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Continuous Lymphoid Cell Lines with Characteristics of B Cells (Bone-Marrow-Derived), Lacking the Epstein–Barr Virus Genome and Derived from Three Human Lymphomas

(membrane receptors/nucleic acid hybridization/nuclear antigen)

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ABSTRACT Three exceptional cell lines have been tested for the presence of the Epstein–Barr virus genome by nucleic acid hybridization (complementary RNA·DNA) and Epstein–Barr virus-determined nuclear antigen tests. Two lines were derived from Swedish lymphoma cases and one from an African Burkitt-like lymphoma biopsy that was negative for Epstein–Barr virus DNA and the virus-determined nuclear antigen. All three lines apparently lacked the viral genome. Two of the three lines clearly had characteristics of B-cells (bone-marrow-derived).

All previously established human lymphoblastoid cell lines of the B-cell (bone-marrow-derived) type were found to carry the genome of the Epstein–Barr virus (EBV), irrespective of origin (1–4). This is closely correlated with the presence of the EBV-determined nuclear antigen (EBNA), which is expressed in the nuclei of all EBV-genome carrying lines tested (5). This carrier state is not surprising, if considered in light of the fact that EBV can transform normal lymphocytes of EBV-negative subjects, or cord blood cells, into virus-genome-carrying, established lines that grow permanently *in vitro* (for a review see ref. 3). When African Burkitt lymphoma tissues that carry the EBV-genome (6–8) are explanted, EBV-genome-carrying lines become established that are representative for the tumor in most cases, since they carry the same clonal markers (9). The few exceptions where the derived, EBV-carrying cells had discordant markers were probably due to the presence of allogeneic lymphocytes in the tumor, derived from transfused blood (9). Explantation of leukemia and myeloma cells usually does not lead to the outgrowth of the leukemia or the myeloma cells; instead, a small minority of EBV-genome-carrying B-lymphoid cells will grow, as a rule, that are thus nonrepresentative for the neoplastic cell in these conditions (10–13). In two very exceptional cases, the myeloma cell itself grew out, however. The two derived cell lines, 266B1 (12) and 8226 Simpson (14) differed from typical lymphoblastoid cell lines with regard to growth and morphology. Analysis of the immunoglobulins secreted by the two lines showed identity with the myeloma protein of the donor. The cells did not carry the EBV genome (5, 15, 16). Even so-called "purified" human T-lymphocyte (thymus-derived) suspensions gave rise, after EBV infection, to EBV-genome-carrying lines with B-cell characteristics (W. Leibold, M. Jondal & G. Klein, unpublished data). This is understandable in view of the fact that only B, but not T, lymphocytes were found to carry receptors for the Epstein–Barr virus (17). Taken together, these findings show that EBV-genome-carrying B-cells possess a great selective advantage under the conditions of *in vitro* suspension cultures. This also implies that the presence of EBV-genome-carrying lymphoid cells in normal or malignant lymphocyte suspensions makes the successful establishment of any other cell type in continuous suspension culture rather unlikely.

The only previously known exception to the rule that all human lymphoblastoid cell lines carry the EBV genome *in vitro* was the Molt-4 line, derived from the peripheral blood of a patient with acute lymphoblastic leukemia (14, 16). Unlike the lines so far mentioned, however, Molt-4 had T-cell characteristics, since it made direct rosettes with sheep red cells and had no surface immunoglobulin or Fc receptor. Moreover, Molt-4 cell extracts contain terminal deoxynucleotidyl transferase activity, an enzyme found in thymus but not in bone marrow (18). It is therefore likely that Molt-4 represents a leukemic T-cell, and the notion that B-cells with a lymphoid morphology cannot grow *in vitro* as established lines without the EBV-genome was still defensible.

The present paper reports the first evidence for established B-cell lines of human origin with lymphoid morphology that lack the EBV genome, as judged by the absence of detectable EBV DNA and EBNA antigen. The establishment of the U-698M and U-715M cell lines, derived from Swedish lymphoma cases, has been described elsewhere (19). The history and the morphological and growth properties of these lines differed from the usual, EBV-genome-carrying human lymphoblastoid cell lines and it was therefore of interest to study whether these lines did carry the viral genome. The same was true for the third line, BJA-B-1, which came from an unusual African Burkitt-like lymphoma case (J. Menézes, W. Leibold & G. Klein, manuscript in preparation). Unlike most other African Burkitt lymphomas that are EBV-genome- and EBNA-carrying clones, the donor from whom this line was derived (JA), although EBV seropositive, did not have detectable EBV genomes or EBNA in her tumors on

Abbreviations: EBV, Epstein–Barr virus; EBNA, nuclear antigen determined by presence of the Epstein–Barr virus; B, bone-marrow-derived; T, thymus-derived; RBC, red blood cells; FITC, fluorescein isothiocyanate; cRNA, RNA complementary to DNA; Fc, the portion of 7S immunoglobulin that forms a crystallizable fragment; EA and VCA, intracellular antigens determined by Epstein–Barr virus.

repeated biopsies (8, 20). It was therefore of great interest to determine whether this cell line was representative for the virus-genome-negative biopsy or was merely the outgrowth of an admixed, nonrepresentative EBV-genome-carrying lymphoid cell-type.

MATERIALS AND METHODS

Cell Lines. Details about the establishment and characteristics of BJA-B-1 and of U-698M and U-715M are described elsewhere (ref. 19 and J. Menézes, W. Leibold & G. Klein, manuscript in preparation). In brief, the BJA-B-1 line was derived from the fresh biopsy material of an African patient (JA) with the diagnosis of Burkitt's lymphoma. Biopsy cells were kept in suspension culture at 37°, 5% CO_2 in air and 80–95% relative humidity. RPMI 1640 (Gibco) supplemented with 20% fetal calf serum, penicillin, streptomycin, Fungizone, and *N*-2-hydroxyethyl piperazine-*N'*-2-ethanesulfonic acid (Hepes) buffer was used as the medium. After 52 days in culture, BJA-B-1 was established as a permanent line. It is maintained under the same conditions by subculturing twice a week.

U-698M and U-715M lines were established by the Spongostan grid organ culture technique previously described (13). Fragmented tissue from a lymphosarcoma tonsil (U-698M) and a lymphoreticuloma lymph node (U-715M) was explanted on the grids, which were placed in 60-mm Falcon petri dishes. Cells that appeared in the medium from the organ cultures during cultivation were transferred at routine medium renewal to 50-ml Erlenmeyer flasks containing a monolayer of adult skin fibroblasts as feeder cells. The U-698M and U-715M cell lines were considered as established 53 and 44 days after *in vitro* explantation, respectively. They were maintained as stationary suspension cultures in Hams F-10 medium supplemented by 10% fetal calf serum and antibiotics (100 IU penicillin per ml, 50 μg of streptomycin per ml, and 25 μg of amphotericin B per ml). Prior to testing, they were fed with the same medium as the BJA-B-1.

Sheep Red Blood Cell (RBC) Rosettes. Fifty microliters of a 1% sheep RBC suspension in 100% fetal calf serum were mixed with 50 μl of cells, at a concentration of 10^7/ml, in 100% fetal calf serum. After shaking, the cells were pelleted, incubated at 37° for 15 min and at 4° for 60 min. The pellet was carefully lifted with a pasteur pipette and transferred to a slide. Rosettes were counted under sealed coverslip with oil immersion at ×800. Two hundred cells were counted.

Fc Rosettes. (Fc is the portion of the 7S antibody that is found in the crystallizable fragment.) Suspended rat RBC (50 μl), covered with maximal subagglutinating quantity of rabbit 7S antibody (purified on Sephadex G-200), were mixed with 50 μl of cells and pelleted. After incubation at 37° for 30 min, and vigorous shaking, 200 cells were counted. No rosettes were observed in the control cell suspension, containing only rat RBC.

Complement Rosettes. Rat RBC (50 μl), covered with a maximal subagglutination titer of rabbit 19S antibody (purified on Sephadex G-200) and subsequently treated with fresh A.SW mouse serum at a dilution of 1:3, were mixed with 50 μl of cells. Rosette formation was read as for Fc rosettes. The controls were treated similarly, but heat inactivated complement was used; no rosettes were observed.

Rosette Tests for EBV Receptor (ref. 17). Virus-producing P3HR-1 cells were membrane-stained with anti-MA-positive fluorescein isothiocyanate (FITC)-conjugated Abwao immunoglobulin, diluted 1:4, as described (17). After incubation for 30 min at 4°, and three washes, 50 μl cells, at a concentration of 10^6 per ml, were added to a pellet of 50 μl of target cells (concentration 10^7/ml) and the mixture was resuspended. After 60-min incubation at 4°, 200 membrane-labeled cells were checked for EBV-rosette formation.

Membrane Fluorescence Tests for Surface Immunoglobulin. Cells (0.5×10^6) of the three lines were labeled with 100 μl of goat antiserum to human immunoglobulin, FITC-conjugated (Hyland). After 30-min incubation at 4°, the cells were washed three times with cold medium. In a second step, they were exposed to an FITC conjugate of rabbit antiserum to goat 7S immunoglobulin (Hyland). After 30-min incubation at 4°, the cells were washed three times in medium and the number of membrane-fluorescence-positive cells was evaluated on a sample of 200 counted cells. Both FITC-conjugates were preabsorbed with human erythrocytes. They did not stain Molt-4, a surface-immunoglobulin-negative, T-cell-derived line (14). In addition, the U-698M and U-715M cells have also been examined by direct immunofluorescence with FITC-conjugated rabbit antisera specific for human α, γ, and μ heavy (H) chains and κ and λ light (L) chains and by an indirect technique employing rabbit antisera against the same H- and L-chains and an FITC conjugated goat antiserum against rabbit immunoglobulin. All sera were purchased from Dakopatts, Copenhagen and Behringwerke, Marburg-Lahn. Prior to use sera were checked for specificity by immunodiffusion. Dilutions were made with fetal calf serum.

Nucleic Acid Hybridization Tests. Hybridization with RNA complementary to EBV DNA (cRNA) (ref. 2) was performed as described (8). Filters containing 10 μg of cellular DNA were incubated with 120 000 cpm ^{32}P-labeled EBV cRNA (7×10^7 cpm/μg) in 0.3 ml of 0.9 M NaCl, 0.09 M sodium citrate, pH 7.5, 50% formamide, for 3 days at 45°.

EBV Antigen Tests. All three lines were tested for the EBV-determined intracellular antigens, EA, VCA, and EBNA. EA and VCA were assessed on acetone-fixed smears, using the FITC-conjugated human immunoglobulins, F-Nathan for EA (dilution 1:40) and F-Agnes for VCA (1:40), as previously described (21). As positive controls, the EA-VCA producer cord blood cell line cb-8-7 was used. EBNA was tested by anticomplement immunofluorescence, as previously described (5). Each test included two EBNA-negative control sera (EK and IE) and two EBNA-positives (OO and PG). All sera were tested in a dilution of 1:8. Smears were prepared and fixed by acetone–methanol as described (5). As positive controls, the EBV-genome-carrying Raji line was used. It gave regularly positive reactions with the OO and the PG sera, but not with EK and IE. Fresh complement of EK was used as the complement source. The Hyland goat anti-human β1 C reagent was used to stain EBNA, at a dilution of 1:40. Raji nuclei were brilliantly stained in 90–95% of the cells, provided positive sera were used.

RESULTS

Membrane Receptors. Table 1 shows the results of membrane receptor tests on the three lines. It appears that both BJA-B-1 and U-698M had clear B-cell characteristics, as judged by

TABLE 1. *Membrane receptor tests**

Cell line designation	Sheep RBC receptors	Fc receptors	Complement receptors	EBV receptors†	Surface immunoglobulin
BJA-B-1	0	0.5	12	93	100
U-698M	0	2.5	0	87	100
U-715M	0	1.5	0	3	‡

* The figures designate the percentage of positive cells.

† Percentage of EBV-MA-positive cells that formed rosettes with the test cell population.

‡ Positive with polyvalent anti-Ig, negative with monovalent anti-H and anti-kappa reagents used. Production of small amounts of alpha heavy chains was detected by a sensitive immunochemical method (ref. 19).

surface immunoglobulin and EBV receptors and the absence of rosette formation with sheep RBC. The results with U-715M are less clear. It has few or no EBV-receptors, no sheep RBC, Fc, or complement receptors. The majority of the cells gave a positive reaction with the polyvalent anti-Ig reagent but failed to react with the monovalent anti-H and anti-kappa reagents used. However, a small amount of alpha heavy chains has been detected in the cell lysate of U-715M (ref. 19). It is conceivable that this line has been derived from a so-called O-cell, rather than a T- or B-cell. Alternatively, U-715M was derived from a B- or T-cell that was drastically changed in its phenotypic expression of normal surface receptors in connection with the malignant transformation.

Nucleic Acid Hybridization. As shown in Table 2, two EBV-DNA-carrying B-cell lines, Raji and NC37, were found to contain 57 and 78 viral genomes per cell by nucleic acid hybridization, in good agreement with the data of Nonoyama and Pagano (2). In contrast, no hybridization above that obtained with negative controls, e.g., HeLa cell DNA and salmon sperm DNA, was observed with the EBV [^{32}P]cRNA and DNA from the cell lines U-698M, U-715M, and BJA-B-1. It is concluded that the three cell lines studied here contain less than two, and probably less than one, EBV genome per cell.

EBV Antigen (EA, VCA, and EBNA) Tests. Table 3 summarizes the results. In view of its importance, the EBNA test was repeated more than a dozen times on U-698M, U-715M, and BJA-B-1, always including Raji as a positive control. Whereas, brilliant EBNA staining was obtained in 90–95% of the Raji cells in every test, no EBNA staining was ever seen in the three test lines. Two other EBV-genome-carrying controls, cb-8-7 and P3HR-1, were also regularly EBNA-positive; they were also positive for EA and VCA. The EBNA-negative U-698M, U-715M, and BJA-B-1 lines were negative for the other two EBV antigens, EA and VCA, as well.

DISCUSSION

In mutual agreement, the nucleic acid hybridization and the EBNA tests showed that the three test lines, U-698M, U-715M and BJA-B-1, did not contain detectable EBV genomes. In addition to the EBV-genome-positive controls shown in Table 2, 13 other lymphoid cell lines of the B-cell type, derived either from Burkitt's lymphoma biopsies or from normal donors, were all found to contain both EBNA and EBV DNA although they did not produce other EBV-associated antigens or EBV particles. The different lines contained 5–60 EBV genome equivalents per cell, with an average value of 25 genomes per cell (T. Lindahl, unpublished results). Similar results have also been obtained by others (15, 22). The present data do not exclude the possibility that the three cell lines studied here were transformed by a fragment of an EBV DNA molecule not coding for or expressing EBNA. Additional data on this point may be obtained by the more sensitive hybridization technique of reassociation kinetics (16).

TABLE 2. *Hybridization between EBV cRNA and DNA from the three cell lines, together with negative and positive controls*

DNA source	No. of determinations	cRNA cpm bound per 10 μg of DNA	EBV genomes per cell
HeLa	4	430	< 2
NC37	3	7219	78
Raji	4	5393	57
U-698M	4	449	< 2
U-715M	2	394	< 2
BJA-B-1	3	427	< 2

At least two of the EBV-DNA-negative and EBNA-negative test lines, BJA-B-1 and U-698M, had clear B-cell characteristics. These results therefore show that it is possible, at least under special circumstances and from exceptional patients, to establish permanent B-cell lines, capable of continuous growth *in vitro*, that apparently do not carry the EBV genome. It must be added, however, that all three cell lines were derived from malignant lymphomas and presumably represent the malignant cell itself. It is conceivable that the same change that has rendered them malignant *in vivo* (another virus?) has also "immortalized" them, i.e., endowed them with the capacity of continuous *in vitro* growth. There is no evidence that the B-lymphocytes of normal individuals can grow as *in vitro* lines, unless they carry the EBV genome.

The history of BJA-B-1 deserves special comments. It has been derived from the biopsy of an African patient with the diagnosis of Burkitt's lymphoma, JA (KCC 1490) (8, 20). In previous nucleic acid hybridization studies and EBNA tests, repeated biopsies of this patient were EBV DNA and EBNA negative. This tumor thus differs from the majority of the African Burkitt lymphomas that are regularly EBV DNA and EBNA positive (6–8). It can be noted, however, that the Burkitt-like lymphomas occurring outside Africa have been found, so far at least, to be negative for EBV DNA (23) and the same is true for other non-Burkitt like lymphomas and

TABLE 3. *EBV antigen tests*

Cell line	Antigen: EA	VCA	EBNA
cb-8-7*	+	+	+
P3HR-1	+	+	+
Raji	−	−	+
U-698M	−	−	−
U-715M	−	−	−
BJA-B-1	−	−	−

* EBV-transformed cord blood cell line.

lymphosarcomas, sometimes derived from patients with high EBV antibody titers (6, 8). It is therefore conceivable that although JA was an African case, the tumor corresponded biologically to the EBV-genome-negative, rare non-African Burkitt-like lymphomas.

The BJA-B-1 line is noteworthy also in another respect. Patient JA had a high serum anti-EBV antibody titer (anti VCA 320, ref. 8) As shown in the present paper, the BJA-B-1 line carries receptors for Epstein-Barr virus. We have also recently found (unpublished) that BJA-B-1 cells can be infected, at least abortively, with EBV *in vitro*. In spite of the presence of the virus in the patient, and of EBV-receptors on the cell, which has a demonstrated susceptibility to viral infection, the cell has nevertheless failed to "pick up" the virus *in vivo*. This shows that EBV does not readily travel along as a passenger in malignant lymphoma cells *in vivo* and further emphasizes the very special relationship between the viral genome and the usual African Burkitt-lymphoma, which is regularly the malignant proliferation of an EBV-genome carrying clone. It appears likely that the virus genome has been present in such lymphomas from their inception, i.e., the malignancy has developed in an EBV-carrying cell. In patients like JA, and the donor of U-715M (anti-VCA titer 160), horizontal infection of the established lymphoma is probably prevented by the relatively high titers of virus-neutralizing antibodies, which would still allow propagation of the viral genome within the clones that have become infected in the course of the primary infection event.

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