Maintenance of Serum Immunoglobulin G Antibodies to Epstein-Barr Virus (EBV) Nuclear Antigen 2 in Healthy Individuals from Different Age Groups in a Japanese Population with a High Childhood Incidence of Asymptomatic Primary EBV Infection

Shizuko Harada,1 Yoshio Kamata,1 Yasuyuki Ishii,1 Hiroyuki Eda, Ryo Kitamura,1 Maya Obayashi,1 Sayuri Ito,1 Fumihiro Ban,1 Jun Kuranari,1 Haruhiko Nakajima,1 Tomoko Kuze,1 Masao Hayashi,1 Nobuhiko Okabe,2,3 Hidenobu Senpuku,4 Nobuyuki Miyasaka,5 Yoshiko Nakamura,6 Hirokazu Kanegane,7 and Kazuo Yanagi1*

Herpesvirus Laboratory, Department of Virology I,1 Infectious Disease Surveillance Center,2 and Department of Bacteriology I,4 National Institute of Infectious Diseases, Toyama 1-23-1, Shinjuku-ku, Tokyo 162-8640, Department of Pediatrics, Kanagawa Prefecture Nursing School Hospital, Yokohama City, Kanagawa 235-0022,3 Department of Autoimmune Diseases and Rheumatology, School of Medicine, Tokyo Medical and Dental University, Yushima 1-5-45, Bunkyo-ku, Tokyo 113-0034,5 Department of Pathology, Showa Medical School Fujigaoka Hospital, Fujigaoka 1-30, Aoba-ku, Yokohama City, Kanagawa 227-0043,6 and Department of Pediatrics, School of Medicine, Toyama Medical and Pharmaceutical University, Sugitani 2630, Toyama City, Toyama 930-0152,7 Japan

Received 24 July 2003/Returned for modification 16 October 2003/Accepted 7 November 2003

Immunoglobulin G (IgG) antibodies to Epstein-Barr virus (EBV) nuclear antigens 2 and 1 (EBNA-2 and EBNA-1, respectively) were studied using sera from healthy individuals of a population with a high incidence of asymptomatic primary EBV infections during infancy or childhood in Japan. Two CHO-K1 cell lines expressing EBNA-2 and EBNA-1 were used for complement and indirect immunofluorescence assays. The positivity rate for EBNA-2 IgG rose in the 1- to 2-year age group, increased and remained at a plateau (~45%) between 3 and 29 years of age (3- to 4-, 5- to 9-, 10- to 14-, and 15- to 29-year age groups), and then reached 98% by age 40 (~≥40-year age group). Both seropositivity for EBNA-1 and seropositivity for EBNA2 in Raji cells (EBNA/Raji) were detected in the 1- to 2-year age group, remained high, and finally reached 100% by age 40. The geometric mean titer (GMT) of EBNA-2 IgG reached a plateau in the 5- to 9- and 10- to 14-year-old groups and remained elevated in the older age groups (15 to 29 and ≥40 years). The GMT of EBNA-1 IgGs increased to a plateau in the 1- to 2-year-old group and remained unchanged in the older age groups. The GMT of EBNA/Raji IgGs also reached a plateau in the 1- to 2-year-old group, remained level throughout the 3- to 14-year age groups, and decreased in the 15- to 29-year-olds. EBNA-2 IgGs emerged earlier than EBNA-1 IgGs in 8 of 10 patients with infectious mononucleosis, who were between 1 and 27 years old, and declined with time in three of eight cases. These results suggest that EBNA-2 IgG antibodies evoked in young children by asymptomatic primary EBV infections remain elevated throughout life, probably because of reactivation of latent and/or exogenous EBV superinfection.

Primary infection of adolescents and young adults with Epstein-Barr virus (EBV) causes infectious mononucleosis (IM) (17, 18, 21, 38). A characteristic serologic feature of IM is a delayed antibody response to the EBV nuclear antigens (EBNAs), which are complexes of six distinct proteins (19, 20, 25, 38). The different time patterns of immunoglobulin G (IgG) antibody responses to EBNA-1 and EBNA-2 are useful for serodiagnosis of IM (19, 41). EBNA-1 is essential for maintenance of latent EBV plasmid DNA in latently infected cells (24, 49, 50), and EBNA-2 is needed for growth transformation of infected cells (14, 25). Analyses of IgG antibodies to EBV viral capsid antigen (VCA) (16, 18) show that the prevalence of EBV varies with both geography and socioeconomic level. In Europe, IM is common in adolescents and young adults. IM is also common in the United States in affluent socioeconomic groups, which have a low prevalence of EBV. IM is uncommon in populations in which EBV is prevalent (3, 15, 16, 38).

Primary infections of infants with EBV are usually asymptomatic (3, 8, 23, 38). Spread within families is thought to be a common route of EBV transmission (10). Antibody responses to EBNA in EBV primary infections of infants in Ghana (3) and infants presenting with minor complaints in the United States (8) are similar to those in subjects with IM. However, antibody responses to EBNA-2 and EBNA-1 in asymptomatic primary infections have been studied only for the unusual situation of primary EBV infection in newborns of mothers infected with human immunodeficiency virus (34). We hypothesized that analyses of sera from different age groups of a population undergoing asymptomatic infection during infancy and young childhood would provide information about the long-term antibody responses to EBNA-2 and...
EBNA-1 following asymptomatic EBV infection. The population that would be most suitable for the analyses would be that with a majority of asymptomatic, primary infections of infants and young children. Such a study could also improve the serodiagnosis of EBV infections in a population with a high EBV prevalence. We have generated EBNA-2- and EBNA-1-expressing CHO-K1 cell lines to distinguish and analyze the antibody responses to EBNA-2 and EBNA-1 by immunofluorescence assays. Detection of antibodies to EBNAs expressed in Raji cells (referred to as EBNA/Raji) by anticomplement immunofluorescence (ACIF) is widely used as a standard test for serodiagnosis (7, 37). The characteristics of long-term antibody responses to EBNA-2, EBNA-1, and EBNA/Raji in individuals with asymptomatic primary EBV infections are poorly defined. In Japan, the incidence of EBV infection in infants is high, but IM is uncommon (22). The characteristic epidemiology of EBV in Japan prompted us to investigate the age-related distribution pattern of IgG antibodies to EBNA-2, EBNA-1, and EBNA/Raji in the general population. IgG antibody responses to EBNA-2 and EBNA-1 in Japanese patients with IM are also reported.

MATERIALS AND METHODS

Vectors and cells. The DNA clone pDF225 with the BamHI K fragment from the genomic DNA of the EBV strain B95-8 at the BamHI site of pBR322 (6) was kindly provided by E. Kieff (Harvard University). The DNA clone P5 with the BamHI C-W-Y-H-F fragment from the genomic B95-8 DNA at the BamHI site of cosmID pHC79 (13) was kindly provided by B. E. Griffin (Imperial Cancer Research Fund, London, England). The vector pSV2gpt (32), a pSV2-derived vector that contains an Ecosgp gene segment downstream from the simian virus 40 (SV40) early transcription promoter and upstream from a DNA segment that is necessary for splicing and polyadenylation, was kindly provided by K. Oda (The University of Tokyo, Tokyo, Japan). pCRKRE2 (31) was from Y. Morimoto (Mitsubishi Chemical, Tokyo, Japan). Both pSV2gpt and pCRKRE2 have the SV40 early promoter. The EBNA-2-expressing vector pCSVE2 was made by subcloning the AccII-DraI fragment from the DNA clone P5 under the control of the SV40 early promoter in pCRKRE2. The EBNA-1-expressing vector pSVEBK was constructed by inserting the BamHI K DNA fragment under the control of the SV40 early promoter in pSV2gpt. The vector pEA2gpt, which contains a subfragment of the EcoRI A fragment of B95-8 EBV DNA (40), was kindly provided by G. Klein (Karolinska Institute, Stockholm, Sweden). The Chinese hamster-derived epithelial cell-like CHO-K1 cell line (36) and the Burkitt lymphoma-derived Raji cell line expressing EBNA (33) were obtained from the American Type Culture Collection (Manassas, Va.) through the Japanese Cancer Research Resources Bank (Tokyo, Japan). Immortalized lymphoblastoid cell (LCL) lines were generated in our laboratory by in vitro infection of human cord blood lymphocytes with the EBV strain B95-8. CHO-K1 cells were cultured in Ham’s F-12 medium (GIBCO) supplemented with 10% fetal bovine serum (Flow Laboratories, McLean, Va.). Both Raji and LCL lines were cultured in RPMI 1640 medium (Nissui, Tokyo, Japan) supplemented with 10% fetal bovine serum at 37°C in a CO2 incubator. Transfection was done as previously described (14). Cells were cotransfected with the EBNA-expressing vectors and pSV2neo at the molecular ratio 10:1 and cultured in the presence of 400 μg of G418 (GIBCO)/ml for selection of drug-resistant cells.

Western blotting and immunofluorescence assays. Western blotting and immunofluorescence assays have been described previously (14). For both ACIF and indirect immunofluorescence, cells were grown in either microslide culture chambers (Belco, Vineland, N.J.) or 12-well 5-mm HTGC glass plates (Erne Scientific, Portsmouth, N.H.) and fixed with cold acetone-methanol (1:1). The human serum KM, which is positive for EBNA, was used as the positive-control primary antiserum. The fluorescein isothiocyanate (FITC)-conjugated rabbit anti-human C3c complement polyclonal antibody (DAKO, Glostrup, Denmark) was used for ACIF. FITC-conjugated F(ab)2 of goat IgG specific for human IgG (MBL, Nagoya, Japan) was used for indirect immunofluorescence.

Serum. A total of 139 sera were collected between 1988 and 1993 from healthy volunteers in the Tokyo, Yokohama, and Chiba metropolitan areas. Upon questioning, the volunteers or their parents or guardians responded negatively about a history of IM or IM-like disorders. Informed consent to the use of the sera in this study was obtained from these volunteers or their parents or guardians. The numbers of individuals in each age group were 16 (1- to 2-year-old group), 22 (3- to 4-year-old group), 19 (5- to 8-year-old group), 21 (10- to 13-year-old group), 43 (14- to 19-year-old group), and 75 (≥40-year-old group). Sequential sera from 10 patients with IM who were between the ages of 1 and 27 years and hospitalized in the Showa University Fujigakoa Hospital (Yokohama City, Kanagawa, Japan) and in the Kanazawa University Hospital (Kanazawa City, Ishikawa, Japan) were used to analyze patterns of antibody responses to EBNA proteins after symptomatic EBV IM. Informed consent was given by the patients or their parents. Titers of IgGs to early antigen (EA) were analyzed by indirect immunofluorescence assays with acetone-fixed Raji cells that were infected with the EBV strain P3HR-1 in the presence of cyclosporin arabinoside. IgG titers to VCA were quantified by indirect immunofluorescence with the cell line P3HR-1. All sera had been stored at −20°C.

Measurement of IgG antibody titers. Titers of IgG antibodies to EBNA-1, EBNA-2, and EBNA/Raji were measured by indirect immunofluorescence assays. Serum specimens from volunteers were first diluted fivefold and then serially diluted twofold for endpoint determination of titers.

Statistical analyses. Statistical tests for significant differences in seropositive rates among various age groups were performed by the χ2 test. Statistical significance tests of differences in geometric mean titers (GMTs) of IgGs between various age groups were performed by the t test.

RESULTS

Expression of full-length EBNA-1 and EBNA-2 proteins in CHO-K1 cells. To titrate the antibodies to EBNA-2 and EBNA-1 by immunofluorescence assays, we constructed EBNA-2- and EBNA-1-expressing cells by transfecting non-EBV-infected cells with the EBNA-2- and EBNA-1-expressing vectors. The CHO-K1, COS-1, and BHK-21 cell lines all expressed EBNA-1 equally well when analyzed by both ACIF and Western blotting (data not shown). The expression of EBNA-2 in the cell line CEKNC1 and of EBNA-1 in the cell line CEW21 was examined by either indirect immunofluorescence or ACIF (Fig. 1). In addition, the sizes of the expressed EBNA-2 and EBNA-1 polypeptides were determined by Western blot analysis. The molecular masses of 84 kDa for EBNA-2 and 78 kDa for EBNA-1 were consistent with the same proteins produced by EBV B95-8-transformed lymphoblastoid B-cell lines (Fig. 1). The CHO-K1 cell line CEKNC expressed EBNA-2 at least as well as, and possibly even better than, the CHO-K1 cells that were transfected with pEAA2gpt or P5 (data not shown). The expression levels of EBNA-1 in the cell line CEW21, detected by both indirect immunofluorescence and Western blot assays, were similar to the EBNA-1 expression levels after transfection of the human B-cell line DG75 with the vector pC43, which carries the BamHI-PvuII subfragment with the left half of BamHI K (14) (data not shown). The EBNA-1 expression in CEW21 was stable until the cells were passaged 30 times. Cells passaged 40 times had declining levels of EBNA-1. In the round lymphoid cells, it is sometimes difficult to visually distinguish nuclear from cytoplasmic fluorescence. However, the round nuclei of the epithelial cell-like CHO-K1 cells could be readily differentiated from the cytoplasm. In addition, the EBNA-1 antigens in the fixed cells were stable, as indicated by the undiminished immunofluorescence of the acetone-methanol-fixed cells after incubation at 37°C for 1 week (data not shown). The properties of the EBNA-2- and EBNA-1-expressing CHO-K1 cell lines made them useful for immunofluorescence assay of EBNA IgGs. Thus, EBNA-1-positive and -negative sera were easily distinguished by indirect immunofluorescence assay as well as ACIF. The growth rates of the
cell lines expressing EBNA-1 or EBNA-2 in the medium containing 0.5% fetal bovine serum were the same as that of the untransfected CHO-K1 cells (data not shown).

Relationship of serum positivity for EBNA IgG antibodies to age in a population with high EBV prevalence. Serum positivity for IgG antibodies to EBNA-2, EBNA-1, and EBNA/Raji in healthy individuals of different ages from a Japanese population with a high EBV prevalence (22) was determined by titration by the indirect immunofluorescence assays. The distribution of titers of all the sera is depicted in Fig. 4, and sera with an IgG antibody titer of $\geq 1:5$ were counted as positive. The positive rates for EBNA-2 IgG antibodies in the different age groups were 19% for the 1- to 2-year-olds, 23% for the 3- to 4-year-olds, 42% for the 5- to 9-year-olds, 48% for the 10- to 14-year-olds, 64% for the 3- to 4-year-olds, 68% for the 5- to 9-year-olds, 81% for the 10- to 14-year-olds, 79% for the 15- to 29-year-olds, and 100% for the $\geq 40$-year-olds. The positive rates for EBNA-2 IgG antibodies were 19% for the 1- to 2-year-olds, 23% for the 3- to 4-year-olds, 42% for the 5- to 9-year-olds, 48% for the 10- to 14-year-olds, 64% for the 3- to 4-year-olds, 68% for the 5- to 9-year-olds, 81% for the 10- to 14-year-olds, 79% for the 15- to 29-year-olds, and 100% for the $\geq 40$-year-olds (Fig. 2A). Sera positive for IgG antibodies to EBNA-2 were detected in the 1- to 2-year-old group; the percentages of positive sera increased from 19 to 23% of the 1- to 2- and 3- to 4-year age groups to 42 to 48% of the 5- to 9-year and 10- to 14-year age groups ($P = 0.045$), were maintained at a plateau of 42 to 48% between 5 and 29 years (5- to 9-, 10- to 14-, and 15- to 29-year-olds), and then increased to 98% in the $\geq 40$-year age group (Fig. 2). The frequency of EBNA-2 antibody-positive sera in the group of subjects $\geq 40$ years of age was significantly different ($P < 0.0001$) from that in the 15- to 29-year-old group. The percentages of sera positive for EBNA-2 IgG antibodies were significantly lower than those of sera positive for EBNA-1 and EBNA/Raji between 1 and 29 years (1- to 2-, 3- to 4-, 5- to 9-, 10- to 14-, and 15- to 29-year-olds) (Fig. 2) and showed a plateau over the 5- to 9-, 10- to 14-, and 15- to 29-year age groups. The percentage of sera positive for EBNA-1 IgG rose in the 1- to 2-year-old group, was similar between 3 and 29 years (3- to 4-, 5- to 9-, 10- to 14-, and 15- to 29-year-olds), and then increased from a frequency of 81% in the 15- to 29-year-old group to 100% in those over 40 years. The frequency of EBNA-1 IgG antibody-positive sera in the $\geq 40$-year-old group was significantly different ($P = 0.0005$) from that in the 15- to 29-year-old group. The percentages of sera positive for IgG antibodies to EBNA/Raji were similar to those sera positive for IgG antibodies to EBNA-1 in all the age groups and also increased to 100% by age 40. The frequency of EBNA/Raji antibody-positive sera in

![FIG. 1. Expression of EBNA-2 and EBNA-1 in CHO-K1 cells. (A) Immunoblots of extracts from the EBNA-2-expressing CEKNC1 cell line and the EBNA-1-expressing CEW21 cell line. Lane 1, CHO-K1 cells transfected with the vector pKCRH2 as a negative control; lane 2, EBNA-2 proteins expressed in the CEKNC1 cells; lane 3, bands of EBNA-2 (arrowhead) and EBNA-1 (arrow) proteins in lymphoblastoid B-cell line LCL-C4; lanes 4 and 5, EBNA-1 proteins in two clones of the CEW21 cell line (arrow). (B) Immunofluorescence of the CEKNC1 and CEW21 cell lines. (a) ACIF of the EBNA-2-expressing CEKNC1 cells with an EBNA-positive human serum and FITC-conjugated rabbit anti-human C3c complement polyclonal antibody; (b) indirect immunofluorescence of the EBNA-1-expressing CEW21 cells with an EBNA-positive human serum and FITC-conjugated goat anti-human IgG polyclonal antibody; (c) ACIF of Raji cells.](image1)

![FIG. 2. Age distribution of percentages of sera positive for IgG antibodies to EBNA-2 (A), EBNA-1 (B), and EBNA/Raji (C). The vertical lines above the bars indicate 95% confidence intervals.](image2)
the group ≥40 years of age was significantly different (P = 0.0002) from that in the 15- to 29-year-old group.

**GMTs of IgGs to EBNA-1, EBNA-2, and EBNA/Raji in the healthy population according to age.** The GMT of EBNA-2 IgGs increased from 0.53 to 0.56 in the group of 1- to 4-year-olds (1- to 2- and 3- to 4-year-old groups) to a plateau (0.81 to 0.91) in the 5- to 29-year-old group (5- to 9- and 10- to 14-year-old groups). The increase in GMT between the 1- to 4-year-old group and the 4- to 14-year-old group is statistically significant (P < 0.0001) (Fig. 3A). The GMT of EBNA-1 IgGs was 1.51 in the 1- to 2-year-old group and remained almost unchanged in all other age groups (2.08 for 3- to 4-year-olds, 1.78 for 5- to 9-year-olds, 1.99 for 10- to 14-year-olds, 1.78 for 15- to 29-year-olds, and 1.77 for ≥40 year-olds) (Fig. 3B). The IgG GMT ratios of EBNA-2 to EBNA-1 by age are also shown (Fig. 3D). These results indicate that the EBNA-2 IgGs peaked later than the EBNA-1 IgGs and remained elevated in all age groups, especially in the group ≥40 years old. The GMT of IgGs to EBNA/Raji was at a plateau throughout the 1- to 14-year-old groups (1.35 for 1- to 2-year-olds, 1.62 for 3- to 4-year-olds, 1.57 for 5- to 9-year-olds, and 1.90 for 10- to 14-year-olds), as was the GMT of EBNA-1 IgGs, but decreased to 1.47 to 1.56 in the older age groups (15- to 29- and 40-year-old groups). The decrease in GMT from 1.90 (10- to 14-year-old group) to 1.47 (15- to 29-year-old group) is statistically significant (P = 0.008) (Fig. 3C). These data suggest that the titers of IgGs to EBNA/Raji are not representative of the titers of IgGs to EBNA-1 or EBNA-2 but reflect the titers to other EBNAs, namely, EBNA-3A, EBNA-3B, EBNA-3C, and EBNA-LP (25), in some age groups.

**Age distribution of ratios of anti-EBNA-2 to anti-EBNA-1, anti-EBNA-2 to anti-EBNA/Raji, and anti-EBNA-1 to anti-EBNA/Raji.** The titer ratios of EBNA-2 to EBNA-1 IgGs, EBNA-2 IgGs to EBNA/Raji IgGs, and EBNA-1 IgGs to EBNA/Raji IgGs in individual serum samples are shown in Fig. 4. Twenty-eight percent of sera (21 of 75 sera) had EBNA-2/EBNA-1 ratios of ≥1.0 in the ≥40-year-old group (Fig. 4A). Forty-six percent of sera (34 of 74 sera) had ratios of EBNA-2 to EBNA/Raji of ≥1.0 in the ≥40-year age group (Fig. 4B). In contrast, 10% of the sera (4 of 39 sera) had titer ratios of EBNA-2 to EBNA-1 of ≥1.0 and 20% of the sera (8 of 41 sera) had titer ratios of EBNA-2 to EBNA/Raji that were ≥1.0 in the age group under 30 years, when ratios were calculated with the exclusion of individual serum samples that were negative for both EBNA-2 and EBNA-1 (Fig. 4Aa to Ae) or for both EBNA-2 and EBNA/Raji (Fig. 4Ba to Be).

The IgG titer correlation coefficient (r) between EBNA-1 and EBNA/Raji was 0.75 (Fig. 4Ci), in contrast to the low correlation coefficients of IgG titers between EBNA-2 and EBNA-1 (0.33) (Fig. 4Af) and between EBNA-2 and EBNA/Raji (0.38) (Fig. 4Bf) in the ≥40-year age group (Fig. 4). The high correlation coefficient of IgG titers between EBNA-1 and EBNA/Raji IgGs in the healthy adult group agrees with the previous reports for European study populations (28).

**Response patterns of IgG antibodies to EBNA-1, EBNA-2, and EBNA/Raji in IM.** The time patterns of IgG antibody responses to EBV antigens in U.S. and European IM patients with symptomatic primary EBV infection have been described previously (19, 38). We analyzed serial sera from 10 Japanese individuals with IM, 5 of whom (cases 1, 5, 6, 8, and 9) were under the age of 8 years, to examine whether similar trends were occurring in Japan. This information is relevant to the interpretation of the experimental results of this study. In 8 of 10 cases (cases 1 to 8) of IM, IgG antibodies to EBNA-2 rose earlier than those to EBNA-1. In three of eight cases (cases 1 to 3), EBNA-2 IgGs declined over time to low or undetectable levels (Table 1). IgG antibodies to EBNA-2 were detected after both EBNA-1 and EBNA/Raji IgG antibodies in 2 of 10 cases (cases 9 and 10). EBNA/Raji IgGs were detected earlier

![FIG. 3. (A to C) Age distribution of GMTs of IgGs to EBNA-2 (A), EBNA-1 (B), and EBNA/Raji (C). The vertical lines on GMT graphs indicate 95% confidence intervals. (D) Ratios of IgG GMTs to EBNA-2 and EBNA-1.](image-url)
FIG. 4. Relationships between antibody titers to EBNA-2, EBNA-1, and EBNA/Raji in different age groups. Each dot represents a titer of serum from a single subject. (A) The anti-EBNA-2 titer is on the ordinate, and the anti-EBNA-1 titer is on the abscissa. (B) The anti-EBNA-2 titer is on the ordinate, and the anti-EBNA/Raji titer is on the abscissa. Subpanels a to f in panels A and B show age groups in years. (C) The anti-EBNA-1 titer is on the ordinate, and the anti-EBNA/Raji titer is on the abscissa. (c to e) 5- to 29-year-old group; (f) ≥40-year-old group. The correlation coefficient (r) is shown for panels A, B, and C.
than EBNA-1 IgGs in four cases (cases 5 to 8), suggesting that IgG antibody to EBNA/Raji was representative of the response of IgGs to EBNA-2. These patterns of antibody responses of Japanese patients including children with IM are similar to those reported for American patients (19).

### DISCUSSION

While the antibody response to the VCA in various geographical regions has been studied in healthy age groups (1, 16, 21, 22, 27, 29, 44), there was little information about the long-term, age-related antibody responses to EBNA/Raji prior to this study. A prospective experimental design could be used to gather such information. However, the high incidence of asymptomatic primary EBV infections during infancy or early childhood and the resulting low incidence of IM in Japan made it feasible to analyze both the long-term and age-related serum antibody responses to EBNA proteins following the asymptomatic primary EBV infections, by a cross-sectional analysis of well-defined age groups.

To ensure that the post-IM antibody responses in Japan were comparable to those that have been described in other

### TABLE 1. Antibody responses to EBNA-1, EBNA-2, and EBNA/Raji in patients with IM

| Case | Age and sex | Interval of serum sample (days) | Titer |
|------|-------------|---------------------------------|-------|
|      |             |                                 | EBNA-2 IgG | EBNA-1 IgG | EBNA/Raji IgG | VCA IgG | VCA IgM | EA IgG |
| 1    | 1, M        | 0                               | 5       | <5       | <5       | 320     | <10     | <10    |
|      |             | 56                              | 10      | <5       | <5       | 640     | <10     | 80     |
|      |             | 67                              | 10      | <5       | 5        | 320     | <10     | 80     |
|      |             | 96                              | 5       | 5        | 5        | 320     | <10     | 40     |
|      |             | 138                             | 5       | 5        | 5        | 320     | <10     | 40     |
| 2    | 21, F       | 0                               | <5      | <5       | <5       | 640     | 80      | ND     |
|      |             | 4                               | 20      | <5       | <5       | 320     | 40      | ND     |
|      |             | 125                             | 5       | 40       | 10       | 320     | <10     | ND     |
| 3    | 24, F       | 0                               | 20      | <5       | <5       | 1,280   | 40      | ND     |
|      |             | 33                              | 10      | <5       | <5       | 1,280   | <10     | 320    |
|      |             | 163                             | <5      | 5        | 10       | 640     | <10     | 40     |
| 4    | 27, M       | 0                               | <10     | <10      | <10      | 1,280   | 80      | 40     |
|      |             | 78                              | 10      | <10      | <10      | 640     | <10     | 20     |
| 5    | 8, F        | 0                               | <5      | <5       | <5       | 320     | 160     | ND     |
|      |             | 1                               | <5      | <5       | <5       | 640     | 80      | ND     |
|      |             | 3                               | <5      | <5       | <5       | 1,280   | 80      | ND     |
|      |             | 15                              | <5      | <5       | <5       | 160     | 20      | ND     |
|      |             | 50                              | <5      | <5       | <5       | 160     | 10      | ND     |
|      |             | 113                             | 10      | 10       | 10       | 320     | <10     | ND     |
| 6    | 4, F        | 0                               | <5      | <5       | <5       | 80      | <10     | ND     |
|      |             | 35                              | <5      | 5        | 10       | 640     | <10     | ND     |
|      |             | 98                              | 20      | <5       | 20       | 640     | <10     | ND     |
| 7    | 21, F       | 0                               | <5      | <5       | <5       | 10,240  | 160     | ND     |
|      |             | 20                              | <5      | <5       | <5       | 2,560   | 320     | 20     |
|      |             | 48                              | <5      | <5       | 10       | 2,560   | 160     | 10     |
|      |             | 89                              | <5      | 10       | 10       | 320     | <10     | 40     |
| 8    | 3, M        | 0                               | 5       | <5       | 5        | 5,120   | 160     | ND     |
|      |             | 11                              | 5       | <5       | 5        | 2,560   | 40      | ND     |
| 9    | 5, F        | 0                               | <5      | <5       | <5       | 320     | 20      | <10    |
|      |             | 19                              | <5      | <5       | <5       | 160     | 20      | 20     |
|      |             | 51                              | <5      | <5       | <5       | 320     | <10     | 20     |
|      |             | 107                             | <5      | <5       | <5       | 320     | <10     | 40     |
|      |             | 135                             | <5      | 5        | 5        | 320     | <10     | 40     |
|      |             | 156                             | <5      | 10       | <5       | 320     | <10     | 20     |
|      |             | 198                             | <5      | 20       | 20       | 320     | <10     | 20     |
| 10   | 19, F       | 0                               | <5      | <5       | <5       | 640     | 40      | ND     |
|      |             | 70                              | <5      | <5       | <5       | 320     | <10     | 10     |
|      |             | 133                             | <5      | <5       | <5       | 160     | <10     | 20     |
|      |             | 245                             | <5      | 40       | 20       | 160     | <10     | 20     |

* a M, male; F, female. Age is in years.
* b ND, not done.
geographic areas, we also analyzed sequential sera from Japanese patients with IM. The early appearance of EBNA-2 IgGs followed by a later EBNA-1 response in Japanese IM patients is consistent with the results in American cases of IM (19).

Our data show that the IgG responses to EBNA-2 and EBNA-1 after the asymptomatic EBV infection have specific characteristics. First, the IgG antibody response to EBNA-2 does not necessarily occur in all of the individuals who undergo asymptomatic primary infection in childhood and may be transient in some individuals. Data showing that the percentages of EBNA-2-positive sera were approximately half (35 to 60%) of those of EBNA-1 and remained at a plateau between 5 and 29 years of age (the 5- to 9-, 10- to 14-, and 15- to 29-year-old groups) support this conclusion. Second, serum IgG positivity for EBNA-2 seems to be acquired between 30 and 39 years in almost all the individuals who are negative for EBNA-2 IgGs. Specifically, the percentage of EBNA-2 IgG-positive sera increases from the plateau of 42 to 48% positivity between 5 and 29 years (the 5- to 9-, 10- to 14-, and 15- to 29-year-old groups) to 98% in the group of ≥40 years. The increases in the percentages of sera positive for EBNA-2 IgG cannot be explained solely by a primary EBV infection because 80% of individuals were already positive for IgGs to EBNA-1 and to EBNA/Raji by the age of 15. Third, the GMTs of EBNA-2 IgA antibodies did not decline in adult age groups, as might be expected from the study of patients with IM (19), but remained elevated throughout the age groups older than 10 years (the 10- to 14-, 15- to 29-, and ≥40-year-old groups) in the population that consists mostly of those with asymptomatic EBV infection during childhood. Furthermore, the number of individual serum samples that have EBNA-2 to EBNA-1 IgG antibody titer ratios of >1.0 increases in the ≥40-year age group.

Fundamentally different immunologic events may be the cause of the differences between antibody responses of children with asymptomatic primary EBV infections and those of young adults with symptomatic primary infections. EBNA-2 IgG antibodies may remain elevated throughout life in approximately half of individuals following asymptomatic primary EBV infections. This interpretation is consistent with the reports that (i) antibody responses to the restricted (EA-R) and diffused (EA-D) components of EBV EAs in primary EBV infections of infants with minor clinical manifestations are different from those in IM (8) and (ii) the serologic responses to VCA and EA are different between infants and children (aged 10 to 48 months) with IM (23).

An alternate explanation, based on the assumption that the IgG response patterns to EBNA-2 and EBNA-1 in asymptomatic and symptomatic EBV infections are similar, is that latently infected EBV reactivates, replicates, and boosts serum EBNA-2 IgG antibodies. Latency and reactivation are common in all human herpesvirus infections (39). In addition, it is conceivable that immune responses evoked by asymptomatic primary EBV infections are not strong enough to prevent reactivation of latent EBV and subsequent virus replication and ultimately decline with time. This hypothesis is supported by the reports showing that EBV is detected at a high incidence (22 to 23%) in mouth washings of seropositive healthy adults in the United States, England, and Japan (9, 26, 43). It is also likely that superinfections with exogenous EBV occur in adults. This possibility is consistent with the reports that infections with more than one EBV type or strain can be detected in both immunocompromised (47, 48) and healthy (4, 10–12, 30, 45, 46) individuals. Furthermore, multiple EBV infections are common in the majority of patients with IM (42), as well as in asymptomatic EBV carriers (42, 45). The isolation of multiple strains from a single individual may indicate either the presence of multiple strains in primary infections as has been described previously for human herpesvirus 6 (35) or successive superinfections as has been reported elsewhere for human cytomegalovirus (2, 5). Moreover, the reactivation and superinfection hypotheses are not mutually exclusive.

It is not known why the antibody response patterns of IgGs to EBNA-2 and to EBNA-1 in acute-convalescent phases of IM are so different and whether the same differences occur in the acute-convalescent phases of asymptomatic EBV infections. Further analyses of antibody responses to EBV antigens in the course of early asymptomatic EBV infections and possible later reactivation and/or reinfection would help researchers to improve serodiagnosis, to design an EBV vaccine, and to better understand the virus-host balance of EBV infections.

ACKNOWLEDGMENTS

We thank K. Yamashita, Infectious Disease Surveillance Center, NIID, for useful advice on statistical evaluation.

Financial support for this research was provided by a grant-in-aid from the Ministry of Health and Welfare.

REFERENCES

1. Albeck, H., T. Bille, H. J. Fenger, U. Narvestad, G. S. Sorensen, G. Henle, W. Henle, N. Hoijgaard Nielsen, and J. P. Hart Hansen. 1985. Epstein-Barr virus infection and serological profile in Greenland Eskimo children. Acta Paediatr. Scand. 74:691–696.
2. Bale, J. F., Jr., S. J. Petheram, I. E. Souza, and J. R. Murph. 1996. Cytomegalovirus reinfection in young children. J. Pediatr. 128:347–352.
3. Biggar, R. J., G. Henle, J. Bocker, E. T. Lennette, G. Fleisher, and W. Henle. 1978. Primary Epstein-Barr virus infections in African infants. II. Clinical and serological observations during seroconversion. Int. J. Cancer 22:244–250.
4. Brooks, J. M., D. S. Croom-Carter, A. M. Leese, R. J. Tierney, G. Habershaw, and A. B. Richmond. 2000. Cytotoxic T-lymphocyte responses to a polymorphic Epstein-Barr virus epitope identify healthy carriers with coresident viral disease. J. Virol. 74:1801–1809.
5. Chandler, S. H., B. H. Handsfield, and J. K. McDougall. 1976. Isolation of multiple strains of cytomegalovirus from women attending a clinic for sexually transmitted disease. J. Infect. Dis. 135:655–660.
6. Dambaugh, T., C. Beisel, M. Hummel, W. King, S. Fennwold, A. Cheung, M. Heller, N. Raab-Traub, and E. Kieff. 1980. Epstein-Barr virus (B95–8) DNA: molecular cloning and detailed mapping. Proc. Natl. Acad. Sci. USA 77:2999–3003.
7. Ernberg, I., and G. Klein. 1979. EB virus-induced antigens. p. 39–60. In M. A. Epstein and B. G. Achong (ed.), The Epstein-Barr virus. Springer-Verlag, Berlin, Germany.
8. Fleisher, G., W. Henle, G. Henle, E. T. Lennette, and R. J. Biggar. 1979. Primary infection with Epstein-Barr virus in infants in the United States: clinical and serologic observations. J. Infect. Dis. 139:553–558.
9. Gopal, R. M., R. J. Thomson, J. Fox, R. S. Tedder, and R. W. Hones, 1990. Detection by PCR of HHV-6 and EBV DNA in blood and oropharynx of healthy adults and HIV-seropositives. Lancet 335:1598–1599.
10. Gratama, J. W., M. A. Osterveer, G. Klein, and I. Ernberg. 1990. EBNA size polymorphism can be used to trace Epstein-Barr virus spread within families. J. Virol. 64:4703–4708.
11. Gratama, J. W., M. A. Osterveer, J. Lepoutre, W. E. Fibbe, O. Ringden, J. M. Vossen, R. Willemze, R. L. Bolhuis, J. J. van Rool, and I. Ernberg. 1992. Epstein-Barr virus infection in allogeneic marrow grafting: lessons for transplant physicians and virologists. Ann. Hematol. 64(Suppl.):A162–A165.
12. Gratama, J. W., M. A. Osterveer, W. Weimar, K. Sint Nicolaas, W. Sizzo, R. L. Bolhuis, and I. Ernberg. 1994. Detection of multiple ‘Epnotypes’ in individual Epstein-Barr virus carriers following lymphocyte transformation by virus derived from peripheral blood and oropharynx. J. Gen. Virol. 75:849–854.
13. Griffin, B. E., and L. Karran. 1984. Immortalization of monkey epithelial cells by specific fragments of Epstein-Barr virus DNA. Nature 309:78–82.
