A Lymphoblastoid Cell Line with High Phagocytic Activity Established from Peripheral Blood of a Patient with Chronic Myelocytic Leukemia in Blast Crisis

Kazuhiro Mitani¹, Fuminori Hyodoh¹, Yoshihito Yawata², and Tetsuo Kimoto¹

Department of Pathology¹ and Medicine,² Kawasaki Medical School, Kurashiki 701-01, Japan

ABSTRACT. A human hematopoietic cell line (K-23-M) was established from a patient with chronic myelocytic leukemia in blast crisis. Morphologically, the cultured cells were lymphoblastoid cells that produced IgA and were Epstein-Barr viral nuclear antigen positive. But they showed high phagocytic activity to glutaraldehyde-treated sheep red cells and had properties of a monocyte or macrophage that included surface Fc receptors, α -naphthyl butyrate esterase positivity blocked by NaF, migration in soft agar and the ability to attach to a glass surface. Lysozyme secretion was absent, and chromosomes were diploid and Ph¹ negative. This cell line is unique in that it has strong phagocytic activity. Its existence shows that lymphoblastoid cell line may be a more important cell line for the study of human hematopoietic cells than previously has been believed.

Almost all human hematopoietic cell lines are "lymphoblastoid cell lines (LCLs)". Because LCLs have EBNA, and produce immunoglobulins, they have been believed to be B-lymphocytic cell lines (22). Kimoto *et al.* (11), however, reported that Mono-1 was morphologically an LCL but had the properties of a monocytic cell. Furthermore, Mono-1-207, a subline of Mono-1, showed lobulation like that of neutrophils in arginine deficient medium (12).

After Kimoto's report, differentiation to granulocytes or macrophages in arginine deficient conditions was demonstrated by Okabe *et al.* (23) using M-1 (10), and by Honma *et al.* (7) using HL-60 (2). Recently, K-562 (15) has been reported to differentiate to monocytic, granulocytic and erythrocytic cells in media gradually depleted of essential nutrients (16). As a result, the possibility has been strengthened that Mono-1-207 is morphologically an LCL, but with the characteristics of the myeloid precursor cell line.

Because we consider that B-lymphocytic characteristics only one aspect of an LCL, we conducted a series of experiments to determine whether there is a cell line among the LCLs with remarkable monocytic, myelocytic, or erythrocytic properties. We

Abbreviations used: ANBE, α -naphthyl butyrate esterase; EBNA, Epstein-Barr viral nuclear antigen; FCS, fetal calf serum; GRC, glutaraldehyde-treated sheep red cells; LCL, lymphoblastoid cell line; Ph¹, Philadelphia chromosome; TdT, terminal deoxyribonucleotidyl transferase; TEM, transmission electron microscope.

here report an interesting LCL (K-23-M) with monocytic properties, particularly strong phagocytic activity.

MATERIALS AND METHODS

Establishment of K-23-M. Peripheral blood was obtained on Oct. 10, 1979 from a patient (55-year-old female) with chronic myelocytic leukemia in blast crisis that morphologically changed to acute promyelocytic leukemia. Mononuclear cells were collected by Ficoll-Hypaque gradient centrifugation then suspended in RPMI 1640 medium with 23% heat-inactivated FCS (Flow Laboratories, U.S.A.). Four-milliliter portions of this suspension (10^6 cells/ml) were placed in 60-mm petri dishes and incubated at 37°C in a 5% CO₂-incubator.

After the 63rd day of culture, a lymphoblastoid cell line, designated K-23-M, was established. After 3 months of culture, RPMI 1640 with 10% FCS was used to maintain the line, and the medium was changed once every 3–5 days.

Cytological and cytochemical observations. Cover slip smears from the cultured cells were stained with Wright-Giemsa for light microscopy. For the TEM examination, the method described previously (18) was used. For the cytochemical study, smears were stained with peroxidase (6), ANBE and naphthol AS-D chloroacetate esterase (14), and acid phosphatase (30). TEM observations also were made on samples stained with peroxidase (29).

Fc and C₃ receptors. Agglutination and rosette-forming tests were performed by a procedure similar to that used in the identification of T- and B-cells and monocytes (32).

Detection of cytoplasmic immunoglobulins. FITC-conjugated goat antisera against human IgG, IgM and IgA were purchased from E-Y Laboratories, Inc., U.S.A. Direct immunofluorescence techniques were used.

Detection of EBNA. A mixture of serum proved anti-EBNA positive by the Special Reference Laboratory, Japan, and fresh serum was used as the first solution. FITC-conjugated goat anti-human C₃ from E-Y Laboratories was the second solution. After treatment with the first solution, the direct immunofluorescence technique was used. P3HR-1, Raji, and EB-3 cells were the positive controls and rat liver cells the negative control.

Assay of lysozyme activity. The lysoplate method (24) was used in which culture supernatants and cell lysates were the samples and lysozyme of egg white (Sigma Chemical Co., St Louis, Mo.) the control.

Chromosomal analysis. Cells were collected after treatment with colchicine for 2 h. The preparations were treated with 0.075 M KCl for 8 min, then fixed with ethanol/acetic acid (3 : 1), flame-dried and stained with Giemsa. One hundred metaphase cells were studied.

TdT assay and monoclonal antibody tests. The indirect immunofluorescence technique was employed. Anti-TdT and anti-human monocyte sera were purchased from Bethesda Research Laboratories, Inc., U.S.A. and anti-human B-lymphocyte serum from Sera-Lab., Co., Ltd., England.

Phagocytic activity. Sheep red cells treated with 2.5% glutaraldehyde for one day (GRC) were washed with a sufficient quantity of PBS then stored at 4°C as a suspension in PBS (27). Suspensions of 2×10^7 GRC and $5\text{--}20 \times 10^5$ line cells were made in centrifuge tubes containing 1 ml of RPMI 1640 with 40% FCS. These were centrifuged for 3 min at $150 \times g$ then kept in a 5% CO₂-incubator 4 h, after which 2–3 drops of trypan blue were added for the count in an improved Neubauer hemocytometer with bright-lines (Kayagaki Irikakogyo Co., Ltd., Japan). Four hundred viable cells were examined with the differential counter and divided into 4 groups: those phagocytizing no cells, those phagocytizing one cell, those

phagocytizing 2–3 cells and those phagocytizing more than 4 cells. Phagocytic activity was measured after 6 months of culture.

RESULTS

Cytological and cytochemical observations. K-23-M cells in culture photographed under phase contrast microscopy are shown in Fig. 1. Some cells formed clumps, some adhered to the glass surface and some were suspended as individual cells. A single cell was approximately 5–25 μm . Most cells had the “hand-mirror” shape with slender pseudopodia at one end (the uropod). Sometimes they showed a strong tendency to clump, the uropods pointing away from the center. When they adhered to the glass surface, they tended to assume a fibroblast shape or the dendritic type of macrophage shape. These findings are consistent with the definition of a “lymphoblastoid cell line” given by Nilsson and Pontén (22). In soft agar some cells showed locomotive mobility. After Wright-Giemsa staining, the pseudopodia cells resembled none of the hematopoietic cells seen *in vivo*. But, there were some cells similar to plasmocytes or macrophages.

TEM examination showed scattered globules and spherical mitochondria as described by Moore *et al.* (19). No herpes virus-like particles were found.

Cytochemical examination disclosed that the α -naphthyl butyrate esterase (ANBE) and acid phosphatase stainings were positive. The peroxidase and naphthol AS-D chloroacetate esterase stainings were negative.

The stainability of ANBE varied; sometimes it was diffusely positive, sometimes it disappeared completely. Usually, however, cells were stained in a weakly positive dot (33). For cells that were positive, treatment with stain containing 0.04 M NaF after treatment with 0.04 M NaF solution for 1 min produced negative stainability. When the stainability was negative without NaF, cells that had been washed 3 times

Fig. 1. K-23-M cells in culture seen through an inverted phase contrast microscope. Cells have characteristics of an LCL. $\times 200$.

Fig. 2. Fc receptors of K-23-M cells cultured for 9 months. Phagocytes also are present. $\times 250$.

with PBS turned positive in some instances.

Fc and C₃ receptors. The presence of Fc receptors varied greatly, from only 2% of the cells at 8 months of culture to 85–97% at 9 months of culture and 24–38% at 11 months of culture. Fc receptors (EA₇₈ rosette-forming test) at 9 months of culture are shown in Fig. 2. C₃ receptors always present in more than 70% of the cultured cells. Spontaneous rosette formation with sheep red cells took place in less than 5%

Fig. 3. GRC phagocytosis of K-23-M cells. A macrophage-like phagocyte is present. $\times 1,600$.

of the cells.

Cytoplasmic immunoglobulins. Some cells were always IgA positive. IgG sometimes was weakly positive and sometimes negative. IgM was always negative.

EBNA. In the early period of culture, K-23-M cells were EBNA negative; but, after 8–12 months of culture, they became EBNA positive.

Lysozyme activity. Up to day 76, the culture supernatants had lysozyme activity. Thereafter, both the cell lysates and supernatants became negative for this activity.

Chromosome analysis. The patient's peripheral blood before the onset of blast crisis was Ph¹ positive, whereas afterwards those few cells that underwent mitosis did not show Ph¹. K-23-M cells were Ph¹ negative. In every group, the chromosome

Fig. 4. Phagocytes on the hemocytometer. (a) $\times 100$, (b) $\times 400$.

Fig. 5. TEM observations confirm the GRC phagocytosis of K-23-M cells. $\times 3,500$.

number was 46: diploid.

TdT assay and monoclonal antibody tests. Anti-TdT, anti-monocyte and anti-B lymphocyte sera were always negative to immunofluorescence against K-23-M.

Phagocytic activity. K-23-M cells showed strong phagocytic activity. GRC phagocytosis by K-23-M cells is shown in Figs. 3, 4 and 5. In Figs. 3 and 4, many lymphoblastoid cells are undergoing marked phagocytosis similar to that seen only in monocytes and macrophages.

The percentage of phagocytosis in the line cells (%phagocytosis) rose in direct proportion to the concentration of FCS added at the start of the incubation for the examination of phagocytosis (Table 1). This ascending curve reached a peak at about 40–60% FCS then maintained a plateau up to 100% FCS.

Results of experiments to determine the necessity of centrifugation and the optimal period of incubation are shown in Table 2. For all subsequent experiments samples were centrifuged at $150\times g$ for 3 min and the incubation time was 4 h.

The %phagocytosis also varied with the medium used before the examination of phagocytosis. Cells cultured in medium with a low FCS content showed a high

TABLE 1. ACTIVATION OF PHAGOCYTOSIS OF K-23-M CELLS WITH FCS^a

FCS (%)	K-23-M cells phagocytizing sheep red cells (%)				% phagocytosis
	0	1	2-3	≥4 ^b	
0	75	8	8	8	25
10	61	16	12	11	39
20	55	20	11	14	45
40	37	22	23	19	63

^a Phagocytic activity was observed after 4 h of incubation in medium that contained 0 to 40% FCS. Phagocytized sheep red cells had been treated with 2.5% glutaraldehyde.

^b Number of red cells phagocytized per cell.

TABLE 2. TIME COURSE OF ACTIVATION OF PHAGOCYTIC ACTIVITY OF K-23-M CELLS WITH 40% FCS

Hour ^c		K-23-M cells phagocytizing sheep red cells (%)				% phagocytosis
		0	1	2-3	≥4	
1	n ^a	95	4	1	0	5
	c	66	15	10	10	34
2	n	85	9	5	1	15
	c	64	14	12	11	36
3	n	71	15	7	7	29
	c	55	15	11	20	46
4	n	68	12	10	10	32
	c	54	15	13	18	47
5	n	64	12	9	16	36
	c	45	16	17	23	55

^a n=no centrifugation. c=centrifugation^b.

^b Before incubation K-23-M cells mixed with glutaraldehyde-treated sheep red cells in RPMI 1640 medium containing 40% FCS were centrifuged at 150×g for 3 min.

^c Incubation period.

TABLE 3. RELATION BETWEEN %PHAGOCYTOSIS AND CONCENTRATION OF FCS IN THE CULTURE MEDIUM^a

FCS (%)	K-23-M cells phagocytizing sheep red cells (%)				%phagocytosis	(cell count ^b ×10 ⁵ /ml)
	0	1	2-3	≥4		
5	68	9	7	17	32	(2.5)
10	75	13	4	9	25	(7.1)
20	80	8	4	7	20	(14.1)
40	79	4	4	13	21	(16.7)

^a After 4 days of incubation in medium containing 5 to 40% FCS, phagocytic activity was checked after 4 h of incubation in medium plus 40% FCS.

^b Cell count on the 5th day of incubation. The cell count on the 1st day was 2×10⁵/ml.

Fig. 6. Changes of %phagocytosis in the medium containing 5% FCS. This experiment was done on the 5th day of incubation in medium containing 40% FCS. After being washed, K-23-M cells were incubated in medium containing 5% FCS. Phagocytic activity was counted daily for 6 days.

%phagocytosis (Table 3). As shown in Fig. 6, the %phagocytosis for K-23-M cells rose continuously in medium with 5% FCS.

The %phagocytosis of the K-23-M cells varied widely (Tables 1, 2, 3 and Fig. 6). It was impossible to obtain stable data for the phagocytic activity of this cell line. The %phagocytosis ranged from a high of 69% to a low of 5%, but there were no significant differences in mean %phagocytosis (about 27%) among the 23 cloned sublines of K-23-M obtained by the soft agar method.

In addition, cytoplasmic IgA was checked with smears of phagocytizing K-23-M cells. Most phagocytizing cells did not produce IgA, and most IgA-positive cells did not phagocytize GRC, but a number of cells showed both phagocytosis and IgA production (Fig. 7). This phenomenon was present in each cloned subline.

DISCUSSION

The term "LCL" was defined by Nilsson and Pontén (22) as a cell line that produces immunoglobulin, is EBNA positive and diploid, and shows characteristic growth as free-floating clumps of pear-shaped cells extending their uropods in a centrifugal direction. Therefore, K-23-M is an LCL.

An LCL is considered a B-cell line, but monocytic properties of LCLs frequently have been reported; phagocytic activity (5, 19, 21), a high density of Fc receptors (8, 11), positive stainability of ANBE that is blocked by NaF (13, 18), migration in soft agar and attachment to glass surfaces with a change in form to the dendritic type of macrophage shape (22). These properties are observed only in monocytic cells under normal conditions, but because LCLs have been considered B-cell lines, these monocytic properties are believed to be acquired characteristics.

Reports of phagocytosis of lymphocytic cells have been made that include normal lymphocytes (9), acute lymphocytic leukemia cells (4) and myeloma cells (3, 17). There also has been a report that acute lymphoblastic leukemia cells autonomously produce parathyroid hormone (28). Therefore, it is not unusual to find lymphocytic

Fig. 7. GRC phagocytosis of IgA-producing K-23-M cells. $\times 1,600$.

cells that have acquired monocytic properties after long term culture.

As reported previously, Mono-1-207 is an LCL, but study of its differentiation has enhanced the possibility of its being a myeloid precursor cell line. There has been no report of immunoglobulin-producing cells differentiating to mature granulocytes so it is extremely difficult to explain the potency of Mono-1-207 according to the previous theory of LCLs.

We observed remarkable monocytic properties in an LCL. There has been no previous report of an LCL with such strong phagocytic activity and high density of Fc receptors. We found that some phagocytizing K-23-M cells are difficult to distinguish from phagocytizing macrophages (Fig. 3). Moreover, the phagocytized particles used in our experiment were GRC. A GRC can not attach to lymphocytes because of its negative surface charge (20, 26). Therefore, if only this one characteristic is considered, K-23-M might be considered a monocytic cell line. This would be incorrect because K-23-M cells can produce IgA.

To explain this phenomenon found in Mono-1-207 and K-23-M cells, we have

TABLE 4. TEST FOR ACTIVATION OF PHAGOCYTOSIS

Activator	K-23-M cells phagocytizing sheep red cells (%)				%phagocytosis
	0	1	2-3	≥4	
40% CS	99	1	0	1	1
Serum free	83	7	5	6	17
40% FCS	65	10	10	15	35

This experiment was done on the 5 th day of incubation with serum free medium containing 5 μ g Insulin and 5 μ g Transferrin/ml.

After being washed, K-23-M cells were incubated in the medium combinations for 4 h.

made a new hypothesis: An LCL is a differentiating cell line. The stem cell is pluripotent and the terminal, differentiated cells are plasmocytic and monocytic cells. Because its differentiated cells are mainly plasmocytic, the LCL has been believed to be a B-cell line.

The proportion of phagocytic cells increased in medium with 5% FCS (Fig. 6). This phenomenon is explainable if K-23-M is a differentiating cell line, as K-23-M cells differentiate in both 5% and 40% FCS media. Because they proliferate slowly in low FCS medium, the percentage of differentiated cells (phagocytes) is relatively high in medium with 5% FCS. Therefore, Fig. 6 shows how the proportion of nonphagocytes and phagocytes changes from the value in medium containing 40% FCS to that in medium containing 5% FCS.

If an LCL is a differentiating cell line, it explains not only the granulocytic differentiation of Mono-1-207 and the phagocytic activity of K-23-M but the other monocytic and lymphocytic properties of an LCL as well. As to immunoglobulin production, very wide variations in the number of immunoglobulin-producing cells in lymphoblastoid cells (2.5–25%) were found by Takahashi *et al.* (31) in cloned sublines of an LCL. This phenomenon also is accounted for if LCLs are considered differentiating cell lines.

As to the percent of phagocytosis increasing in direct proportion to the concentration of FCS, the same phenomenon has been reported for mouse macrophages (27). This leads us to the conclusion that FCS has phagocyte activating activity. This activity was found for FCS, but not for calf serum (Table 4).

Even if an LCL is not a differentiating cell line and its monocytic properties are acquired characteristics, there is the fact that B-lymphocytes easily acquire monocytic properties. Another important fact is that almost no LCLs produce IgM, the most common immunoglobulin produced by B-lymphocytes.

Because of its remarkable monocytic properties, K-23-M may be useful for proving our hypothesis or for accounting for contradictory facts stated above.

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