

test was used to compare positive rates of gene expression. A regression analysis was used to compare the expression of each gene and the EBV DNA load. *P*-values <0.05 were deemed to be statistically significant.

ACKNOWLEDGEMENTS

We thank the following people for their contributions to this study: Kayoko Matsunaga (Fujita Health University); Masaki Ito, Atsushi Kikuta, and Mitsuaki Hosoya (Fukushima Medical University); Tomohiro Kinoshita (Nagoya University); Kazuhiko Shirota (National Hospital Organization Shizuoka Medical Center); Mariko Seishima (Ogaki Municipal Hospital); Masashi Shiomi (Osaka City General Hospital); Hajime Katsumata and Yasuo Horikoshi (Shizuoka Children's Hospital); Tsuyoshi Ito (Toyohashi City Hospital); and Sachiyo Kamimura (University of Miyazaki). This study was supported in part by a grant from the Ministry of Education, Culture, Sports, Science and Technology, Japan (19591247).

REFERENCES

- Arrand, J. R. & Rymo, L. (1982). Characterization of the major Epstein-Barr virus-specific RNA in Burkitt lymphoma-derived cells. *J Virol* **41**, 376-389.
- Bell, A. I., Groves, K., Kelly, G. L., Croom-Carter, D., Hui, E., Chan, A. T. & Rickinson, A. B. (2006). Analysis of Epstein-Barr virus latent gene expression in endemic Burkitt's lymphoma and nasopharyngeal carcinoma tumour cells by using quantitative real-time PCR assays. *J Gen Virol* **87**, 2885-2890.
- Bollard, C. M., Aguilar, L., Straathof, K. C., Gahn, B., Huls, M. H., Rousseau, A., Sixbey, J., Gresik, M. V., Carrum, G. & other authors (2004). Cytotoxic T lymphocyte therapy for Epstein-Barr virus⁺ Hodgkin's disease. *J Exp Med* **200**, 1623-1633.
- Bollard, C. M., Gottschalk, S., Leen, A. M., Weiss, H., Straathof, K. C., Carrum, G., Khalil, M., Wu, M. F., Huls, M. H. & other authors (2007). Complete responses of relapsed lymphoma following genetic modification of tumor-antigen presenting cells and T-lymphocyte transfer. *Blood* **110**, 2838-2845.
- Brooks, L., Yao, Q. Y., Rickinson, A. B. & Young, L. S. (1992). Epstein-Barr virus latent gene transcription in nasopharyngeal carcinoma cells: coexpression of EBNA1, LMP1, and LMP2 transcripts. *J Virol* **66**, 2689-2697.
- Cohen, J. I. (2000). Epstein-Barr virus infection. *N Engl J Med* **343**, 481-492.
- Deacon, E. M., Pallesen, G., Niedobitek, G., Crocker, J., Brooks, L., Rickinson, A. B. & Young, L. S. (1993). Epstein-Barr virus and Hodgkin's disease: transcriptional analysis of virus latency in the malignant cells. *J Exp Med* **177**, 339-349.
- Fujii, N., Takenaka, K., Hiraki, A., Maeda, Y., Ikeda, K., Shinagawa, K., Ashiba, A., Munemasa, M., Sunami, K. & other authors (2000). Allogeneic peripheral blood stem cell transplantation for the treatment of chronic active Epstein-Barr virus infection. *Bone Marrow Transplant* **26**, 805-808.
- Gotoh, K., Ito, Y., Shibata-Watanabe, Y., Kawada, J., Takahashi, Y., Yagasaki, H., Kojima, S., Nishiyama, Y. & Kimura, H. (2008). Clinical and virological characteristics of 15 patients with chronic active Epstein-Barr virus infection treated with hematopoietic stem cell transplantation. *Clin Infect Dis* **46**, 1525-1534.
- Hagihara, M., Tsuchiya, T., Hyodo, O., Ueda, Y., Tazume, K., Masui, A., Kanemura, A., Yoshida, F., Takashimizu, S. & other authors (2003). Clinical effects of infusing anti-Epstein-Barr virus (EBV)-specific cytotoxic T-lymphocytes into patients with severe chronic active EBV infection. *Int J Hematol* **78**, 62-68.
- Heslop, H. E., Ng, C. Y., Li, C., Smith, C. A., Loftin, S. K., Krance, R. A., Brenner, M. K. & Rooney, C. M. (1996). Long-term restoration of immunity against Epstein-Barr virus infection by adoptive transfer of gene-modified virus-specific T lymphocytes. *Nat Med* **2**, 551-555.
- Hislop, A. D., Taylor, G. S., Sauce, D. & Rickinson, A. B. (2007). Cellular responses to viral infection in humans: lessons from Epstein-Barr virus. *Annu Rev Immunol* **25**, 587-617.
- Kanegane, H., Nomura, K., Miyawaki, T. & Tosato, G. (2002). Biological aspects of Epstein-Barr virus (EBV)-infected lymphocytes in chronic active EBV infection and associated malignancies. *Crit Rev Oncol Hematol* **44**, 239-249.
- Kieff, E. D. & Rickinson, A. V. (2007). Epstein-Barr virus and its replication. In *Fields Virology*, pp. 2603-2654. Edited by D. M. Knipe & P. M. Howley. Philadelphia: Lippincott Williams & Wilkins.
- Kimura, H. (2006). Pathogenesis of chronic active Epstein-Barr virus infection: is this an infectious disease, lymphoproliferative disorder, or immunodeficiency? *Rev Med Virol* **16**, 251-261.
- Kimura, H., Morita, M., Yabuta, Y., Kuzushima, K., Kato, K., Kojima, S., Matsuyama, T. & Morishima, T. (1999). Quantitative analysis of Epstein-Barr virus load by using a real-time PCR assay. *J Clin Microbiol* **37**, 132-136.
- Kimura, H., Hoshino, Y., Kanegane, H., Tsuge, I., Okamura, T., Kawa, K. & Morishima, T. (2001). Clinical and virologic characteristics of chronic active Epstein-Barr virus infection. *Blood* **98**, 280-286.
- Kimura, H., Morishima, T., Kanegane, H., Ohga, S., Hoshino, Y., Maeda, A., Imai, S., Okano, M., Morio, T. & other authors (2003). Prognostic factors for chronic active Epstein-Barr virus infection. *J Infect Dis* **187**, 527-533.
- Kimura, H., Hoshino, Y., Hara, S., Sugaya, N., Kawada, J., Shibata, Y., Kojima, S., Nagasaka, T., Kuzushima, K. & Morishima, T. (2005). Differences between T cell-type and natural killer cell-type chronic active Epstein-Barr virus infection. *J Infect Dis* **191**, 531-539.
- Kubota, N., Wada, K., Ito, Y., Shimoyama, Y., Nakamura, S., Nishiyama, Y. & Kimura, H. (2008). One-step multiplex real-time PCR assay to analyse the latency patterns of Epstein-Barr virus infection. *J Virol Methods* **147**, 26-36.
- Kuzushima, K., Yamamoto, M., Kimura, H., Ando, Y., Kudo, T., Tsuge, I. & Morishima, T. (1996). Establishment of anti-Epstein-Barr virus (EBV) cellular immunity by adoptive transfer of virus-specific cytotoxic T lymphocytes from an HLA-matched sibling to a patient with severe chronic active EBV infection. *Clin Exp Immunol* **103**, 192-198.
- Kwon, J. M., Park, Y. H., Kang, J. H., Kim, K., Ko, Y. H., Ryou, B. Y., Lee, S. S., Lee, S. I., Koo, H. B. & Kim, W. S. (2006). The effect of Epstein-Barr virus status on clinical outcome in Hodgkin's lymphoma. *Ann Hematol* **85**, 463-468.
- Leenman, E. E., Panzer-Grumayer, R. E., Fischer, S., Leitch, H. A., Horsman, D. E., Lion, T., Gadner, H., Ambros, P. F. & Lestou, V. S. (2004). Rapid determination of Epstein-Barr virus latent or lytic infection in single human cells using *in situ* hybridization. *Mod Pathol* **17**, 1564-1572.
- Okamura, T., Hattakawa, Y., Arai, H., Inoue, M. & Kawa, K. (2000). Blood stem-cell transplantation for chronic active Epstein-Barr virus with lymphoproliferation. *Lancet* **356**, 223-224.
- Okano, M., Kawa, K., Kimura, H., Yachie, A., Wakiguchi, H., Maeda, A., Imai, S., Ohga, S., Kanegane, H. & other authors (2005). Treatment guidelines for diagnosing chronic active Epstein-Barr virus infection. *Ann J Hematol* **80**, 64-69.
- Patel, K., Whelan, P. J., Prescott, S., Brownhill, S. C., Johnston, C. F., Selby, P. J. & Burchill, S. A. (2004). The use of real-time reverse

transcription-PCR for prostate-specific antigen mRNA to discriminate between blood samples from healthy volunteers and from patients with metastatic prostate cancer. *Clin Cancer Res* 10, 7511–7519.

Quintanilla-Martinez, L., Kumar, S., Fend, F., Reyes, E., Teruya-Feldstein, J., Kingma, D. W., Sorbara, L., Raffeld, M., Straus, S. E. & Jaffe, E. S. (2000). Fulminant EBV(+) T-cell lymphoproliferative disorder following acute/chronic EBV infection: a distinct clinicopathologic syndrome. *Blood* 96, 443–451.

Rickinson, A. B. & Kieff, E. (2007). Epstein–Barr virus. In *Fields Virology*, pp. 2655–2700. Edited by D. M. Knipe & P. M. Howley. Philadelphia: Lippincott Williams & Wilkins.

Rooney, C. M., Smith, C. A., Ng, C. Y., Loffin, S. K., Sixbey, J. W., Gan, Y., Srivastava, D. K., Bowman, L. C., Krance, R. A. & other authors (1998). Infusion of cytotoxic T cells for the prevention and treatment of Epstein–Barr virus-induced lymphoma in allogeneic transplant recipients. *Blood* 92, 1549–1555.

Saridakis, V., Sheng, Y., Sarkari, F., Holowaty, M. N., Shire, K., Nguyen, T., Zhang, R. G., Liao, J., Lee, W. & other authors (2005). Structure of the p53 binding domain of HAUSP/USP7 bound to Epstein–Barr nuclear antigen 1 implicates for EBV-mediated immortalization. *Mol Cell* 18, 25–36.

Savoldo, B., Huls, M. H., Liu, Z., Okamura, T., Volk, H. D., Reinke, P., Sabat, R., Babel, N., Jones, J. F. & other authors (2002). Autologous Epstein–Barr virus (EBV)-specific cytotoxic T cells for the treatment of persistent active EBV infection. *Blood* 100, 4059–4066.

Straathof, K. C., Leen, A. M., Buza, E. L., Taylor, G., Huls, M. H., Heslop, H. E., Rooney, C. M. & Bollard, C. M. (2005). Characterization of latent membrane protein 2 specificity in CTL lines from patients with EBV-positive nasopharyngeal carcinoma and lymphoma. *J Immunol* 175, 4137–4147.

Straus, S. E. (1988). The chronic mononucleosis syndrome. *J Infect Dis* 157, 405–412.

Sugaya, N., Kimura, H., Hara, S., Hoshino, Y., Kojima, S., Morishima, T., Tsurumi, T. & Kuzushima, K. (2004). Quantitative analysis of Epstein–Barr virus (EBV)-specific CD8⁺ T cells in patients with chronic active EBV infection. *J Infect Dis* 190, 985–988.

Taketani, T., Kikuchi, A., Inatomi, J., Hanada, R., Kawaguchi, H., Ida, K., Oh-Ishi, T., Arai, T., Kishimoto, H. & Yamamoto, K. (2002).

Chronic active Epstein–Barr virus infection (CAEBV) successfully treated with allogeneic peripheral blood stem cell transplantation. *Bone Marrow Transplant* 29, 531–533.

Tao, Q., Robertson, K. D., Manns, A., Hildesheim, A. & Ambinder, R. F. (1998). Epstein–Barr virus (EBV) in endemic Burkitt's lymphoma: molecular analysis of primary tumor tissue. *Blood* 91, 1373–1381.

Thorley-Lawson, D. A. & Gross, A. (2004). Persistence of the Epstein–Barr virus and the origins of associated lymphomas. *N Engl J Med* 350, 1328–1337.

Tosato, G., Straus, S., Henle, W., Pike, S. E. & Blaese, R. M. (1985). Characteristic T cell dysfunction in patients with chronic active Epstein–Barr virus infection (chronic infectious mononucleosis). *J Immunol* 134, 3082–3088.

Tsang, N. M., Chuang, C. C., Tseng, C. K., Hao, S. P., Kuo, T. T., Lin, C. Y. & Pai, P. C. (2003). Presence of the latent membrane protein 1 gene in nasopharyngeal swabs from patients with mucosal recurrent nasopharyngeal carcinoma. *Cancer* 98, 2385–2392.

Tsuge, I., Morishima, T., Morita, M., Kimura, H., Kuzushima, K. & Matsuoka, H. (1999). Characterization of Epstein–Barr virus (EBV)-infected natural killer (NK) cell proliferation in patients with severe mosquito allergy; establishment of an IL-2-dependent NK-like cell line. *Clin Exp Immunol* 115, 385–392.

Weinberger, B., Plentz, A., Weinberger, K. M., Hahn, J., Holler, E. & Jilg, W. (2004). Quantitation of Epstein–Barr virus mRNA using reverse transcription and real-time PCR. *J Med Virol* 74, 612–618.

Williams, H. & Crawford, D. H. (2006). Epstein–Barr virus: the impact of scientific advances on clinical practice. *Blood* 107, 862–869.

Young, L., Alfieri, C., Hennessy, K., Evans, H., O'Hara, C., Anderson, K. C., Ritz, J., Shapiro, R. S., Rickinson, A. & other authors (1989). Expression of Epstein–Barr virus transformation-associated genes in tissues of patients with EBV lymphoproliferative disease. *N Engl J Med* 321, 1080–1085.

Zhang, Y., Nagata, H., Ikeuchi, T., Mukai, H., Oyoshi, M. K., Demachi, A., Morio, T., Wakiguchi, H., Kimura, N. & other authors (2003). Common cytological and cytogenetic features of Epstein–Barr virus (EBV)-positive natural killer (NK) cells and cell lines derived from patients with nasal T/NK-cell lymphomas, chronic active EBV infection and hydroa vacciniforme-like eruptions. *Br J Haematol* 121, 805–814.

blood

2010 116: 4546-4559
Prepublished online Aug 10, 2010;
doi:10.1182/blood-2010-05-285452

Detection of EBV genomes in plasmablasts/plasma cells and non-B cells in the blood of most patients with EBV lymphoproliferative disorders by using Immuno-FISH

Sara Calattini, Irini Sereti, Philip Scheinberg, Hiroshi Kimura, Richard W. Childs and Jeffrey I. Cohen

Updated information and services can be found at:
<http://bloodjournal.hematologylibrary.org/cgi/content/full/116/22/4546>

Articles on similar topics may be found in the following *Blood* collections:
Lymphoid Neoplasia (640 articles)

Information about reproducing this article in parts or in its entirety may be found online at:
http://bloodjournal.hematologylibrary.org/misc/rights.dtl#repub_requests

Information about ordering reprints may be found online at:
<http://bloodjournal.hematologylibrary.org/misc/rights.dtl#reprints>

Information about subscriptions and ASH membership may be found online at:
<http://bloodjournal.hematologylibrary.org/subscriptions/index.dtl>

Blood (print ISSN 0006-4971, online ISSN 1528-0020), is published weekly by the American Society of Hematology, 2021 L St, NW, Suite 900, Washington DC 20036.
Copyright 2011 by The American Society of Hematology; all rights reserved.



Detection of EBV genomes in plasmablasts/plasma cells and non-B cells in the blood of most patients with EBV lymphoproliferative disorders by using Immuno-FISH

Sara Calattini,¹ Irini Sereti,² Philip Scheinberg,³ Hiroshi Kimura,⁴ Richard W. Childs,³ and Jeffrey I. Cohen¹

¹Medical Virology Section, Laboratory of Infectious Diseases, ²Laboratory of Immunoregulation, National Institute of Allergy and Infectious Diseases, and ³Hematology Branch, National Heart Lung and Blood Institute, National Institutes of Health, Bethesda, MD; and ⁴Department of Virology, Nagoya University Graduate School of Medicine, Nagoya, Japan

Epstein-Barr virus (EBV) is present in B cells in the blood of healthy people; few studies have looked for EBV in other cell types in blood from patients with lymphoproliferative disorders. We use a new technique combining immunofluorescent cell-surface staining and fluorescent *in situ* hybridization to quantify both EBV copy number per cell and cell types in blood from patients with high EBV DNA loads. In addition to CD20⁺

B cells, EBV was present in plasmablast/plasma cells in the blood of 50% of patients, in monocytes or T cells in a small proportion of patients, and in "non-B, non-T, non-monocytes" in 69% of patients. The mean EBV copy number in B cells was significantly higher than in plasmablast/plasma cells. There was no correlation between EBV load and virus copy number per cell. Although we detected CD21, the EBV B-cell receptor,

on EBV-infected B cells, we could not detect it on virus-infected T cells. These findings expand the range of cell types infected in the blood. Determining the number of EBV genomes per cell and the type of cells infected in patients with high EBV loads may provide additional prognostic information for the development of EBV lymphoproliferative diseases. (*Blood*. 2010;116(22):4546-4559)

Introduction

Epstein-Barr virus (EBV) infects more than 90% of the human population.¹ In immunocompetent hosts, the virus is latent in B cells of the peripheral blood and is not associated with disease.²⁻⁴ However, in immunocompromised patients, immune surveillance to the virus is often impaired, a larger number of B cells are infected with EBV, and the virus can contribute to lymphoproliferative disease. Approximately 1%-20% of transplant recipients can develop posttransplantation lymphoproliferative disease (PTLD) during the first year after transplantation, and approximately 90% of these cases are EBV positive.⁵ Persons with AIDS have a 60-fold increased risk of developing lymphoma, compared with the general population, and virtually all Hodgkin and non-Hodgkin lymphomas that occur in the late stages of HIV infection are EBV positive.⁶

Although EBV establishes a latent infection in peripheral blood B cells of healthy people, less is known about the phenotype of virus-infected cells in the blood of immunocompromised persons with high EBV DNA loads. Most studies have focused on the phenotype of virus-infected B cells in transplant recipients.^{2,7-11} However, EBV can infect cells other than B cells, including T cells, natural killer (NK) cells, monocytes, and pre-Langerhans cells.¹²⁻¹⁶

Several techniques have been developed to detect EBV in cells. *In situ* hybridization using a probe that detects the EBV-encoded RNAs (EBERs) is considered the best test for localizing latent EBV in tissue samples.¹⁷ Combined staining for EBERs and antibodies to cell-surface markers for tissues on microscope slides, or for peripheral blood by flow cytometry,¹⁸ has been used to determine the phenotype of the EBV-infected cells.

Although detection of EBERs indicates that cells are infected with EBV, this test cannot provide an estimate of the number of EBV genomes present per cell.

We describe a new technique (Immuno-FISH) that combines immunofluorescent staining for surface proteins (using antibodies directly conjugated to fluorochromes) and fluorescent *in situ* hybridization for EBV DNA. This technique allows the simultaneous determination of the cell type infected by EBV and quantification of EBV copy number in the infected cell. We show that EBV is present not only in B cells, but also in a large percentage of other cell types in the peripheral blood of patients with high EBV DNA loads. In addition, we correlate the number of EBV genomes per cell with the phenotype of the infected cells.

Methods

Study participants

Patients had blood drawn after informed consent was obtained in accordance with the Declaration of Helsinki under protocols approved by the Institutional Review Boards of the National Institute of Allergy and Infectious Diseases, the National Cancer Institute, the National Heart, Lung, and Blood Institute (patients 1-23), Nagoya University Hospital (patients 24-27), or the University of Maryland and the National Institute of Allergy and Infectious Diseases (patients 28-29). For patients from the United States, we selected those whose EBV DNA loads were more than 5000 copies per million cells (normal is < 200 copies/million cells) and, for patients from Japan, more than 50 000 copies per μ g of DNA.

Submitted May 14, 2010; accepted July 25, 2010. Prepublished online as *Blood* First Edition paper, August 10, 2010; DOI 10.1182/blood-2010-05-285452.

The online version of this article contains a data supplement.

The publication costs of this article were defrayed in part by page charge payment. Therefore, and solely to indicate this fact, this article is hereby marked "advertisement" in accordance with 18 USC section 1734.

Measurement of EBV DNA in blood

For patients 1-23 and 28-29, the EBV DNA load data were reported as the number of EBV genomes per 10^6 cells. Peripheral blood mononuclear cells (PBMCs) were lysed and EBV quantitative real-time polymerase chain reaction (qPCR) was performed as previously described¹⁹ (see supplemental data, available on the *Blood* Web site; see the Supplemental Materials link at the top of the online article).

Immuno-FISH procedure

Cryopreserved PBMCs were thawed at 37°C, washed once in media and once in phosphate-buffered saline (PBS), and then resuspended in a solution of 0.2N acetic acid, 0.02N HCl, in Tris HCl 0.1M (adjusted to pH 3.7) for 15 minutes at room temperature. The cells were washed twice in PBS and incubated with fluorochrome-conjugated monoclonal antibodies (to stain cell-surface proteins) in suspension for 30 minutes at 4°C. Cells were washed 2× with PBS and applied to Superfrost Plus microscope slides (Thermo Fisher Scientific) by cytospin centrifugation at 500g for 5 minutes.

The cells were then fixed with 5mM BS₃ (Thermo Fisher Scientific) for 30 minutes at room temperature and permeabilized with Triton X-100 (Sigma-Aldrich) at 0.5% in PBS for 10 minutes at room temperature. The slides were washed with PBS and incubated at 37°C for 1 hour in a solution of 2× saline-sodium citrate (SSC) containing 10 U/mL RNase T1 (Roche Applied Science) and 15 U/mL RNase A (Sigma-Aldrich). The slides were then washed once in 0.1M Tris-HCl (pH 7.5), incubated for 5 minutes in a solution of 0.1M Tris-HCl (pH 7.5)/0.1M glycine, and then washed in 2× SSC.

FISH was performed with a biotinylated EBV BioProbe (Enzo Life Sciences) specific for the BamHI W region of EBV. There are 5-12 BamHI W repeats per EBV genome, depending on the virus strain.²⁰ The probe was diluted to a final concentration of 0.25 ng/μL in hybridization buffer (Enzo Life Sciences) and denatured at 94°C for 10 minutes. The slides were then incubated with the denatured probe at 85°C for 6 minutes under a coverslip sealed with rubber cement and then hybridized overnight at 37°C. Slides were washed 2× for 15 minutes in a solution of 0.1× SSC, 0.1% Tween, and 1% BSA at 42°C, washed once in 0.1× SSC, and washed 3 times in Tris-HCl (0.1M; pH 7.5). Slides were incubated with streptavidin-conjugated Alexa 488 or streptavidin-conjugated Alexa 594 for 15 minutes at a final concentration of 1 μg/mL, washed in PBS/Tween 0.1% for 5 minutes, washed 3× in Tris-HCl (0.1M; pH 7.5), and mounted with Fluoromount-G medium (Southern-Biotech). The slides were visualized with a Leica SP5 confocal microscope (Carl Zeiss), with a 63×/1.4 numerical aperture magnification objective. For each field, photographs of serial z-stack sections (0.5 μm thick) were obtained throughout the cells and then overlapped to quantify the total number of fluorescent spots (each spot corresponds to one EBV genome copy) in the cells.

When FISH alone was performed, the cells were incubated for 15 minutes with a solution of 0.2N acetic acid, 0.02N HCl, in Tris HCl (0.1M; adjusted to pH 3.7), then applied to the slides, fixed with BS₃, permeabilized with Triton X-100, and FISH was performed as described above.

Cell-surface markers, fluorochromes, and EBV antibodies

After incubation with acetic acid and HCl, cells were resuspended in PBS at a concentration of 1×10^6 cells per 100 μL and stained with mouse anti-human monoclonal antibodies to label cell-surface markers.

For the sorting procedure, the CD19 phycoerythrin (PE)-Cy7 antibody was used to identify B cells. The CD3 allophycocyanin (APC)-H7 and the CD56 APC antibodies were used to identify the T and NK subpopulations, respectively. All of these antibodies were purchased from BD Biosciences.

For each patient sample, cells were stained with different combinations of monoclonal antibodies. The first combination contained antibodies that recognize CD3 T cells (Alexa 594), B cells (Alexa 647), and monocytes (V450); the second combination included antibodies that recognize CD3 T cells (Alexa 594), NK cells (Alexa 647), and monocytes (V450), and the third combination contained antibodies that recognize the B-cell surface

marker, CD20 (Alexa 488), together with the κ and λ light chains of immunoglobulin (Ig; Alexa 647). Staining with antibodies that recognize the κ and λ chains was performed after permeabilization of the cells to allow staining of intracytoplasmic immunoglobulin. The biotinylated FISH EBV probe was detected using streptavidin-conjugated Alexa 488 for the first and second combinations, and with streptavidin-conjugated Alexa 594 for the third combination.

For samples in which EBV was detected in CD3 T cells, another aliquot of cells was stained with Alexa 647-conjugated anti-CD4 antibody and Alexa 488-conjugated anti-CD8 antibody, and the FISH probe was detected using streptavidin-conjugated Alexa 594.

To detect lytic replication of EBV, PBMCs were stained with a combination of antibodies for B and T cells (CD3 Alexa 594 and CD19/CD20 Alexa 647), applied to Superfrost Plus microscope slides, and fixed and permeabilized as previously described. Cells were then incubated for 30 minutes with BZLF1 monoclonal antibody (Meridian Life Science) conjugated with Alexa 488. Slides were then washed 3× in PBS and mounted with Fluoromount-G medium. B95-8 and BJAB cells were stained with BZLF1 antibody as positive and negative controls for virus lytic replication, respectively.

Results

FISH and Immuno-FISH assays are sensitive and specific

To determine the sensitivity and specificity of our FISH assay, we diluted EBV-positive X50-7 cells with EBV-negative BJAB cells and performed FISH. A mixture of 10% of X50-7 cells and 90% BJAB cells yielded 9.6% (11/114) EBV-positive cells by FISH, whereas a mixture of 1% X50-7 cells and 99% BJAB cells showed 1.1% (8/745) EBV-positive cells by FISH (representative example in supplemental Figure S1A-B). As controls, one slide of 100% EBV-positive X50-7 cells and one of 100% EBV-negative BJAB cells were also stained by FISH, and EBV episomes were detected in all of the EBV-positive cells and none of the EBV-negative cells (supplemental Figure 1C-D). As an additional negative control, Immuno-FISH staining of cells from patients with very low or undetectable EBV viral loads by PCR did not detect any FISH-positive cells (data not shown). Combining Immuno-FISH with DAPI (4',6'-diamidino-2-phenylindole) staining, we found EBV DNA only in the nucleus of the cells (data not shown). These results indicate that the FISH procedure is specific, and that it can detect at least 1% of EBV-positive cells in a mixture of EBV-negative cells.

We also quantified the number of EBV genomes per cell using the FISH procedure. EBV-positive cells were analyzed by confocal microscopy; serial z-stack sections were imaged every 0.5 μm, overlapped, and the number of fluorescent spots, each corresponding to a single EBV episome, was counted. Analysis of 65 individual X-50-7 cells from 5 separate experiments showed a median of 7 EBV genomes per cell, with a mean of 9.4 genomes per cell (range, 2-18 genomes per cell). These results are similar to those previously reported, indicating that X50-7 cells contain approximately 5 EBV genomes per cell.²¹ To confirm the sensitivity of the FISH assay, we tested the Namalwa cell line, which contains 2 copies of EBV integrated in each cellular genome.²² Analysis of 411 cells in 5 separate experiments showed a median of 1 EBV genome per cell and a mean of 0.76 genomes per cell (range, 0-4 copies per cell) (supplemental Figure 1E). This value is slightly lower than the expected number and could be a result of the reduced accessibility of the probe to hybridize to the target when integrated in genomic DNA. Moreover, the 2 copies of EBV are integrated in tandem in the cellular genome of Namalwa cells.²³

Table 1. Phenotype of EBV-infected PBMCs from subjects

Patient	Diagnosis	EBV DNA load/10 ⁶ cells	Percent of EBV-positive cells (no. positive/total no. cells)				
			B cells	Plasmablast/ plasma cells	Non-B, non-T, nonmonocyte cells	T cells	Monocytes
1	HIV	12 000	75 (3/4)	ND	25 (1/4)	0	0
2	HIV	4300	89 (8/9)	0	11 (1/9)	0	0
3	HIV	18 000	91 (10/11)	0	9 (1/11)	0	0
4	HIV	130,000	71 (12/17)	67 (6/9)	29 (5/17)	0	0
5	HIV	9400	25 (1/4)	0	25 (1/4)	25 (1/4)	25 (1/4)
6	HIV	5100	100 (11/11)	0	0	0	0
7	HIV	22 000	67 (10/15)	50 (4/8)	20 (3/15)	0	13 (2/15)
8	HIV	6300	82 (9/11)	17 (1/6)	18 (2/11)	0	0
9	Tx	NA	100 (4/4)	0	0	0	0
10	Tx	30 000	92 (12/13)	0	8 (1/13)	0	0
11	Tx	6200	83 (5/6)	ND	17 (1/6)	0	0
12	LyGHL	170 000	64 (49/77)	0	6 (5/77)	27 (21/77)	3 (2/77)
13	Severe primary EBV infection	280 000	90 (30/33)	0	10 (3/33)	0	0
14	PTLD	1 300 000	55 (11/20)	25 (2/8)	45 (9/20)	0	0
15	ATL EBV-LPD	190 000	79 (19/24)	21 (3/14)	21 (5/24)	0	0
16	LyG	28 000	79 (15/19)	14 (1/7)	21 (4/19)	0	0
17	ICL	110 000	97 (28/29)	0	3 (1/29)	0	0
18	AA	64 000	80 (8/10)	ND	20 (2/10)	0	0
19	AA	110 000	89 (17/19)	20 (1/5)	0	0	5 (1/19)
20	AA	6 500 000	52 (24/46)	37 (6/16)	48 (22/46)	0	0
21	B-cell CAEBV	15 000	73 (11/15)	20 (1/5)	27 (4/15)	0	0
22	B-cell CAEBV	30 900	97 (29/30)	0	3 (1/30)	0	0
23	B-cell CAEBV	310 000	80 (12/15)	25 (2/8)	20 (3/15)	0	0
24	T-cell CAEBV	54 666/μg	0	ND	0	100 (88/88)	0
25	T-cell CAEBV	67 612/μg	0	ND	0	100 (21/21)	0
26	T-cell CAEBV	230 000/μg	0	ND	0	100 (78/78)	0
27	T-cell CAEBV	4154/μg	0	ND	0	100 (18/18)	0
28	IM	1700	83 (10/12)	17 (1/6)	0	0	0
29	IM	10 651	100 (11/11)	0	0	0	0

For some patients, the percentages for each subpopulation total to > 100%, because the same cell marker (eg, CD20 for B cells) was used in more than one combination (eg, CD20 with CD3 and CD14 in one combination, CD20 with kappa/lambda in another combination). Patients with HIV infection had CD4 T-cell counts < 400/μL and no EBV-positive lymphomas. Patient 21 received rituximab therapy 1 month before blood was drawn; patients 10, 11, 18, 19, and 20 received anti-thymocyte globulin 5 days to 1 month before blood was drawn (patient 18 received rabbit anti-thymocyte globulin, and patients 10, 11, 18, 19, and 20 received horse anti-thymocyte globulin).

ND indicates not done; Tx, allogeneic hematopoietic stem cell transplant recipient; LyGHL, lymphomatous/granulomatous/Hodgkin lymphoma; ATL EBV-LPD, adult T-cell leukemia and EBV-lymphoproliferative disease; ICL, idiopathic CD4 lymphopenia; AA, aplastic anemia receiving antithymocyte globulin; and IM, EBV infectious mononucleosis.

and we may not have been able to resolve the 2 genomes using our Immuno-FISH procedure.

To determine whether the immunofluorescent staining component of Immuno-FISH was sensitive and specific when followed by FISH, we isolated fresh PBMCs and sorted B cells (CD19⁺), NK cells (CD56⁺/CD3⁻) and T cells (CD3⁺) by flow cytometry. We stained aliquots of each of the 4 subpopulations with Alexa-conjugated antibodies specific for each subpopulation (eg, T cells with anti-CD3 antibody) and with antibodies that should not recognize the subpopulation (eg, T cells with anti-CD19/anti-CD20). The purity of the staining was more than 97% in all of the combinations tested (supplemental Table 1).

EBV is detected in circulating B cells

Immuno-FISH was used to characterize the phenotype of the cells infected by EBV from 29 patients with elevated EBV DNA levels in the blood (Table 1). We defined B cells as cells recognized by a combination of anti-CD19 and anti-CD20 antibodies. EBV was detected in B lymphocytes from all 29 patients tested (Figure 1; Table 1), except for the 4 patients with T-cell chronic active EBV (CAEBV) from Japan. In the other 25 patients, B cells represented the majority of cells containing EBV, except for one patient (patient 5) with HIV infection. The median number of EBV genomes in B cells for all the other patients was 13.9 per cell, with a mean of 14.2 per cell (range, 1-40) (Table 2; Figure 2).

EBV is detected in circulating plasmablasts/plasma cells

Although EBV has been detected in latently infected plasmablastic lymphomas²⁴ and plasmacytomas in immunocompromised patients with HIV,²⁵ EBV has not been reported in circulating plasmablasts or plasma cells. Plasmablasts and plasma cells have a prominent cytoplasm containing abundant κ or λ light-chain Ig and lack CD20 on their surface. To determine whether EBV is present in circulating plasmablasts/plasma cells, Immuno-FISH was performed using PBMCs stained with a combination of antibodies to κ and λ chains of Ig and CD20. Surprisingly, we detected EBV in plasmablast/plasma cells in 50% (11 of 22) of the patients tested (Figure 3A; Table 1). Nearly all of the patient groups had some patients with EBV-positive plasmablast/plasma cells. The median number of EBV genomes in plasmablast/plasma cells was 8.3 copies per cell, with a mean of 9.2 copies per cell (range, 3-21), which was lower than the median (13.9) or mean (14.2) number of EBV genomes in B cells (Table 2; Figure 2).

EBV is detected in circulating T cells in some patients, and in CD3⁺, CD4⁻, and CD8⁻ T cells in Japanese patients with T-cell CAEBV

EBV has been reported in circulating T cells from patients with HIV infection in 1 study based on EBV PCR of sorted T cells²⁶ and in patients with T-cell CAEBV.¹² Excluding the patients with T-cell

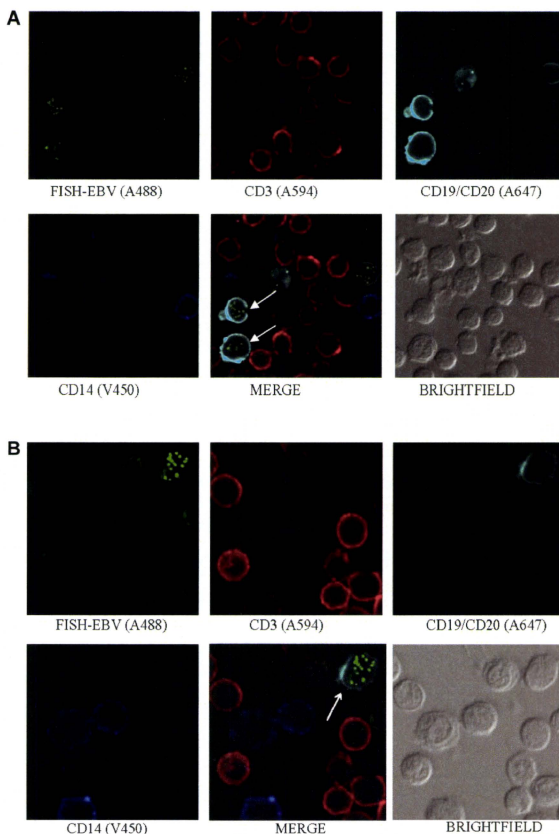


Figure 1. EBV is detected in B cells. Photomicrographs of PBMCs stained by Immuno-FISH from patients 17 (A) and 8 (B) show EBV in the nucleus of B cells (arrows). EBV DNA was identified with the biotinylated FISH probe and streptavidin-conjugated Alexa 488, B cells using a combination of Alexa 647-conjugated anti-CD19 and anti-CD20 antibodies (Biollegend), T cells with Alexa 594-conjugated anti-CD3 antibody (Invitrogen), and monocytes with V450-conjugated anti-CD14 antibody (BD Biosciences). Arrows indicate EBV-positive cells.

CAEBV, we detected EBV in T (CD3⁺) cells from 2 patients: 1 with HIV (patient 5) and 1 with Hodgkin lymphoma (patient 12) (Figure 3B; Table 1). Interestingly, in these patients, the EBV-positive T cells represented more than 25% of their EBV-positive cells. These cells contained a median of 7.85 genomes per cell, with a mean of 9 genomes per cell (range, 2-40). Sufficient cells were available from the patient with Hodgkin lymphoma to perform additional staining of CD4 and CD8 T cells; EBV was only detected in CD4⁺ T cells, each of which had more than 30 EBV genomes per cell (data not shown). EBV was not detected in T cells from the 7 other patients with HIV.

The EBV receptor in B cells is CD21,²⁷ whereas in T cells, the virus receptor has not been identified. Although EBV was detected in CD21⁺ B cells from patient 12 (Figure 4A), none of

the EBV-positive, CD3⁺ cells were also positive for CD21 (Figure 4B).

We also studied 4 patients with T-cell CAEBV from Japan (patients 24-27). Three of 4 patients showed EBV only in CD3⁺, CD4⁻, and CD8⁻ cells (supplemental Figure 2A-B; Table 1). Although the staining for CD4 and CD8 is performed separately from staining for CD3, we can assume that the CD4⁻, CD8⁻, and EBV-positive cells (supplemental Figure 2B) are CD3⁺, because no EBV-positive B cells were detected in these patients. One patient had EBV in CD3⁺, CD8⁺, and CD4⁻ cells (supplemental Figure 2C). The median number of EBV genomes in T cells for the T-cell CAEBV patients was 8.5 copies per cell, with a mean of 9.1 copies per cell (range, 1-26). Combining the results from all 6 patients in whom we detected EBV in T cells,

Table 2. Mean number of EBV genomes per cell for each cell subpopulation

Patient	Diagnosis	EBV DNA load/ 10 ⁶ cells	Average number of EBV genomes per cell (range)				
			B cells	Plasmablast/ plasma cells	Non-B, non-T, non-monocyte cells	T cells	Monocytes
1	HIV	12 000	22.25 (5-40)	ND	21 (21)	0	0
2	HIV	4300	14.75 (6-23)	0	19 (19)	0	0
3	HIV	18 000	12.3 (4-24)	0	21 (21)	0	0
4	HIV	130 000	15.75 (1-38)	9.3 (6-16)	10.4 (6-15)	0	0
5	HIV	9400	10 (10)	0	2 (2)	14 (14)	2 (2)
6	HIV	5100	10.9 (4-20)	0	0	0	0
7	HIV	22 000	10.4 (6-17)	8.7 (4-16)	8.75 (6-14)	0	1 (1)
8	HIV	6300	22.4 (5-35)	16 (16)	7 (7)	0	0
9	Tx	NA	7.3 (4-11)	0	0	0	0
10	Tx	30 000	14.25 (6-30)	0	28 (28)	0	0
11	Tx	6200	16.2 (2-27)	ND	3 (3)	0	0
12	LyG/HL	170 000	17.8 (4-38)	0	11.2 (7-21)	8.8 (2-40)	1.5 (1-2)
13	Severe primary EBV infection	280 000	17.3 (5-35)	0	9.6 (9-10)	0	0
14	PTLD	1 300 000	19.2 (3-35)	7.5 (3-12)	13 (7-27)	0	0
15	ATL EBV-LPD	190 000	10.7 (2-23)	8.3 (7-10)	12.5 (10-15)	0	0
16	LyG	28 000	8.26 (2-16)	3 (3)	18 (8-34)	0	0
17	ICL	110 000	12.9 (3-28)	0	25 (25)	0	0
18	AA	64 000	16.5 (7-26)	ND	7 (7)	0	0
19	AA	110 000	14.58 (2-34)	21 (21)	0	0	3 (3)
20	AA	6 500 000	17.04 (3-35)	10.8 (4-17)	12.75 (5-21)	0	0
21	B-cell CAEBV	15 000	11.18 (4-22)	6 (6)	6.3 (2-9)	0	0
22	B-cell CAEBV	30 900	13.17 (4-33)	0	5 (5)	0	0
23	B-cell CAEBV	310 000	13.9 (6-23)	5.5 (5-6)	6 (6)	0	0
24	T-cell CAEBV	54 666/μg	0	ND	0	7.25 (1-22)	0
25	T-cell CAEBV	67 612/μg	0	ND	0	7.66 (1-17)	0
26	T-cell CAEBV	230 000/μg	0	ND	0	10.24 (2-26)	0
27	T-cell CAEBV	4154/μg	0	ND	0	9.88 (3-21)	0
28	IM	1700	12.5 (9-22)	5 (4-6)	0	0	0
29	IM	10 651	12.63 (5-27)	0	0	0	0

the mean copy number of EBV genomes in T cells was lower than the mean copy number for B cells; however, the difference was not significant (paired *t* test, *P* = .77; Figure 2).

EBV is detected in circulating monocytes

EBV has been reported to infect circulating monocytes of patients with HIV.¹⁵ We detected EBV in monocytes (CD14⁺ cells) from 4 patients: 2 HIV-positive patients, 1 with Hodgkin lymphoma, and 1 receiving antithymocyte globulin for aplastic anemia (Figure 5A; Table 1). The median number of EBV genomes in monocytes for the 4 patients was 1.75 copies per cell, with a mean of 1.85 copies per cell (range, 1-3). This was significantly lower than the mean copy number for B cells (paired *t* test; *P* = .0083) (Figure 2).

EBV is not detected in circulating NK cells in the patients in this study

EBV has been detected in circulating NK cells of patients with NK-cell CAEBV.¹² When PBMCs from the patients were stained with a combination of antibodies to CD16 and CD56, NK cells (CD16⁺/CD56⁺ and CD3⁻) were detected in each of the patients; however, none contained EBV.

EBV is detected in circulating “non-B, non-T, and non-monocyte cells”

Surprisingly, in 69% of the patients, we detected EBV in PBMCs that were not recognized by B-cell (CD19, CD20), T-cell (CD3), or monocyte (CD14) monoclonal antibodies (Figure 5B; Table 1). Several patients, including some with HIV (patients 1, 4, 5, and 7), PTLD (patient 14), aplastic anemia

receiving antithymocyte globulin (patients 18 and 20), and B-cell CAEBV (patients 21 and 23) had EBV in non-B, non-T, and non-monocyte cells that represented more than 20% of EBV-positive cells. EBV was detected in this cell population in each of the groups of patients we studied, except for patients with T-cell CAEBV from Japan and patients with infectious mononucleosis (IM). The median number of EBV genomes in non-B, non-T, non-monocyte cells for all the patients was 10.8 copies per cell, with a mean of 12.3 copies per cell (range, 2-34). Comparison of the mean copy number in EBV-positive B cells versus EBV-positive non-B, non-T, non-monocyte cells showed no significant difference (paired *t* test; *P* = .197; Figure 2).

EBV lytic protein BZLF1 is detected in circulating PBMCs

To determine whether we could detect evidence for lytic replication of EBV in virus-infected PBMCs, we performed immunofluorescence staining for EBV BZLF1 (an immediate-early protein that mediates the switch between the latent and the lytic forms of EBV infection), together with T- and B-cell markers (CD3 and CD19/CD20). Sufficient cells were available to assay 15 of the 29 patients (patients 8, 11, 12, 14, 15, 19-23, and 25-29). We detected BZLF1 in 87% (13 of 15) of these patients tested, indicating lytic EBV replication. The cell-surface phenotype of the BZLF1-positive cells was variable: in 4 patients, some of the BZLF1-positive cells were CD19⁺/CD20⁺, whereas other cells were negative for both CD19/CD20 and CD3 (patients 8, 14, 15, and 21); in 3 patients, some BZLF1-positive cells were CD3⁺, whereas others were negative for both CD19/CD20 and CD3 (patients 12, 26, and 27) (Figure 6). In 6 patients (patients 11, 19, 22, 23, 28, and

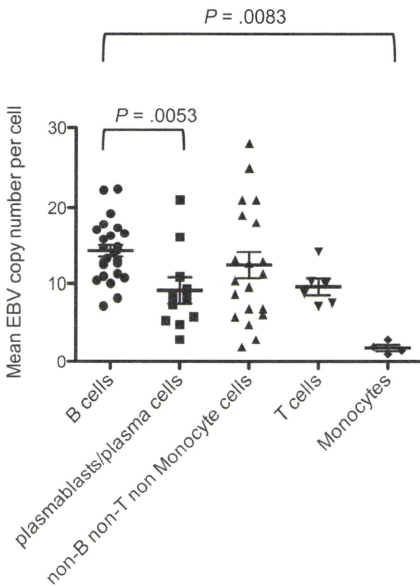


Figure 2. Mean EBV copy number per cell for each cell subpopulation for each patient. P values were obtained with the t test. The difference in mean EBV copy number between B cells and non-B, non-T, non-monocyte cells ($P = .2878$) and between B and T cells ($P = .77$) was not significant.

29), all of the BZLF1-positive cells were negative for CD3 and CD19/CD20.

Correlation between EBV copy number per cell and underlying disease

We analyzed the distribution of EBV copy numbers per cell for each of the 29 patients (Tables 2-3). Half of the patients infected with HIV had cells carrying more than 30 EBV genomes per cell; however, high copy numbers per cell did not correlate with the EBV DNA load in the blood (supplemental Figure S3). Some patients with high EBV DNA loads (patients 7 and 10 with 22 000 and 30 000 EBV genome copies per million cells, respectively) had no cells with more than 30 EBV genome copies, whereas others with lower EBV DNA loads (patients 6 and 8 with 5100 and 6300 EBV genome copies per million cells, respectively) had cells with more than 30 EBV genome copies (Table 3). Moreover, the percentage of cells with more than 30 EBV genome copies in some patients with a lower EBV DNA load (patient 1 with 20% of cells with more than 30 EBV genomes and 12 000 copies of EBV per million cells) was higher than in patients with a high EBV DNA load (patient 4 with 8% of cells with more than 30 EBV genomes and 130 000 copies of EBV per million cells). Only one patient in our study, HIV patient 1, had a majority of EBV-positive cells with more than 20 EBV genomes per cell.

Only patients with HIV (patients 1, 3, and 8) and those receiving antithymocyte globulin for aplastic anemia (patients 19 and 20) had

more than 25% of EBV-positive cells with more than 21 EBV genomes per cell.

Analysis of the 7 patients with B- or T-cell CAEBV showed that only one of these patients (patient 22) had more than 30 EBV genomes per cell. Despite the high EBV loads in these patients, 6 of 7 patients had a majority of EBV-positive cells with only 1-10 EBV genomes per cell. Thus, in these patients, the high EBV DNA load was caused by a large number of EBV-infected cells, rather than a high copy number per cell. Similarly, the 2 patients with IM also had a majority of EBV-positive cells with 10 or fewer EBV genomes per cell.

We also analyzed the distribution of the EBV genome copies per cell based on their phenotype. The mean number of EBV genome copies per cell for each of the different cell types showed no correlation with the total EBV DNA load in the blood (Figure 7). A more detailed analysis of the distribution of the number of EBV copies in B cells also showed no correlation with the EBV DNA load in the blood (Table 3; supplemental Figure S4). Taken together, these results suggest that patients with higher EBV DNA loads have a higher number of virus-infected cells, rather than a higher number of EBV genome copies per cell.

Discussion

Using the Immuno-FISH procedure, we analyzed 29 patients with different diseases that had high EBV DNA loads in the peripheral blood. All of the patients, except for the 4 T-cell CAEBV patients from Japan, were expected to have a B-cell EBV lymphoproliferative process. In all but 1 of these 25 patients (HIV patient 5), the majority of the EBV-positive cells were B cells; however, there was a wide range (52%-100% of the EBV-positive cells were B cells). Using this technique, EBV was detected in both B and non-B cells in 88% of these patients. Thus, the generally accepted belief that EBV is usually present only in B cells is not the case for patients with elevated EBV DNA loads.

Circulating plasmablast/plasma cells are detected in the blood of healthy persons, although they represent approximately 0.14% of PBMCs.²⁸ Plasmablast/plasma cells have been detected in the blood in patients with virus infections,²⁹ and these cells represent 5.5% of B cells in the peripheral blood of patients with HIV viremia.³⁰ EBV has been detected in plasma cells in human tonsils; approximately 10%-20% of these cells undergo lytic virus replication and most do not complete the full cycle.³¹ We detected EBV in plasmablasts/plasma cells in the blood of approximately 50% of the patients. Therefore, in patients with lymphoproliferative diseases and high EBV DNA loads, EBV is present in circulating plasmablasts/plasma cells, in addition to B cells.

We detected EBV in peripheral blood T cells in a patient with HIV and in one with Hodgkin lymphoma, as well as in the 4 Japanese patients with T-cell CAEBV. Using cell sorting followed by PCR, EBV was found in circulating T cells in HIV-1-infected children and adolescents with elevated EBV DNA loads.²⁶ EBV was found in CD4⁺ and CD8⁺ cells at approximately 10-fold lower EBV copy numbers than in CD19⁺ cells. In addition to T-cell CAEBV,¹²⁻¹⁵ EBV has been detected in T cells from patients with EBV-associated hemophagocytic lymphohistiocytosis³² and from patients with T-cell lymphoma.^{33,34} In contrast to the EBV-infected B cells, we were unable to detect CD21 on EBV-infected T cells. CD21 has been detected on the surface of T-cell lines³⁵ and at low levels on primary T cells.³⁶ Infection of a human T-lymphoblastic cell line resulted in virus internalization and viral

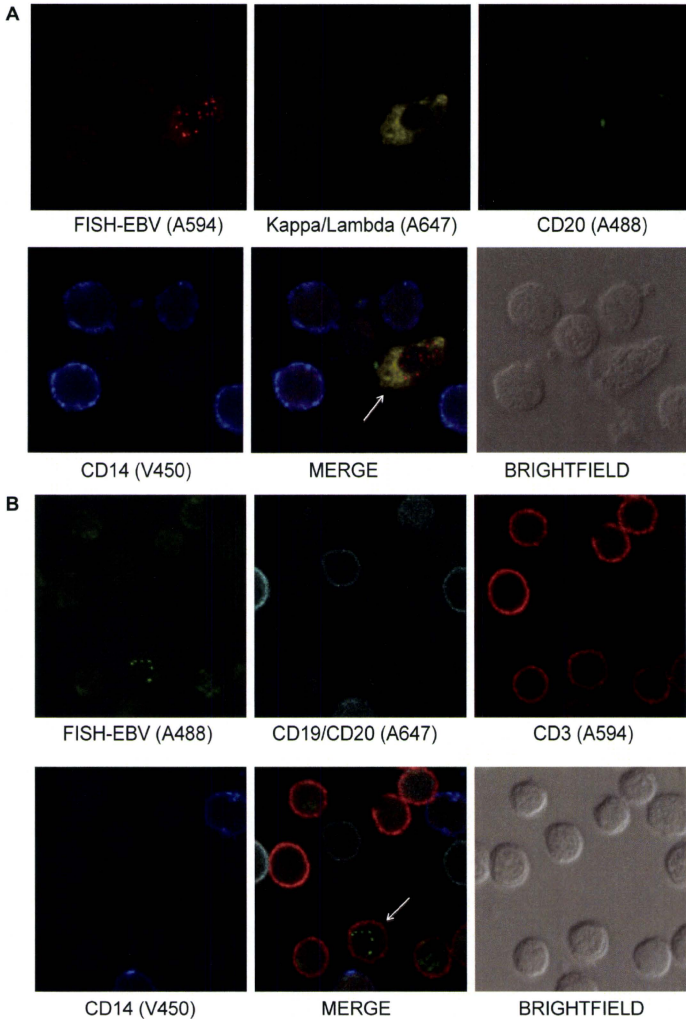


Figure 3. EBV is detected in plasmablasts/plasma cells and T cells. (A) Photomicrographs of PBMCs stained by Immuno-FISH from patient 4 shows EBV in plasmablasts/plasma cells identified using Alexa 647–conjugated anti- κ and anti- λ chain immunoglobulin antibodies. The EBV-positive cells are negative for CD20 using Alexa 488–conjugated anti-CD20 antibody. EBV DNA was identified with the biotinylated FISH probe and streptavidin-conjugated Alexa 594 and monocytes with V450-conjugated anti-CD14 antibody. Although most of the EBV DNA was detected in the nucleus, some viral DNA was detected in the cytoplasm, suggesting that EBV lytic infection was present in the cell. (B) Photomicrographs of PBMCs stained by Immuno-FISH from patient 12 shows EBV in CD3⁺ T cells. EBV DNA was identified with biotinylated FISH probe and streptavidin-conjugated Alexa 488, B cells using a combination of Alexa 647–conjugated anti-CD19 and anti-CD20 antibodies, T cells with Alexa 594–conjugated anti-CD3 antibody, and monocytes with V450-conjugated anti-CD14 antibody. Arrows indicate EBV-positive cells.

gene expression.^{37,38} It is possible that EBV infected T cells of CD21; alternatively, EBV may have used a different receptor using CD21 in our patients and down-regulated the expression than CD21 to enter the T cells.

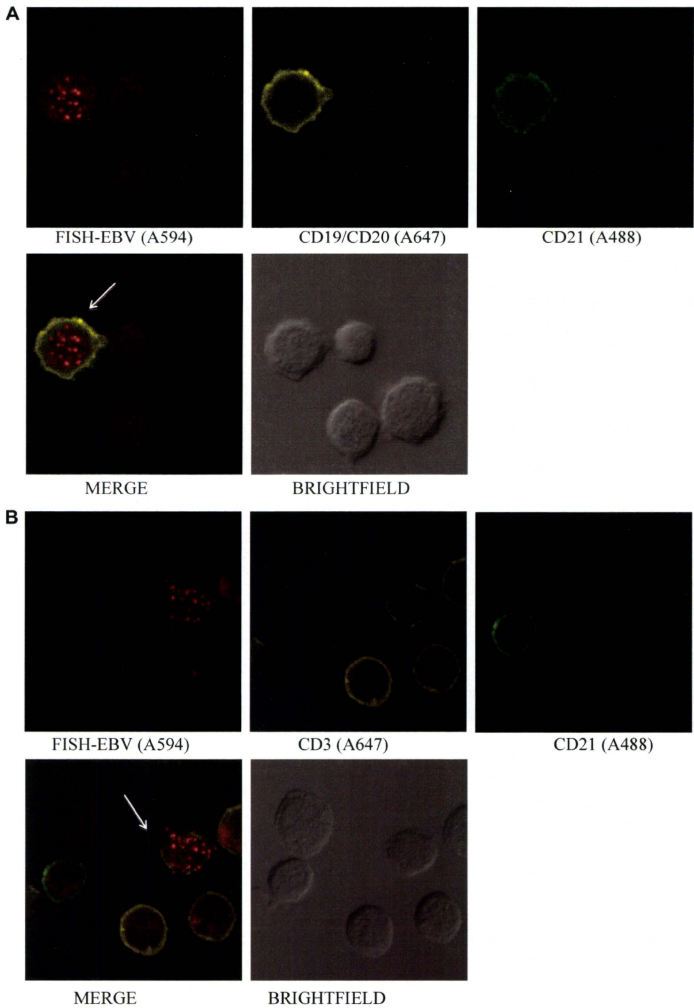


Figure 4. EBV is detected in CD21⁺ B cells and in CD21⁻ T cells. (A) Photomicrographs of PBMCs stained by Immuno-FISH from patient 12. EBV is detected in B cells that are CD21⁺. (B) EBV is detected in CD3⁺ T cells that do not express CD21. EBV DNA was identified with the biotinylated FISH probe and streptavidin-conjugated Alexa 594. B cells using a combination of Alexa 647-conjugated anti-CD19 and anti-CD20 antibodies, T cells with Alexa 647-conjugated anti-CD3 antibody, and CD21 with Alexa 488-conjugated anti-CD21 antibody. Arrows indicate EBV-positive cells.

We detected EBV in monocytes from 4 patients. EBV infection of peripheral blood monocytes has been described in patients with HIV based on sorting of cells by flow cytometry and EBV DNA PCR.¹⁵ EBV has also been reported to infect monocytes *in vitro* and result in the activation of the viral lytic cycle.³⁹⁻⁴¹ Our observation that very few monocytes were

infected by EBV, and that the viral copy number per cell was very low (ranging from 1 to 3 copies per cell), is consistent with the hypothesis that EBV infection of monocytes might be very transient with lytic replication, resulting in the rapid death of infected cells, or that monocytes have a shorter lifespan than B cells in the circulation.

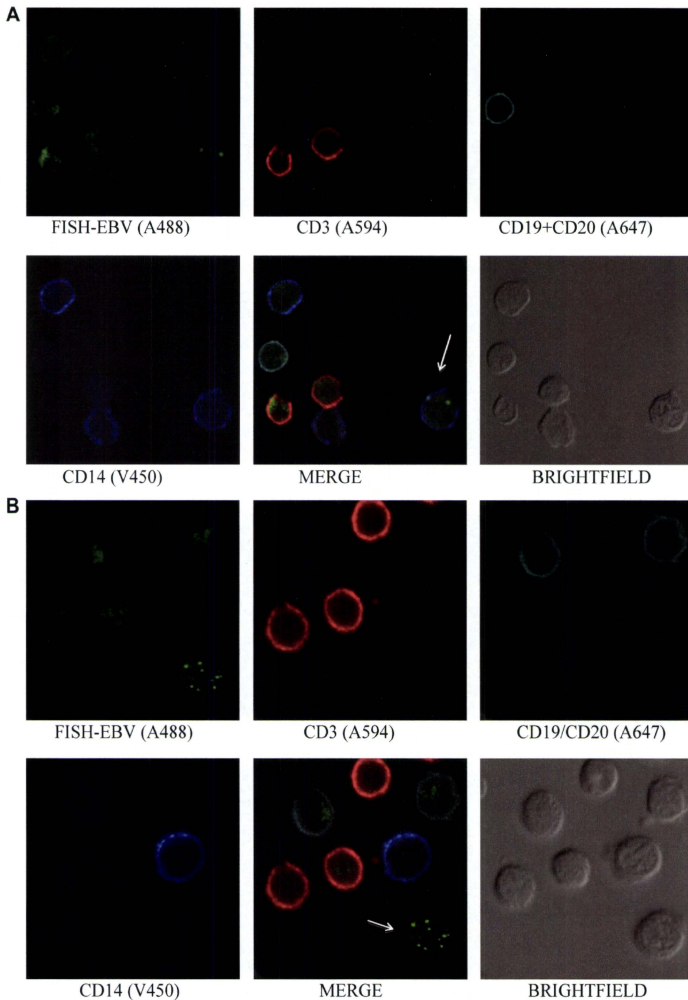


Figure 5. EBV is detected in monocytes and in non-B, non-T, and non-monocyte cells. (A) PBMCs stained by Immuno-FISH from patient 5 shows EBV in monocytes. EBV DNA was identified with the biotinylated FISH probe and streptavidin-conjugated Alexa 488. T cells using Alexa 594-conjugated anti-CD3. B cells using a combination of Alexa 647-conjugated anti-CD19 and anti-CD20 antibodies, and monocytes with V450-conjugated anti-CD14 antibody. Arrow indicates EBV-positive cell. (B) PBMCs stained by Immuno-FISH from patient 6 shows EBV in non-B, non-T, and non-monocyte cells. Arrow indicates EBV-positive cell.

We found that 69% (20 of 29) of the patients had EBV in circulating non-B, non-T, non-monocyte cells. These cells might represent one or more of several cell types. First, these non-B, non-T, non-monocyte cells might be plasmablasts/plasma cells. Approximately half (9 of 17) of the patients with EBV in non-B, non-T, non-monocyte cells had virus in plasmablasts/plasma cells

(CD20⁻, positive for κ and λ immunoglobulin light chains). Therefore, some of the non-B, non-T, non-monocyte cells may have been plasmablasts/plasma cells that were not stained with antibody to κ and λ immunoglobulin light chains. These cells, however, have a very prominent cytoplasm (Figure 3A), and most of the non-B, non-T, non-monocyte cells did not have a prominent

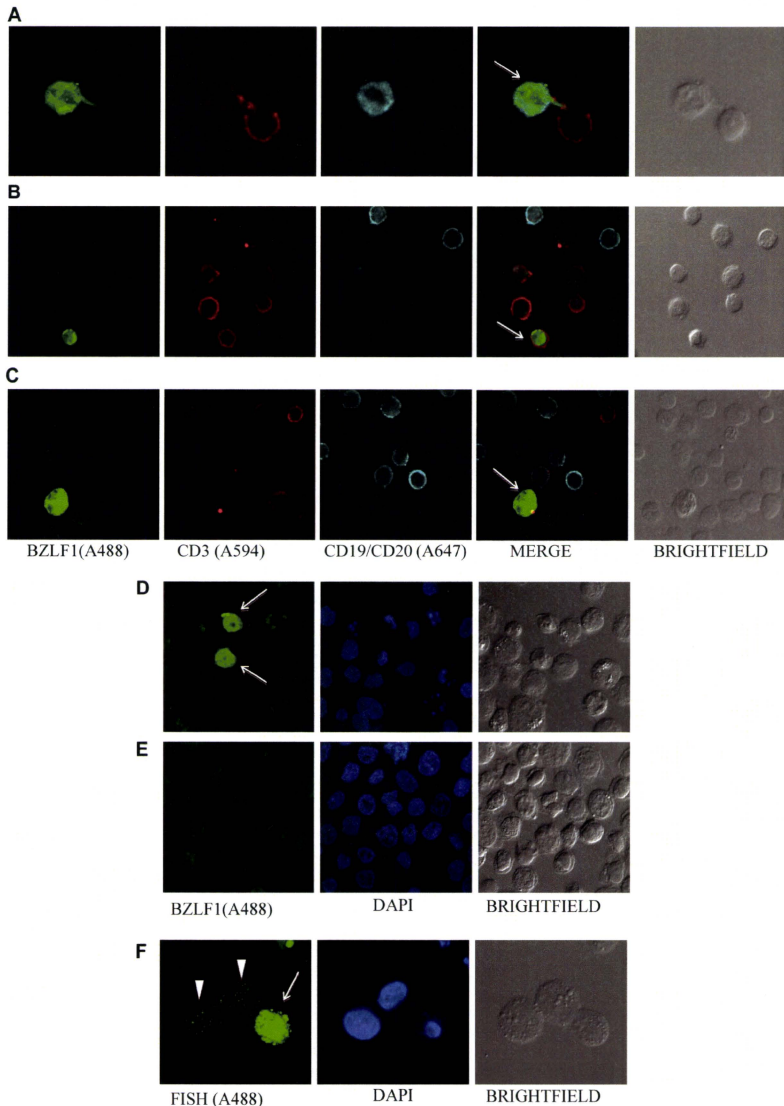


Figure 6. EBV lytic protein BZLF1 is detected in PBMCs. Photomicrographs of PBMCs stained for EBV BZLF1 shows virus lytic infection in B cells (patient 21, A), T cells (patient 12, B), and non-B, non-T cells (patient 25, C). BZLF1 was directly conjugated to Alexa 488. T cells were stained by using Alexa 594-conjugated anti-CD3, and B cells by using a combination of Alexa 647-conjugated anti-CD19 and anti-CD20 antibodies. BZLF1 staining of EBV-positive B95-8 (D) and EBV-negative BJAB (E) cells served as positive and negative controls, respectively, for EBV lytic infection. Arrows indicate BZLF1-positive cells. (F) B95-8 cells stimulated with butyric acid and TPA (phorbol 12-myristate 13-acetate) served as an additional control for lytic infection; cells undergoing lytic infection show a homogeneous pattern with high levels of EBV DNA (arrow), and latently infected cells show a punctate pattern (arrowheads).

Table 3. Distribution of the number of EBV genomes per cell

Patient	Diagnosis	EBV DNA load/10 ⁶ cells	% of range of EBV genomes per cell				Total EBV ⁺ cells counted
			1-10	11-20	21-30	> 30	
1	HIV	12 000	10	30	40	20	10
2	HIV	4300	34	44	22	0	9
3	HIV	18 000	31	31	38	0	13
4	HIV	130 000	50	38	4	8	24
5	HIV	9400	75	25	0	0	4
6	HIV	5100	43	43	7	7	14
7	HIV	22 000	58	42	0	0	19
8	HIV	6300	25	38	25	12	16
9	Tx	NA	75	25	0	0	4
10	Tx	30 000	54	38	8	0	24
11	Tx	6200	40	40	20	0	10
12	LyG/HL	170 000	64	27	6	3	77
13	Severe primary EBV infection	280 000	27	54	11	8	37
14	PTLD	1 300 000	36	29	9	16	33
15	ATL EBV-LPD	190 000	48	44	4	4	25
16	LyG	28 000	70	25	0	5	20
17	ICL	110 000	45	31	21	3	38
18	AA	64 000	69	23	8	0	13
19	AA	110 000	46	21	25	8	24
20	AA	6 500 000	36	37	18	9	55
21	B-cell CAEBV	15 000	65	30	5	0	20
22	B-cell CAEBV	30 900	31	54	9	6	35
23	B-cell CAEBV	310 000	54	33	13	0	15
24	T-cell CAEBV	54 666/μg	75	24	1	0	88
25	T-cell CAEBV	67 612/μg	71	29	0	0	21
26	T-cell CAEBV	230 000/μg	56	41	3	0	78
27	T-cell CAEBV	4154/μg	54	39	7	0	18
28	IM	1700	65	29	6	0	17
29	IM	10 651	57	29	14	0	14

Additional cell subsets (eg, "non-T, non-NK, non-monocytes") that are not represented in Tables 1 and 2 are included in the counts for this table.

cytoplasm. Second, some of the non-B, non-T, non-monocyte cells might be B cells with very low levels of CD20 that are undetectable by antibody staining. This seems unlikely, based on the sensitivity of Immuno-FISH to detect B cells after sorting for B cells (supplemental Table 1). Furthermore, only 1 of the 29 patients (patient 21) had received anti-CD20 antibody (rituximab), and EBV was still detected in his B cells. Third, EBV could be present in cells other than T, B, NK cells, and monocytes. For example, EBV infects pre-Langerhans cells in the peripheral blood.¹⁶ However, we did not detect EBV DNA in pre-Langerhans cells (CD1a⁺ CD11c⁺, and CD14⁻) or in basophils (CD123⁺) in PBMCs from 4 patients who had EBV in non-B, non-T, non-monocyte cells (patients 12, 15, 16, and 20), using Immuno-FISH (data not shown). Infection of non-B, non-T, non-monocyte cells may be more common in immunocompromised persons. The only groups of patients for whom we did not detect EBV in these cells were patients with T-cell CAEBV and IM; the latter were otherwise healthy persons. In addition, the 1 HIV patient without EBV in non-B, non-T, and non-monocyte cells had the highest CD4 T-cell count (354 cells per μL); the other HIV patients all had CD4 T-cell counts less than 250 cells per μL.

We detected evidence of lytic EBV infection in nearly all of the patients for whom we performed immunofluorescence staining for BZLF1 protein. BZLF1 is the first viral protein expressed during the reactivation of EBV in B cells; it initiates a cascade of viral lytic gene expression, culminating with the death of the infected cells.⁴² It is interesting to note that although some of the BZLF1-positive cells were CD19/CD20⁺, others were negative when stained with antibodies for B and T cells. These cells could be plasmablast/plasma cells (negative for CD19/CD20), because EBV-positive

plasmablast/plasma cells were detected by Immuno-FISH in most of these patients. BZLF1 was previously detected in lymphocytes in tonsils of patients with IM; most of these cells were CD20⁻ and VS38c⁺ with morphologic features indicative of plasma cells, whereas only rare cells were CD20⁺.⁴³ The BZLF1 promoter in B cells is activated only after they differentiate into plasma cells.³¹ In the latter study, BZLF1 was detected almost exclusively in CD20^{lo} plasma cells in human tonsils. Plasma cells in the bone marrow are CD20⁻, whereas plasma cells in tonsils express low levels of CD20.⁴⁴

Immuno-FISH allows one to determine both the EBV genome copy number and phenotypic characterization of individual cells in the peripheral blood. This procedure combines the highly sensitive, specific FISH technique with multicolor immunofluorescence using monoclonal antibodies directly conjugated to Alexa dyes. These fluorochromes are very stable at high temperatures and low pH and therefore maintain their activity during the FISH procedure. Moreover, the use of directly conjugated antibodies obviates the problems of the cross-reacting secondary antibodies and permits the detection of multiple surface markers in the same slide.

Other studies have analyzed EBV copy numbers in various cell populations by sorting cells with monoclonal antibodies to cell surface markers and then performing PCR on the sorted populations. Although this technique has been useful,^{2,4} it has several limitations. First, the sensitivity has been estimated to be approximately 90%²; therefore, one cannot detect cell populations containing less than 10% of a given cell phenotype. Second, the specificity of cell sorting followed by PCR amplification has limitations; if very rare EBV PCR-positive cells are detected in sorted cells, this could be a result of a low level of

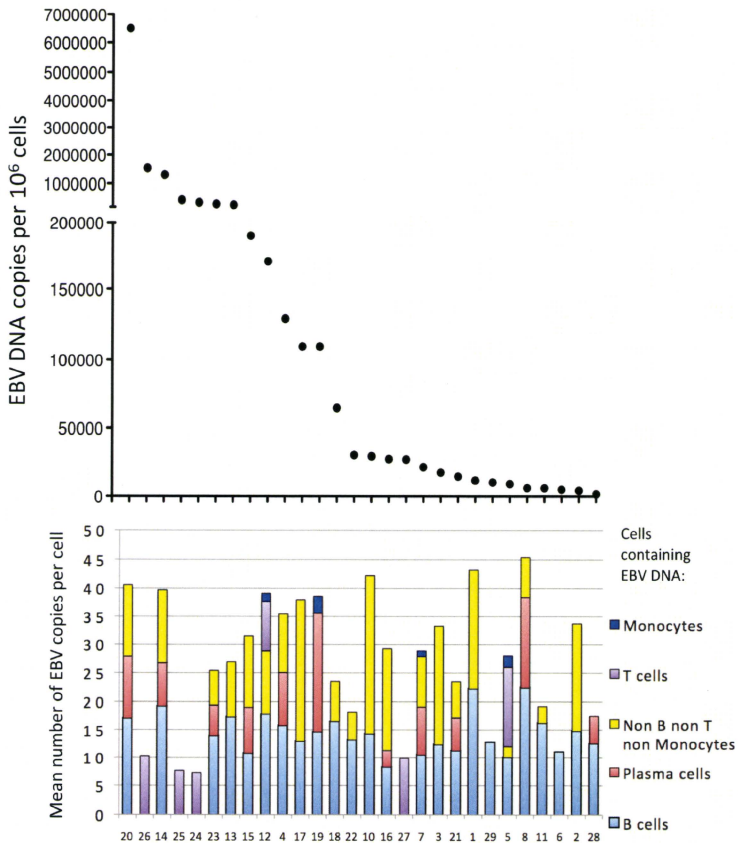


Figure 7. Correlation between the EBV DNA load in peripheral blood and the mean number of EBV DNA copies per cell for each cell subpopulation for each patient. Patient numbers are indicated at the bottom of the figure, and the vertical bars for each patient indicate the mean EBV DNA copy number per cell type.

EBV-positive B cells contaminating these sorted cells. Third, the number of EBV genomes per cell cannot be determined. Recently, combined cell-surface staining and in situ hybridization for EBER followed by flow cytometry has been used to identify EBV-positive T-cell subsets in the blood¹⁸; however, the number of the EBV genomes per cell could not be determined. Fourth, sorting cells requires a large number of cells and may not be feasible in patients with low numbers of PBMCs. Although labor intensive, the Immuno-FISH procedure has improved specificity, compared with the cell sorting and PCR procedure, and allows one to accurately measure the number of EBV genomes per cell. Using this procedure, we were able to determine the phenotype of EBV-infected cells with a specificity of more than 97% (supplemental Table 1; supplemental Figure 1).

Immuno-FISH might be used to better characterize the cell types infected with EBV and viral copy number of individual cells

in the peripheral blood of immunocompromised patients. Immuno-FISH does not require tissue biopsies and therefore might be used to noninvasively monitor transplant patients or other immunocompromised patients who have high EBV DNA loads. Although an elevated EBV DNA load is often predictive of EBV PTLD in recipients of T cell–depleted allogeneic hematopoietic cell transplants, EBV DNA loads in non-T cell–depleted allogeneic hematopoietic cell and solid organ transplant recipients may remain persistently elevated without disease and often does not require treatment with potentially toxic agents⁵; currently, there is no noninvasive procedure to predict which of these patients with high EBV DNA loads would develop PTLD. Determination of the number of EBV genomes per cell and the subtype of B (or non-B) cells infected with the virus might provide additional prognostic information for the development of PTLD in patients with high EBV DNA loads. Immuno-FISH should be useful to identify

EBV-infected cell populations in the blood of persons with CAEBV whose B, T, or NK cells are infected with the virus. Immuno-FISH might also be useful for studies of other virus infections in the blood.

In conclusion, using Immuno-FISH, we found that EBV is present in B cells as well as in plasmablasts/plasma cells and non-B, non-T, non-monoocyte cells in the blood of patients with lymphoproliferative diseases. In patients with high EBV loads who are immunocompromised, the number of genomes per cell and the type of cells infected may provide additional prognostic information for the development of EBV lymphoproliferative diseases. Moreover, the identification of EBV-positive, CD20-negative cells in the blood may have important implications for rituximab therapy in these patients.

Acknowledgments

We thank Owen Schwartz, Lily Koo, and Steven Becker for assistance with confocal microscopy; Mark Connor and Charles Brown for advice on the FISH procedure; Gary Fahle for measur-

ing EBV DNA in the blood; Jing Qin for statistical analyses; Susan Moir and Pratip Chattopadhyay for advice on B cells and antibody staining; and Gregg Roby, Gilbu Uzel, and Wyndham Wilson for help in obtaining clinical samples.

This study was supported by the Intramural Research Program of the National Institute of Allergy and Infectious Diseases, the National Heart, Lung, and Blood Institute, and the National Cancer Institute.

Authorship

Contribution: S.C. performed the experiments, analyzed the data, and wrote the manuscript; I.S., P.S., H.K., and R.W.C. provided PBMC samples and critically reviewed the manuscript; and J.I.C. designed the study, analyzed the data, and wrote the manuscript.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

Correspondence: Jeffrey I. Cohen, Laboratory of Infectious Diseases, National Institutes of Health, 50 South Dr, Building 50, Room 6134, Bethesda, MD 20892; e-mail: jcohen@niaid.nih.gov.

References

- Cohen JI. Epstein-Barr virus infection. *N Engl J Med*. 2000;343(7):481-492.
- Babcock GJ, Decker LL, Freeman RB, Thorley-Lawson DA. Epstein-Barr virus-infected resting memory B cells, not proliferating lymphoblasts, accumulate in the peripheral blood of immunosuppressed patients. *J Exp Med*. 1999;190(4):567-576.
- Babcock GJ, Decker LL, Volk M, Thorley-Lawson DA. EBV persistence in memory B cells in vivo. *Immunity*. 1998;9(3):395-404.
- Souza TA, Stollar BD, Sullivan JL, Luzuriaga K, Thorley-Lawson DA. Peripheral B cells latently infected with Epstein-Barr virus display molecular hallmarks of classical antigen-selected memory B cells. *Proc Natl Acad Sci U S A*. 2005;102(50):18093-18098.
- Gottschalk S, Rooney CM, Heslop HE. Post-transplant lymphoproliferative disorders. *Annu Rev Med*. 2005;56:29-44.
- Carbone A. Emerging pathways in the development of AIDS-related lymphomas. *Lancet Oncol*. 2003;4(1):22-29.
- Hopwood PA, Brooks L, Parratt R, et al. Persistent Epstein-Barr virus infection: unrestricted latent and lytic viral gene expression in healthy immunosuppressed transplant recipients. *Transplantation*. 2002;74(2):194-202.
- Rose C, Green M, Webber S, et al. Detection of Epstein-Barr virus genomes in peripheral blood cells from solid-organ transplant recipients by fluorescence in situ hybridization. *J Clin Microbiol*. 2002;40(7):2533-2544.
- Schauer E, Webber S, Green M, Rowe D. Surface immunoglobulin-deficient Epstein-Barr virus-infected B cells in the peripheral blood of pediatric solid-organ transplant recipients. *J Clin Microbiol*. 2004;42(12):5802-5810.
- Schauer E, Webber S, Kingsley L, Green M, Rowe D. Increased Ig-nuB lymphocytes in the peripheral blood of pediatric solid organ transplant recipients with elevated Epstein-Barr virus loads. *Pediatr Transp*. 2009;13(3):311-318.
- Snow AL, Martinez OM. Epstein-Barr virus: evasive maneuvers in the development of PTL. *Am J Transp*. 2007;7(2):271-277.
- Kimura H, Hoshino Y, Hara S, et al. Differences between T-cell-type and natural killer cell-type chronic active Epstein-Barr virus infection. *J Infect Dis*. 2005;191(4):531-539.
- Quintanilla-Martinez L, Kumar S, Fend F, et al. Fulminant EBV (+) T-cell lymphoproliferative disorder following acute/chronic EBV infection: a distinct clinicopathologic syndrome. *Blood*. 2000;96(2):443-451.
- Jaffe ES. Nasal and nasal-type T/NK cell lymphoma: a unique form of lymphoma associated with the Epstein-Barr virus. *Histopathology*. 1995;27(6):581-583.
- Tugizov S, Herrera R, Veluppillai P, Greenspan J, Greenspan D, Palefsky JM. Epstein-Barr virus (EBV)-infected monocytes facilitate dissemination of EBV within the oral mucosal epithelium. *J Virol*. 2007;81(11):5484-5496.
- Walling DM, Ray AJ, Nichols JE, Flatz CM, Nichols CM. Epstein-Barr virus infection of Langrethans cell precursors as a mechanism of oral epithelial entry, persistence, and reactivation. *J Virol*. 2007;81(13):7249-7268.
- Gulley ML, Glaser SL, Craig FE, et al. Guidelines for interpreting EBER in situ hybridization and LMP1 immunohistochemical tests for detecting Epstein-Barr virus in Hodgkin lymphoma. *Am J Clin Pathol*. 2002;117(2):259-267.
- Kimura H, Miyake K, Yamauchi Y, et al. Identification of Epstein-Barr virus (EBV)-infected lymphocyte subtypes by flow cytometric in situ hybridization in EBV-associated lymphoproliferative diseases. *J Infect Dis*. 2009;200(7):1078-1087.
- Feng WH, Cohen JI, Fischer S, et al. Reactivation of latent Epstein-Barr virus by methotrexate: a potential contributor to methotrexate-associated lymphomas. *J Natl Cancer Inst*. 2004;96(22):1691-1702.
- Allan GJ, Rowe DT. Size and stability of the Epstein-Barr virus major internal repeat (IR-1) in Burkitt's lymphoma and lymphoblastoid cell lines. *Virology*. 1989;173(2):489-498.
- Arribas JR, Clifford BD, Fichtenbaum CJ, Roberts RL, Powderly WG, Storch GA. Detection of Epstein-Barr virus DNA in cerebrospinal fluid for diagnosis of AIDS-related central nervous system lymphoma. *J Clin Microbiol*. 1995;33(6):1580-1583.
- Henderson A, Ripley S, Heller M, Kieff E. Chromosome site for Epstein-Barr virus DNA in a Burkitt tumor cell line and in lymphocytes growth-transformed in vitro. *Proc Natl Acad Sci U S A*. 1983;80(7):1987-1991.
- Szeles A, Falk KI, Imreh S, Klein G. Visualization of alternative Epstein-Barr virus expression programs by fluorescein in situ hybridization at the cell level. *J Virol*. 1999;73(6):5064-5069.
- Dong HY, Scadden DT, de Leval L, Tang Z, Isaacson PG, Harris NL. Plasmablastic lymphoma in HIV-positive patients: an aggressive Epstein-Barr virus-associated extramedullary plasmacytic neoplasm. *Am J Surg Pathol*. 2005;29(12):1633-1641.
- Richendollar BG, Hsi ED, Cook JR. Extramedullary plasmacytoma-like post-transplantation lymphoproliferative disorders: clinical and pathologic features. *Am J Clin Pathol*. 2009;132(4):581-588.
- Bekker V, Scherpbier H, Beld M, et al. Epstein-Barr virus infects B and non-B lymphocytes in HIV-1-infected children and adolescents. *J Infect Dis*. 2006;194(9):1323-1330.
- Fingerhuth DJ, Wels JG, Tedder TF, Strominger JL, Biro PA, Fearon DT. Epstein-Barr virus receptor of human B lymphocytes is the Cd3 receptor, CR2. *Proc Natl Acad Sci U S A*. 1984;81(14):4510-4514.
- Mei HE, Yoshida T, Sime W, et al. Blood-borne human plasma cells in steady state are derived from mucosal immune responses. *Blood*. 2009;113(11):2461-2469.
- James MC, Rojas OL, Kunkel EJ, et al. Maturation and trafficking markers on rotavirus-specific B cells during acute infection and convalescence in children. *J Virol*. 2004;78(20):10967-10976.
- Moir S, Ho J, Malaspina A, et al. Evidence for HIV-associated B-cell exhaustion in a dysfunctional memory B-cell compartment in HIV-infected seropositive individuals. *J Exp Med*. 2006;205(8):1379-1805.
- Laichalk LL, Thorley-Lawson DA. Terminal differentiation into plasma cells initiates the replicative cycle of Epstein-Barr virus in vivo. *J Virol*. 2005;79(2):1296-1307.
- Kasahara Y, Yachie A, Takei K, et al. Differential cellular targets of Epstein-Barr virus (EBV) infection between acute EBV-associated hemophagocytic lymphohistiocytosis and chronic active EBV infection. *Blood*. 2001;98(6):1882-1888.
- George TJ, Jeng M, Berquist W, Cherry AM, Link MP, Arber DA. Epstein-Barr virus-associated peripheral T-cell lymphoma and hemophagocytic syndrome arising after liver transplantation: case report and review of the literature. *Pediatr Blood Cancer*. 2005;44(3):270-276.

34. Pallesen G, Hamilton-Dutoit SJ, Zhou X. The association of Epstein-Barr virus (EBV) with T-cell lymphoproliferations and Hodgkin's disease: two new developments in the EBV field. *Adv Cancer Res.* 1993;62:179-239.
35. Fingeroth JD, Clabby ML, Strominger JD. Characterization of a T-lymphocyte Epstein-Barr virus/C3d receptor (CD21). *J Virol.* 1988;62(4):1442-1447.
36. Fischer E, Delibrias C, Kazatchkine MD. Expression of CR2 (the C3dg/EBV receptor, CD21) on normal human peripheral blood T lymphocytes. *J Immunol.* 1991;146(3):865-869.
37. Paterson RL, Kelleher C, Amankonah TD, et al. Model of Epstein-Barr virus infection of human thymocytes: expression of viral genome and impact on cellular receptor expression in the T-lymphoblastic cell line, HPB-ALL. *Blood.* 1995;85(2):456-464.
38. Paterson RL, Kelleher CA, Streib JE, et al. Activation of human thymocytes after infection by EBV. *J Immunol.* 1995;154(3):1440-1449.
39. Savard M, Belanger C, Tardif M, Gourde P, Flamand L, Gosselin J. Infection of primary human monocytes by Epstein-Barr virus. *J Virol.* 2000;74(6):2612-2619.
40. Guerreiro-Cacais AO, Li L, Donati D, et al. Capacity of Epstein-Barr virus to infect monocytes and inhibit their development into dendritic cells is affected by the cell type supporting virus replication. *J Gen Virol.* 2004;85(Pt 10):2767-2778.
41. Masy E, Adriaenssens E, Montpellier C, et al. Human monocytic cell lines transformed in vitro by Epstein-Barr virus display a type II latency and LMP-1-dependent proliferation. *J Virol.* 2002;76(13):6460-6472.
42. Speck SH, Chatila T, Flemington E. Reactivation of Epstein-Barr virus: regulation and function of the BZLF1 gene. *Trends Microbiol.* 1997;5(10):399-405.
43. Niedobitek G, Agathangelou A, Herbst H, Whitehead L, Wright DH, Young LS. Epstein-Barr virus (EBV) infection in infectious mononucleosis: virus latency, replication, and phenotype of EBV-infected cells. *J Pathol.* 1997;182(2):151-159.
44. Medina F, Segundo C, Campos-Caro A, Gonzalez-Garcia I, Brieva JA. The heterogeneity shown by human plasma cells from tonsil, blood, and bone marrow reveals graded stages of increasing maturity, but local profiles of adhesion molecule expression. *Blood.* 2002;99(6):2154-2161.

Immunologic and Virologic Analyses in Pediatric Liver Transplant Recipients with Chronic High Epstein-Barr Virus Loads

Kensei Gotoh,¹ Yoshinori Ito,¹ Rieko Ohta,¹ Seiko Iwata,² Yukihiro Nishiyama,² Taro Nakamura,³ Kenitiro Kaneko,⁴ Tetsuya Kiuchi,³ Hisami Ando,⁴ and Hiroshi Kimura²

Departments of ¹Pediatrics, ²Virology, ³Transplant Surgery, and ⁴Pediatric Surgery, Nagoya University Graduate School of Medicine, Nagoya, Japan

Background. Long-term Epstein-Barr virus (EBV) monitoring for potentially life-threatening posttransplant lymphoproliferative disorder (PTLD) has identified asymptomatic patients who maintain high EBV loads over long periods.

Methods. Thirty-one pediatric liver transplant recipients were designated as 11 chronic high EBV load carriers (EBV DNA level >5000 copies/mL of whole blood for >6 months) and 20 control recipients. Serial quantification of EBV DNA, measurement of interleukin 10 (IL-10) concentrations, EBV-specific tetramer staining, and relative quantification of EBV gene expression in peripheral blood mononuclear cells were performed.

Results. Most of the chronic high EBV load carriers were seronegative at transplant, the median time to resolution of a chronic high EBV load was 23 months, and no recipient developed late-onset PTLD. EBV DNA was detected predominantly in CD19⁺ cells. The plasma concentration of IL-10 and the EBV-specific CD8⁺ cell frequency did not differ significantly between the chronic high EBV load carriers and the control recipients. Analysis of gene expression showed that EBV-encoded small RNA 1, *BamHI A* rightward transcripts, and latent membrane protein 2 were positive in peripheral blood mononuclear cells from chronic high EBV load carriers.

Conclusions. EBV-infected cells in the blood of chronic high EBV load carriers expressed a highly restricted set of latency genes, suggesting that the EBV-infected cells escaped from a T cell response.

Epstein-Barr virus (EBV) is a ubiquitous virus that usually infects humans by early adulthood and can cause benign or severe disease. EBV often persists in infected cells, and all EBV-positive cells exhibit 1 of 4 latency types, distinguished by the pattern of expressed EBV antigens. In latency type 0, all antigens are suppressed, as in a healthy virus carrier. In latency type 1, only EBV-encoded nuclear antigen 1 (EBNA1) is expressed, as in Burkitt lymphoma. Latent membrane protein 1

(LMP1) and LMP2, as well as EBNA1, are expressed in latency type 2, as in Hodgkin disease. In latency type 3, highly immunogenic EBNA3 genes (EBNA3A, EBNA3B, and EBNA3C) are expressed, together with other EBV-latent antigens, as in posttransplant lymphoproliferative disorder (PTLD). The noncoding EBV-encoded small RNAs (EBERs) and the *BamHI A* rightward transcripts (BARTs) are expressed in all latency types [1–3]. EBV-specific immune control is mediated by innate and adaptive immune responses. Disruption of the balance of antiviral immunity may lead to the development of EBV-associated disease [4].

EBV-related PTLD is a significant cause of morbidity and mortality after solid-organ transplant in children. It has been reported that elevated levels of EBV DNA are a predictive factor for PTLD, and monitoring EBV loads in the peripheral blood of transplant recipients by polymerase chain reaction (PCR) can help identify patients at risk for developing PTLD before the onset of clinical signs [5–7]. Recently, serial viral load monitoring has identified a population of children who subsequently develop and maintain very high EBV loads

Received 16 July 2009; accepted 10 February 2010; electronically published 18 June 2010.

Potential conflicts of interest: none reported.

Financial support: Japan Society for the Promotion of Science (Grants in Aid for Scientific Research [C] [20591276] to Y.I.); Ministry of Health, Labor, and Welfare of Japan (grant for research on measures for emerging and reemerging infections [Intractable Infectious Diseases in Organ Transplant Recipients, H21-Shinko-Ippan-009] to H.K.).

Reprints or correspondence: Dr Yoshinori Ito, Dept of Virology, Nagoya University Graduate School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya 466-8550, Japan (yoshi-i@med.nagoya-u.ac.jp).

The Journal of Infectious Diseases 2010;202(3):461–469

© 2010 by the Infectious Diseases Society of America. All rights reserved.
0022-1899/2010/20203-0018\$15.00

DOI: 10.1086/653737

in the absence of clinical symptoms [8–12]. This persistent presence of EBV DNA may increase the risk of late-onset PTLD. Previous reports have described the clinical courses among these patients after liver transplant, and a small number of them developed late-onset PTLD [10, 12]; however, no pathophysiological analysis has been reported.

METHODS

Patients. Thirty-three pediatric liver transplant recipients who underwent living-donor liver transplant at Nagoya University School of Medicine from 1999 through 2007 were registered for this study. The primary diseases were biliary atresia ($n = 25$), fulminant hepatitis ($n = 4$), Alagille syndrome ($n = 1$), congenital absence of portal vein ($n = 1$), metastatic liver tumor ($n = 1$), and ornithine transcarbamylase deficiency ($n = 1$). We performed a prospective analysis of EBV load in whole blood over the course of 6 months. Blood samples for the quantification of EBV DNA were obtained weekly until hospital discharge (median, 10 weeks after transplant; range, 6–37 weeks after transplant) and subsequently at each outpatient visit (monthly for 6 months) thereafter or until the EBV load resolved. Two patients were excluded from this study because they continued to be EBV seronegative throughout the study period. Chronic high EBV load was defined as the continuous presence of EBV loads >5000 copies/mL of whole blood for a minimum of 6 months after asymptomatic EBV infection or after complete resolution of symptomatic EBV infection; 11 patients met this criteria. Twenty pediatric liver transplant recipients who were not chronic high EBV load carriers were studied as control recipients. Blood samples for cell sorting, measurement of interleukin 10 (IL-10) concentration, tetramer staining, and real-time reverse-transcription PCR (RT-PCR) assay were obtained over the time of chronic high EBV load states. At the time of blood sampling, all patients were clinically asymptomatic. The results of these analyses were compared with those from 2 patients who developed EBV-related PTLD after a bone marrow transplant, 15 pediatric immunocompetent patients with acute-phase infectious mononucleosis (IM), and 25 healthy EBV-seropositive adult carriers.

The standard immunosuppressive regimen consisted of tacrolimus and prednisolone, as reported elsewhere [13]. Target tacrolimus trough levels in plasma were as follows: 12–15 ng/mL for the first 2 weeks after transplant, 10 ng/mL for the second through fourth weeks, 5–8 ng/mL for the first through sixth months, 5 ng/mL for the sixth through 12th months, and 2–3 ng/mL after the 12th month. When a liver transplant recipient who was positive for EBV developed clinical symptoms or the whole-blood EBV load increased to >5000 copies/mL, immunosuppression with tacrolimus was gradually decreased and kept at the minimum considered safe. Oral acyclovir (30–60 mg/kg/day) was administered until the EBV load decreased

to <5000 copies/mL. No patient received antiviral prophylaxis in this study, and a preemptive approach was adopted to treat patients at risk for PTLD; indeed, no disease occurred.

Informed consent was obtained from all patients or their parents. This study was approved by the University of Nagoya Institutional Review Board.

Quantification of EBV DNA. Viral DNA was extracted from either 200 μ L of whole blood or 10^6 peripheral blood mononuclear cells (PBMCs), using QIAamp DNA blood kits (Qiagen). A real-time quantitative PCR assay was performed, as described elsewhere [14, 15]. The minimum detection level was 2 copies per reaction, which is equivalent to ~ 20 copies/ μ g of DNA for PBMCs and 100 copies/mL for whole blood.

Determination of EBV-infected cells. To determine which cells harbored EBV, PBMCs were fractionated into CD3⁺, CD19⁺, and CD56⁺ cells by means of Dynabeads (Invitrogen). The fractionated cells were analyzed by real-time quantitative PCR. EBV-infected cell fractions were defined as having larger amounts of EBV DNA than of unfractionated PBMCs [14].

Plasma concentration of IL-10. The plasma concentration of IL-10 was measured using enzyme-linked immunoassay kits (Quantikine HS Human IL-10 Immunoassay; R&D Systems). Normal plasma concentrations in healthy persons are 5 pg/mL or less. The samples for measurement of IL-10 were the same as those used for EBV load measurements. The mean times of blood sampling \pm standard deviation (SD) were 1.6 ± 1.2 years after the onset of high EBV load status (3.0 ± 1.8 years after liver transplant) among chronic high EBV load carriers and 4.3 ± 2.3 years after liver transplant among control recipients.

Tetramer staining. Fresh PBMCs from HLA-A2–positive and HLA-A24–positive persons were stained with a phycoerythrin-labeled major histocompatibility complex (MHC) class I tetrameric complex (Medical & Biological Laboratories) and a fluorescein isothiocyanate–labeled anti-CD8 monoclonal antibody (clone B9.11; Immunotech) and analyzed using a FACS-Calibur flow cytometer (Becton Dickinson). For each analysis, CD8⁺ T cells were gated, and 30,000–150,000 events were acquired. The limit of detection was 0.01% of CD8⁺ T cells. The mean times of blood sampling \pm SD were 3.0 ± 1.8 years after the onset of a high EBV load state (1.6 ± 1.2 years after liver transplant) among chronic high EBV load carriers and 4.3 ± 2.3 years after liver transplant among control recipients. The timing of sampling for tetramer staining was after the manipulation of immune suppression in all of the chronic high EBV load carriers. Five of 10 healthy control subjects and 6 of 11 chronic high EBV load carriers were analyzed at least twice during high EBV load periods to determine the stability of subpopulation frequencies.

RNA purification and real-time RT-PCR. RNA was extracted from 2×10^6 PBMCs, using a QIAamp RNeasy Mini kit (Qiagen). Viral mRNA expression was quantified by 1-step

Table 1. Characteristics of Chronic High Epstein-Barr Virus (EBV) Load Carriers

Patient (sex)	Age at LTx, years	Post-LTx follow-up, years	EBV serology ^a	Post-LTx time to initial EBV, ^b months	EBV-related symptoms	Maximum EBV load, copies/mL	Sustained high EBV load period, ^c months
1 (M)	0.5	0.8	R ⁻ /D ⁺	1.0	Elevated liver enzyme	1.0 × 10 ⁵	12 ^d
2 (M)	1.0	2.8	R ⁻ /D ⁺	1.5	...	1.6 × 10 ⁵	12
3 (F)	1.7	2.3	R ⁻ /D ⁺	1.2	Elevated liver enzyme	1.2 × 10 ⁵	29
4 (F)	0.6	3.5	R ⁻ /D ⁺	0.8	...	2.6 × 10 ⁵	24
5 (M)	0.6	4.3	R ⁻ /D ⁺	4.5	...	1.4 × 10 ⁵	8
6 (F)	1.4	3.8	R ⁻ /D ⁺	10.4	Fever, diarrhea	1.1 × 10 ⁵	36 ^d
7 (M)	5.3	1.2	R ⁻ /D ⁺	1.0	...	6.8 × 10 ⁴	15 ^d
8 (F)	1.6	5.0	R ⁻ /D ⁺	1.5	...	5.8 × 10 ⁴	14
9 (M)	1.2	6.0	R ⁻ /D ⁺	2.4	Cervical lymphadenopathy	3.7 × 10 ⁴	23
10 (M)	1.3	6.5	R ⁻ /D ⁺	2.9	Fever, diarrhea	1.1 × 10 ⁶	73 ^d
11 (F)	1.8	7.4	R ⁺ /D ⁺	53.2	Atypical lymphocytosis	1.6 × 10 ⁵	33

NOTE. LTx, liver transplant.

^a EBV serostatus of recipient/donor (R/D) at transplant (+, positive; -, negative).

^b Time when EBV DNA in whole blood was positive for the first time.

^c Continuous detection in whole blood of >5000 EBV DNA copies/mL.

^d Chronic high EBV load carrier state is ongoing without any symptoms.

multiplex real-time RT-PCR, using the Mx3000P real-time PCR system (Stratagene) as described elsewhere [16]. The stably expressed housekeeping gene β_2 -microglobulin (β_2M) was used as an endogenous control and reference gene for relative quantification [17]. The detection limits for EBNA1, EBNA2, LMP1, LMP2, EBER1, BARTs, BamHI Z leftward reading frame 1 (BZLF1), and gp350/220 were 10³, 10³, 10², 10², 10¹, 10², 10⁴, and 10⁴ EBV-positive LCL cells of 10⁶ EBV-negative BJAB cells, respectively. All chronic high EBV load carriers were analyzed at least twice over a 12-month period, and the results in all patients were similar.

Statistical analyses. Statistical analyses were conducted using StatView software (version 5.0; SAS Institute). The Fisher exact test or the Mann-Whitney *U* test was used for comparisons of 2 groups of patients. For comparisons of >3 groups, the Kruskal-Wallis test was used; if the result of the Kruskal-Wallis test was significant, the Tukey-Kramer test was used as a post hoc test. For the negative samples, the default value, defined as the lowest level of expression for a particular gene, was used for the calculation. The default values for the undetected genes were 10⁻⁸. Differences with *P* < .05 were deemed to be statistically significant.

RESULTS

Clinical characteristics and viral loads in peripheral blood from chronic high EBV load carriers. Characteristics of the 11 chronic high EBV load carriers are shown in Table 1. In six

patients, the onset of the high-load carrier state was preceded by EBV-related symptoms. These symptoms were transient and resolved after the reduction of immunosuppression in all patients. During the study period, the chronic high EBV load resolved without progression to PTLD in 7 (64%) of the 11 carriers, whereas the chronic high EBV load state continued with no symptoms in the other 4 carriers (36%). The median time to resolution in those whose high EBV load resolved was 23 months. Longitudinal analysis of EBV load in whole blood

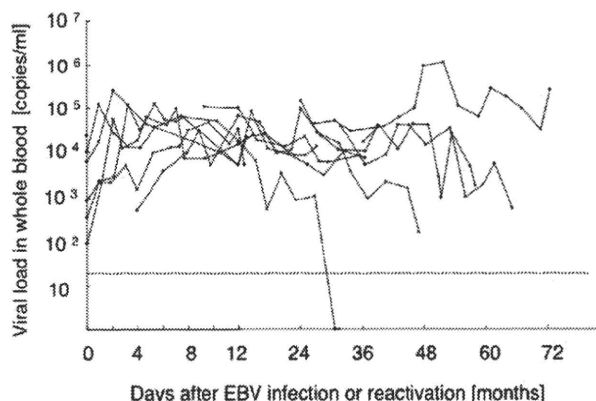


Figure 1. Time courses of Epstein-Barr virus (EBV) loads in whole blood from chronic high EBV load carriers. EBV load was serially measured by real-time polymerase chain reaction. Each line indicates the viral load of an individual patient. The dotted line indicates the detection limit.