Growth and Spread in Nude Mice of Epstein-Barr Virus Transformed B-Cells from a Chronic Lymphocytic Leukemia Patient


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ABSTRACT

Subcutaneous inoculation of Epstein-Barr virus (EBV) transformed peripheral blood B-lymphocytes (PBL) from an untreated chronic lymphocytic leukemia (CLL) patient produced progressively growing lethal tumors in 4 of 11 whole irradiated (440 rads) nude mice. In one tumor bearing mouse there was splenomegaly and generalized enlargement of lymph nodes. Chromosomal analysis and membrane immunofluorescence revealed that cells in all the 4 s.c. tumors and a proportion of cells in the enlarged spleen and lymph nodes had human chromosomes and contained human \( \alpha \) or \( \lambda \) chains demonstrating that these were polyclonal human B-cells. Epstein-Barr virus associated nuclear antigen could be detected in 100% of cells in all the 4 EBV transformed B-cell lines in vitro and aliquots of cells from several s.c. tumors and metastatic lesions examined. Successful serial transplantation into irradiated nude mice was possible for at least 3 generations with one of the 4 s.c. tumors. During serial transplantation, spread of tumor cells to the spleen and lymph nodes could be detected in all the 3 passage mice investigated; however, there was no evidence in any mouse of dissemination of tumor cells into the bloodstream or into any organ other than lymph nodes and spleen. s.c. tumors also developed in a proportion of irradiated nude mice after inoculation of cells from two other s.c. tumors and the metastatic spleen and lymph nodes, but all these tumors regressed during the first or second transplant passage.

Two % of PBL from the untreated patient and 4% of EBV transformed PBL maintained in vitro were found to have trisomy of chromosome 12 which is the most frequently reported anomaly associated with human CLL B-cells. It is highly probable that the cells with trisomy were derived from the leukemic clone of this patient. Cells with this trisomy predominated in most metastatic sites compared to the parent s.c. tumors.

Inoculation of irradiated nude mice with EBV transformed PBL from this patient after chlorambucil therapy (100% metaphase plates with 46,XY,11q+ karyotype) or with EBV transformed PBL from 2 normal adults failed to produce any progressively growing tumor in a total of 12 irradiated animals observed >300 days. Although there are several reports of EBV induced immortalization of CLL B-cells in vitro, we have not seen any previous report on the successful serial transplantation and dissemination of EBV transformed CLL B-cells in nude mice.

INTRODUCTION

Human CLL is characterized by expansion of monoclonal B- (and occasionally T-) lymphocytes (1). Studies on the biological behavior and response to therapy of human CLL B-cells have been hampered to a certain extent by the difficulty in propagating these cells as xenografts in nude mice. EBV induced transformation has been widely used to "immortalize" both normal B- and CLL B-cells in vitro (2-6) even though CLL B-cells are usually somewhat refractory to the full effects of EBV infection when compared to adult normal B-cells (7, 8); however, the consensus has been that EBV immortalized normal B-cells do not behave as malignant cells in vivo because they neither show progressive lethal growth in immunocompetent patients (9) nor do they usually grow s.c. in athymic nude or immunosuppressed mice after s.c. transplantation (10, 11). Furthermore, although a variety of human tumors have been successfully transplanted in nude mice (12), transplantation of even established human leukemia and lymphoma lines in nude mice has been difficult (13) and succeeded only when tumor cells were inoculated into immunologically privileged sites, e.g., inside the cranium (14) or after subjecting recipients to one or more immunosuppressive measures, e.g., whole body X-irradiation, administration of immunosuppressive agents, and splenectomy (15-17). Regarding metastasis, s.c. xenotransplants of both primary and metastatic human tumors have only limited potential for metastasis in nude mice (18). Metastasis of established human leukemia/lymphoma lines is still more rare even in X-irradiated nude mice (13, 15). We report here the progressive lethal growth along with nodal and splenic metastases in irradiated nude mice of EBV transformed peripheral B-cells from a CLL patient. Chromosomal analysis of xenotransplanted B-cells revealed that cells with trisomy of chromosome 12 (a marker commonly observed in human CLL B-cells) (19-22) predominated in most metastatic lesions compared to the proportion of cells with this marker in the parent s.c. tumors.

EBV transformed PBL from the same patient after therapy with chlorambucil, an alkylating agent, or EBV transformed PBL from 2 normal healthy donors failed to produce any progressive tumor in irradiated nude mice.

MATERIALS AND METHODS

Establishment of B-Cell Lines. The 2 cell lines EBV-CLL(1) and EBV-CLL(3) were established from the PBL of a 53-yr-old Caucasian male suffering from B-cell CLL. For EBV-CLL(1), PBL were obtained before the initiation of any treatment. The patient at this time had generalized lymphadenopathy, splenomegaly, and a PBL count of 27,000 cells/mm\(^3\). The majority of PBL had surface immunoglobulin and receptors for C3 and were therefore B-cells. A bone marrow aspirate and the biopsy of an enlarged lymph node revealed diffuse infiltration of small lymphocytes in these tissues, features compatible with CLL Rai Stage II (23). For EBV CCL(3), PBL were obtained approximately 1 mo after stopping treatment with chlorambucil (4 mg daily for a period of 4 mo). His PBL count at this time had dropped to 7500 cells/mm\(^3\), but he still had splenomegaly and generalized lymphadenopathy. The lymphoblastoid cell lines EBV-David and EBV-Paul were established from the PBL of 2 normal Caucasian males (18 and 22 yr old, respectively).

Lymphocytes were separated from other blood elements by Ficoll-Isoopaque density gradient (24, 25). PBL were incubated for 1 h in EBV enriched supernatant of the culture medium in which EBV transformed B95-8 marmoset cells were grown (10\(^7\) PBL/ml culture medium) after which the cells were pelleted (250 \(\times\) g for 10 min), resuspended in RPMI supplemented with 10% FCS (Flow Laboratories, McLean, VA), and incubated at 37°C in a humidified 5% CO\(_2\) atmosphere (7).

Transmission and Scanning Electron Microscopy. Suspensions of cultures of cells were washed twice in phosphate buffered saline, pelleted (250 \(\times\) g for 10 min) and then fixed and postfixed in 2% glutaraldehyde and 1% osmium tetroxide, respectively. After dehydration, the cells were embedded in epoxy resin, sectioned approximately at 70 Å, stained with uranyl acetate and lead citrate and examined with a Philips.
EM300. For scanning, suspensions of washed cells (2 x 10^6 cells/ml RPMI) were layered on polycarbonate filters, fixed and postfixed in 2% glutaraldehyde and 1% osmium tetroxide, subjected to critical point drying in liquid CO₂, coated with gold palladium, and then examined with a Cambridge S150 model scanning electron microscope.

Assays for Membrane Markers, EBNA, and Free Immunoglobulin. Membrane-bound immunoglobulins were detected by direct membrane immunofluorescence (26) using fluorescently labeled F(ab)₂ fragments of antibodies against human γ, μ, α, δ, ε, κ, and λ chains (Behring-Werke, A.G., Marburg, Germany). The immunofluorescence method of Reedman and Klein (27) was used for the detection of EBNA. For immunoglobulin production, a culture supernatant from various cell lines was concentrated by membrane ultrafiltration (X c 300; Amicon Corp., Lexington, MA) and then assayed by Ouchterlony double diffusion as well as immunoelectrophoresis (26) using specific antisera against various human light and heavy immunoglobulin chains (Behring-Werke, A.G.).

Clonogenic Assay in Soft Agar. The 2-layer agar system of Salmon (28) in 35- x 10-mm Petri dishes was used. Cells suspended in 0.3% Bacto-agar in 20% FCS supplemented RPMI (10^5 or 10^6 cells/ml) were layered on a prehardened underlayer formed by 2 ml of 0.5% agar in FCS supplemented RPMI. The cultures were incubated in humidified 5% CO₂ at 37°C. Clonal growth (>40 cells/colony) was determined with the aid of an inverted microscope.

Xenotransplantation of Cells into Athymic Mice. Female, athymic (nu/nu) BALB/c mice (Life Science Inc., St. Petersburg, FL) weighing 25-28 g were subjected to 440 rads of whole body irradiation from a 300 keV G. E. Maxitron machine at the rate of 170 rads/min. Approximately 48 h later, exponentially growing lymphoblastoid cells, shown to be mycoplasma free, were inoculated s.c. into the flank (5 x 10^6 to 10^7 trypan blue impermeable cells/mouse). Animals maintained under germ free conditions (29) were examined and tumors (if any) were measured 3 times/wk.

Cytogenetic Studies. Chromosomal analysis was carried out on aliquots of (a) PBL populations before EBV induced transformation (these cells were incubated with pokeweed mitogen (GIBCO, Grand Island, NY) in FCS supplemented RPMI for 5 days before harvesting; (b) exponentially growing EBV transformed lymphoblastoid cells before inoculation into mice; and (c) cells derived from s.c. tumors, lymph nodes, and spleen of tumor bearing mice. Surgical blades were used to mince s.c. tumors, spleen, and lymph nodes into a homogenous suspension of cells in RPMI supplemented with 20% PCS. The cultures were incubated in humidified 5% CO₂ at 37°C. Clonal growth (>40 cells/colony) was determined by Ouchterlony double diffusion as well as immunoelectrophoresis (26) using specific antisera against various human light and heavy immunoglobulin chains (Behring-Werke, A.G.).

RESULTS

Characterization of EBV Transformed B-Cell Lines. Morphological changes and rapid growth of PBL in suspension culture could be seen within 2 to 3 wk after exposure to EBV. EBV transformed lymphoblastoid cells were larger (10 to 15 μm diameter) than untransformed human PBL (6 to 7 μm diameter). Transmission electron microscopy revealed strands of endoplasmic reticulum in many of the lymphoblastoid cells. Scanning electron microscopy did not show any morphological difference between EBV CLL(1) and the other 3 lymphoblastoid cell lines. All lymphoblastoid cells had a smooth surface. Anticomplement immunofluorescence revealed EBNA in all 4 lymphoblastoid cell lines. The PBL from the 3 donors were negative for EBNA before exposure to EBV. Various proportions of cells from each of the 4 cell lines reacted with γ, μ, α, δ, ε, κ, and λ specific antibodies demonstrating that the lymphoblastoid cell lines were polyclonal.

Clonogenic Assay. EBV CLL(1) cells formed visible colonies 7 to 14 days after plating 10⁴ cells/ml. Plating efficiency was <0.01%. The average colony size on day 14 was 300 cells indicating approximately 8 cell divisions. EBV-CLL(3) and EBV-Paul formed no visible colonies when plated at concentrations of 1 x 10⁵ or 5 x 10⁵ cells/ml.

Growth of EBV Transformed B-Cells in Irradiated Nude Mice. Of 11 irradiated nude mice inoculated s.c. with EBV CLL(1) cells maintained in vitro for 20 or fewer passages, 1 died within a week of tumor inoculation, 4 did not develop any tumor and remained healthy during an observation period of 300 days, and 6 developed visible tumors at the site of inoculation after a latent period of 15 to 20 days. Two of these 6 tumors eventually regressed after reaching a size of approximately 1 cm diameter. The mice with progressively growing tumors were sacrificed when they became moribund. In one mouse (Fig. 1, mouse 2) there was splenomegaly and generalized enlargement of lymph nodes; this mouse was sacrificed 107 days after tumor inoculation (Fig. 2). Chromosomal analysis and membrane immunofluorescence assay revealed that the tumor cells at the site of inoculation and a proportion of cells in the lymph nodes and spleen had human karyotype and contained human immunoglobulin confirming that these were human B-cells. Inoculation of irradiated nude mice with EBV CLL(3) cells in their 56th in vitro passage or EBV-Paul in the 78th in vitro passage produced...
small tumors in 2 of 6 and 1 of 4 mice after a latent period of about 30 days. All of these tumors completely regressed. Inoculation of EBV-David cells in their ninth in vitro passage did not produce any tumor in 2 irradiated nude mice.

Cytogenetic Findings. Trisomy of chromosome 12 was seen in 2% of pokeweed mitogen stimulated PBL population from which EBV CLL(1) originated. At the time of inoculation into mice, 3 of 80 metaphase plates of EBV CLL(1) cells examined (i.e., approximately 4%) had trisomy of chromosome 12. In the mouse that had metastases in the spleen and lymph nodes (Fig. 1, mouse 2) 30% of cells in s.c. tumor, 38% of cells in the nodes, and 100% of spleen cells had trisomy 12 (Fig. 3). No chromosomal abnormality could be seen in the lymphoblastoid lines EBV-Paul and EBV-David but all the EBV CLL(3) cells analyzed had 46,XY,11q+ karyotype (Fig. 4) at the time of inoculation.

Serial Passage of EBV CLL(1) Cells in Irradiated Nude Mice. With the first tumor (Fig. 1), the number of viable cells were adequate for direct inoculation into one mouse. The recipient mouse died 10 days later.

Dissociated cells from the s.c. tumor, spleen, and lymph nodes of the mouse that had enlarged spleen and lymph nodes (mouse 2) were subcultured 3 times so as to establish their human origin and eliminate contamination of mouse cells and then were inoculated s.c. into irradiated nude mice. Two of 3 mice given cells from the s.c. tumor did not develop any tumor; one had a tumor that subsequently regressed. The one mouse given the cultured lymph node cells also developed a tumor that regressed. Four of 5 mice given spleen derived cells developed tumor at the site of inoculation. Two of these tumors subsequently regressed. One mouse had a static tumor when the mouse was sacrificed. The other mouse had a progressively growing tumor and was killed 98 days after tumor inoculation when the mouse was moribund. After 2 passages in vitro 2 irradiated mice were inoculated with cells derived from s.c. tumor. None of these mice developed any tumor.

Three mice were given injections of cells from the tumor in the third mouse; 2 of these 3 mice developed tumor but one tumor eventually regressed. The mouse with the tumor died of perirectal infection. No attempt was made to further passage this tumor.

Inoculation of tumor cells from the s.c. tumor of the fourth mouse into another irradiated nude mouse produced a progressively growing tumor. This mouse was killed 104 days after inoculation. The lymph nodes and spleen were not enlarged; however, cells with EBV CLL(1) morphology were observed in the dissociated spleen and lymph node cultures on the third and seventh day, respectively. These observations were later confirmed by chromosome analysis. Inoculation of cells from
the s.c. tumor into 3 irradiated nude mice did not produce any
tumor. On the other hand, 2 irradiated nude mice inoculated with
cells from the spleen of the passage 1 mouse (Fig. 1) developed tumors within 2 wk. One of the 2 mice died of tumor
66 days after inoculation. No attempt was made to recover
tumor cells from the dead mouse. The remaining tumor bearing
cell was sacrificed 73 days after tumor inoculation. The
axillary lymph nodes of this mouse were enlarged, but the
spleen was of normal size. Cells with human chromosomes were identified in the cultures derived from dissociated lymph
nodes and spleen of this mouse after 7 and 14 days, respectively.
Cells from the s.c. tumor of this mouse were inoculated (after
10 days passage in vitro) into 3 irradiated nude mice. All 3 mice
developed progressive tumors. One of these 3 mice was sacrificed
28 days after tumor inoculation. There was enlargement of
the ipsilateral lymph nodes in this mouse, but the spleen was
of normal size. Cells with human karyotype were identified in the
dissociated lymph nodal cells before passage in vitro and in
spleen cells harvested on the 11th day of culture.

Examination of aliquots of cells from the s.c. tumor, spleen,
and lymph nodes of mouse 2 and from the third transplantation
passage from the tumor derived from mouse 4 revealed that
100% of cells in all the aliquots were EBNA positive but all the
aliquots contained cells that were positive either for X or a light
chain. The proportion of tumor cells with trisomy of chromosome
12 varied from mouse to mouse and in a given mouse from
lesion to lesion (Fig. 1).

Examination of Blood and Other Tissues. There was neither
any cell with abnormal morphology nor any change in the
number of leukocytes in the peripheral blood of the tumor
bearing mice. Apart from the spleen and the regional lymph
nodes indicated in Fig. 1, there was no histological evidence of
tumor spread to any other tissue in any of these mice.

DISCUSSION

Trisomy of chromosome 12, the most frequently reported
abnormality in CLL B-cells (19–22), was seen in 2% of pokeweed
mitogen stimulated metaphases from the patient's PBL
used for establishing EBV CLL(1) and in 4% of EBV CLL(1)
cells inoculated into nude mice (Fig. 1). It is thus highly
probable that the cells with trisomy 12 in the PBL of this
patient and in the mouse xenografts were derived from the
leukemic clone. We have not seen any previous report on serial
transplantation and dissemination of EBV transformed human
CLL B-cells in nude mice.

The clinical stability and slow progression of human B-cell
CLL (32), suggest that human CLL B-cells represent an early
phase in tumor progression (1). The growth and aggressive
behavior of EBV-CLL(1) cells in our nude mice may be due to
the EBV induced stimulus for uncontrolled proliferation. An
additive or synergistic effect of different oncogenic stimuli in
the progression of cancer is well documented (33–35).

The proportion of cells with trisomy 12 increased from 4% in
tumor inocula to 13 to 100% in s.c. xenografts of EBV-
CLL(1) in mouse 2 and the second and third passages of the
tumor from mouse 4 (Fig. 1); furthermore, the proportion of
these trisomic cells was higher in splenic and lymph nodal
metastases than in the parent s.c. tumor (Fig. 1). These
observations suggest that CLL cells with trisomy 12 had an advantage
in regard to proliferation and spread in our nude mice. The
same may also be true in human CLL patients (36); however,
it is also possible that rapid multiplication of the leukemic clone
increases the probability for acquiring trisomy 12. EBV trans-
formed normal B-cells may also acquire trisomy 12 after pro-
longed culture; but this is not associated with their tumorigenicity
in immunosuppressed mice (11); therefore, the emergence of
trisomy 12 in human B-cells appears to be associated with
increased tumorigenicity, proliferative activity, and aggressiveness
only when the B-cells had undergone prior malignant
transformation. Transformation associated plasma membrane
changes have been well documented in CLL B-cells even though
they are indistinguishable from normal B-cells by conventional
morphology (1).

It is possible that the cells with 46,XY,11q+ anomaly in the
EBV-CLL(3) line arose from a karyotypically normal leukemic
cell or from a leukemic cell that had lost the trisomic chromo-
some 12 by nondisjunction; however, it is more probable that
the anomaly originated in a nonneoplastic B-cell because (a)
the PBL for this line was obtained after chemotherapy induced
reduction in the patient's leukocyte count and disappearance of
PBL with trisomy 12 and (b) in contrast to the observed
progressority of leukemic cells in immunosuppressed mice
(11, 15–17, 37) EBV CLL(3) failed to produce any progressive
tumor after s.c. inoculation into irradiated nude mice. The
11q+ anomaly in lymphoblastoid cells is neither associated
with tumorigenicity (11) nor is very common in long-term
cultures of B-cells (38). Chlorambucil therapy could have
contributed to this anomaly in EBV-CLL(3) cells.

Human CLL usually involves monoclonal proliferation of B-
cells (1). All the EBV-CLL(1) xenografts and their metastases
investigated, contained both X or a chain positive cells indicating
that they were polyclonal. This suggests that EBV transformed
nonleukemic B-cells could also progressively grow and spread
in irradiated nude mice. Fatal polyclonal proliferation of EBV
transformed B-cells has also been observed in immunoinco-
pentent patients (9, 39–41). It appears that EBV transformed
normal B-cells are usually not allowed to behave as malignant
cells in vivo by mechanisms mediated by T- and/or natural killer
cells (42, 43) and in nude mice probably also by a radiosensitive
non-T-cellular population (42). The failure of EBV transformed
normal B-cells, e.g., EBV-Paul and EBV-David to produce any
progressive tumor after S.c. inoculation into irradiated nude
mice in contrast to the s.c. growth, dissemination, and trans-
plantability of polyclonal CLL(1) cells suggests that successful
growth of the EBV transformed leukemic clone could have
provided the milieu (e.g., angiogenic factor) for the progressive
growth of other less advantaged lymphoblastoid cell popula-
ations.

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