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Human cell lines containing Epstein–Barr virus but distinct from the common B cell lymphoblastoid lines

(immunoglobulin/ontogeny/Fc receptors/C3 receptors)

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ABSTRACT **A group of very similar cell lines was established from peripheral blood or bone marrow of 12 patients with a variety of disorders. The cells in these cell lines were uniform and round in shape. They grew as single-cell suspensions or as aggregates of small numbers of cells in stationary culture. The most striking characteristic of these lines was the lack of cells with surface immunoglobulin or with demonstrable immunoglobulin synthesis. This lack of immunoglobulin synthesis and their special growth characteristics distinguished them from the lymphoblastoid B cell lines previously described. The cells of these unusual cell lines had strong Fc receptors and C3 receptors and expressed Ia antigens. They did not form rosettes with sheep erythrocytes and did not have detectable levels of terminal deoxynucleotidyltransferase. They did not secrete lysozyme and failed to stain for peroxidase. The presence of the Epstein–Barr virus nuclear antigen in the cells indicated the presence of Epstein–Barr viral genome. The possibility that these cells represent some type of precursor cell in the B cell lineage is discussed, but the exact cellular origin remains to be ascertained.**

In the majority of the lymphoblastoid cell lines obtained by transformation of human lymphocytes with Epstein–Barr virus (EBV), the cells are highly variable in shape and they grow in clumps in stationary cultures (1). They express many characteristics of normal B lymphocytes (2, 3). In particular, synthesis of immunoglobulin (Ig) has been the unique feature by which the B cell origin of these lymphoblastoid lines has been ascertained. In the present studies, a number of unusual lymphoblastoid lines were obtained with growth characteristics and cell morphology markedly different from those displayed by the common lymphoblastoid cell lines of the B cell type. These unusual cell lines were found to contain the EBV genome, but immunoglobulin synthesis was not demonstrable in any of them.

MATERIALS AND METHODS

Patients, Cell Preparation, Cell Culture, and Initiation of Cell Lines. Patients with various primary hematological malignancies, immunodeficiencies, and rheumatological disorders were from either the Rockefeller University Hospital or the Memorial Sloan–Kettering Cancer Center. Bone marrow samples, which were dispersed with sterile needles and peripheral blood, were subjected to Ficoll/Hypaque gradient centrifugation. Cell preparations from which the majority of the red cells and mature granulocytes were depleted were obtained from the interphase.

The culture medium was RPMI-1640 (Microbiological Associates, Bethesda, MD) with L-glutamine supplemented with 2 mM additional glutamine, penicillin at 100 units/ml, and streptomycin at 100 μg/ml (GIBCO). Fetal calf serum (lot P60512, Reheis, Phoenix, AZ) was used at 20% at the beginning of the cultures and later decreased to 10%. The source of EBV was culture supernatants from marmoset cell line B95-8 (4). Four-day culture supernatants from the B95-8 cell line were filtered through a 0.8-μm filter (Nalge, Rochester, NY) and used without storage. Cell lines were initiated in 16-mm tissue culture plates (FB-16-24-TC, Linbro, Hamden, CT) with 2×10^6 cells in 1 ml of medium. In some cultures, 1 ml of filtered B95-8 culture supernatant was added to the wells. Cultures were kept at 37°C and 5% CO_2 in a humid air atmosphere and were fed twice weekly. After the establishment of cell lines, the cells were split 3:1 every third day. Seed samples of freshly established cell lines were cryopreserved in RPMI-1640, 15% fetal calf serum, and 10% dimethyl sulfoxide in liquid nitrogen.

Immunofluorescence, Hemagglutination, and Anti-Ig Rosetting Reactions. $F(ab')_2$ fragments of affinity-column-purified antibodies specific for IgM, IgG, IgA, and κ and λ light chains were used. Preparation of these antibodies was detailed elsewhere (5). Rhodamine-conjugated $F(ab')_2$ fragments with various specificities were used for immunofluorescence. Surface and intracellular immunofluorescent staining for Ig was performed as described (6). Hemagglutination and hemagglutination inhibition was carried out as described (6). For anti-Ig rosetting reactions, F(ab') fragments of the purified antibodies were coupled to bovine erythrocytes with $CrCl_3$ and rosette formation with cells of cell lines was performed essentially as described by Ling *et al.* (7). Direct hemagglutination assay for Ig with anti-Ig antibodies coupled to bovine erythrocytes was carried out in microtiter plates as follows: 0.05-ml aliquots of culture supernatants in 1:2 dilutions were incubated at room temperature with 0.05 ml of 1% anti-Ig antibody-coupled bovine erythrocytes. After 2–3 hr, hemagglutination patterns were read. Isolated myeloma proteins of various classes of Ig and Bence Jones proteins were used as standards in these assays.

Fc Receptors, C3 Receptors, and Other Methods. Bovine erythrocytes were coated with the IgG fraction (2 mg/ml) of an antiserum made against stroma of bovine erythrocytes. Equal volumes (0.05 ml) of cells (10^7/ml) and 1% coated bovine erythrocytes were incubated at room temperature for 30 min after centrifugation at $100 \times g$ for 3 min. Cell pellets were suspended and rosettes were read after the nucleated cells were stained with toluidine blue. Cells with more than three erythrocytes attached were identified as rosette-forming cells. C3 receptors were assayed by the rosetting method with bovine erythrocytes sensitized with IgM antibodies and C5-deficient mouse serum as a complement source (EACm) according to the method described by Ross and Polley (8).

Rosette formation with sheep erythrocytes was performed as described (9). Peroxidase staining was determined by a modification of the technique of Kaplow (10), with 3,3'-dimethoxybenzidine (Sigma). The presence of EBV nuclear an-

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Abbreviations: EBV, Epstein–Barr virus; EA_{IgG}, ox erythrocytes coated with rabbit IgG specific for ox erythrocyte; EACm, ox erythrocytes coated with IgM antibodies and mouse complement.

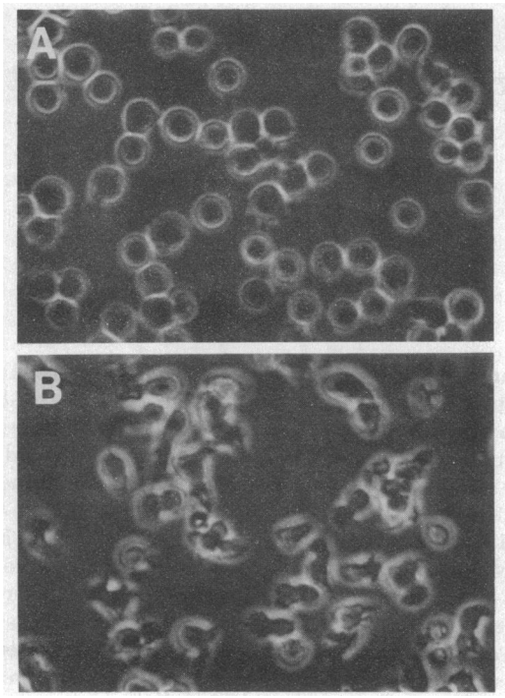

FIG. 1. Phase-contrast micrographs of cells in stationary cultures of cell lines: a round cell line, SNE 1 (*A*), and a B cell lymphoblastoid line, SNE 3 (*B*), demonstrating contrasting cell shapes of the two types of cell lines. (×240.)

tigen was tested by the method of Reedman and Klein (11). Lysozyme secretion was assayed according to Osserman and Lawlor (12). Assays for terminal deoxynucleotidyltransferase were kindly performed by the laboratory of R. Mertlesman (Memorial Sloan-Kettering Cancer Center).

RESULTS

Establishment of Cell Lines. In the course of developing lymphoblastoid cell lines from a variety of patients, a series of nonadherent cell lines with unusual characteristics was encountered. The cells of these lines had a uniform and round shape (Fig. 1*A*). In the vast majority of the cell lines, cells in the exponential growth phase grew either as single cells or as groups of 4–10 cells in stationary cultures. Big cell clumps were observed in only one of 42 cell lines (Chem 7 in Table 1). The cells grew to a density of 2–3 × 10^6/ml.

Cell lines of this type with round cells will be referred to as round cell lines. They were obtained from 12 patients with various diagnoses (Table 1). Except for patients SNE, Josh, and RKZ, all patients had primary hematological malignancies. Six cell lines from five patients were obtained from bone marrow. No EBV-containing supernatants were added to the cultures during the establishment of these lines. In general, continuously growing cell lines of this type were established in 4–12 weeks. Homogeneity in cell shape was striking when these cell lines were established. Several of these cell lines were maintained in continuous cultures over a year. All of them were cryopreserved and could be recovered readily.

Over 1000 lymphoblastoid lines were established in this laboratory from peripheral blood and bone marrow from normal individuals and from patients with various disorders with and without the aid of EBV-containing supernatants from cell line B95-8 (13). In general, only 2–4 weeks were required for the derivation of these lymphoblastoid lines. The vast majority of the lymphoblastoid lines obtained in this manner contained cells with highly variable shapes (Fig. 1*B*). The cells grew in visible clumps of varying sizes to a density of 1–2 × 10^6 cells per ml. In 8 of the 12 patients from which round cell lines were established, one or more lymphoblastoid cell lines of this common type were also established. Five of these (SNE 3, Josh 5, Shaw 2, TG 41, and GTL 2) are listed in Tables 2 and 3.

Lack of Ig Synthesis. Nineteen round cell lines were recovered from cells that were cryopreserved shortly after their establishment. Continuously growing cell lines were reestablished immediately. Cells from these 19 lines were analyzed by immunofluorescence for Ig synthesis. Viable cells were stained for surface Ig with a polyvalent anti-human Ig $F(ab')_2$ reagent. The same reagent was used to stain fixed cells for intracellular Ig. None of the cells in any of the 19 lines stained for either surface Ig or intracellular Ig. Twelve of these lines were studied in greater detail with rhodamine-conjugated reagents specific for human IgM, IgG, IgA, and κ and λ chains. Similar results demonstrating the lack of Ig synthesis were also obtained. Supernatants from these 12 lines were assayed for the presence of Ig by a semiquantitative passive hemagglutination inhibition assay, and none was detected. Results of these studies on five representative lines of this round cell type are listed in the upper portion of Table 2.

Three of these five lines (SNE 1, Josh 7, and Shaw 2) were analyzed further with more sensitive methods. A direct anti-Ig rosetting method was used for the detection of surface Ig. Ox erythrocytes coated with $F(ab')_2$ fragments of antibodies to various classes of human Ig isolated by affinity column chromatography formed rosettes readily with lymphocytes known to have surface Ig. This rosetting reaction assay had been reported to be more sensitive than direct immunofluorescence (14). None of the cells in the three cell lines tested formed rosettes with the coated ox erythrocytes. Supernatants of these

Table 1. Summary of donor's diagnosis and tissue origin of cell lines with cells of uniform and round shape

Cell line	Diagnosis	Tissue origin	No. of round cell lines
SNE 1, 2	Systemic lupus erythematosus	Bone marrow	2
Josh 7	X-linked agammaglobulinemia	Bone marrow	1
GTL 7	Multiple myeloma	Bone marrow	1
AVA 10	Prolymphocytic leukemia	Bone marrow	1
RKZ 1	Amyloidosis	Bone marrow	1
Shaw 1	Chronic lymphocytic leukemia	Peripheral blood	1
Lee 1, 2	Chronic lymphocytic leukemia	Peripheral blood	2
Sau 1, 2	Chronic lymphocytic leukemia	Peripheral blood	2
JML 1–4	Chronic lymphocytic leukemia	Peripheral blood	4
Chem 7	Waldenström macroglobulinemia	Peripheral blood	1
TG 1–22	Acute myelomonoblastic leukemia	Peripheral blood	22
KLM 1–4	Acute monoblastic leukemia	Peripheral blood	4

Table 2. Analysis of Ig synthesis of representative cell lines

Cell lines	Surface Ig, %*					Intracellular Ig, %*					Ig in supernatant, μg/ml†			
	IgM	IgG	IgA	κ	λ	IgM	IgG	IgA	κ	λ	IgM	IgG	κ	λ
Round cells														
SNE 1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Josh 7	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Shaw 1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
TG 17	0	0	0	0	0	0	0	0	0	0	0	0	0	0
GTL 7	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Varied cell shapes														
SNE 3	5	2	0.1	15	2	22	1	0.1	14	13	3.2	0.4	1.6	1.6
Josh 5	0	0	0	0	0	1	0	0	2	2	0	0	0	0
Shaw 2	15	3	0.1	34	3	13	6	1	5	10	1.6	0.8	3.2	0.4
TG 41	35	10	0	40	5	22	2	0	24	0.1	3.2	0	0.8	0
GTL 2	2	1	0	2	12	2	5‡	0	2	7‡	0	1.6	0	3.2

* Ig was determined by immunofluorescence. Percent of positive cells is listed.
† Estimated by the semiquantitative method of passive hemagglutination inhibition. 0 indicates less than 0.1–0.2 μg/ml in the supernatant.
‡ Over 90% of the fixed cells gave varied staining intensities, suggesting a moderate rate of intracellular synthesis.

three cell lines were assayed for the presence of Ig by direct agglutination of ox erythrocytes coated with anti-Ig antibodies. None of the supernatants agglutinated ox erythrocytes coated with purified antibodies to IgM, IgG, or κ or λ light chains. This assay can detect 10–20 ng of Ig per ml and is 10–20 times more sensitive than the passive hemagglutination inhibition assay.

For comparison, results of analysis of immunoglobulin synthesis of five common B lymphoblastoid lines are summarized in the lower portion of Table 2. Ig synthesis was detectable by all three methods in four of the five lymphoblastoid lines. For Josh 5, a few of the cells were stained brightly for intracellular Ig with the anti-IgM, anti-κ, and anti-λ reagents. Eighty-nine additional common B lymphoblastoid lines were found to be positive for Ig synthesis. In only one lymphoblastoid line (Josh 6, derived from the patient with X-linked agammaglobulinemia) no Ig synthesis was detected although the growth characteristics of this cell line were indistinguishable from any of the common B lymphoblastoid cell lines.

Fc and C3 Receptors. Ox erythrocytes coated with isolated IgG from a rabbit antiserum against ox erythrocytes (EA_{IgG}) were used to detect Fc receptors on the surface of cells. In 19 round cell lines, the vast majority of the cells formed EA_{IgG} rosettes (range 71–95%). In general, strong rosettes were formed with more than 10 erythrocytes per rosette. Results of percentages of EA_{IgG} rosette-forming cells in five representative round cell lines are given in Table 3. In contrast, many fewer cells formed EA_{IgG} rosettes (5–60%) and most EA_{IgG} rosettes were formed with three or four erythrocytes in 10 common B lymphoblastoid lines. Results on five of these cell lines are listed in Table 3.

Table 3. Expression of Fc and C3 receptors by representative cell lines

Cell line	Fc receptor (EA_{IgG} rosette-forming cells),* %	C3 receptor (EACm rosette-forming cells),† %
Round cells		
SNE 1	79	85
Josh 7	72	81
Shaw 1	90	85
TG 17	95	85
GTL 7	87	82
Varied cell shapes		
SNE 3	28	52
Josh 5	5	33
Shaw 2	27	38
TG 41	22	30
GTL 2	33	45

* Fc receptors on cells were detected by a rosette formation method with EA_{IgG}.
† C3 receptors were detected by a rosette formation method with EACm.

For C3 receptor detection, ox erythrocytes coated with rabbit IgM anti-ox erythrocyte antibodies were incubated with C5-deficient mouse serum to generate EACm. High percentages (64–98%) of the cells in 19 round cell lines formed EACm rosettes, with the vast majority of the rosettes formed by more than 10 erythrocytes. Lower percentages of cells (30–80%) in 10 common B lymphoblastoid lines formed EACm rosettes, with the majority of rosettes formed by 3–14 erythrocytes. In 9 out of 10 lines tested, 30–50% of cells were EACm rosette-forming cells (Table 3).

Other Studies. Although no exogenous EBV was added to aid the establishment of the round cell lines, 19 of the lines were analyzed and all were found to be positive for the EBV nuclear antigen. The cells of these lines also expressed Ia antigens on their surface, and few if any of the cells formed rosettes with sheep erythrocytes. Supernatants of the cell lines did not contain detectable amounts of lysozyme. None of the cells in the 19 round lines tested stained for peroxidase histochemically and none internalized erythrocytes coated with IgG. Four of the round cell lines were tested and found to be negative for terminal deoxynucleotidyltransferase.

DISCUSSION

Cell lines with unusual growth and cell membrane characteristics have been described in the present studies. These round cell lines could readily be distinguished from the majority of the lymphoblastoid lines of the common type obtained by EBV transformation. The cells of these cell lines appeared to be uniform and round in shape. They grew as single cells or as small groups of cells. They also appeared to grow faster and to a higher cell density. However, the most striking feature of these unusual cell lines was the complete lack of Ig synthesis. Multiple methods were used in the present investigation to document this finding. No surface Ig was found by either immunofluorescence or the more sensitive direct anti-Ig rosetting reaction. No intracellular Ig was stainable by immunofluorescence. In addition, Ig was not detectable in the supernatants of the cell lines by either passive hemagglutination inhibition or direct agglutination of anti-Ig antibody-coated erythrocytes. This finding stands in striking contrast to the results for the common B lymphoblastoid cell lines in which cells with surface Ig and others secreting Ig are characteristic.

Another feature of the round cell lines was the finding of higher percentages of cells forming rosettes with ox erythrocytes

coated with IgG antibodies and ox erythrocytes coated with IgM antibodies and complement. It appeared that either these cell lines contained cells with higher densities of Fc and C3 receptors or these receptors had higher affinities. In addition to the presence of the EBV nuclear antigen, these two features also distinguish these round cell lines from the cell lines established from patients with the acute non-T, non-B lymphocytic leukemia (15).

The round cell lines also shared common features with the common B lymphoblastoid lines. Cells of both types of cell lines were positive for the EBV nuclear antigen. They were nonadherent and nonpagocytic. They expressed the human Ia antigens and failed to form rosettes with sheep erythrocytes. In addition, they did not have a detectable level of terminal deoxynucleotidyltransferase (2, 3).

Although the majority of the round cell lines were derived from tissues obtained from patients with various hematological neoplasm, the results of marker studies suggest that these cell lines did not originate from the malignant blood cells but that they were probably derived from residual normal cells with the following characteristics: Ig^-, Ia^+, negative for terminal deoxynucleotidyltransferase, positive for C3 and Fc receptors, and negative for receptors for sheep erythrocytes, lysozyme secretion, and peroxidase. The round cell lines obtained from SNE, a patient with systemic lupus erythematosus, and Josh, a patient with X-linked agammaglobulinemia, were certainly derived from nonmalignant cells.

The cells of round cell lines described in this report expressed many characteristics of human B cells: i.e., receptors for Fc portions of IgG and complement, expression of Ia antigens, and lack of rosette formation with sheep erythrocytes. However, none of these traits is specific for B cells. Because all round cell lines were positive for EBV nuclear antigen, it appears that the cells from which these lines originated must possess receptors for EBV. Recent studies have suggested that the presence of receptors for EBV could be considered as a B cell marker (16, 17). Previous studies involving isolated cell populations from human peripheral blood have indicated that only B cells had both EBV and C3 receptors and could be transformed by EBV as measured by DNA synthesis and induction of expression of EBV nuclear antigen (18). By these criteria the cell origin of the round cell lines would be of the B cell series. Alternatively, these lines might well come from an unrecognized minor cell population not in the B cell series but resembling B cells in certain aspects. However, definitive assignment of round cell lines to the B cell series can be made only if Ig synthesis can be induced or if they can be shown to share a unique antigenic structure with cells in the B cell lineage.

If one accepts a tentative assignment of B cell origin of these Ig^- cell lines, the cells from which the transformed cell line arose must have been at a stage of B cell development prior to that of pre-B cells in which Ig genes have been activated. Studies of lymphoblasts of four patients with acute lymphoblastic leukemia and a lymphoblastoid cell line with pre-B cell characteristics concluded that C3 receptors were not expressed on pre-B cells (19, 20). The presence of C3 receptors on the cell surface of these Ig^- cell lines would suggest that C3 receptors disappear during certain stages of B cell development and reappear again in later stages (i.e., on containing surface B cells Ig). This type of appearance, disappearance, and reappearance of a surface molecule is not unique and has been demonstrated on human T cells for human Ia antigen (reviewed in ref. 21). In T cells, Ia antigens are present on the stem cells, disappear from mature peripheral T cells, and reappear after these mature peripheral T cells are activated.

Although 42 round cell lines were obtained from 12 patients, they still represent an unusual occurrence. Many cultures were set up without exogenous EBV and yielded either no cell lines or common B lymphoblastoid lines. The exact reasons why round cell lines were obtained from these 12 patients remain obscure. For patients with X-linked agammaglobulinemia, few if any mature B cells were demonstrated in the peripheral blood and bone marrow (22, 23). Thus, there were far fewer cells to be transformed into the usual B cell lymphoblastoid lines which ordinarily would overgrow the cultures. This was also the case with some of the malignancies where round cell lines were established; malignant cell proliferation decreased the number of normal B cells and perhaps permitted round cell lines to be established.

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