Epstein-Barr Virus Induced Lymphoproliferative Tumors in Severe Combined Immunodeficient Mice Are Oligoclonal

By Pavel Pisa, Martin J. Cannon, Eva K. Pisa, Neil R. Cooper, and Robert I. Fox

Severe combined immunodeficient (SCID) mice reconstituted with lymphocytes from Epstein-Barr virus (EBV) negative human donors develop aggressive tumors after the chimeric mice are infected with EBV. The tumors were composed of human B cells that expressed EBV encoded antigens (latent membrane protein and EBV nuclear antigen-2). Southern blot analysis of DNA from 16 SCID/hu tumors with human Ig gene probes showed that each tumor contained multiple heavy and light chain gene rearrangements. Ig κ gene rearrangements were frequent, while clonal λ gene rearrangements were infrequent. Analysis of EBV terminal repeat sequence indicated two or more fused termini in each tumor, consistent with a multiclonal origin. Linear terminal repeat segments and viral antigens (EA-D and EA-R) associated with EBV replication were not detected in the tumors. High levels of human lgs in the SCID/hu serum were oligoclonal and primarily contained κ light chains. Before the appearance of overt tumors, circulating cells with human and EBV DNA could be detected in the SCID/hu mice by the polymerase chain reaction. We conclude that EBV infection in SCID/hu chimeric mice produces a limited number of transformation events, which give rise to oligoclonal tumors resembling EBV-associated lymphoproliferative disorders in some immune-deficient patients.

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EPSTEIN-BARR VIRUS (EBV) infection can lead to a variety of different outcomes including asymptomatic latency and periodic reactivation, polyclonal lymphoproliferation, and Frank malignancy. It is likely that the clinical outcome is determined in part by natural killer (NK) cells and EBV-specific T lymphocytes. In patients with disease-associated or iatrogenic immunosuppression, EBV-associated lymphoproliferative tumors are a serious and often fatal complication. Lymp nodes and other tissues are infiltrated by B lymphocytes that express EBV encoded antigens and contain EBV DNA.

There are several important differences between these EBV lymphoproliferative tumors and most cases of non-Hodgkin’s lymphoma (NHL). First, the EBV lymphoproliferation regresses when immunosuppressive medications are removed, while malignant lymphomas are generally progressive disorders. Second, most NHL tumors are monoclonal, while the EBV-associated lymphoproliferative tumors are generally oligoclonal or polyclonal. Third, many cases of NHL exhibit karyotypic alterations such as translocation of the bcl-2 or c-myc proto-oncogenes, while most EBV lymphoproliferative lesions lack such chromosomal aberrations. Finally, most NHLs lack EBV DNA and viral antigens, while the EBV lymphoproliferative tumors contain viral DNA and express viral encoded antigens such as latent membrane protein (LMP) and EBV nuclear antigen-2 (EBNA-2). A further understanding of these EBV-related lymphoproliferative disorders is important to improve current therapy and to elucidate the normal mechanisms of immune control of endogenous viruses.

The study of EBV infection in vivo previously has been limited to humans and to cotton-top tamarins, an endangered species. Recently, mice with severe combined immune deficiency (SCID) have been reconstituted with human lymphocytes from EBV positive donors and the resulting SCID/hu chimeric mice were noted to develop aggressive human B-cell tumors containing viral DNA. We and others have recently described a modification of this animal model, in which SCID mice were first transplanted with lymphocytes from EBV-negative donors and subsequently inoculated with EBV, B95-8 isolate. Within 6 weeks, these SCID/hu chimeric animals developed fatal lymphoproliferative disease of human B-cell origin that contained EBV DNA. These SCID/hu tumors have a histopathologic appearance that is similar to B-cell tumors arising in some immunodeficient patients.

In the present report, we extend our previous studies by examining the clonality of tumors developing in the SCID/hu mice inoculated with EBV. We found that multiple Ig gene rearrangements in each tumor and different tumors in same animal exhibited different gene rearrangements. Studies of EBV terminal repeat DNA also showed a multiclonal origin of the tumors. There was little evidence for EBV replication in these tumors, based on evaluation of terminal repeat segments and viral antigen (EA-R, EA-D) expression. The data suggest that EBV infects a limited number of B cells that undergo clonal expansion. We conclude that the tumors arising in SCID/hu chimeras after inoculation of EBV may provide insight into the EBV-associated lymphoproliferative disorders that occur in some immunosuppressed humans.

MATERIALS AND METHODS

Human lymphocytes. Palatine tonsil or peripheral blood from six immunologically normal donors was obtained after informed consent from all donors or their parents, using procedures ap-
proved by our human subject review committee. These individuals had no prior exposure to EBV based on their lack of antibodies to EBV capsid antigens. Lymphocytes were prepared by Ficoll-Hypaque (Sigma, St Louis, MO) gradient centrifugation.

Reconstitution and EBV infection of SCID mice. C.B.-17 scid (SCID) mice, ages 8 to 12 weeks, were provided by a breeding colony maintained at the Scripps Research Institute. The colony is free of mouse hepatitis virus. SCID mice were reconstituted by intraperitoneal (ip) injection of 5 x 10^7 human lymphocytes from EBV seronegative individuals.

Cell line B95-8 was maintained in tissue culture using medium RPMI-1640 containing 10% calf serum. Cell-free supernatants of phorbol myristate acetate (PMA) treated B95-8 cell cultures were used as a source of EBV to infect SCID mice.25 One week after human lymphocyte transfer, the SCID/hu chimeras were injected ip with EBV as previously described.25 They were bled from the tail vein before EBV inoculation and then at weekly intervals. When tumors were detected in the SCID/hu chimeras, a complete autopsy of each mouse was performed. All mice developed tumors within 45 days after EBV inoculation. Sixteen tumors from six mice were analyzed in detail as described below.

Solid-phase enzyme-linked immunosassay (ELISA) for total human Ig. Quantitation of human Ig (IgG, IgA, and IgM) was performed using ELISA with peroxidase or alkaline phosphatase conjugated rabbit anti-human IgG (Tago, Burlingame, CA). After precipitating with phosphate-buffered saline (PBS) plus 0.05% Tween, microtiter plates were incubated with serial dilutions of SCID/hu serum samples or standard human sera in PBS.28 The reaction was developed using substrate ABTS (Sigma) or Sigma 104 phosphatase substrate (Sigma) and absorbance read on an ELISA plate reader at 414 nm or 405 nm, respectively.

Detection of EBV and human antigens in SCID/hu tumors. Samples of clinically abnormal tissues (ie, tumors) as well as normal tissues were removed for routine histology and frozen tissue immunohistologic evaluation.27 Also, a cell suspension was prepared from a portion of each tumor and cytospin preparations were acetone fixed after immunohistologic staining.28 Frozen tissue sections (0.5 μm) were stained with murine monoclonal antibodies (MoAbs) against EBV-encoded antigens including early diffuse (EA-D) and early restricted (EA-R) viral antigens, LMP,29 and EBNA-2.30 Murine MoAbs were used to detect human antigens including HLA class I (HLA-A), HLA-class II (HLA-DR), IgG (γ), IgM (μ), IgA (α), κ, λ, CD3, CD4, and CD8 (Becton-Dickinson, Mountain View, CA). Reactivity was detected with a biotin conjugated antimouse Ig antibody and peroxidase conjugated avidin (Tago).31

For two-color immunohistologic staining, a single cell suspension from the tumors was stained with a rabbit antibody specific for human HLA-DR antigen and murine MoAbs against the EBV antigens described previously. After rinsing, the cells were stained with fluorescein conjugated goat antirabbit Ig (Tago) and phycoerythrin rat antimouse Ig (Becton-Dickinson). A cytospin preparation of the stained cells was examined using a fluorescent microscope as previously described.25

Serum protein electrophoresis and immunoelectrophoresis. Serum electrophoresis was performed using a Titan Gel kit (Helena Laboratories, Beaumont, TX) and immunoelectrophoresis using High Resolution Protein Electrophoresis Film Agarose (Ciba Corning, Palo Alto, CA) with specific antisera (Meloy, Springfield, VA) according to manufacturer’s specifications.

Southern blot analysis. Genomic DNA was extracted from SCID/hu tumors as previously described,26 digested with BamHI or HindIII restriction endonucleases (Stratagene, San Diego, CA), electrophoretically separated in a 0.7% agarose gel, and transferred by the alkaline blot method to nylon membranes (Oncor, Gaithersburg, MD) that were hybridized at 65°C with 32P-labeled DNA probes. Nylon filters were then washed in 0.1X SSC, 0.1% sodium dodecyl sulfate (SDS) first at room temperature and then for 1 hour at 64°C. Autoradiography was routinely performed for 4 days at -80°C with a single intensifying screen. DNA probes for the Ig heavy and light chain genes (λ, C, and C)26 were kindly provided by Dr P. Leder (Harvard Medical School, Cambridge, MA).

For analysis of EBV terminal repeat (TR) sequences, the tumor DNA was digested with restriction endonuclease BamHI (Stratagene), separated on agarose gels, transferred to nylon membrane, and reacted with 32P-labeled Xho I 1.9 probe provided by Dr N. Raab-Traub (University of North Carolina, Chapel Hill).32,33

Polymerase chain reaction (PCR) for detection of EBV and HLA-DQA DNA. DNA for PCR was prepared from pelletted cells from the tailbleeds as follows: red blood cells were removed with lysis buffer (0.32 mol/L sucrose, 10 mmol/L Tris-HCl pH 7.5, 5 mmol/L MgCl2, 1% Triton-100). Subsequently, 0.5 ml of extract was added containing: 50 mmol/L KCl, 10 mmol/L Tris-HCl (pH 8.3), 2.5 mmol/L MgCl2, 0.1 mg/mL gelatin, 0.45% Nonidet P40, 0.45% Tween 20, and 100 μg/mL Proteinase K. After a 16-hour incubation at 55°C, samples were incubated at 95°C for 10 minutes to inactivate the protease and then stored at -70°C until assayed. Previously published primers and probes were used for amplification and detection of EBV BamW sequences34 and a chain of HLA-DQα1.35 HLA-DQα was used as a marker for human DNA.36

Five microliters of sample DNA was amplified for 35 cycles in a total volume of 50 μL covered with mineral oil, using a DNA Thermal Cycler (Perkin Elmer-Cetus, Norwalk, CT) and 1.25 U of Taq DNA polymerase (Perkin Elmer-Cetus). Reaction conditions were: annealing 1 minute at 55°C, extension 1.5 minutes at 72°C, and denaturation 1 minute at 95°C. As a control of internal consistency, specific positive and negative control standards were included in every PCR assay.

One fifth of the amplified product was electrophoretically separated in a 1.6% agarose gel and transferred by the alkaline blot method onto nylon membranes that were hybridized with 32P labeled oligonucleotide probes at 42°C for 16 hours. Nylon filters were then washed in 0.2X SSPE (1X SSPE: 0.18 mol/L NaCl, 10 mmol/L NaH2PO4, 1 mmol/L EDTA, pH 7.4), 0.1% SDS first at room temperature and then for 10 minutes at 42°C. Autoradiography was routinely performed for 4 hours at -80°C.

RESULTS

Induction of SCID/hu tumors. SCID mice were injected with lymphocytes from peripheral blood (PBL) or palatine tonsils (lymph nodes, LN) from EBV-seronegative individuals. A total of six SCID mice were reconstituted, including two with PBL and four with LN cells. The SCID/hu mice were infected with EBV 7 days later. All EBV-infected SCID/hu mice developed clinical signs of tumor 34 to 45 days after EBV injection. Autopsies on the six killed mice showed 16 solid tumors in the abdomen, thymus, and thoracic lymph nodes. These tumors had the pathologic appearance of a high-grade immunoblastic non-Hodgkin's B-cell lymphoma and had normal karyotype.37 Six additional SCID mice were reconstituted with LN cells but were not infected with EBV; these SCID/hu chimeras failed to develop tumors up to day 70 postreconstitution.

Immunohistologic characterization of SCID/hu tumors. Frozen tissue sections of SCID/hu tumors were stained with MoAbs directed against EBV-associated antigens...
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using immunoperoxidase methods. The majority of tumor cells expressed the EBV-associated antigens EBNA-2 and LMP. To confirm that these viral antigens were present in human cells, suspensions of the tumor cells were analyzed by two-color immunohistologic methods to show that cells containing viral antigen also contained human HLA-DR antigen. In comparison, EBV antigens associated with lytic replication (EA-D and EA-R) and capsid viral antigen gp200/350 were rarely seen (<0.5% of tumor cells). As controls, the monoclonal anti-EBV antibodies reacted with PMA induced B95-8 cells, but not with uninfected human cells nor with uninfected SCID mouse tissues.

Immunohistologic evaluation of SCID/hu tumors showed a predominance of cells containing cytoplasmic human IgG (>80%) with lower numbers of IgA (approximately 10%) and IgM (approximately 10%) located within the same tumor nodule. Also, lymphocytes containing cytoplasmic κ (approximately 80% of cells) and λ (2% to 20%) were present within the same tumor. Human T cells and their subsets were evaluated using anti-CD3, CD4, and CD8 antibodies. The proportion of lymphoid cells in SCID tumors reacting with anti-CD3 was less than 3% (ranging from <0.1% to 2.5% in five tumors evaluated) and the majority of these cells were CD8⁺.

Detection of human Ig in SCID/hu chimeras. Human Ig was detectable in SCID/hu chimeric mice 1 week after inoculation of EBV (Fig 1). The total Ig level increased rapidly and high levels (up to 70 mg/mL) were present at the time of appearance of tumors at 4 to 6 weeks. One week after EBV infection, the SCID/hu sera contained approximately 60% IgM, 30% IgG, and 10% IgA human Ig. By 4 weeks after EBV infection, the SCID/hu mice sera contained greater than 85% IgG with approximately 5% IgA and 10% IgM. Rheumatoid factor, which can artefactually alter measurement of IgG levels, was not detected in the SCID/hu sera. Human Ig also was detected in the reconstituted but noninfected control groups of SCID/hu mice. However, the latter levels were at least 10-fold lower than in the EBV-infected groups at 2 weeks after reconstitution and the levels decreased over the subsequent weeks after EBV infection. Finally, murine Ig was not detected in SCID mice before reconstitution with human lymphocytes or after EBV infection (data not shown).

Consecutive weekly samples of SCID/hu serum were evaluated by serum electrophoresis and showed small clonal IgG bands appearing 3 weeks after EBV inoculation (Fig 2A, lane 3). By 4 weeks (lane 4), an additional IgG
band was present. By 5 weeks (lane 5), the IgG band with fastest mobility had disappeared and other IgG bands were beginning to increase in intensity. This pattern of "clonal" Ig bands that first appeared and then receded was seen in the consecutive sera of all SCID/hu mice infected with EBV. To examine the light chains in these mice, sera samples from SCID/hu mice 5 weeks after EBV infection were studied by immunofixation (Fig 2B). Two representative mice sera show presence of "oligoclonal" k bands but only a diffuse distribution of reactivity with \( \lambda \) was noted.

Oligoclonal human Ig gene rearrangements in SCID/hu tumors. DNA from 16 SCID/hu tumors was analyzed by Southern blotting for rearrangements of the heavy and light Ig chain genes. Examples of such analyses are shown in Fig 3. To detect heavy chain gene rearrangements, DNA was digested with restriction enzyme (HindIII) and hybridized

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**Fig 3. Southern blot analysis of human Ig gene rearrangements in SCID/hu tumors.** (A) Normal genomic human DNA, SCID mouse DNA, and SCID/hu tumor DNA were digested with restriction enzyme HindIII and hybridized with a human J\(_k\) probe. (B) DNA was digested with BamHI and hybridized with C\(_k\). (C) The DNA was digested with HindIII and hybridized with C\(_\lambda\) probe. The DNA samples in each lane are the same in panels A through C. In lane 1, DNA was from human peripheral granulocytes of normal donor 1 and shows the most common germ-line fragments: 11 kb for J\(_k\) gene; 12 kb for C\(_k\), 12, 9.5, and 8.5 kb for C\(_\lambda\). In lane 2, SCID mouse tail DNA was used. Lanes 3a and 3b contain SCID/hu tumor DNA from two anatomically isolated tumors from one SCID/hu mouse, reconstituted with lymphocytes from donor 1. Lanes 4a and 4b contain DNA from two tumors arising in a second mouse reconstituted with lymphocytes from donor 1. Lanes 5a through 5d and 6a through 6c contain DNA from anatomically distinct tumors developing in two SCID/hu chimeric mice reconstituted with tonsil lymphocytes from donor 2; DNA from this donor's granulocytes (lane 5a) exhibited a less common germ-line restriction fragment pattern that included an additional 5-kb band in the germine configuration. Lane 7 in (C) contains DNA from a third mouse developing tumors after reconstitution with cells from donor 2.
with a \( J_\kappa \) probe (Fig 3A). Lane 1 shows the germline configuration of the human \( J_\kappa \) that was present in all donors and lane 2 indicates that the human \( J_\kappa \) probe does not hybridize with murine SCID DNA. The Southern blot analysis shows multiple \( J_\kappa \) rearrangements in all SCID/hu tumors. All tumors expressed four or more rearranged bands. Some tumors (eg, Fig 3A, lanes 4b and 5b) had as many as seven different \( J_\kappa \) rearrangements.

Multiple rearrangements of the \( \kappa \) light chain gene were shown when SCID/hu tumor DNA was digested with \textit{Bam}H\textsubscript{I} restriction endonuclease and hybridized with human \( C_\kappa \) probe (Fig 3, panel b). Germline for \( C_\kappa \) in all donors had a 12-kb fragment (example shown in Fig 3B-lane 1). Some tumors had up to five visible clonal rearrangements (Fig 3B, lane 6a).

\( C_\kappa \) rearrangements were analyzed after \textit{Hind}III digestion (Fig 3C). In most individuals, genomic restriction fragment length polymorphism for \( C_\kappa \) (RFLP) includes 12-kb and 9.5-kb bands. As an example, DNA from donor 1 is shown in lane 1. This pattern of RFLP was present in five different donors. A less frequent normal genomic RFLP pattern includes an additional 5-kb band (Fig 3C, lane 5a).19 In SCID/hu tumors from four different donors, no clonal rearrangements of the \( C_\kappa \) were noted (examples shown in Fig 3C, lanes 3a and 3b). In SCID/hu tumors from two donors, possible minor oligoclonal \( C_\kappa \) bands were detected. Examples are shown in panel C, where lanes 4b and 5d contain faint bands in addition to the donors’ \( C_\kappa \) genomic RFLP pattern. Lanes 6 and 7 show additional SCID/hu tumors derived from the same human donor shown in lane 5.

**EBV TR analysis.** DNA from SCID/hu tumors was digested with restriction enzyme \textit{Bam}H\textsubscript{I} and reacted with \textit{Xho} I 1.9 to the EBV TR region.25 Figure 4 shows that this probe does not react with uninfected SCID tissues (lane 1) and that most tumors (lanes 2 to 4) have two or more bands with size greater than 8 kb. In one SCID tumor (lane 2a) a single predominant form of fused termini was noted, but a separate tumor from the same mouse (lane 2b) showed two bands. In comparison with B95-8 cells (lanes 5a and 5b), the SCID/hu tumors did not show the lower molecular weight (ie, 6 kb or lower) TR segments associated with EBV replication.

**Circulating human EBV-positive cells in SCID/hu chimeras.** Blood samples from SCID/hu mice were tested by PCR for the presence of EBV DNA (Fig 5A) and a single copy human gene (HLA-DQ\( \alpha \)) DNA (Fig 5B). Under the conditions of PCR amplification used, the intensity of the HLA-DQ\( \alpha \) signal on the autoradiography is proportional to the number of human cells.27 Human DNA was detectable in SCID blood shortly after transplantation of human lymphocytes and gradually increased until the animal’s death (Fig 5B, lanes 1 through 4). However, EBV DNA was not detected in the circulation until 3 weeks after inoculation of EBV and then increased until the animal’s death. In lane 5, DNA derived from the SCID/hu tumor contains both HLA-DQ\( \alpha \) and EBV DNA in high levels.

**DISCUSSION**

Recently, several different models of creating mouse/human chimeras have been described. Mosier et al19 and others21-22 have injected human lymphocytes from EBV-positive donors into SCID mice and noted the development of aggressive B-cell tumors containing EBV DNA. In a modification of this model termed SCID/hu (EBV), we23 and others27 have reconstituted SCID mice with lympho-
cytes from EBV-negative donors followed by subsequent EBV infection. In the latter model, lymphoid tumors of human origin develop 4 to 8 weeks after infection. We now extend these initial observations by showing that these lymphoid tumors are multiclonal based on rearrangements of their Ig genes and their EBV TR sequences. EBV antigens associated with viral transformation (EBNA-2 and LMP) were present in the tumors, but viral antigens associated with lytic replication (EA-D, EA-R and gp 200/350) were only rarely detected. These results suggest that EBV infection leads to viral transformation of several B cells in the SCID/hu chimera and that these cells subsequently expand to form tumor nodules.

The fate of lymphocytes in the SCID/hu (EBV) model was different from normal lymphocytes infected in vitro by EBV. First, a high frequency of IgG producing cells was noted in the SCID/hu tumors, in contrast to the predominance of IgM containing cells derived from in vitro infection. Second, the lymphoid tumors grew as large nodules containing multiple clones of B lymphocytes rather than as the small clusters of lymphoid cells after in vitro infection. These results suggest that factors provided by the SCID microenvironment promote the progressive growth and differentiation of IgG producing B lymphocytes. Further characterization of these specific factors may provide insight into mechanisms of B-cell-derived maturation and perhaps lymphomagenesis in immunodeficient patients. Third, the SCID/hu (EBV) model provides a method to analyze the interaction of EBV with B lymphocytes in the absence of pre-existing anti-EBV T cells. This situation is analogous to immunodeficient children in whom initial EBV infection may lead to overwhelming lymphoproliferative disease.

The molecular analysis of these SCID/hu tumors confirmed the serologic results, showing multiple rearrangements of the IgG heavy and κ light chain genes, but only infrequent clonal rearrangements of the λ genes. These rearrangements were unique for every tumor. In a single mouse, multiple tumors were often present and each tumor had a distinct pattern of Ig gene rearrangements. The specific variable region Ig gene segments used by these tumors are currently being studied by DNA sequence methods, but preliminary results using a panel of anti-idiotypic antibodies to immunohistologically examine the tumors did not indicate any preferential utilization (unpublished observations, April 1991).

During the subclinical stages of lymphoproliferation, circulating human cells were found 1 week after reconstitution and circulating EBV-infected human cells were detected by 3 weeks. This suggests the possibility of metastatic EBV tumor cells throughout the body at the time of death. However different, lymphoid nodules within the same animal generally had distinct patterns of Ig gene rearrangement. This suggests that an initial tissue distribution of human lymphoid cells occurred in the SCID/hu chimera and that EBV infected these cells in situ. The EBV-infected B cells probably undergo clonal expansion in situ to form the tumor nodules detected at autopsy. In this regard, the SCID/hu (EBV) tumors have similarity to EBV-infected tamarins that develop multiple tumors of different B-cell origin. In addition to human B cells in the SCID/hu (EBV) tumors, a small number of human CD3+ CD8+ T cells were detected. The specific function of these T cells remains unknown, but we have not detected evidence of T-cell reactivity against EBV-associated antigens (unpublished observations, April 1991).

Tumors in the SCID/hu (EBV) model differ from those in the SCID/hu model of Mosier et al. In the SCID/hu (EBV) tumors, only rare cells expressed EBV antigens associated with viral replication (EA-D and EA-R), while such antigens were frequently detected in the Mosier model (and unpublished observations, April 1991). Also, the low molecular weight TR fragments associated with EBV replication were not detected in the SCID/hu (EBV) tumors, but were uniformly present in the tumors from Mosier model SCID/hu mice (unpublished observations, April 1991). In summary, the SCID/hu (EBV) model may provide interesting insights into the lymphoproliferation induced by EBV and the cell-cell interactions that occur in the tumors of some immunodeficient patients.

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