ABSTRACT

Subcutaneous inoculation of Epstein-Barr virus (EBV) transformed peripheral blood lymphocytes (PBL) from an untreated chronic lymphocytic leukemia (CLL) patient produced progressively growing lethal tumors in 4 of 11 whole body irradiated (440 rad) 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\(^3\) 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 CLL (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-Isoaque 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 × 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. Suspension cultures of cells were washed twice in phosphate buffered saline, pelleted (250 × 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 × 10⁶ 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 fluoresceinated 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 (Xc 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- × 10-mm Petri dishes was used. Cells suspended in 0.3% Bacto-agar in 20% FCS supplemented RPMI (10⁵ or 10⁶ 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/culture) 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 20 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 × 10⁴ to 10⁵ 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 inoculated 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% FCS. For harvesting metaphases, cells were first exposed for approximately 2 h to Colcemid, 0.05 µg/ml (GIBCO) and then to a 0.075 M KCl solution for 20 min. The cells were fixed in 3 changes of a 3:1 mixture of absolute methanol and eosin for histological examination. 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, and 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 × 10⁵ or 5 × 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

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. Characterization (1978) recommendation (31).
GROWTH OF EBV-TRANSFORMED CLL CELLS IN NUDE MICE

Fig. 2. X-irradiated nude mouse (mouse 2 in Fig. 1) approximately 100 days after s.c. inoculation of $5 \times 10^6$ EBV CLL(1) cells grown in vitro. The tumor shows extensive ulceration (thick arrow). The right and left axillary lymph nodes and the contralateral inguinal lymph node are grossly enlarged (thin arrows).

Fig. 3. Metaphase plate of a CLL(1) cell obtained from the metastatic lesion in the spleen of the mouse in Fig. 2. Arrows, human chromosome 12.

Fig. 4. Karyotype of a G-banded EBV CLL(3) cell showing 46,XY,11q+ chromosomal complement. Arrow, the 11q+ chromosome.

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 mouse 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 λ or κ 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 leukemia 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 leukemia clone increases the probability for acquiring trisomy 12. EBV-transformed normal B-cells may also acquire trisomy 12 after prolonged 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 chromosome 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 tumorigenicity 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 κ or λ 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 immunocompetent patients (9, 39–41). It appears that EBV transformed normal B-cells are usually not allowed to behave as malignant cells in vitro 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 transplantability 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 populations.

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REFERENCES

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