EBNA Size Polymorphism Can Be Used To Trace Epstein-Barr Virus Spread within Families

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The Epstein-Barr virus (EBV)-determined nuclear antigens EBNA 1, 2, 3, 4, and 6, regularly expressed in EBV-transformed lymphoblastoid cell lines, vary in size among viral strains. We have used this characteristic to trace the spread of the virus within seven families by using an approach called Ebnotyping. Among 33 evaluable individuals, 3 were EBV seronegative, and 17 different EBV strains could be isolated from the peripheral blood or throat washes of the remaining 30. All unrelated persons carried different strains. The EBV strain carried by 19 persons was also found in 1 or more family members. The same viral strain was carried by two members in five families, by three members in the sixth, and by five members in the seventh. The paternal strain was isolated from one child in two families, and the maternal strain was isolated from one or more children in three families. EBV was isolated from both blood and throat wash in six individuals. The Ebnotypes of both derived lymphoblastoid cell lines were identical within each individual. These results indicate that spread within families may be a relatively common route of EBV transmission. The number of horizontal transmission events required to generate diversification of the Ebnotype will require larger epidemiological studies.

The principle of fingerprinting herpesvirus strains for epidemiologic studies has been pioneered for herpes simplex virus at the DNA (19) and protein (22) levels. Similarly, analysis of restriction fragment length polymorphism of Epstein-Barr virus (EBV) has allowed the distinction between natural virus isolates (15, 16, 20). We report here on the use of the size polymorphism of EBV nuclear antigens (EBNA) for epidemiological purposes.

Seven EBV-encoded proteins are known to be expressed in EBV-transformed lymphoblastoid cell lines (LCL). Six of them are nuclear antigens (EBNA 1 to 6), and the seventh is a membrane protein designated LMP (1). In different isolates, EBNA 1 varies between 65 and 97 kilodaltons (kDa) as a result of differences in the length of the internal repeat sequence located in the *Bam*HI-K region (2, 3, 7a, 8). EBNA 2A varies in size from 75 to 95 kDa. EBNA 3 and 6 occur as different-size molecules in the range of 130 to 180 kDa (12, 26), whereas EBNA 4 shows relatively little size variation (around 140 kDa) (24). The size variability of EBNA 2, 3, 4, and 6 is probably due to variations in the primary amino acid sequences, as is the case with EBNA 1. Repeated isolations of virus from a single individual over time periods ranging from 3 to 7 years have yielded the same EBNA size pattern as the original virus isolate (4). Established LCL cultured for several years have also maintained stable EBNA size patterns (4). In contrast, EBNA 5 shows a large size variation (ranging from 20 and 130 kDa) between different LCL carrying the same EBV strain, probably as a result of posttranscriptional processing (6). Therefore, the size variation of EBNA 5 cannot be used to distinguish between different EBV strains.

The combined size variations of EBNA 1, 2, 3, 4, and 6 have been used to distinguish between different EBV strains

(3, 8, 12, 24, 26). We refer to this approach as Ebnotyping and had used it earlier to distinguish between donor and recipient virus in bone marrow-grafted patients (7a, 8). In the course of this study, it became apparent that four of six human leukocyte antigen (HLA)-identical sibling donor-recipient combinations carried identical EBV strains. This observation prompted us to examine the spread of EBV within some families.

MATERIALS AND METHODS

Subjects. We studied seven families of middle to high socioeconomic status residing either in The Netherlands (six families) or in Sweden (one family). The ages and genders of these family members are shown in the pedigrees depicted in Fig. 1 to 6.

Family 1 was studied to trace the source of EBV infection in the grandmother. As a medical researcher, she had served as an EBV-negative serum donor at our laboratory for at least 15 years. At the age of 60, she developed acute infectious mononucleosis (IM), confirmed by the presence of heterophile antibodies, immunoglobulin M (IgM) antibodies to EBV capsid antigen (VCA) and early antigen complex (EA), and the absence of EBNA antibodies. She had regularly taken care of her 2-year-old grandson during the 4 weeks before the onset of IM. The child was at a daycare center 5 days a week. The grandmother prepared food for the child on several occasions and tasted it repeatedly while feeding him. Salivary transmission by food tasting has been suggested as a possible mode of transmission between young children and adults (11). The child and other family members were clinically healthy during the whole period. During the convalescence of the grandmother, a few drops of saliva from the grandchild were diluted in RPMI 1640 medium and added to cord blood cells. Transforming activity was detectable after dilution up to 1,000-fold.

Families 2 to 6 were investigated in connection with our study of EBV infection in bone marrow transplant (BMT) recipients. Bone marrow was received as follows: in family

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2, sibling J from sibling M; in family 4, sibling F from sibling E; in family 5, sibling D from sibling C; in family 6, sibling F from sibling G; and in family 7, sibling D from sibling C. The diagnostic indication for BMT was acute leukemia in remission in all five cases. All donor-recipient pairs were HLA-A,B,C,DR identical and mixed leukocyte reaction nonreactive. Family 3 was related to family 2 via sibling F, who was the husband of sibling J in family 2.

Serological and virus isolation studies. Sera were tested at 10-fold serial dilutions starting from 1:10 for the presence of IgG VCA antibodies (10) by indirect immunofluorescence.

EBV was isolated either from the oropharynx by throat washing or by establishing EBV-carrying LCL from the peripheral blood. Throat washes were obtained by having subjects gargle for 1 min with 10 ml RPMI 1640 medium. Bacteria and debris were removed by centrifugation at $600 \times g$ for 10 min. Fetal calf serum (GIBCO Laboratories, Grand Island, N.Y.) was added (end concentration, 10%) to the throat washes, after which they were stored at -20°C . After thawing, 3-ml samples were incubated for 3 to 6 h with 12×10^6 cord blood mononuclear cells that had been freshly obtained from full-time deliveries. After washing, the cells were distributed over 60 wells of a 96-well flat-bottom Falcon microtiter plate (Becton Dickinson Labware, Oxnard, Calif.) in RPMI 1640 medium (0.2 ml per well) supplemented with 10% fetal calf serum, glutamine, and antibiotics and were cultured for 8 to 12 weeks at 37°C in a 5% CO_2 -containing atmosphere with biweekly feeding.

Fresh or cryopreserved and thawed peripheral blood mononuclear cells (PBMC) were cultured in 96-well flat-bottom microtiter plates. Per well, 2×10^5 viable PBMC were cultured in 200 μl of RPMI 1640 medium containing glutamine, antibiotics, 10% fetal calf serum, and cyclosporin (0.5 $\mu\text{g}/\text{ml}$; Sandoz, Basel, Switzerland) at 37°C . The cultures were fed biweekly with cyclosporin-free medium and maintained for 8 to 12 weeks. Between 1 and 2 weeks after initiation of the cultures, 10^5 fresh cord blood lymphocytes were added to each well to rescue EBV released from virus-carrying cells (17).

From these two types of cultures, wells with growing cells were harvested from each tissue culture plate and pooled, and the resulting LCL were expanded.

Immunoblotting. Lymphoblastoid cells were harvested, washed once in ice-cold phosphate-buffered saline (PBS), dissolved in electrophoresis sample buffer, sonicated, and boiled for 5 min. Samples corresponding to 10^6 lymphoblastoid cells per lane were loaded for electrophoresis in gels containing 7.5% polyacrylamide and sodium dodecyl sulfate; a discontinuous buffer system was used. The following molecular mass markers were used: myosin (200 kilodaltons [kDa]), *Escherichia coli* β -galactosidase (116 kDa), rabbit muscle phosphorylase *b* (97 kDa), bovine serum albumin (66 kDa), and hen egg white ovalbumin (43 kDa) (Bio-Rad Laboratories, Richmond, Calif.). The proteins were transferred from gel to nitrocellulose filter (Schleicher & Schuell, Dassel, Federal Republic of Germany) in Tris-glycine buffer containing 20% methanol at 10 V/cm during 4 h. The positions of the molecular weight markers were noted after staining with Ponceau red. Excess protein-binding sites on the filters were blocked by incubation in PBS containing 5% nonfat dry milk (PBSM) for 2 h. Subsequently, the filters were incubated with serum diluted in PBSM for 16 h at 4°C . Serum from a patient with chronic lymphocytic leukemia (18) was used at a 1:20 dilution for the detection of EBNA 1, 2, 3, 4, and 6. This serum also reacted with EBNA 5, seen as multiple weak bands in the 22- to 90-kDa region. The EBV

antibody titers of this serum in immunofluorescence assays were as follows: VCA, IgA <10 and IgG 5,120; EA (diffuse), IgA 20 and IgG 320; EA (restricted), IgG <5 ; EBNA 40. After washing with PBSM, the filters were incubated for 90 min at room temperature with rabbit anti-human IgG conjugated to alkaline phosphatase (Sigma Chemical Co., St. Louis, Mo.) and diluted 1:1,000 in PBSM. Thereafter, they were washed with PBSM containing 0.5% Tween 20, and bound antibody was detected by alpha-naphthyl phosphate and fast red TR salt.

RESULTS

Of the total of 45 individuals (see pedigrees in Fig. 1 to 6), 41 were investigated. Three were EBV seronegative and had negative viral cultures (family 2, sibling M; family 3, mother B; family 4, sibling G). LCL could be established from 30 of the 38 EBV-seropositive individuals. The virus was derived from the blood only in 18 persons, from the throat wash only

FIG. 1. Pedigree of family 1 and immunoblot of the established LCL from the family members, probed with the polyspecific PG serum (18). Males and females are indicated by squares and circles, respectively. Their ages at the time of initial investigation are indicated below these symbols. Letters above the lanes correspond with those in the pedigree. Numbers to the right of the immunoblot indicate molecular masses in kilodaltons. The Ramos cell line was used as an EBV-negative control. EBNA 1, 2, 3, and 6 were assigned to their corresponding bands after probing with monospecific antisera as described in references 1 (EBNA 1) and 23 (EBNA 3 and 6). The assignment of EBNA 4 was done by exclusion, since no EBNA 4-monospecific antiserum was available. EBNA 2 was identified by using a serum (KF) from which the EBNA 1 antibodies had been removed by absorption to the EBNA 2-negative P3HR-1 cell line. Letters below the lanes denote the different EBV strains. E, Epstein-Barr virus nuclear antigen. Sibling H was not studied.

FIG. 2. Pedigrees and immunoblots of families 2 and 3. r, Bone marrow recipient; d, bone marrow donor; J1 and J2, LCL derived from blood obtained at day 9 pre-BMT and day 1820 post-BMT, respectively. Mother B and sibling M were EBV seronegative. Siblings D and E were not studied. Other details are as in the legend to Fig. 1.

in 6, and from both blood and throat wash in the remaining 6. Seventeen different EBV strains, designated a to s, could be distinguished by their Ebnotypes (see the immunoblots in Fig. 1 to 6). The Ebnotypes of the LCL derived from throat wash and peripheral blood were identical in all six individuals for whom both cultures had been successful (data not shown). All unrelated persons carried individually distinct viral strains. Consequently, no strain was encountered in more than one family. However, different members of the same family (including husband F and wife J linking families 3 and 2, respectively) could carry the same EBV strain. In families 1, 2, 5, and 6, both parents were tested, and individually distinct EBV strains were isolated from each of them. In contrast, a single EBV strain could be isolated from more than one individual within all seven families.

In family 1 (Fig. 1), the a strain was isolated from three individuals: the maternal grandmother (B), the father (C), and the grandson (G) (see also Materials and Methods). The mother (D) and her sister (F) carried different strains (b and c, respectively).

In family 2 (Fig. 2), sibling J carried the same EBV strain (d) as did her father until she became EBV seronegative after the bone marrow graft from her EBV-seronegative brother M (7a). After a 4-year period of seronegativity, she seroconverted again, with transient symptoms of mild cervical lymphadenopathy and mild liver function disturbances. Her new EBV strain (f) was identical to the one carried by her husband. In the husband's family (family 3; Fig. 2), strain f was also isolated from his younger sister (C). The father carried an EBNA 2B strain (g); the PG serum, reactive only with EBNA 2A-type EBV strains, detected only EBNA 1 and 3. Probing with an EBNA 2B-specific serum revealed an

FIG. 3. Pedigree and immunoblot of family 4. r, Bone marrow recipient; d, bone marrow donor; F, LCL derived from blood obtained at day 9 pre-BMT. Sibling G was EBV seronegative. Other details are as in the legend to Fig. 1.

EBNA 2B band at 75 kDa (not shown). The mother was still seronegative at the age of 63.

In family 4 (Fig. 3), the mother's strain (h) could be isolated from four of the five younger children (E, F, H, and J). Sibling G, aged 29, was EBV seronegative. The two oldest siblings (C and D) carried a different strain (j). Attempts to isolate the virus from the father were unsuccessful. Sibling F received a bone marrow graft from his EBV-identical brother E; the same EBV strain (h) could be isolated from sibling F's peripheral blood at 1 and 5 years post-BMT (data not shown).

In family 5 (Fig. 4), sibling C carried the same EBV strain as did his mother (l), whereas the father and his younger brother (D, isolate 1) carried different strains (k and m, respectively). Sibling C donated marrow to his brother (7a). After BMT, sibling D's original EBV strain (m) was eradicated and replaced by his brother's virus (l; isolates 2 and 3 obtained at days 252 and 915 post-BMT, respectively).

In family 6 (Fig. 5), the father and the mother carried different EBV strains (n and p, respectively). The mother's strain was also isolated from siblings F and G. Sibling D carried a different strain (q). Sibling F was tested 6 years after transplantation of marrow from his sister G. Attempts to isolate his pretransplant EBV strain were unsuccessful.

The father in family 7 (Fig. 6) carried a EBV strain (r) that differed from the one isolated from his sons (s). Sibling D had received a marrow graft from his brother; his EBV isolates (1 and 2) were obtained at 50 and 208 days post-BMT and were identical to the strain of his donor. Attempts to isolate his pretransplant EBV strain have been unsuccessful.

FIG. 4. Pedigree and immunoblot of family 5. r, Bone marrow recipient; d, bone marrow donor; D1, D2, and D3, LCL derived from blood obtained at day 9 pre-BMT, day 252 post-BMT, and day 915 post-BMT, respectively. Other details are as in the legend to Fig. 1.

DISCUSSION

We have used the Ebnotyping method to analyze the intrafamilial spread of EBV in seven families. This study was prompted by our observation that four of six HLA-identical sibling bone marrow donor-recipient pairs carried the same EBV strain, whereas 18 unrelated marrow donors carried an equal number of different strains (7a). The latter observation was confirmed in this study: a given EBV strain was observed only within a single family.

Our results show that EBV can be transmitted between family members. Previous serological and clinical studies have suggested that transmission of disease, such as IM, is relatively rare within families and between other close contacts (7, 14, 21, 29). These observations may be explained by the fact that IM can be transmitted only to EBV-seronegative persons, who constitute a minority of adolescent and adult populations. The transmission of virus, not the disease, may be much more frequent under suboptimal hygienic conditions and when children are involved, since the frequency of seronegative individuals among children is much higher than among adults (11). A study of 112 sibling and 165 adult contacts of children with serologically confirmed IM revealed serological evidence for recent primary EBV infection in 19 sibling contacts and recent reactivation of EBV in 26 adults, as suggested by high antibody titers to VCA or early antigens (27). The Ebnotyping method permits a distinction between (re)infection with exogenous virus or activation of endogenous virus.

The nature (i.e., salivary contact) rather than the frequency of the contact appears to be pivotal for EBV trans-

FIG. 5. Pedigree and immunoblot of family 6. r, Bone marrow recipient; d, bone marrow donor; F, LCL derived from blood obtained at day 1967 post-BMT. Other details are as in the legend to Fig. 1.

mission. The first indications of the role of kissing in the transmission of IM came from the observations of Hoagland (13) and Evans (5). Even a single salivary transmission event from an infectious person can be sufficient for effective virus transmission. This contention is borne out by our prospective study of the incubation period of IM in an adolescent after kissing a girl who developed IM 2 days later (28) and the case of the grandmother in family 1, who developed IM after tasting the food rejected by her grandson. In contrast, transmission of the virus has not been observed in situations in which salivary contact is unlikely to occur; studies at a university and a military academy have shown no higher incidence of EBV seroconversion among seronegative roommates of IM patients than among unselected EBV-seronegative persons (9, 25).

The results presented here indicate the usefulness of the Ebnotyping method for epidemiological studies. In particular, this approach may be helpful in unraveling the factors contributing to the differential rates at which the prevalence of EBV antibodies increases with age as a function of socioeconomic status (11). The isolation of the same EBV strain from up to five related persons and from the same individuals over periods of up to seven years (data not shown) indicate that genetic changes of the virus as detected by Ebnotyping may become detectable only after several and possibly numerous horizontal transmission events.

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FIG. 6. Pedigree and immunoblot of family 7. r, Bone marrow recipient; d, bone marrow donor. D1 and D2, LCL derived from blood obtained at days 50 and 208 post-BMT, respectively. Other details are as in the legend to Fig. 1.

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