2, 5′-CCGCAATAGTCATCATTCC-3′ (forward) and 5′-CAGCTCTTCTGACGTAGCA-3′ (reverse); GAPDH, 5′-AAGACAGCTTACATCAGC-3′ (forward) and 5′-GTTGTCCTGGAAGGACTCATG-3′ (reverse); DR7B probe, 5′-FAM-ATACCGCCAGATCTGCGCTCTG-TAMRA-3′; Id2 probe, 5′-FAM-CCAGAGCTTGGTGCTGGGGA-TAMRA-3′; GAPDH probe, 5′-FAM-TGCAACCCAATCTGAGCACT-TAMRA-3′. Each amplified fragment was cloned into the pCR2.1 TOPO plasmid (Invitrogen) using a TA cloning strategy. These constructs provided standard DNAs for calibration of gene copy number. Serial 10-fold dilutions of each recombinant construction were made to prepare stocks containing 1 to 10⁶ copies/μL. Real-time PCR was done with 1× LightCycler FastStart DNA Master HybProbe kit (Roche Diagnostics) in a LightCycler instrument (Roche). Amplification conditions were as follows: for DR7B, 4 mmol/L MgCl₂, 600 mmol/L each primer, and 400 mmol/L probe; for Id2, 4 mmol/L MgCl₂, 750 mmol/L each primer, and 400 mmol/L probe; and for GAPDH, 3 mmol/L MgCl₂, 520 mmol/L each primer, and 100 mmol/L probe. After initial denaturation at 95°C for 10 minutes, 50 cycles with denaturation at 95°C for 10 seconds and annealing at 60°C for 1 minute were carried out. A final cooling step at 40°C for 1 minute was added. Specificity, sensitivity, and reproducibility criteria were checked to validate the method.

Western blotting

Transfected cell pellets were suspended in lysis buffer [0.5% NP40, 0.5 mol/L NaCl, 10 mmol/L Tris-HCl (pH 7.5)] and centrifuged. Supernatant was collected, and after reading at 595 nm according to the Bradford method, total proteins were separated on 15% SDS-polyacrylamide gels and transferred to nitrocellulose membranes (Sigma-Aldrich). Membranes were blotted overnight at 4°C with various primary antibodies after blocking with 5% skim milk. Antibody dilutions were 1:2,560, 1:10, and 1:25,000 for anti-DR7B, anti-Id2 (Invitrogen), or anti-GAPDH (Ambion), respectively. Blots were then revealed with 1:1,000 anti-rabbit IgG-biotin (Sigma) for DR7B and Id2 or with 1:1,000 anti-mouse IgG-biotin (Sigma) for GAPDH. After addition of enhanced chemiluminescence reagents (Amersham Pharmacia Biotech), blots were exposed to X-ray film.
Statistical analysis

Labeling for DR7B or gp116/64/54 in RS cells was compared between patients as a function of sex, stages, and remission using the $\chi^2$ test. The Student's $t$ and Mann-Whitney tests were used to compare labeling as a function of age and HHV-6 quantification, respectively. Multivariate analysis (stepwise descending logistic regression) was carried out on factors such as HHV-6 quantification, age, sex, stages, remission, and immunohistochemical labeling with antibodies against DR7B and/or gp116/64/54 complex to identify prognostic factors. For NFkB, p53, and Id2 studies, differences in gene expression or protein activity were analyzed by paired $t$ test. P values of $\leq 0.05$ were considered as statistically significant.

Results

Detection of HHV-6 and its DR7B oncoprotein in RS cells from EBV-negative lymph node sections

DR7B protein was detected in the cytoplasm of RS cells (Fig. 1A and B) in 28 of 38 (73.7%) HHV-6–positive and LMP1-negative patients tested. All RS cells staining with anti-DR7B antibody were stained with anti-CD30 antibody (Fig. 1C-E). In 17 of these 28 patients (60.7%), DR7B was detected only in RS cells. All characteristics of patients with DR7B-positive or DR7B-negative RS cells are reported in Table 1. A slightly higher mean age and higher stage at diagnosis were found for patients with RS cells testing positive for DR7B compared with those testing negative. Median values for HHV-6 quantification were similar in patients with and without DR7B in their RS cells. Staining intensity was not correlated with any of the parameters studied. The strongest labeling was observed in the only patient with a lymphocyte-rich subtype. Mummified cells (HL cells that display features of nonclassic apoptosis) were positively labeled for DR7B in 9 of 28 cases (32.1%), this staining being more frequent in stages I and II (77.8%) than in stages III and IV (22.2%). These cells were associated with remission in eight of nine cases (88.9%).

In 17 patients (44.7%), DR7B was detected in infiltrating cells (lymphocytes, plasmocytes, polymorphonuclear cells, or histiocites). These patients were significantly ($P = 0.0059$) younger (25.8 ± 12.7 years) than those testing negative (40.6 ± 17.4 years).

About the HHV-6 gp116/64/54 complex, infiltrating cells tested positive in all patients, whereas RS cells tested positive in only 15 patients (39.5%). RS labeling was significantly more frequent ($P = 0.0154$) at stages III and IV (60%) than at stages I and II (20%).

Colocalization of DR7B from HHV-6 and LMP1 from EBV oncoproteins in RS cells

DR7B was detected in RS cells from all nine patients positive for HHV-6 and EBV with the NS subtype of cHL. For six of them, DR7B was present only in RS cells, whereas for the three others, DR7B was also found in infiltrating cells. Double immunohistochemical labeling showed that all RS cells stained by anti-DR7B were also positive for LMP1 (Fig. 2). For the only mixed cellularity subtype cHL, DR7B was detected in infiltrating cells and not in RS cells, which, in contrast, were stained by the anti-LMP1 antibody. Despite the low number of patients, some trends seem to appear. The six patients who presented DR7B labeling that occurred only in RS cells were all males, whereas the sex ratio was 0.33 for the other three patients. Ann Arbor stages at diagnosis were notably higher for these six patients compared with the others (five of six versus one of three patients were at stage III/IV). Finally, long-term remission occurred more frequently when DR7B was expressed in both infiltrating and RS cells (two of three versus three of six patients).

Fig. 2. A, rabbit anti-DR7B primary antibody was revealed with Alexa Fluor 488–conjugated goat anti-rabbit IgG secondary antibody. B, mouse anti-LMP1 primary antibody was revealed with Alexa Fluor 594–conjugated goat anti-mouse IgG secondary antibody. C, double labeling of a RS cell with antibodies against DR7B and LMP1. Images were taken with a Nikon Eclipse microscope at ×600 magnification.
To test for potential interactions between viral proteins DR7 (A or B) and human p53 protein, full-length DR7 (A or B) genes fused to the activation domain of VP16 in pACT vector and full-length p53 cDNA fused to the GAL4-binding site in pBIND vector were transfected into HEp-2 cells at the same time as pG5luc. Overall results obtained for luciferase reporter gene expression 24 hours after transfection of the three plasmids—pBIND, pACT, and pG5luc—are presented in Fig. 3 and revealed significant binding ($P \leq 0.01$) between DR7B and p53 similar to DR7A.

**Upregulation of p105-p50 and p65 promoters by DR7B viral protein**

To detect upregulation of p65 and p105-p50 promoters by DR7B, the expression plasmid DR7-pCI was cotransfected with p105-p50 or p65 promoter constructs. Normalized results reported in Fig. 4 showed a statistically significant increase in luciferase reporter gene activity for both p65 (58.2%, $P = 0.03$) and p105-p50 (43.9%, $P = 0.05$) promoters when DR7B viral protein was expressed.

**Stimulation of NFκB activity by DR7B viral protein**

Because p105-p50 and p65 promoters were regulated by DR7B viral protein expression, we wondered if these proteins could also induce a change in NFκB activity. For this purpose, we cotransfected DR7-pCI with pNFκB-luc or pTAL-luc vector into HEp-2 cells. As reported in Fig. 4, DR7B viral protein expression induced a significant increase ($P = 0.05$) in luciferase reporter gene activation and therefore NFκB activity (90%).

**Overexpression of Id2 in BJAB cells stably transfected by DR7B**

DR7B mRNA was expressed in large amounts ($\sim 30 \times 10^7$ copies/μg RNA) in BJAB cells stably transfected by the DR7B gene cultured in the absence of doxycycline. No expression was detected when these cells were exposed to 1 μg/mL doxycycline (Fig. 5A), nor in untransfected BJAB cells or BJAB cells stably transfected with plasmid alone. Similar findings were observed for DR7B protein expression by Western blot assay (data not shown).

Id2 mRNA was quantified in similar amounts in untransfected BJAB cells, BJAB cells transfected with control plasmid, and DR7B-transfected cells cultured with 1 μg/mL doxycycline. Inversely, in DR7B-transfected cells cultured without doxycycline (overexpression of DR7B), Id2 expression was largely increased ($\sim 270$-fold). Similar results were observed by Western blot, showing higher Id2 protein levels in DR7B stably transfected BJAB cells than in controls, although GAPDH protein levels were similar for each condition (Fig. 5B).

**Discussion**

This report revealed, for the first time, the presence of HHV-6 variant B and the expression of DR7B protein in RS cells from HL patient lymph nodes. In patients dually infected by HHV-6 and EBV, both viruses were localized in the same cells. We provided evidence that DR7B is able to bind to human p53 and induce NFκB transactivation and that it can increase Id2 expression after DR7B stable transfection.

In this preliminary study, conducted on a cohort of heterogeneous cHL patients, HHV-6 was detected in RS cells in 39.5% of LMP1-negative patients tested by the viral anti-gp116/64/54 antibody. To our knowledge, HHV-6 detection in RS cells has been reported only once and in a very small number of cases (38). Here, we tested a large population in which 44.7% were young adults (18-40 years) with NS cHL. A much larger number of patients (73.7%) had RS cells testing positive for DR7B. The CD30 phenotype was identified by double labeling of DR7B-labeled RS cells for each patient, although RS cells were recognizable on the basis of their morphology. Only a few patients had RS cells testing positive for DR7B and gp116/64/54. Discrepancies in the labeling patterns...
obtained with these antibodies did not result from differences in sensitivity. Anti-DR7B antibodies labeled a larger number of RS cells and a smaller number of environmental cells than anti-gp116/64/54 antibodies. gp116/64/54 antibodies detects glycoproteins produced late during the replication cycle and therefore can be used to monitor complete viral replication in infected cells. Inversely, DR7B antibodies label a protein produced early in the viral cycle. Furthermore, the median amounts of HHV-6 DNA were similar, regardless of the labeling pattern. Whereas all patients exhibited gp116/64/54 labeling of at least some infiltrating cells, DR7B protein was observed in infiltrating cells from only 17 patients. These patients were significantly younger than those with no DR7B in infiltrating cells, and labeling was not associated with particular features of HL. HHV-6 DR7B positivity in tumor and bystander cells, or only in one type, seemed to show different mechanisms of pathogenic pathways and different environmental influences on the tumor cells in different age groups of HL. This finding may possibly have some important pathogenic implications.

Interestingly, a large percentage of patients with DR7B-positive RS cells were in remission, although they were older age and had a higher stage—two prognostic factors classically considered as negative. For these patients, treatment, whatever it was, seemed to be more effective than for DR7B-negative patients. Inversely to classic RS cells, mumified cells were largely DR7B positive at early disease stages. These observations (older age and higher stage), not found when gp116/64/54 antibodies were used, led us to suppose that DR7B oncprotein accumulates in RS cells at advanced stages of the disease and in older patients, as if viral replication stopped at an early step.

The accumulation of DR7B in RS cells may be related to cell proliferation by binding to the human tumor suppressor protein p53, preventing its elimination. In cancer, p53 function may be affected in different ways, notably by direct interaction with viral proteins, leading to p53

![Fig. 4. Transactivation and increase in the activity of NFκB when DR7B protein is expressed. A, results obtained for p50 and p65 promoter activation following DR7B (+DR7) viral protein expression. Reactions are compared with similar transfection assays done without viral protein expression (−DR7). The very low amount of luciferase luminescence detected in the different negative controls was used as background. B, comparison of results obtained for NFκB activation with DR7B protein (+DR7) expression or without viral protein expression (−DR7). Columns, mean; bars, SD. *, P ≤ 0.05.](image-url)
inactivation (39). Human papillomavirus 16 and 18 oncoproteins E6, SV40 oncoprotein large T antigen, and adenovirus E1B 55-kDa protein have all been shown to interact with p53 protein, inhibiting its control of cell cycle arrest after DNA damage. The finding that DR7B was able to bind to human p53 suggests that this interaction may induce or participate in lymphomagenesis, considering the fact that HHV-6 is able to retain p53 within the cytoplasm, thus protecting infected cells from apoptosis (40, 41). This leads to p53 accumulation and upregulation in the presence of HHV-6 (42).

We explored nine NS cHL patients positive for both LMP1 oncoprotein of EBV and DR7B; double immunohistochemical labeling showed that all DR7B-positive RS cells were also positive for LMP1. Both viruses could interact, either directly or more probably indirectly particularly through p53 (40) and/or NFκB pathway involvement (43). It was previously suggested that HHV-6 can activate EBV replication and may contribute to the pathogenesis of EBV-associated diseases (44). For the only mixed cellularity HL patient who had DR7B in infiltrating cells and not in RS cells as opposed to LMP1, the mechanism is certainly different.

Constitutive activation of NFκB complex seems to be a common feature in H/RS cells from HL (5), thus involving resistance to cell death and enhancing proliferation. An obvious mechanism through which NFκB can promote tumorigenesis is by inducing expression of antiapoptotic genes. Other mechanisms also exist: The cyclin D1 promoter, for example, was identified as a target of NFκB (45), promoting G1-to-S phase progression in the cell cycle and resulting in cyclin D1 expression. NFκB can also stimulate c-Myc promoter activity. Different mechanisms are likely to be implicated in NFκB constitutive activation. It has been shown that expression of a single viral protein such as Tax from human T-cell lymphotropic virus type 1, HBx from hepatitis B virus, Rta from HHV-8, and EBNA-2 and LMP1 from EBV (46, 47) can activate NFκB. All these proteins have transactivating or signal-inducing functions on their own. Most of these viruses either can be carcinogenic themselves or are associated with tumor development. In this study, we showed that DR7 protein from HHV-6B is able to transactivate p105-p50 and p65 NFκB subunits in HEP-2 cells and to activate the NFκB complex. Therefore, HHV-6B is able to induce or reinforce NFκB constitutive transactivation in H/RS cells.

Because DR7B protein was found to be expressed in RS cells of cHL and because the virus was implicated in lymphomagenesis pathways (p53 and NFκB), it seemed interesting to examine the modifications conferred by DR7B expression in B-cell progenitors of RS cells. It was therefore important to construct a model, and a Burkitt’s lymphoma cell line (BJAB) negative for EBV and HHV-6 was chosen. The pTRIN76 plasmid allowed us to stably transfect the BJAB cell line and to modulate DR7B expression by the use of doxycycline concentrations. Id2 factor, an inhibitor of E2A transcription factor, has been reported to be involved in reprogramming of the B-cell phenotype of RS cells and consecutive proliferation of these cells (48). In the present study, stable expression of the DR7B protein caused overexpression of Id2. This interesting result can give rise to different hypotheses about DR7B action on cell signaling pathway deregulation such as the transforming growth factor-β signaling pathway (49). In fact, RS cells possess both transforming growth factor-β ligand and receptor, so autocrine signaling could contribute to aberrant Id2 expression. Furthermore, Id2 upregulation probably results from AP-1 deregulation (50).

In summary, our results revealed the presence and accumulation of HHV-6B in RS cells of patients suffering from HL. The DR7B binding to p53 and the ability of DR7B to

![Fig. 5. Increased expression of Id2 when DR7B is expressed.](Image)

**Fig. 5.** Increased expression of Id2 when DR7B is expressed. A, Id2 mRNAs are overexpressed in BJAB cells when DR7B mRNAs are produced as quantified by Taqman technology. BJAB cells were stably transfected with DR7B gene and cultured without doxycycline (DR7 0) or in the presence of 1 μg/mL doxycycline for 48 h (DR7 1). A cellular control (BJAB untransfected cells) and a control vector (BJAB cells stably transfected by the pTRIN76 plasmid alone) were shown. The values are expressed relative to that of GAPDH mRNAs. Columns, mean of three separate assays; bars, SD. B, upregulation of Id2 protein expression is observed in DR7B stably transfected BJAB cells cultured without doxycycline (DR7 0) by Western blot. Id2 protein is weakly detected in the diverse controls (BJAB, pTRIN76, and DR7 1). **, P ≤ 0.01.
transactivate NF-κB and to overexpress Id2 are different arguments in favor of an implication of HHV-6 in NS cHL. Finally, in this study, the detection of DR7B in the RS cells of EBV-negative cHL patients may be considered as a surrogate marker for the identification of patients with a high probability of remission.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**References**

Involvement of Human Herpesvirus-6 Variant B in Classic Hodgkin's Lymphoma via DR7 Oncoprotein

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