CONCISE COMMUNICATIONS

# Immunologic Properties of Epstein-Barr Virus–Seronegative Adults

**W. J. Jabs, H. J. Wagner, P. Neustock, H. Klüter, and H. Kirchner**

*Institute of Immunology and Transfusion Medicine and Department of Pediatrics, University of Lübeck School of Medicine, Lübeck, Germany*

**Epstein-Barr virus (EBV) seronegativity is rare in people >20 years old. However, some persons remain EBV-seronegative for nearly their whole lives. The aim of this study was to examine properties of the immune system of EBV-seronegative adults that could contribute to long-term EBV seronegativity. Therefore, differential blood cell counts and lymphocyte subpopulations were determined, and the production of interferon (IFN)-$\alpha$ and -$\gamma$ and interleukin (IL)-6 and -2 in a whole blood assay was investigated. Whereas no differences in the distribution of lymphocyte subpopulations between EBV-seronegative and -positive adults were found, a significant higher percentage of monocytes in EBV-seronegative adults was observed. Significantly more IFN-$\alpha$ and IL-6 were detected in culture supernatants of EBV-seronegative persons after stimulation with Newcastle disease virus. In contrast, no differences in the induction of the lymphokines IFN-$\gamma$ and IL-2 were seen. These data suggest that faster and higher production of IFN-$\alpha$ and IL-6 may protect EBV-seronegative adults against EBV infection.**

The Epstein-Barr virus (EBV) is a widespread human herpesvirus with a tropism for B lymphocytes. Its potency of B cell immortalization into lymphoblastoid cell lines in vitro and its association with several malignancies, such as Burkitt's lymphoma, nasopharyngeal carcinoma, and B cell lymphoma in immunosuppressed persons is well documented (reviewed in [1]).

The virus is transmitted primarily by saliva and infects epithelial cells of the oropharynx [2]. From this initial site, the virus also infects circulating B lymphocytes through the complement receptor CR2 (CD21), specific for the C3d component [1]. After primary infection, the virus persists in the host for life. The defense and the immune surveillance of the virus includes specific, HLA-restricted T cell and NK cell cytotoxicity and neutralizing antibodies [1, 3]. Cytokines, such as interferon (IFN)-$\alpha$ and -$\gamma$ and interleukin (IL)-2 and -6, modulate the inflammatory response to the virus [4, 5]. Most persons are infected in early childhood and have no symptoms. By age 20, >90% of humans are seropositive for EBV, indicating former infection [6]. However, some individuals do not seroconvert for a long time [6], although EBV is a ubiquitous pathogen that is shed in the saliva of 90% of latently infected persons [2] and all patients with infectious mononucleosis, a self-limiting lymphoproliferative disorder of primary EBV infection [3].

Received 14 September 1995; revised 27 December 1995.

Reprints or correspondence: Dr. Wolfram J. Jabs, Institute of Immunology and Transfusion Medicine, University of Lübeck School of Medicine, Ratzeburger Allee 160, 23538 Lübeck, Germany.

**The Journal of Infectious Diseases 1996;173:1248–51**

0022–1899/96/7305–0026$01.00

The phenomenon of long-term EBV seronegativity can be explained by two hypotheses: One is that teenaged persons are more likely to date and kiss extensively than people >20 years old who are settling down socially. This could explain the low seroconversion rate after age 20 (lacking-exposure hypothesis). On the other hand, immunologic differences between EBV-seronegative and -seropositive persons, either innate or acquired, which provide resistance to the virus, are conceivable (intrinsic-resistance hypothesis). Although either explanation is plausible, we prefer the second.

To investigate this latter hypothesis, we examined immune parameters in EBV-seronegative adults that are thought to play a role in the defense of EBV [1, 3–5]. The monokine system was analyzed with special regard to IFN-$\alpha$ production and the T lymphokine system with its products IFN-$\gamma$ and IL-2.

## Materials and Methods

*Study and control population.* Blood samples were taken from 30 healthy volunteers (24 men, 6 women, 19–58 years old; median, 31), who were negative for IgG antibodies to EBV tested in an ELISA (Enzygnost Anti-EBV/IgG; Behring, Marburg, Germany). Additionally, EBV seronegativity of the study population was confirmed by standard indirect immunofluorescence technique (IFT) (Fresenius, Oberursel, Germany). Sera were screened for IgG antibodies to Epstein-Barr viral capsid antigen (EB-VCA), Epstein-Barr nuclear antigen (EBNA), and EBV early antigen (EBV-EA) as well as for IgM antibodies to EB-VCA. Persons were regarded to be EBV-seronegative with EB-VCA IgG <1:40, EB-VCA IgM <1:10, EBNA IgG <1:10, and EBV-EA <1:20.

Samples from 31 healthy blood donors (24 men, 7 women, 19–61 years old; median, 36 years) and from 26 blood donors (20 men, 6 women, 20–61 years old; median, 28) served as controls

for flow cytometry and the whole blood assays, respectively. Each person in the control population was EBV-seropositive by IFT (EB-VCA IgG ≥1:40, EB-VCA IgM <1:10, and EBNA IgG ≥1:10).

*Flow cytometry.* EDTA-anticoagulated blood samples were prepared at a Q-Prep Epics immunology work station (Coulter, Krefeld, Germany). The following lymphocyte subpopulations were determined by staining leukocytes with fluorescent monoclonal antibodies: T lymphocytes, B lymphocytes, T helper cells, cytotoxic T cells, and NK cells. The following antibodies were used: Mo2-RD1/KC56-FITC, CD3-ECD/T4-RD1/T8-FITC, CD3-ECD, CD16-FITC, NKH-1-RD1, and CD3(IgG1)-FITC/B4-RD1. As controls, MsIgG2b-ECD/MsIgG1-RD1/MsIgG1-FITC, MsIgG2a-RD1, and Ms IgG1-FITC were used (all antibodies were from Coulter). Flow cytometry of lymphocyte subpopulations was done with a Coulter EPICS XL. The differential blood cell count was measured on a Coulter MAX-M flow cytometer.

*Whole blood assay.* The whole blood assay is an effective method for analyzing the production of interferons and interleukins and provides an appropriate model of the in vivo situation. For mitogenic induction of IL-2 and IFN-$\gamma$, we used the plant lectin phytohemagglutinin (PHA; Burroughs Wellcome, Dartford, UK) in a final concentration of 5 $\mu$g/mL. For IL-6 and IFN-$\alpha$ stimulation, Newcastle disease virus (NDV, strain Kumarov; provided by R. Zawatzky, German Cancer Research Center, Heidelberg) in a final concentration of 1 hemagglutinating unit/mL was used. The whole blood assay was done as previously described [7]. Cell-free supernatants were harvested immediately after stimulation and on days 1, 2, 4, and 7 after induction. The samples were stored at −80°C until assayed.

*Cytokine determination.* Cytokine concentrations were analyzed using the following quantitative ELISAs: for IFN-$\alpha$, Cytoscreen Human IFN-$\alpha$ Immunoassay (BioSource International, Camarillo, CA); for IL-6 and IL-2, Quantikine Human IL-6 (or IL-2) Immunoassay (R&D Systems, Minneapolis); and for IFN-$\gamma$, TiterZyme Interferon-$\gamma$ Immunoassay (PerSeptive Diagnostic, Cambridge, MA).

*Statistical analysis.* Absolute numbers of leukocytes and data from the whole blood stimulation technique are expressed as mean ± SD; percentages of leukocytes and lymphocyte subpopulations are given as medians. Significance was determined by using the Mann-Whitney $U$ test. $P < .05$ was considered significant.

## Results

*Lymphocyte subpopulations and differential blood cell counts.* The distribution of the lymphocyte subpopulations as shown in table 1 showed no significant differences between the 2 study populations. With regard to the blood cell counts, we found a significantly higher percentage of monocytes in the peripheral blood of EBV-seronegative adults ($P < .03$); other leukocyte values were within normal ranges (table 1).

*Production of cytokines.* We investigated the production of IFN-$\alpha$ and IL-6 after stimulation with NDV over 7 days. The data from day 0 served as a negative control to rule out measurable cytokine levels in culture medium, stimulants, or venous blood. The kinetics of monokine release are depicted in figure 1. IFN-$\alpha$ production was higher in EBV-seronegative than -seropositive adults throughout the cultivation time. Significant differences in mean values of IFN-$\alpha$ were detected on days 1, 4, and 7 ($P < .01$, $P < .04$, and $P < .004$, respectively). Production of IL-6 increased during the incubation period. The increase of IL-6 was consistently higher in EBV-seronegative than -seropositive adults; differences were significant on days 2, 4, and 7 of cultivation ($P < .05$, $P < .003$, and $P < .001$, respectively).

Whereas monokines were stimulated by viral induction (NDV), lymphokine production was investigated after mitogenic induction (PHA). The release of IFN-$\gamma$ increased during the incubation period, whereas IL-2 release increased until day 2 and then decreased to the initial level. In contrast to the elevated levels of monokines found in culture supernatants of EBV-seronegative adults, production of the lymphokines IFN-$\gamma$ and IL-2 did not differ significantly between study groups.

## Discussion

Epidemiologic data from various regions of the world demonstrate a rapid increase in positive antibody titers to EBV during the first 2 decades of life. Later, EBV seroconversion occurs, but infrequently [6]. Thus, EBV seronegativity in adults is rare, although adults normally are in close contact with the ubiquitous virus.

A number of reasons for this phenomenon, such as a lack of the EBV receptor CD21 on B cells, hypogammaglobulinemia, or selective IgG deficiency, are conceivable and each of them, although unlikely, should be considered possible. Gervais et al. [8] reported a relative lack of EBV receptors on B cells of persistently EBV-seronegative adults. However, this result was true of only 0.5% of persistently EBV-seronegative adults and might therefore be of only minor importance in

**Table 1.** Differential blood cell counts and lymphocyte subpopulations of EBV-seronegative and -seropositive adults.

| | EBV-seronegative ($n$ = 30) | EBV-seropositive ($n$ = 31) |
|---|---|---|
| Differential blood cell counts | | |
| Leukocytes/$\mu$L | 5763 ± 1510 | 6348 ± 2117 |
| Monocytes (%) | 9.7* ± 2.5 | 8.0* ± 1.4 |
| Neutrophils (%) | 59.0 ± 8.1 | 59.8 ± 7.1 |
| Lymphocytes (%) | 27.7 ± 6.6 | 27.9 ± 6.6 |
| Lymphocyte subpopulation (%) | | |
| CD3 T cells | 75.0 ± 6.2 | 73.2 ± 5.0 |
| CD4 T cells | 47.4 ± 6.7 | 48.4 ± 8.3 |
| CD8 T cells | 23.3 ± 6.1 | 22.8 ± 7.7 |
| CD4:CD8 cell ratio | 2.05 ± 0.8 | 2.1 ± 1.3 |
| CD19 B cells | 14.6 ± 4.3 | 12.5 ± 3.7 |
| CD16, CD56, NK cells | 9.4 ± 4.7 | 8.0 ± 3.5 |

NOTE. Leukocyte values are mean ± SD; percentages are median ± SD.
* $P < .03$.

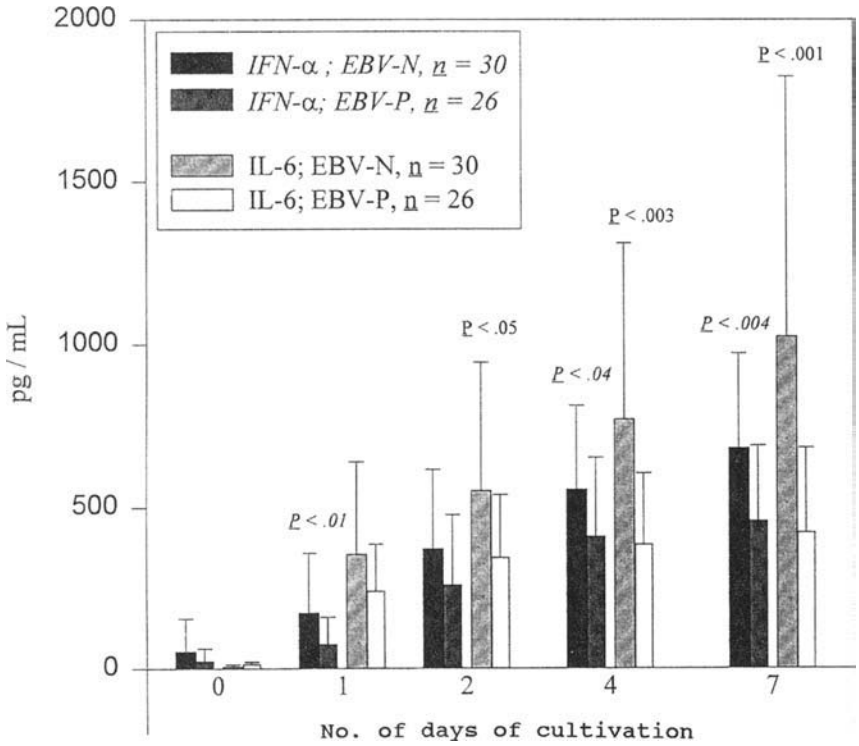


**Figure 1.** Monokine production in whole blood assay after stimulation with Newcastle disease virus (NDV); EBV–seronegative (EBV-N) vs. EBV-seropositive (EBV-P) adults; mean ± SD and *P* values are shown.

explaining EBV seronegativity. We determined serum immunoglobulin levels for all study participants. Since IgG (and subclasses), IgA, and IgM levels in EBV-seronegative adults were in the normal range, we excluded hypogammaglobulinemia or selective IgG deficiency as reasons for undetectable antibodies to EBV (data not published). Moreover, viral IL-10, a known monocyte inhibitory factor [9], could be considered a critical factor for slight immunosuppression in EBV-infected persons. Therefore, the differences shown between EBV-seronegative and -positive adults are perhaps a result of EBV infection rather than the cause of EBV seronegativity. Nevertheless, with support of our data, the most probable reason for long-term EBV seronegativity are interindividual immunologic differences, as described previously [10]. In that study, IFN-$\alpha$ production varied by donor (high and low IFN-$\alpha$ producers could be distinguished) and by age (young vs. elderly).

Lymphocyte subpopulations were not significantly different between study groups. However, we found a significantly higher percentage of monocytes in the peripheral blood of EBV-seronegative adults. Monocytes play a central role in the early host defense against viral infections by producing a number of proinflammatory cytokines, such as IFN-$\alpha$, IL-1$\beta$, and IL-6. The production of monokines was significantly higher in EBV-seronegative than in -seropositive adults. IFN-$\alpha$ production increased faster in EBV-seronegative than in EBV-seropositive persons, since we found significant differences in the release of IFN-$\alpha$ on day 1 after stimulation. This is consistent with data from Lotz et al. [5], who described an early release of IFN-$\alpha$ (within 24 h) after EBV infection in vitro.

IFN-$\alpha$ is a cytokine with antiproliferative and immunomodulatory activities and inhibits viral gene transcription and replication [11]. These effects were also shown for EBV: Thorley-Lawson [12] reported that in vitro transformation of lymphocytes by EBV is inhibited by human leukocyte IFN. Our results suggest that the increased release of monokines produced during confrontation with the virus may be an efficient mechanism for preventing infection with EBV in vivo.

It remains to be clarified how IFN-$\alpha$ mediates its effect on B cells to prevent them from EBV-induced immortalization in vitro. There is growing evidence that IFN-$\alpha$ not only inhibits B cell outgrowth due to EBV infection but even prevents infection with EBV itself [13, 14]. Delcayre et al. [15] described a sequence motif within the IFN-$\alpha$ molecule similar to the CD21 binding site on complement fragment C3d and demonstrated that IFN-$\alpha$ could bind to CD21. Further investigations showed that binding of IFN-$\alpha$ to CD21 inhibited capping of EBV/CD21 complexes after incubation of purified B lymphocytes with EBV. The internalization of virus-receptor complexes was subsequently inhibited [13]. These results reflected a new role for IFN-$\alpha$ in prevention of EBV infection in vitro.

The production of the lymphokines IFN-$\gamma$ and IL-2 was not elevated in EBV-seronegative adults. Since IFN-$\gamma$ and IL-2 are released mainly by activated T cells, our data suggest that the antigen-specific T cell response in EBV-seronegative adults is not increased.

In conclusion, this study shows that EBV-seronegative adults differ from -seropositive adults in some immunologic functions. Long-term EBV seronegativity is probably not due to

lack of exposure to EBV but might reflect increased monokine activity of the cellular immune system. The reason that most persons finally do seroconvert in response to EBV [6] still remains a mystery but could be explained by a slow decrease of IFN-$\alpha$ production over a lifetime [10] and other immunologic dysfunctions during aging. This might lead to impaired immunity against EBV and increased susceptibility to infection with EBV.

**References**


1. Miller G. Epstein-Barr virus: physiology, pathogenesis, and medical aspects. In: Fields BN, ed. Virology. New York: Raven Press, **1990**:1921–58.
2. Yao QY, Rickinson AB, Epstein MA. A reexamination of Epstein-Barr virus carrier state in healthy seropositive individuals. Int J Cancer **1985**;35:35–42.
3. Schuster V, Kreth HW. Epstein-Barr virus infection and associated diseases in children. Eur J Pediatr **1992**;151:718–25.
4. Foss HD, Herbst H, Hummel M, et al. Patterns of cytokine gene expression in infectious mononucleosis. Blood **1994**;83:707–12.
5. Lotz M, Tsoukas CD, Fong S, Dinarello CA, Carson DA, Vaughan JH. Release of lymphokines after Epstein-Barr virus infection in vitro. I. Sources and kinetics of production of interferons and interleukins in normal humans. J Immunol **1986**;136:3636–42.
6. de-Thé G, Day NE, Geser A, et al. Seroepidemiology of the Epstein-Barr virus: preliminary analysis of an international study—a review. In: de-Thé G, Epstein MA, zur Hausen H, eds. Oncogenesis and herpesviruses II. Lyon, France: IARC Scientific Publication, **1975**;11:3–18.
7. Kirchner H, Kleinicke C, Digel W. A whole blood technique for testing production of human interferon by leukocytes. J Immunol Methods **1982**;48:213–9.
8. Gervais F, Willis A, Leyritz M, Lebrun A, Joncas JH. Relative lack of Epstein-Barr virus (EBV) receptors on B cells from persistently EBV seronegative adults. J Immunol **1981**;126:897–900.
9. O'Garra A, de-Waal-Malefyt R, Vieira P, Mosmann TR. Interleukin-10. Annu Rev Immunol **1993**;11:165–90.
10. Katschinski DM, Neustock P, Klüter H, Kirchner H. Influence of various factors on interferon-$\alpha$ production in cultures of human lymphocytes. 1. J Interferon Res **1994**;14:105–10.
11. Kirchner H. The interferon system as an integral part of the defense system against infections. Antiviral Res **1986**;6:1–17.
12. Thorley-Lawson DA. The transformation of adult but not newborn human lymphocytes by Epstein-Barr virus and phytohemagglutinin is inhibited by interferon: the early suppression by T cells of Epstein-Barr virus infection is mediated by interferon. J Immunol **1981**;126:829–33.
13. Delcayre AX, Lotz M, Lernhardt W. Inhibition of Epstein-Barr virus–mediated capping of CD21/CR2 by alpha interferon (IFN-$\alpha$): immediate antiviral activity of IFN-$\alpha$ during the early phase of infection. J Virol **1993**;67:2918–21.
14. Kure S, Tada K, Wada J, Yoshie O. Inhibition of Epstein-Barr virus infection in vitro by recombinant human interferon alpha and gamma. Virus Res **1986**;5:377–88.
15. Delcayre AX, Salas F, Mathur S, Kovats K, Lotz M, Lernhardt W. Epstein-Barr virus/complement C3d receptor is an interferon $\alpha$ receptor. EMBO J **1991**;10:919–26.