

EPSTEIN-BARR VIRUS TRANSFORMATION OF HUMAN PRE-B CELLS*

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Epstein-Barr virus (EBV)¹ shows a very restricted target cell tropism, in that only infection of human and primate B lymphocytes results in establishment of continuously growing lymphoblastoid cell lines (LCL) (1, 2). After infection of B lymphocytes, the EBV nuclear antigen (EBNA) appears within 10–12 h, cellular DNA synthesis after 40 h, and mitosis after 48 h (3). An initial intracellular increase of IgM (κ and λ) is seen in the infected cells 40–50 h after infection (4).² B lymphocytes that express all five Ig classes seem susceptible to infection (5). Cell lines derived from cord blood exclusively express IgM and IgD, reflecting the phenotypically more immature lymphocytes circulating in the neonate (6). Little is known about B cell ontogeny in man. The first surface IgM (sIgM)-positive cells have been identified in the fetal liver as early as the 9th wk of gestation (7). Since the bone marrow becomes a site for fetal hematopoiesis after the 12th week, the B cell development appears to gradually move from the liver to the bone marrow. In a recent study (8), Kamps and Cooper concluded that among cells belonging to B cell lineage, ~60 were of sIg⁻ pre-B type in the liver and bone marrow of a 12–15-wk-old human fetus. Their study indicates that the first pre-B cell would express only μ chain in the cytoplasm, and then light chain, before any IgM could be found on the surface. Such cells can still be found in the liver and bone marrow of the human fetus before the 30th wk of gestation. Since the degree of B cell maturity necessary for EBV infection is not known, valuable information regarding the infectability of pre-B cells could be obtained by using bone marrow and liver of fetal origin.

Materials and Methods

Establishment of Cell Lines. Bone marrow and, in two cases, liver were obtained from 14–20-wk-old fetuses after prostaglandin E₂-induced abortions within a clinical trial at the Karolinska Hospital. Femurs were flushed with RPMI medium and livers were gently minced with scissors, whereafter single cell suspensions were centrifuged over a Ficoll-

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¹ *Abbreviations used in this paper:* EBNA, EBV nuclear antigen; EBV, Epstein-Barr virus; FCS, fetal calf serum; FITC, fluorescein isothiocyanate; LCL, lymphoblastoid cell line; sIg, surface immunoglobulin; X-LA, x-linked agammaglobulinemia.

² Ernberg, J., D. Killander, A. Rosén, and G. Klein. Epstein-Barr virus induced immunoglobulin synthesis. Manuscript submitted for publication.

Isopaque one-step gradient and the whole mononuclear population was collected and used. The cells were exposed to EBV-B95-8 for 1 h, whereafter they were cultured in RPMI medium containing 10% fetal calf serum (FCS) under standard tissue culture conditions. Bone marrows from 13 different fetuses were infected, and in all cases EBV-positive cell lines were obtained. Uninfected control cultures did not give rise to any cell lines. One line was lost, so we present data on 12 lines, one subclone, and 2 lines from fetal liver. The cell lines were considered as established after 3–4 wk in culture, when they were given a serial number and designated as follows: Fe, fetal; BM, bone marrow derived; Li, liver derived; E, EBV transformed; 95, derived from B95-8 strain (9) of EBV; and Sto, cell lines established at the Department of Tumor Biology, Karolinska Institutet.

Immunofluorescence Assays. Presence of surface or intracellular Ig was detected with fluorescein isothiocyanate (FITC)-conjugated rabbit anti-human heavy or light chain-specific sera (Dakopatts, Copenhagen) on 5×10^5 live cells (for sIg) or 10^4 fixed cells on cytosmeared (intracellular Ig). At least 200 cells were examined in a Leitz fluorescence microscope, and each cell line was tested in three or more separate experiments. Reactivity with the monoclonal antibodies was detected with a fluoresceinated second rabbit antibody (Litton Bionetics Inc., Kensington, MD), directed against mouse IgM (for LB-1 and BB-1) or mouse Ig (for B2). The second antibody was diluted 1:10 with balanced salt solution (BSS) in 10% EBV-negative sera.

Assay for Fc and C3 Receptors. Presence of Fc and C3 receptors was assayed in parallel with standard rosetting techniques using ox erythrocytes (10).

Cloning in Soft Agarose. The cloning frequency was determined by plating the cells in soft agarose as described previously (11).

Results and Discussion

In this paper we describe the establishment and characterization of 12 lymphoblastoid cell lines from fetal bone marrow and 2 from fetal liver, by EBV transformation. The easily identifiable events of the primary infection, appearance of EBNA and induction of DNA synthesis, showed a pattern similar to that in cord blood and adult B lymphocytes. In these experiments we have used the whole heterogeneous population of bone marrow cells as targets for the infection. The selection of immortalized cells is thus based on the distribution of EBV receptors and sensitivity to virus transformation among these cells.

There is no information available to allow preselection of stem cells or pre-B cells in the human bone marrow, and nothing is yet known about the EBV receptor distribution on such cells. The fresh fetal bone marrow cells that were infected consisted of immature hematopoietic cells, out of which 8–17% could be identified as B cells by their sIgM expression. Thus, most of the established cell lines (10/15) carried sIgM with variable levels of sIgD and equal proportions of kappa- and lambda-expressing cells (Table I). Since no attempt was made to clone the EBV-infected cells, except in one case (FeBM-14-E95-Sto), the lines are polyclonal as expected. Five cell lines derived from the younger fetuses (≤ 16 wk) expressed no sIg at all, but all belonged to the B cell lineage, as they synthesized intracellular Ig, either μ chain alone, or μ heavy chain and κ or λ light chain, a pattern characteristic for pre-B cells (Fig. 1 and Table I).

These cell lines clearly represent at least two major stages in B cell differentiation; the sIg⁻ pre-B cell and the sIgM⁺/IgD⁺ fetal B lymphocyte. Among the cell lines representing the pre-B cell stage, one of them (FeBM-11-E95-Sto) may be of early pre-B type, since it expresses only μ chain and no light chain in its cytoplasm. As can be seen in Table I, the frequency of positive cells varies between the lines. There was always a large proportion of cells scored as Ig⁻, as

TABLE I
Expression of Ig in Fetal Bone Marrow Cell Lines

Cell line	Age of fetus*	Percent sIg ⁺ cells [‡]						Percent cytoplasmic Ig ⁺ cells		
		μ	d	γ	α	κ	λ	μ	κ	λ
FeBM-1-E95-STO	18	22-24	0-7	0	0	8-20	14-30	ND	ND	ND
FeBM-2-E95-STO	16	18-52	5-12	0	0	0-42	4-39	ND	ND	ND
FeBM-5-E95-STO	20	16-27	7-10	0	0	9-11	10-12	ND	ND	ND
FeBM-6-E95-STO	20	12-38	4-6	0	0	11-17	13-15	ND	ND	ND
FeBM-7-E95-STO	20	20-35	4-5	0	0	8-15	8-13	ND	ND	ND
FeBM-8-E95-STO	16	0	0	0	0	0	0	32	0	25
FeBM-9-E95-STO	20	13-16	7-14	0	0	8-24	13-14	ND	ND	ND
FeBM-10-E95-STO	18	7-10	0-6	0	0	2-12	3-9	ND	ND	ND
FeBM-11-E95-STO	14	0	0	0	0	0	0	13	0 [§]	0
FeBM-12-E95-STO	16	0	0	0	0	0	0	21	0	12
FeLi-12-E95-STO	16	0-3	0	0	0	0	0	23	9	0
FeBM-13-E95-STO	16	0	0	0	0	0	0	26	16	1
FeLi-13-E95-STO	16	14	0	0	0	14	2	ND	ND	ND
FeBM-14-E95-STO	20	10	1	0	0	11	0	49	18	0
FeBM-14-E95-STO clone 1 [¶]	20	17-76	13	0	0	0	19-70	9	0	15

* The ages of the fetuses were estimated according to the following formula (where F = length of foot in millimeters): $[(F + 24)/11] \times 4 =$ gestation week.

[‡] Ranges of the test results are presented.

[§] In one experiment, 6% of the cells stained weakly positive for presence of κ-light chains. ND, not determined.

[¶] Clones were obtained by plating, immediately after infection, an average of one cell/well in a 96-well microplate. Established growing clones were harvested and subcultured as described for the polyclonal cell lines.

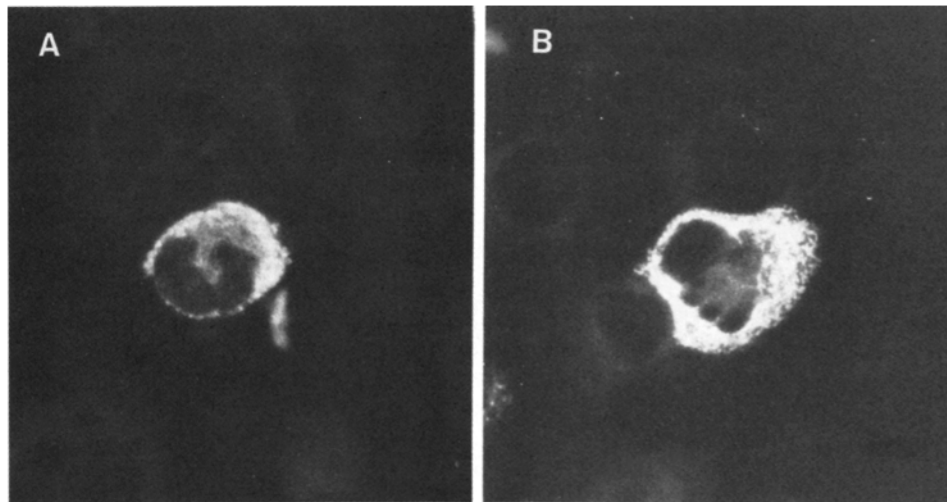


FIGURE 1. Micrograph of intracellular immunofluorescence in FeBM-8-E95-Sto, stained for presence of μ-heavy chains (A) and λ-light chains (B).

judged from both intracellular and surface Ig staining. It is unlikely, but still possible, in these polyclonal lines that several "clones" frozen at different levels of B differentiation are growing independently of each other. One argument against this is the fact that the one clone analyzed (FeBM-14-E95-Sto clone 1)

TABLE II
Expression of Various Surface Markers and Cloning Frequencies on the Fetal Bone Marrow Cell Lines

Cell line	sIg*	Percent positive cells					Cloning frequency [†] (%)
		LB-1 [‡]	BB-1 [‡]	B-2 [‡]	FcR [§]	C3R [§]	
FeBM-1-E95-STO	+	70	44	ND	17	69	<2.7
FeBM-2-E95-STO	+	36	28	ND	0	69	<1.2
FeBM-5-E95-STO	+	61	53	ND	7	70	<1.3
FeBM-6-E95-STO	+	58	64	ND	4	84	<0.3
FeBM-7-E95-STO	+	58	71	ND	13	80	<0.3
FeBM-9-E95-STO	+	39	53	ND	0	57	<0.9
FeBM-10-E95-STO	+	59	31	ND	0	92	<1.1
FeBM-14-E95-STO	+	70	50	17	0	93	ND
FeBM-14-E95-STO clone 1	+	41	56	14	0	93	ND
FeLi-13-E95-STO	+	ND	ND	5	35	85	ND
FeBM-8-E95-STO	-	53	37	31	2	77	<0.3
FeBM-11-E95-STO	-	44	36	8	1	65	<1.2
FeBM-12-E95-STO	-	55	21	2	5	70	<1.0
FeLi-12-E95-STO	-	52	21	9	7	77	<0.7
FeBM-13-E95-STO	-	70	26	4	5	93	<2.1

* Ig expression according to Table I.

[‡] Mean percent positive cells of two to three experiments on each cell line. ND, not done.

[§] As controls for optimal rosetting conditions, K562 and Daudi were used in each experiment; they were 100% Fc or C3 receptor positive, respectively.

[†] As controls in each cloning experiment, the Burkitt line, Raji, was used, showing a cloning frequency of 62%; a randomly chosen adult B-LCL was also included, with a mean cloning efficiency of 3.5%.

also contained negative cells. Thus, there may be a variation of Ig expression within the individual clones, whereas 100% of the cells in all lines were positive for the HLA-associated beta-2 microglobulin (data not shown).

In vitro infection of adult peripheral blood lymphocyte (PBL)-derived B lymphocytes with EBV usually results in an increase in Ig synthesis and in some cells maturing into Ig-secreting plasma cells (4). It is possible that the pre-B cells in the human fetus fail to undergo any differentiation upon EBV infection due to their immunoincompetence. In the mouse system, information on B cell antigens confined to stages of differentiation is being obtained with the use of monoclonal antibodies (12). Recently some monoclonal antibodies that recognize human B cell antigens have become available; further characterization of our EBV-transformed fetal lines has involved the use of three such monoclonal antibodies (Table II). LB-1 has been described by Youkochi and co-workers (13) as detecting a lymphoblastoid antigen present on LCL of B cell origin and mitogen-activated lymphocytes, but not on a pre-B leukemia (NALM-6), Burkitt lymphoma-derived cell lines, or plasma cell-like myelomas. BB-1 shows a similar pattern of reactivity except that it detects a B lymphoblastoid antigen on mitogen-activated sIg⁺ cells and also reacts with Burkitt lines. The antigen recognized by the other monoclonal, B2, is present on both normal resting B cells and malignant early sIg⁺ B cells, but is lost during maturation and is absent on plasma cell-like malignancies (14). Our data suggest that the two antigens detected by LB-1 and BB-1 are also induced on the sIg⁻ pre-B cells by EBV transformation (Table II), but are not detected on fresh fetal BM cells (data not shown). All the fetal cell

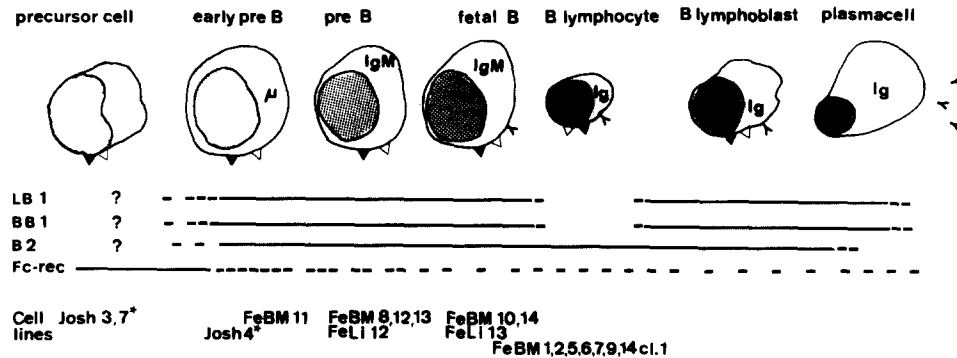


FIGURE 2. A tentative scheme for B cell differentiation stages. The surface markers such as the EBV receptor (Δ), C3-receptor (\blacktriangle), and Ig molecules (Y) are indicated on the cells. Other markers that have been identified on cell lines (5, 13-16, this paper) are listed below and the presence of a marker is illustrated with a solid or dotted line, whereas absence of the line indicates absence of the marker. The fetal cell lines described here are listed at the approximate differentiation stages they may represent, together with the Josh cell lines (15) that represent earlier stages. The EBV-transformed adult B lymphocytes would, according to this scheme, be positioned at the B lymphoblast stages.

lines tested were positive for both these antigens whether they belonged to the sIg⁺ or sIg⁻ group (Table II). The B2 antigen showed a much weaker staining and fewer positive cells overall but no obvious difference between the more immature lines and the sIg⁺ cell lines. The presence of Fc receptors varied among the cell lines while the C3 receptors that were tested in parallel were strongly expressed on all cell lines (Table II). Although the presence or absence of Fc receptors cannot be used to define a certain maturation stage, it is interesting to note that our sIg⁻ cell lines all have very low amounts of Fc receptor-positive cells. This is in contrast to the pre-B cell lines established from the patients with X-linked agammaglobulinemia (X-LA) described by Fu et al. (15), which were the most immature lines, of possibly precursor type, and which had a high percentage of Fc receptor-positive cells.

Some EBV-associated Burkitt lymphoma tumor lines are usually thought to represent an immature B cell since they lack sIgD and do not secrete Ig (16). These are highly malignant monoclonal tumors with a high cloning efficiency in soft agar. In vitro transformed peripheral B lymphocytes have at least a 10-fold lower cloning efficiency (17). To find out whether these in vitro transformed fetal lines would be more "Burkitt-like" because of their immaturity, we also tested their cloning capacity in soft agar (Table II). We found no difference between the two groups of fetal cell lines, and they did not differ in their cloning capacity from the more mature conventional adult B-LCL.

In Fig. 2 we have summarized some of the current information on B cell development in man, together with the information on EBV susceptibility gained from our study. It is a probable assumption that the EBV-positive lines (Josh 7 and Josh 3) from a patient with X-LA that lack both intracellular heavy and light chain (15), represent a precursor B cell that is also present in healthy individuals. The next identifiable step in human B cell development would be synthesis of only intracellular μ chain, represented by the lines Josh 4 and FeBM-11-E95-Sto.

The establishment of these FeBM lines supports the suggestion by Fu et al. (15) that normal pre B cells also can be transformed by EBV.

Before the pre-B cell matures into a surface IgM⁺ B lymphocyte, both heavy and light chains appear in the cytoplasm. Fc and C3 receptors, although not unique for the B cell lineage, are indicated in Fig. 2 as they appear or disappear during B cell ontogeny. In addition to previous information concerning the distribution of antigens detected by monoclonal antibodies and the presence of the EBV receptor, we have added the new information that these are also present on pre-B cell lines. EBV-transformed fetal cell lines could serve as the normal counterpart to the more often studied pre-B cell characteristics on cells from malignancies that affect the B cell lineage. With regard to defining both certain cell surface markers and differentiation steps, the transformed normal pre-B cells are advantageous since many of these malignant cells can not be cultured *in vitro*, a prerequisite for such studies. In conclusion, this study shows that fetal bone marrow and liver contain cells that can serve as targets for EBV. The morphology, EBNA expression, growth pattern, and cloning efficiency in soft agar were similar to that of normal adult B-LCL. Both sIg⁺ and sIg⁻ B cells have the EBV receptor, indicating that the transformed cell lines represent normal B cell maturation stages.

Summary

In vitro infection of human B lymphocytes with Epstein-Barr virus (EBV) results in establishment of B lymphoblastoid cell lines that reflect normal B cell phenotypes. In this study we have investigated whether immature B cells from fetal bone marrow and liver can serve as targets for EBV. The fetal bone marrow cells were readily transformed by EBV. Among the resulting cell lines, five were surface Ig (sIg)-negative. Three B cell-associated antigens defined by monoclonal antibodies were expressed to the same extent on the fetal cell lines, whether they belonged to the sIg⁻ or sIg⁺ group. The various differentiation stages that these cell lines may represent are discussed.

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