PRODUCTION OF A B CELL LYMPHOBLASTOID LINE FROM LONG-TERM HUMAN BONE MARROW SUSPENSION CULTURE

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A model which can be used to study hematopoiesis and immunogenesis consists of hematopoietic and lymphoid cell lines, investigation of which can reveal the characteristics of differentiation and regulation of individual clones of a cell population. Lymphoblastoid lines can be obtained from hematopoietic and lymphoid cells of healthy subjects and patients with hemoblastosis, as a result of their culture in the presence of a virus or with the addition of exogenous stimulating factors of varied origin to the culture [1, 5-7].

In the investigation described below a factor-independent human lymphoblastoid cell line, arising during culture of normal bone marrow cells on a supporting layer consisting of bone marrow cells from a patient with chronic myeloid leukemia (CML), was obtained.

EXPERIMENTAL METHOD

Bone marrow cells from a patient with CML with a Ph'-chromosome, in the progressive stage of the disease before treatment, and bone marrow from a healthy woman were obtained by sternal puncture and placed in a flask with heparin (50 U/ml). After sedimentation of the erythrocytes, the film containing myelokaryocytes was transformed to a flask with McCoy's medium. Nucleated cells were cultured by the method in [3] in flasks (Nunc, Denmark), the bottom of which had an area of 25 cm^2, at 37°C on McCoy's medium containing sodium bicarbonate (7.5%) – 1%, sodium pyruvate (100 ×) – 1%, vitamins (100 ×) – 1%, amino acids: essential (50 ×) – 0.8%, nonessential (100 ×) – 0.4%, and L-glutamine (100 ×) – 1%, embryonic calf serum 6.25%, horse serum 12.5% (all components from Flow Laboratories, USA), AN (IV) serum 6.25%, hydrocortisone hemisuccinate 10^{-6} M, penicillin 100 U/ml, and streptomycin 100 μg/ml. Bone marrow cells from the patient with CML were cultured initially in a concentration of $0.66 \cdot 10^6$/ml, and 3 weeks later normal bone marrow cells were added in a concentration of $0.25 \cdot 10^6$/ml. The time of the second transplantation was taken as the zero point for counting the duration of culture. Every day half of the nutrient medium was replaced by fresh and the number of living cells in the suspension was counted. The morphological analysis was carried out on films stained by Nocht's method. Cytochemical tests were undertaken for myeloperoxidase, naphthol-ASD-chloroacetate esterase, nonspecific α-naphthyl acetate esterase with or without the addition of sodium fluoride, acid phosphatase, and the PAS reaction. The method in [2], with certain modifications, was used for the karyologic analysis. Immunologic phenotyping of the cells was carried out by determining antigens and receptors on surface membranes of living cells in culture, washed five times with medium 199. Antigens were determined by the indirect immunofluorescence (IF) test on preparations of cells sedimented on a polylysine film with the aid of commercial mouse monoclonal antibodies and fluorescein isothiocyanate (FITC)-labeled antibodies against mouse IgG (Ortho, USA). The presence of surface immunoglobulins (SIG) was judged by staining of the cells in the indirect IF test with FITC-labeled $F(ab')_2$-fragments of antibodies to human immunoglobulins (Ortho). Cytoplasmic immunoglobulins (CIG) were detected by the direct IF method, using FITC-labeled antibodies to heavy (μ, δ, γ) and light (κ, λ) chains of human immunoglobulins (Hoechst, West Germany) on preparations of cells fixed for 20 min at −20°C. Receptors for sheep and mouse erythrocytes were identified by the spontaneous rosette formation test with sheep and mouse

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TABLE 1. Immunologic Phenotyping of Cells of Lymphoblastoid Cell Line

Cell markers	Specificity	Expression of markers	
		% of positive cells	density
Antigens			
M1 (OKM1)	Monocytes, granulocytes	0	–
T3 (OKT3)	T cells	0	–
B7 (OKB7)	B cells	32	+/±
HLA-DR (OKIa1)	DR antigens on B cells, on activated T cells	78	++++/+++/++/+
T10 (OKT10)	Stem cells, thymocytes, 0 cells	73	+/++/±
Surface immunoglobulins – $F(ab')_2$	B cells	67	+
Cytoplasmic immunoglobulins			
μ-chains	Early B cells	31	+
δ-chains	The same	36	+
μ-chains	Late B cells	20	++/+++
γ-chains	The same	9	++/+++
λ-chains	» »	18	++/+++
Receptors for sheep's erythrocytes	T cells	0	–
	B cells	35	+/++
For mouse erythrocytes $F_c\gamma$	B cells, T suppressor cells, monocytes, granulocytes	1	+

erythrocytes, respectively [10]. Receptors for the F_c fragment of IgG ($F_c\gamma$-receptors) were identified by the indirect rosette formation test with bovine erythrocytes, sensitized with IgG-antibodies [8].

EXPERIMENTAL RESULTS

During the first weeks of culture of bone marrow cells from a patient with CML a decrease in the number of hematopoietic cells and the formation of a sublayer of elongated fibroblast-like cells, endothelial cells on the bottom of the flask were observed. After the second transplantation of normal bone marrow cells into the supporting layer thus formed, a sharp decrease took place in the number of hematopoietic cells in the first 2 weeks of culture. Meanwhile small groups of hematopoietic cells began to appear on the supporting layer, they grew in size, and became spherical in shape. By the 4th week they were numerous. This coincided with complete elimination of the supporting layer. Thus after 3-4 weeks of culture after the second transplantation a call line growing in the form of spherical aggregates, going into the suspension and not requiring interaction with the supporting layer, was formed.

Cells of the line were able to maintain themselves for more than 10 months (over 40 passages) without exogenous stimulation. Growth of the cell line was unchanged when the mixture of embryonic, calf, human AB, and horse sera was replaced by one of them in a concentration of 5 to 20%. The greatest increase in cell mass was observed with concentrations of $0.03 \cdot 10^6$-$0.12 \cdot 10^6$ cells/ml. The doubling time, determined from the increase in the number of cells during 48 h of the exponential phase of growth, was 24 h.

Cytologic analysis revealed the heterogeneity of the cell population. Most of the cells were undifferentiated cells containing a large nucleus with a delicate chromatin structure, 1-3 nucleoli, and vacuolated basophilic cytoplasm. Cells with cytoplasmic outgrowths also were found. In the total population there were cells of "small lymphocyte" type and also cells with two or three nuclei.

All cells of the line were negative for myeloperoxidase and naphthol-ASD-chloroacetate esterase; 100% of the cells were positive for nonspecific α-naphthyl acetate esterase, and the reaction was not inhibited by sodium fluoride. About 60% of cells were positive for acid phosphatase. Nearly all cells contained PAS-positive material and the reaction product was distributed diffusely in the cell cytoplasm. The cytochemical data are evidence of the lymphoid nature of cells of this line.

Karyologic analysis showed that dividing cells of this line have the 46 XX karyotype. No structural chromosomal or chromatid aberrations were found.

As a result of immunologic phenotyping of cells of this line (Table 1) SIG and CIG, B7-antigen, and receptors for mouse erythrocytes were found. It can therefore be reliably concluded that the cells of this line are B cells. Heterogeneous expression of HLA-DR antigens and T10 antigen and also the different fraction of cells carrying other B-cell markers confirm

in this case that the line consists of B cells at different levels of differentiation, starting from the earliest stages (pre-B cells) and ending with late stages, corresponding to plasmacytoid cells and plasma cells. The number of early B cells was evidently about 30%, as shown by weak staining of the cytoplasm of the cells by antisera against μ- and δ-chains of human immunoglobulins, and also by the large proportion of cells expressing T10 and Ia antigens with high density. Cells with bright staining of their cytoplasm were probably B cells in the late stages of maturation, for they were characterized by absence of SIG, a lower density of HLA-DR antigens, and a high content of CIG, which are monoclonal with respect to the light chain (only the λ-chain is present). The presence of a few cells of the γ-chain also points to late stages of B-cell differentiation, when a switch takes place to the synthesis of "later" immunoglobulins. The number of these cells was about 20%. The remaining cells (about 50%) were evidently various transitional forms and ordinary mature B cells, corresponding in level of maturation to the virgin B cell, characterized by the presence of a receptor for mouse erythrocytes.

The analysis thus showed that a lymphoblastoid line consisting of B cells at different stages of differentiation was obtained in long-term human bone marrow suspension culture. This line arose from normal bone marrow cells, as was confirmed by karyologic analysis, on a supporting layer of fibroblast-like, endothelial, and macrophagal cells from a patient with CML, 3-4 weeks after retransplantation. It must be emphasized that addition of an exogenous stimulating factor to the culture was not necessary for proliferation and differentiation of the calls of this line. Meanwhile the formation of a B-cell line on a stromal supporting layer may indicate the need for close contact between the "parental" cells of the line and macrophages and fibroblast-like cells in order to activate their primary proliferation. The possibility likewise cannot be ruled out that the line may be formed in this system as a result of spontaneous infection of the cells with Epstein–Barr virus, although karyologic analysis did not reveal the characteristic disturbances in the cell genome. However, the frequency of origin of lines from cells transformed by Epstein–Barr virus is very small in this culture system (one of 32 cases) [9]. On the whole, the factor-independent B-cell line obtained as described above can be used to study the biochemical characteristics of B cells as one of the partners in order to obtain hybridoma cells for the culture of some viruses.

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