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Deficiency in Epstein-Barr Virus Receptors on B-Lymphocytes of Preleukemia Patients[1]

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ABSTRACT

Lymphocytes from eight preleukemia patients were exposed to Epstein-Barr virus (EBV) *in vitro* in an attempt to establish lymphoblastoid cell lines. No signs of viral infection were detected, and no cell lines were obtained. Studies using fluorescein-labeled EBV and flow cytometry revealed an unusual and consistent deficiency in EBV receptors in all patients examined. In control studies, about 15% of the unseparated lymphocytes from healthy donors bound fluorescein-labeled EBV. In spite of the lack of EBV receptors, B-lymphocytes amounted to 10 to 20% of the preleukemia lymphocyte populations, a proportion similar to that in healthy donors. When lymphocytes from preleukemic patients were first implanted with functional EBV receptors and then exposed to EBV, synthesis of EBV-determined nuclear, early, and viral capsid antigens was induced. Subsequently, several cell lines originating from preleukemic patients' lymphocytes were established. These lines are of a B-lymphocyte origin and carry EBV genome. They will provide experimental material for the molecular analysis of lymphocytic defects in preleukemia and their possible role in the transition to acute leukemia.

INTRODUCTION

The preleukemia syndrome is characterized by a variety of clinical symptoms, the most consistent among them being pancytopenia, hypercellular bone marrow, and maturational abnormalities in erythrocytic, granulocytic, and megakaryocytic progenitor populations (8, 13). No evidence of overt leukemia is present; however, 50 to 75% of these patients ultimately develop acute nonlymphocytic leukemia (9).

The wide range of abnormalities in preleukemia implies that the basic defect(s) responsible for the development of an acute leukemia may reside at the level of the multipotential hematopoietic stem cell. Sufficient data have been accumulated to indicate that cytogenetically aberrant clones are often present in the bone marrow of the preleukemic patients (11, 14). The karyotypic changes are nonrandom, including mainly trisomy for chromosomes 1q, 8, 9, and 21 and loss of all or part of chromosomes 5, 7, 20q, and iso-17q (11, 14). The chromosomes most often involved are Nos. 5 (17.1%), 7 (22.4%), 8 (37.1%), 17 (19.6%), and 21 (23.4%). Sometimes, more unusual chromosome features are reported, such as monosomy 8 and rearrangements involving chromosomes 11, 12, and 16 (23). Nonrandom chromosomal abnormalities of the sort described above accompany the development of most human tumors. It is almost a consensus among oncologists that genetic aberrations are major ingredients, if not the driving force, of the carcinogenesis process (2, 7, 16). Chromosomal aberrations in bone marrow cells of the preleukemic patients may thus account for the increased risk for the ultimate development of leukemia.

Current concepts regarding the differentiation of lymphoid and myeloid cells imply that both lineages originate from a common pluripotent stem cell in the bone marrow (4, 5). The pluripotent stem cell gives rise to the committed lymphoid and myeloid stem cells, and these differentiate into the circulating blood elements (4, 5). If the genetic aberrations in preleukemia happen in the pluripotent bone marrow stem cells, they may lead to a gradual expansion of myeloid and lymphoid clones. Thus, although preleukemia usually converts into acute nonlymphocytic leukemia (8, 13), one may expect to find aberrant preleukemia lymphocytes as well. Abnormalities in the latter could lead to a decreased immune activity and could predispose to the transition from preleukemia to acute leukemia. Analysis of the preleukemic lymphocytes, on the other hand, may help to evaluate the nature of the molecular defects underlying this syndrome.

The preliminary characterization of preleukemic patients' lymphocytes in our laboratory included analysis of surface markers and attempts to establish lymphoblastoid cell lines. Human B-lymphocyte cultures can usually be obtained by transforming cells with EBV (Footnote 3; Ref. 10). Only human and certain primate B-lymphocytes express EBV receptors and can serve as targets for EBV-mediated transformation (10, 22). Here, we report our inability to establish cell lines even by repeated exposure to EBV of cells derived from 8 patients with preleukemia. Subsequent studies revealed that B-lymphocytes from all 8 patients were deficient in EBV receptors. When, however, functional EBV receptors were implanted onto these cells (21), preleukemic patients' lymphocytes could then be infected and transformed by EBV; permanent cell lines were established.

MATERIALS AND METHODS

Patients. The 8 patients studied satisfied the diagnostic criteria for preleukemic syndrome of Linman and Bagby (9). Their peripheral blood smears showed anisocytosis, poikilocytosis, and macrocytes; marrow aspirates revealed megaloblastoid erythropoiesis and in some instances ringed sideroblasts. There was no evidence of overt acute leukemia. Bone marrow of these patients was hypercellular, with maturational abnormalities in at least one cell lineage, and included fewer than 5% blast cells. Megaloblastic anemia, secondary to vitamin B_{12} and folate deficiencies, was ruled out in each case. In several patients, levels of fetal hemoglobin were increased. The ages of the patients ranged from 19 to 83 years, and none had received any cytotoxic drugs. WBC and differentials from the preleukemic patients showed varying degrees of granulocytopenia (Table 1). One patient (Patient 1) evolved into frank

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[3] The abbreviations used are: EBV, Epstein-Barr virus; EBNA, Epstein-Barr virus-determined nuclear antigen; FITC-EBV, fluorescein isothiocyanate-labeled Epstein-Barr virus; EA, Epstein-Barr virus-determined early antigen; VCA, Epstein-Barr virus-determined virus capsid antigen.

Table 1
Percentage of differential WBC of preleukemic patients

	Age (yr)	Sex	WBC	Band neutrophils	Segmented neutrophils	Lymphocytes	Monocytes	Eosinophils	Basophils
Patient									
1	83	M	2,900	9	51	27	8	1	0
2	79	M	2,500	0	61	14	12	9	4
3	19	F	4,700	1	41	48	7	0	0
4	80	F	9,800	11	70	8	4	5	2
5	62	M	1,300	4	40	52	0	0	0
6	82	F	3,900	19	22	42	15	1	1
7	73	M	3,600	3	24	68	5	0	0
8	75	M	1,300	4	40	52	0	0	0
Controls			4,000–11,000	0–10	43–74	13–43	2–12	0–6	0–2

acute nonlymphocytic leukemia several months after being studied, but the remainder are clinically stable.

Lymphocyte Separation. Following informed consent, 10 to 20 ml of blood were taken by venipuncture. Peripheral blood leukocytes were separated by Ficoll:Isopaque (Pharmacia, Uppsala, Sweden) density sedimentation and used immediately.

EBV and Sendai Virus. The transforming B-95-8 substrain of EBV (B-EBV) was obtained from supernatants of starving B-95-8 cell cultures after the cells and debris were removed by low-speed centrifugation. The virus was purified and concentrated to 0.2% of the initial volume. The infectivity of B-EBV was assessed by its ability to induce EBNA in Ramos cells, an EBV genome-negative cell line of Burkitt's cell line origin. To prepare FITC-EBV, the 500 times-concentrated virus in 0.5 ml 160 mM NaCl:10 mM phosphate buffer, pH 7.5, was supplemented with fluorescein isothiocyanate (Sigma Chemical Co., St. Louis, Mo.). The viral protein:stain ratio was 2:1. After 1 hr at room temperature, the labeled virus was separated from an unbound stain by 2 passages through Sephadex G-25 (Pharmacia) columns and ultracentrifugation. The virus remained biologically active, as assessed by its ability to induce EBNA. Sendai virus was propagated in 10-day-old fertilized chicken eggs, isolated from allantoic fluid, and tested for the fusion and agglutination activities as described previously (22).

Transplantation of EBV Receptors. Isolation of Sendai virus envelopes by solubilization of whole virions with Triton X-100 (Sigma) and coreconstitution with purified Lukes cell (EBV receptor-positive cells of the American Burkitt's lymphoma origin) membranes was as described previously (22). The coreconstituted hybrid Sendai virus-EBV receptor vesicles were used for the receptor implantation as follows; 10×10^6 lymphocytes in 0.5 ml of buffer containing 160 mM NaCl, 20 mM *N*-tris(hydroxymethyl)methylglycine, and NaOH, pH 7.2, (fusion buffer) were supplemented with 20 μg of the reconstituted vesicles at 4°. After 10 min, the agglutinated cell:vesicle mixture was transferred to 37° for 30 min, followed by 2 washes in Roswell Park Memorial Institute Tissue Culture Medium 1640 (plus 10% fetal calf serum; Grand Island Biological Co., Grand Island, N. Y.). The receptor-implanted cells were then exposed to EBV and cultured under standard conditions of 5% CO_2:95% air.

Analytical Assays. EBV receptors and surface immunoglobulin (B-cell markers) and OKT-11 receptors (T-cell marker) were detected by flow cytometry using an Ortho cytofluorograph, FITC-EBV, fluorescein-conjugated $F(AB')_2$ fragment, goat anti-human immunoglobulins (IgA, IgG, IgM), and monoclonal OKT-11 antibodies (Ortho Pharmaceutical Corp., Rahway, N. J.). EBNA was detected by an anticomplement immunofluorescence method as described by Reedman and Klein (15). The immortalization of lymphocytes by EBV was assessed morphologically (detection of large colonies of living cells 2 to 4 weeks after application of the virus) and by detecting EBNA in the majority of the 2- to 4-week-old cells.

RESULTS

Lack of EBV Receptors on Lymphocytes from Preleukemic Patients. All preleukemia patients examined displayed a lack of

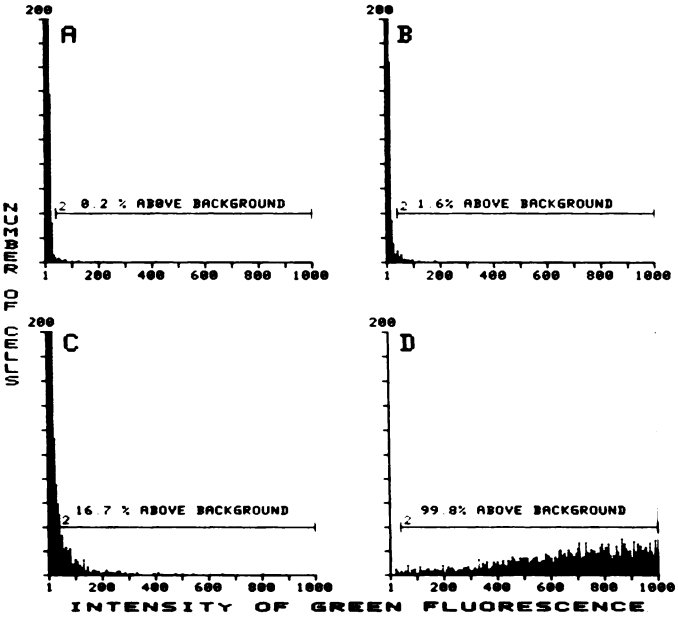

Chart 1. Assay for EBV receptors on normal and preleukemic patients' lymphocytes using FITC-EBV and flow cytometry. EBV was labeled with fluorescein isothiocyanate as described in "Materials and Methods." FITC-EBV receptor binding assay: aliquots of FITC-EBV were added to 5×10^6 cells in 50 μl of Roswell Park Memorial Institute Tissue Culture Medium 1640 (plus 10% fetal calf serum) and incubated in darkness for 60 min at 20°. Following viral adsorption, cells were centrifuged through a 5% sucrose cushion, and the pellets were washed once and analyzed in an Ortho cytofluorograph. *A* and *B*, different preleukemic patients; *C*, normal donor; *D*, Lukes' cell line of the American Burkitt's lymphoma origin.

EBV receptors on their lymphocytes, as assessed by the absence of specific FITC-EBV association with the cells (Chart 1, *A* and *B*; Table 2). Since the EBV receptors are considered as specific surface markers for the mature normal human B-lymphocytes, the preleukemia cells were analyzed for their relative proportion of B-cells. As shown in Table 2, the surface immunoglobulin-expressing cells, which consist mostly of B-lymphocytes, amounted to 10 to 20% of the preleukemic lymphocyte population, a proportion similar to that of healthy donors. The preleukemic cells were also positive for complement C3d receptors and monoclonal B-1 marker (not shown). Plasma immunoglobulin levels of the patients were within normal limits, indicating unimpaired B-cell secretory function (1). The T-cell subpopulation in preleukemia, as indicated by OKT-11 and OKT-3 positivity, ranged between 13 and 80% of the total lymphocyte population (Table 2). Unlike the lymphocytes from preleukemic patients, about 15% of the unseparated lymphocytes from healthy donors were found to bind FITC-EBV (Table 2). This is in agreement with the proportion of cells that bound the surface immunoglob-

ulin (used as a B-cell marker). The specificity of the EBV receptor detection assay was demonstrated using homogeneous receptor-positive and -negative cell lines. Originating from human T-cell leukemia, 1301 cells did not significantly bind FITC-EBV. In contrast, EBV was found adsorbed to virtually all of the Lukes cells which originated from the American Burkitt's lymphoma, of EBV cell origin (Chart 1*D*). Several patients were tested repeatedly for the presence of EBV receptors on their lymphocytes over a period of 4 months, always with the negative results as described above.

Interaction of Preleukemia Lymphocytes with EBV. Exposure of normal human B-lymphocytes to EBV *in vitro* results in the virus adsorption, its penetration into the cells, expression of EBNA within 12 to 24 hr, induction of cellular DNA synthesis, and subsequent transformation into a lymphoblastoid cell line (10). None of these events occurred when preleukemia lymphocytes were exposed to EBV (Table 2). Control cells obtained from healthy individuals showed all the stages of virally induced transformation described above (Table 2). Clearly, the lack of interaction of EBV with preleukemia lymphocytes resulted in the first place from the lack of viral adsorption to the cells (Chart 1). It was not certain, however, whether the receptor-negative B-cells of preleukemia patients would also prove to be defective in their capacity to express EBV DNA, if the latter could be introduced into the cells by bioengineering techniques. We have shown recently that functional EBV receptors can be implanted onto membranes of the receptor-negative cells using reconstituted Sendai virus envelopes as the transplantation vehicles (21, 22). EBV receptor-implanted cells become, in most cases, suitable targets for EBV infection. We therefore applied the EBV receptor implantation technique to convert preleukemia lymphocytes temporarily into EBV receptor-positive target cells. The manipulated cells bound FITC-EBV (not shown). Their exposure to the transforming B-EBV resulted in both EBNA induction and a subsequent cell transformation in 3 of 4 cases (Table 2). The lines originating from preleukemic patients' lymphocytes were found to be heterogeneous with respect to the expression of several surface markers as assessed by flow cytometry: 99% of cells were surface immunoglobulin positive; 70% expressed complement C3d receptors; and no cells presented T-cell markers. These findings imply that the cell lines are of a B-cell lineage. With respect to their EBV status, all cells expressed EBNA, 4 to 5% of cells expressed EA, and less than 0.5% were VCA positive.

DISCUSSION

The preleukemic syndrome is characterized by a very heterogeneous set of signs and symptoms, most prominent among these being abnormalities in the myeloid cell population (8, 9, 13). Its etiology remains unknown. There have been as yet no cell lines originating from patients with preleukemia that could be used for the detection of possible pathogenetic molecular defects. Moreover, due to the uncertain clinical diagnosis of preleukemia, there has been no treatment aimed at preventing the conversion from the preleukemia to the full acute leukemia state.

The present work describes an abnormal B-lymphocyte feature, the deficiency in EBV receptors, in 8 preleukemic patients. In contrast, normal mature human B-lymphocytes have EBV receptors and can be transformed by the virus (6, 12, 24). The receptors, like receptors for complement (C3d), may be regarded as differentiation antigens marking a population of immunologically active B-lymphocytes. Neither lymphoid stem cells and immature B-cells nor the mature plasma cells possess EBV and C3d receptors, nor can they be infected by the virus (6, 12, 24). The absence of viral receptors on B-lymphocytes from preleukemic patients may thus indicate a deficiency in certain active immunocompetent B-cell subsets. The relevance of this observation to certain clinical manifestations in preleukemic patients, such as the reduced resistance to bacterial infection (13), is not

Table 2

Analysis of preleukemia lymphocytes for the presence of EBV receptors, B- and T-cell surface markers, and transformability by EBV

	Surface markers (% of positive cells)			Exposure of nonmanipulated cells to EBV[c] EBNA-positive cells (%)			Exposure of EBV receptor-implanted cells to EBV[c] EBNA-positive cells (%)		
	EBV receptor[b]	Surface immunoglobulin	OKT-11 or OKT-3 receptors	3 days	4 wk[d]	Immortalization	3 days	4 wk	Immortalization
Patient									
1	0.8	20.4	55.2[e]	0		–	2	100	+
2	0.8	15.1	47.4	0		–	1.5	0	–
3	0	9.5	26.4	0		–	1.0	100	+
4	0	10.5	35.3[e]	NT[f]			NT		NT
5	0.3	14.6	47.6[e]	0		–	2.0	100	+
6	NT	10.7	75.4	0		–	NT		NT
7	NT	NT	82.1	0		–	NT		NT
8	0	9.6	13.3[e]	0		–	NT		NT
Healthy donors									
1	15.3	19.0	83.4	3	100	+			
2	15.4	12.9	67.9[e]	5	100	+			
3	11.4	10.1	67.9	2	100	+			

[a] Immortalization, establishment of continuously growing cell line within 2 to 4 weeks after the infection.
[b] EBV receptors were assayed using FITC-EBV as shown in Chart 1. The values in the table represent specific FITC-EBV binding, which was obtained by subtracting a nonspecific adsorption of the virus to T-lymphocytic cell lines, such as 1301 or YAC-1, (usually 1.5%) from the absolute adsorption values as exemplified in Chart 1.
[c] EBV receptor-implanted and nonimplanted preleukemia or control lymphocytes were suspended in Roswell Park Memorial Institute Tissue Culture medium 1640 (plus 10% fetal calf serum and antibiotics) at the cell concentration of 2×10^6/ml. Following addition of EBV, the virus:cell mixture was incubated for 1 hr at 37°, washed, and cultured under standard conditions. Medium was changed once a week. Lymphocytes from healthy donors were not submitted to receptor implantation.
[d] All nonmanipulated, EBV-exposed preleukemic cells died within 2 to 3 weeks and were not tested for EBNA.
[e] OKT-3 receptor.
[f] NT, not tested.

yet clear. Previously, it was assumed that neutropenia or granulocyte dysfunction was responsible for these infections (9). Analysis of other B-cell differentiation markers such as the intracellular, surface, and secreted immunoglobulin, Fc, C-3, and insulin receptors may show further abnormalities in the differentiation status of the preleukemia *versus* normal B-lymphocytes. The presence of surface immunoglobulin taken with the absence of EBV receptors on B-cells (Table 2) indicates that preleukemia lymphocytes may belong to the category of lymphoplasmacyte-plasma cells, according to the criteria for the normal B-cell lineage (12).

Although the lymphocytes from preleukemic patients are EBV receptor negative, they can be infected and transformed by EBV following EBV receptor implantation, a technique that allows introduction of viral DNA into the cytoplasm (Table 2). Using this approach, several cell lines originating from the preleukemia cells were established (20). All the cell lines were of B-cell origin (20), which indicated that the EBV-infected T-lymphocytes were eliminated by the viral lytic cycle, as reported elsewhere (21). The results of the present study confirm our previous finding that virtually all human and animal cells can be infected by EBV, if the membrane barrier resulting from the lack of EBV receptors is bypassed by receptor implantation (17, 18, 20–22). Clearly, however, viral infection appears to be followed by cell transformation only in human B-lymphocytes and, possibly, in normal epithelial cells (17, 18). The approach described here may also be helpful in other cases, such as Hodgkin's disease and chronic lymphocytic leukemia, where the establishment of B-lymphoblastoid cell lines by standard methods seems impossible.

The expression of EBV DNA in preleukemic patients' lymphocytes differed from that in normal human cells. EBV-infected normal human lymphocytes usually display a tight control over the EBV life cycle, allowing only for a latent expression of viral functions as reflected by the total block of the viral reproductive cycle, exclusive expression of EBNA, and cell immortalization (10). After prolonged cultivation *in vitro*, a small proportion (1 to 2%) of the EBV-transformed lymphoblastoid cell lines loses its control over the viral lytic pathway, starts to express EA and VCA, and releases small quantities of new viral particles (10). In contrast, when the cells from preleukemic patients were implanted with receptors and infected by EBV, a significant proportion of the cells expressed EA and VCA as early as 3 days after the infection. Replicated viral DNA and new EBV particles could be detected by cytohybridization and electron microscopy, respectively, 3 to 4 weeks after the infection. Cell lines established from preleukemic lymphocytes are now used for a regular production of infectious EBV in our laboratory.

All the preleukemic patients tested had serum antibodies against EBV antigens, just as do the healthy individuals who have had infectious mononucleosis and developed a life-long immunity to EBV (3). However, the lymphocytes from preleukemic patients, unlike the cells of healthy seropositive persons, are deficient in EBV receptors. It is likely that the individuals with preleukemia did have EBV receptor-positive cells when they were originally exposed to EBV. These cells might subsequently have been lost or altered during the progression of cellular molecular changes related to the development of preleukemia. The resulting lymphoid stem cells of preleukemic patients may have as yet undefined molecular defects at the level of gene expression or its control, as reflected by the lack of EBV receptor on the differentiated circulating B-lymphocytes and an incomplete control of EBV infection. This confirms our hypothesis that genetical aberrations in pluripotential stem cells in the bone marrow of preleukemia patients (11, 14, 23) may be transmitted to lymphoid stem cells and circulating lymphocytes. Additional indication is our recent finding that preleukemia patients' T-lymphocytes have a generally low or absent natural killer cell activity, as well as an aberrant T-regulatory cell function (1). The newly established lymphoblastoid cell lines originating from preleukemic patients will provide ample experimental material for the molecular studies devoted to exploring the above hypothesis.

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