Possible role of human herpesvirus 8 in the lymphoproliferative disorders in common variable immunodeficiency

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Patients who have common variable immunodeficiency (CVID) and granulomatous/lymphocytic interstitial lung disease (GLILD) are at high risk for early mortality and B cell lymphomas. Infection with human herpes virus type 8 (HHV8), a B cell lymphotrophic virus, is linked to lymphoproliferative disorders in people who have secondary immunodeficiencies. Therefore, we determined the prevalence of HHV8 infection in CVID patients with GLILD. Genomic DNA isolated from peripheral blood mononuclear cells was screened by nested- and real time-quantitative PCR (QRT–PCR) for the presence of HHV8 genome. It was positive in 6/9 CVID patients with GLILD (CVID–GLILD), 1/21 CVID patients without GLILD (CVID–control), and no patients receiving intravenous gamma globulin (n = 13) or normal blood donors (n = 20). Immunohistochemistry (IHC) demonstrated expression of the latency-associated nuclear antigen-1 (LANA-1) in the biopsies of the lung, liver, and bone marrow of four patients with CVID–GLILD. One CVID–GLILD patient developed a B cell lymphoma during the course of the study. QRT–PCR demonstrated high copy number of HHV8 genome and IHC showed diffuse staining for LANA-1 in the malignant lymph node. HHV8 infection may be an important factor in the pathogenesis of the interstitial lung disease and lymphoproliferative disorders in patients with CVID.
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The following groups of people were recruited prospectively for participation in the study (Table I): normal blood donors (n = 20); patients receiving intravenous gamma globulin but without CVID (n = 13); CVID patients with GLILD (CVID-GLILD n = 9); and CVID patients without GLILD (CVID-control: n = 21). All patients with CVID-GLILD underwent an open lung biopsy that demonstrated one or more of the following histologic patterns: granulomatous disease, lymphocytic interstitial pneumonitis, lymphoid hyperplasia, or follicular bronchiolitis. These different histologic patterns frequently are present concomitantly in the individual lung biopsies of CVID patients (2), which suggests that these are histologic variations of the same disease. Therefore, we have collectively termed these histologic patterns as GLILD. Lymphocyte enumeration studies demonstrated that patients with CVID-GLILD exhibited a >40% reduction in the numbers of CD4+ T cells compared with CVID-control patients (P < 0.03; Table II). There was no difference in the numbers of CD8+ T cells or B cells between the two groups.

Most patients with CVID-GLILD are infected with HHV8 as determined by nested PCR and QRT-PCR

Genomic DNA isolated from PBMCs was used as the template to screen for HHV8 sequences by nested PCR from all participants in the study (Fig. 1). Presumptive evidence of HHV8 infection required that the PCR generated amplicons from ORF 26 and ORFK9. The prevalence of HHV8 infection among the CVID-GLILD patients was markedly higher than among the CVID-control patients (6/9 versus 1/21; P < 0.0009; Fig. 1). No normal blood donors (n = 20; Fig. S1, available at http://www.jem.org/cgi/content/full/jem.20050381/DC1) or patients receiving intravenous gamma globulin for other disorders (n = 13) were positive for HHV8 infection by nested PCR. The nested PCRs for HHV8 ORF 26 and ORFK9 were performed on three separate occasions for each subject, and the results (negative or positive for HHV8 amplicons) were consistent. At no time did we observe HHV8 amplicons in our negative control or in patient samples that initially tested negative. DNA sequencing of the ORF26 amplicon was performed in five patients. Analysis of the DNA sequences at strain-variable positions established that all patients were infected with the A strain of HHV8 (1086-C, Table I.

Table I. Study population

| Group             | Gender | Age mean ± SEM |
|-------------------|--------|----------------|
| CVID-control      | 14/7   | 47.2 ± 3.5     |
| CVID-GLILD        | 4/5    | 42.1 ± 4.4     |
| XLA               | 0/4    | 36.5 ± 4.0     |
| Other hypogammaglobulinemia | 2/4 | 50.4 ± 7.5 |
| SCID              | 1/0    | 19.0           |
| WAS               | 0/1    | 27.0           |
| HIM               | 0/1    | 64.0           |
| NBD               | 8/12   | 33 ± 2.8       |

HIM, hyper IgM syndrome; NBD, normal blood donor; SCID, severe combined immunodeficiency; WAS, Wiskott-Aldrich syndrome; XLA, X-linked agammaglobulinemia.

Table II. Lymphocyte enumeration in CVID patients

| Variables | CVID-GLILD | CVID-control | p-value |
|-----------|------------|--------------|---------|
| n         | 9          | 21           |         |
| CD3+      | 825.9 ± 173.2 | 1,136.0 ± 95.5 | 0.1254 |
| CD3+/CD4+ | 381.4 ± 66.4 | 653.1 ± 61.4 | 0.0319 |
| CD3+/CD8+ | 429.2 ± 136.3 | 475.5 ± 68.2 | 0.2995 |
| CD19      | 143.3 ± 51.4 | 198.9 ± 35.7 | 0.2887 |

Figure 1. Detection of HHV8 genomes by nested PCR from PBMCs. Dotted lines separate patient/subject groups. The cohorts of patients included: “Normal” (normal blood donors), “IVIG” (patients receiving intravenous immunoglobulin for disorders other than CVID), “CVID-control” (CVID patients without GLILD), and “CVID-GLILD” (CVID patients with GLILD). ● (lane 12) is BCBL-1, an HHV8-infected B cell lymphoma cell line (positive control; lane 1) is an H2O template negative control. ORF26 and ORFK9 indicate HHV8 open reading frames 26 and K9, respectively. B-actin indicates PCR of patient DNA using primers specific for B-actin. HHV8 amplicons were detected from DNA by nested PCR (PBMC DNA) or nonnested PCR (BCBL-1 DNA [lane 12]). White lines indicate that intervening lanes have been spliced out.
This is the most prevalent strain in the United States and accounts for 60% of HHV8 infections (5). Results using real time-quantitative PCR (QRT-PCR) confirmed the results obtained by nested PCR, and demonstrated variable copy numbers of HHV8 genome in the DNA derived from PBMCs (Table III). QRT-PCR was consistently negative for HHV8 infection on subjects that tested negative for HHV8 infection by nested PCR (unpublished data).

Detection of LANA-1 by IHC in tissues from patients with CVID–GLILD

Using formalin-fixed, paraffin-embedded tissue biopsies (lung, liver, lymph nodes, bone marrow, small intestine), we performed immunohistochemistry (IHC) to detect the LANA-1 in the available tissue biopsies of five patients with CVID–GLILD (Fig. 2; Table III). LANA-1 was chosen because it is expressed in the proliferative lesions that are known to be associated with HHV8 infection (4). Three patients demonstrated evidence of HHV8 infection by IHC (Table III). HHV8 infection was detected in CVID–GLILD patients by IHC in multiple tissues, including lung, liver, lymph node, and bone marrow. QRT-PCR confirmed the presence of HHV8 genome in all tissues that were positive for LANA-1 by IHC.

One CVID–GLILD patient (patient 42), died from granulomatous hepatitis just before the initiation of this study. IHC for LANA-1 of this patient’s liver biopsy demonstrated that many of the hepatocytes were infected with HHV8 (Fig. 2 G). Insufficient liver tissue precluded an analysis of this liver biopsy by QRT-PCR. Liver biopsies from three other patients in the prospective CVID–GLILD cohort of patients also demonstrated granulomatous hepatitis. These biopsies were positive by nested and QRT-PCR for HHV8 genome, but negative for LANA-1 by IHC; this reflected the relative insensitivity of IHC to detect evidence of HHV8 infection (Table III).

During the course of the study, B cell lymphomas occurred in 22% (2 of 9) of CVID–GLILD patients (B cell NHL in patient 11 and B cell lymphoma of mucosal-associated lymphoid tissue [MALT] in patient 53). In patient 11, several lines of evidence suggested that HHV8 infection contributed to the development of the lymphoma. HHV8 infection of the malignant lymph node was demonstrated by diffuse staining for LANA-1 by IHC (Fig. 2 C) and a conspicuously high copy number of HHV8 genome by QRT-PCR (>15,000 copies/μg DNA; Table III). Multiple tissues were infected by

Table III. HHV8 copy number in HHV8-infected patients

| Patient no. | Tissue     | Diagnosis         | Mean HHV8 copy no. ± SD (95% confidence interval) | IHC (number/μg DNA) |
|------------|------------|-------------------|-------------------------------------------------|---------------------|
| 23         | PBMC       | CVID-control      | 42.0 ± 20.6 (21.8–62.2) | NA                  |
| 10         | PBMC       | CVID–GLILD        | 43.5 ± 16.5 (27.3–59.7) | NA                  |
| 11         | PBMC       | CVID–GLILD        | 12.3 ± 8.7 (3.8–20.7) | NA                  |
| 16         | PBMC       | CVID–GLILD        | 16.8 ± 5.7 (11.2–22.3) | NA                  |
| 27         | PBMC       | CVID–GLILD        | 19.7 ± 9.2 (10.7–28.7) | NA                  |
| 29         | PBMC       | CVID–GLILD        | 28.8 ± 12.7 (16.3–41.2) | NA                  |
| NA         | lung       | HIV-1, KS         | 255 ± 141 (116–393) | +                   |
| 10         | small intestine | CVID–GLILD    | 633 ± 501 (142–1,123) | –                   |
| 10         | colon      | CVID–GLILD        | 286 ± 117 (171–401) | –                   |
| 10         | bone marrow | CVID–GLILD      | 414 ± 119 (297–531) | +                   |
| 10         | liver      | CVID–GLILD        | 375 ± 264 (117–634) | –                   |
| 10         | lung       | CVID–GLILD        | 405 ± 203 (206–604) | +                   |
| 11         | liver      | CVID–GLILD        | 434 ± 11 (423–445) | –                   |
| 11         | lung       | CVID–GLILD        | 17 ± 7 (10–24) | +                   |
| 11         | lymph node | CVID–GLILD (NHL)  | 15,356 ± 1,100 (14,279–16,434) | +        |
| 16         | lung       | CVID–GLILD        | 5 ± 3 (1.7–7.6) | –                   |
| 29         | lung       | CVID–GLILD        | 65 ± 41 (25–106) | –                   |
| 51         | lung       | CVID–GLILD        | 48 ± 22 (26–70) | –                   |
| 51         | lymph node | CVID–GLILD       | 19 ± 12 (7–32) | –                   |
| 51         | liver      | CVID–GLILD        | 87 ± 73 (16–159) | –                   |

*a* Patients with infection in PBMCs and tissues.

*b* Kaposi’s sarcoma (positive control).

NA, not applicable.
HHV8 as determined by QRT-PCR and IHC (lymph node, lung) or QRT-PCR alone (liver). The malignant lymph node was negative for EBV infection as determined by in situ hybridization for EBV-encoded small RNAs. Finally, the GLILD and granulomatous hepatitis preceded the development of the NHL in this patient by ≥3 yr. Evidence for a role of HHV8 infection in the development of the MALT (patient 53) was equivocal. Examination of the malignant tissue from the MALT lymphoma was positive for HHV8 infection as determined by nested PCR, but not QRT-PCR or IHC (Fig. 2 H). The genomic DNA from the PBMCs of patient 53 also was negative for HHV8 infection by nested PCR (Fig. 1). Because of the ambiguous results in patient 53, we did not score this patient as infected with HHV8 in the final analysis. The MALT lymphoma also was negative for EBV-encoded small RNAs by in situ hybridization.

With the advent of high-dose intravenous gamma globulin therapy, noninfectious complications of CVID are an increasing cause of morbidity and mortality in this group of patients (6). In particular, B cell lymphomas and progressive interstitial lung disease are the major causes of death in this population (1, 2, 7). CVID patients with GLILD exhibit lymphocytic infiltration of the lung parenchyma, splenomegaly, diffuse lymphadenopathy, CD4 lymphopenia (2, 8), and have evidence of HHV8 infection in several organ systems (Fig. 2; Table III). We hypothesize that GLILD represents the pulmonary component of more generalized lymphoproliferative disease that is promulgated by infection with HHV8. In addition to the lymphoproliferative disease, our data suggest that the granulomatus hepatitis, which is found frequently in CVID-GLILD patients (2, 8), may be caused by HHV8 infection.

The finding that HHV8 is able to infect cells in several different organ systems in patients with CVID-GLILD is in agreement with recent observations on the host range of HHV8. Bechtel et al. (9) demonstrated that HHV8 is able to infect an assortment of human and nonhuman cell lines of epithelial, endothelial, and mesenchymal origin. Additionally, we recently reported the presence of HHV8 in a variety of cell types in the plexiform lesions of patients with primary pulmonary hypertension (10). In total, these observations suggest that the host range for HHV8 infection may be broader than previously believed.

Earlier studies indicate that serology is more sensitive than nested PCR, using genomic DNA derived from PBMC in detecting HHV8 infection. However, because patients with CVID are unable to make specific antibodies and receive pooled human IgG as therapy, serologic studies cannot be performed in this population. The sensitivity of nested PCR, using genomic DNA derived from PBMCs varies from <50% in patients with KS to >80% in multicentric Castleman’s disease, an aggressive lymphoproliferative disorder (11, 12). Patients with CVID-GLILD have generalized lymphadenopathy with HHV8 infection in several organ systems. Therefore, it is not surprising that the prevalence (67%) of HHV8 infection as determined by nested PCR, using genomic DNA from PBMCs in the CVID-GLILD cohort is closer to Castleman’s disease than KS. The absence of

Figure 2. Detection of LANA-1 by IHC in tissue of CVID-GLILD patients. (A) IHC for LANA-1 of lung tissue from patient 11 with lymphocytic interstitial pneumonitis; 200×. The interstitium is expanded by a population of lymphocytes. Many of the cells express LANA-1 protein by IHC (brown staining). The inset (1,000×) highlights the punctate nuclear staining that characterizes the presence LANA-1. (B) Same patient’s lung coimmunostained for CD3 (T cell marker, brown) and CD20 (B cell marker, red). The mononuclear cells of the interstitial infiltrates are composed of a mixed infiltrate of B and T lymphocytes; 1,000×. (C) Same patient’s cervical lymph node. The biopsy was diagnosed as NHL. The IHC demonstrates abundant LANA-1–positive cells (brown); 1,000×. (D) CD3 and CD20 coimmunostain of this patient’s lymph node biopsy. Most of the cells are red, indicating a predominance of B cells in this lymphoma. Occasional T lymphocytes are present (brown); 200×. The inset highlights the predominance of B cells; 1,000×. (E) A bone marrow biopsy from patient 10 demonstrates ~50% of cells within the bone marrow are positive for LANA-1; 400× and 1,000×. (F) Transbronchial biopsy (patient 10) demonstrating patchy positivity by LANA-1 IHC, primarily within the lymphocytes of the bronchial wall, 200×. The inset demonstrates that ~50% of the cells within this lymphoid aggregate are positive for LANA-1; 1,000×. (G) Liver biopsy from a patient diagnosed with granulomatous hepatitis (patient 42). There are scattered hepatocytes positive for LANA-1; 200×. The inset shows positive nuclear staining in a hepatocyte nucleus; 1,000×. (H) Lung sample from a patient 58 diagnosed with a MALT-type lymphoma. The lung tissue was negative for LANA-1 by IHC, but positive by PCR; 200×.
HHV8 infection in groups other than the patients with CVID is consistent with epidemiologic studies in the United States that demonstrated a low prevalence of HHV8 infection (0.1–3%) in HIV-1 uninfected blood donors (13).

By QRT-PCR, the HHV8 viral load in the DNA from PBMCs from CVID-GLILD patients ranged between 12 and 46 copies per μg of DNA. Assuming 10^6 cells yields ~3 μg DNA, there were between 36 and 138 copies of HHV8 genome per 10^6 cells; this is similar to the HHV8 copy numbers observed in PBMCs of patients with KS and multicentric Castleman’s disease (14, 15). The copy number of HHV8 genome in the DNA from the tissue biopsies from patients with CVID-GLILD were similar to that observed in the tissues of other HHV8-induced diseases (16).

The high prevalence of HHV8 infection in the CVID-GLILD cohort raises questions as to the source of the virus and mode of infection. Intravenous immunoglobulin (IVIG) is one possible source of transmission of HHV8. However, the low prevalence (4.8%) of HHV8 infection in patients with CVID, but without GLILD, and the absence of HHV8 infection in patients receiving IVIG for reasons other than CVID argues against this possibility. One HHV8-positive patient with CVID-GLILD (subject 29) first received IVIG 3 yr before entry in the study. Sterilization processes of IVIG over the last decade inactivate enveloped viruses, such as HHV8 (17, 18). It is clear that further epidemiologic studies are required to define the source and modes of transmission for HHV8 infection in patients with CVID.

There are several possible reasons why HHV8 may be an opportunistic pathogen in patients with CVID. The inability to make specific antibodies along with the CD4 lymphopenia, which was a prominent feature in patients with CVID-GLILD, likely are important. However, we speculate that the overproduction of inflammatory cytokines also may be involved. Subgroups of patients with CVID overproduce inflammatory cytokines, such as IL-6 and TNF-α. This dysregulation of cytokine production has been attributed to polymorphisms in the respective promoters of these cytokine genes (19). The overproduction of TNF-α, which occurs in the subgroup of CVID patients with splenomegaly, granulomatous lung disease, and CD4 lymphopenia, has been hypothesized to contribute to the formation and maintenance of granulomas in these patients. We have not determined serum levels of TNF-α in our cohort of CVID-GLILD patients, but multisystemic granulomatous disease, CD4 lymphopenia, and splenomegaly are prominent features in our CVID-GLILD cohort of patients (reference 2; Table I).

Levels of inflammatory cytokines, such as IL-6 and TNF-α, are also elevated in HHV8-induced malignancies (20). IL-6 enhances the replication of HHV8 and TNF-α and IL-6 may play an important role in the promotion of HHV8-induced malignancies (20). Polymorphisms within the promoter of the IL-6 gene have been linked to an increased propensity to develop KS in people infected with HIV-1 and patients who have undergone renal transplants (21, 22). Therefore, we hypothesize that the granulomatous and lymphoproliferative disease that is seen in the CVID-GLILD cohort of patients, is due to the unique interplay of HHV8 infection, cellular and humoral immunodeficiency, and polymorphisms within the promoters of inflammatory cytokine genes (e.g., TNF-α and IL-6). Studies are in progress to address this possibility.

It is possible that other viruses, in addition to HHV8, are involved in the pathogenesis of GLILD and lymphoproliferative disorders in CVID. For example, EBV and CMV are opportunist pathogens that have been implicated as a cause of idiopathic pulmonary fibrosis (23, 24). EBV is found in a small number of B cell lymphomas in patients with CVID, including in one patient with an HHV8-negative, primary effusion lymphoma (25). Prospective studies that examine larger numbers of patients need to be performed to determine the role of these and other transformation-competent viruses in the etiology of GLILD and lymphomas that occur in the context of CVID.

In summary, our results suggest that HHV8 infection may underlie the poor prognosis, diffuse interstitial lung disease, and increased prevalence of lymphoproliferative disorders in patients with CVID. These observations raise the possibility that HHV8 infection may be more prevalent than previously believed, especially in people with primary immunodeficiencies. Lymphoproliferative disorders of unclear etiology are increased in other primary immunodeficiencies; this points out the importance of systematically studying the prevalence of HHV8 infection in these disorders as well.

**MATERIALS AND METHODS**

**Subjects.** This study was approved by the Institutional Review Board at National Jewish Medical and Research Center (Denver, CO). All subjects signed informed consent before participation in the study. Patients with CVID or receiving IVIG for reasons other than CVID were recruited from the Adult or Pediatric Immunodeficiency Clinic at the National Jewish Medical and Research Center. Normal blood donors were recruited from healthy employees at National Jewish Medical and Research Center. The criteria used for the diagnosis of CVID was as described previously (2). Patients with CVID were screened for HIV-1, hepatitis B virus, and HCV infection by PCR, and were negative. Lymphocyte subsets from peripheral blood of patients with CVID were enumerated by FACS using the following CD antigens: total T cells (CD3+), T helper cells (CD3+/CD4+), T suppressor/cytotoxic cells (CD3+CD8+), and B cells (CD19+). Lymphocyte numbers were expressed as cells per cubic milliliter and compared with established control values.

**Nested and QRT-PCR and DNA sequencing.** Genomic DNA from PBMC was extracted using the PAXgene Blood DNA Validation Kit per the manufacturer’s instructions (QIAGEN). Genomic DNA from tissue was isolated from formalin-fixed, paraffin-embedded tissues as described previously (26). For detection of HHV8 genome by nested PCR, primer sets were designed to amplify two highly conserved open reading frames: ORF26 (minor capsid antigen) and ORFK9 (viral interferon regulatory factor 1; reference 27). The HHV8-ORF26 amplicon was sequenced using the ABI Prism 3100 Genetic Analyzer (Applied Biosystems). QRT-PCR was performed as described previously (28). Input DNA concentrations were normalized to human β-actin using TaqMan β-Actin Control Reagents kit per the manufacturer’s instructions (Applied Biosystems).

**IHC.** IHC on tissue biopsies was performed using a monoclonal antibody specific for HHV8–LANA-1 (Advanced Biotechnologies Inc.) as previously described (10).
**Statistical analysis.** Fisher’s exact test was used to compare dichotomous variables between the CVID-GILD patients and CVID-control patients. For quantitative variables, the Wilcoxon rank-sum test was used to compare the two CVID groups. A two-tailed P value of less than 0.05 is considered statistically significant.

**Online supplemental material.** Fig. S1 shows additional nested PCRs for HHV8 using genomic DNA isolated from PBMCs on 10 additional normal blood donors. Fig. S2 shows additional positive (KS) and negative (lung, liver, lymph node, bone marrow) IHC for LANA-1. Precautions that were used to prevent contamination of genomic DNA obtained from patients, and a complete description of the histopathologic studies of the malignant lymph node obtained from patient 11 are included in online supplemental Materials and methods. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20050381/DC1.

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