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MONOCYTIC PROPERTIES OF FOUR LYMPHOBLASTOID CELL LINES ESTABLISHED FROM THREE PATIENTS WITH NON-LYMPHOCYTIC LEUKEMIA

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Abstract

Four human hematopoietic cell lines, designated as K-21, K-22, K-23-L and K-23-M, were established from three patients with non-lymphocytic leukemia. All of them were morphologically "lymphoblastoid cell lines". But these cell lines had properties like the monocyte or macrophage, such as the presence of Fc receptor sometimes up to 97% (K-23-M), positive stainability of α-naphthyl butyrate esterase that was blocked by NaF, and ability to migrate on soft agar, to attach to glass surface, and to phagocytize certain particles, polystyrene latex particles, opsonized or glutaraldehyde-treated sheep red cells. Among these monocytic properties examined so far in these 4 cell lines, the phagocytic activity of the K-23-M was the most remarkable finding. Consequently, these observations raise a question on the theory that lymphoblastoid cell lines originated from B-lymphocyte.

INTRODUCTION

For many years attempts have been made to establish long-term cultures of leukemic cells from patients with non-lymphocytic leukemia.

In 1964 Iwakata and Grace[1] reported the new cell line established from a patient with acute myelocytic leukemia. At first their report was considered as the first success at growing human myelocytic leukemia cells, and since then many cell lines have been established from leukemic patients by various researchers.

In 1966 Tanigaki et al.[2] reported that immunoglobulins were produced by these "myelocytic leukemia cell lines", and in 1967 Moore et al.[3] reported that immunoglobulins were produced by cultured normal human leukocytes, so it became doubtful whether all cell lines established from patients with myelocytic

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leukemia were leukemia cell lines.

After many experimental approaches, a unique theory was established. Namely, that most non-lymphocytic leukemia cells could not be cultured for a long time, and that even if almost all cells were leukemia cells at the time of primary culture, B-lymphocytes could be transformed by Epstein-Barr viral infection into cell lines[4]. Morphologically and functionally these established cell lines were similar to each other, so that these cell lines were defined as "lymphoblastoid cell lines" by Nilsson (1975)[5].

Since the report by Lozzio (1975)[6] on a cell line with Philadelphia (Ph^1) chromosome (K-562), many non-lymphocytic human hematopoietic cell lines have been established, e.g. U-937 (Sundström 1976)[7], HL-60 (Collins, 1977)[8], Line-230 (Karpas, 1978)[9], KG-1 (Koeffler, 1978)[10], and TPH-1 (Tsuchiya, 1980)[11]. But all these non-lymphocytic cell lines were clearly different from the lymphoblastoid cell lines as defined by Nilsson thus the concept that lymphoblastoid cell lines occupying over 90% of the human hematopoietic cell lines are B-lymphocytic cell lines remains intact.

In contrast, Mono-1 reported by kimoto et al. in 1976[12], in spite of being morphologically a lymphoblastoid cell line, has the property of a monocytic cell, and Mono-1-207, its subline, showed lobulation like neutrophils in arginine deficient medium (Kimoto et al., 1978)[13]. Differentiation to granulocyte or macrophage in arginine deficient condition has been demonstrated by Okabe (1979)[14] using M-1, the cell line from spontaneous myeloid leukemia of SL-strain mice established by Ichikawa (1969)[15], and by Honma (1980)[16] using HL-60. As a result, the possibility that Mono-1-207 is morphologically a lymphoblastoid cell line, but also has characteristics of myeloid precursor cell line, has been strengthened.

Therefore, I have studied to clarify whether or not there exists a cell line having distinct myelocytic, monocytic or erythrocytic properties, or capable of differentiating into granulocyte, macrophage or erythrocyte in the lymphoblastoid cell lines established from peripheral blood of patients with non-lymphocytic leukemia. This research has shown that some lymphoblastoid cell lines have monocytic properties and they are reported here.

MATERIALS AND METHODS

Establishment of four cell lines

Through the courtesy of Prof. Yoshito Yawata, Department of Medicine, Kawasaki Medical School, the peripheral blood of three patients was obtained. The first sample was aspirated from a 36-year old man with acute myelocytic leukemia on July 12, 1979, the second sample was aspirated from a 41-year

old man with acute monocytic leukemia on July 21, 1979, and the third sample was aspirated from a 55-year old woman with chronic myelocytic leukemia in blast crisis on October 10, 1979.

By Ficoll-Hypaque gradient centrifugation mononuclear cells of each blood were collected, whose concentration was adjusted to about 10^6 cells/ml suspended in RPMI-1640 medium supplemented with 23% heat-inactivated fetal calf serum (FCS). Four ml of this suspension was placed in 60 mm Petri dishes, and incubated at 37°C in a 5% CO_2-incubator. About 1 ml of RPMI-1640 supplemented with 23% FCS was added to each cultured medium weekly by the time when a lot of clusters in each dish was found.

As for the first sample, the first subculture was done on the 42nd culture day. For the second sample, the first subculture was done on the 33rd culture day, and for the third sample, the first subculture was done on the 60th culture day. In addition, from the third sample, a 4th cell line was estabished by creating a subculture on the second culture day and then continuing subculture to the 5th, 9th, 19th and 28th culture day. This subline was described in detail in another report.

As described above, four lymphoblastoid cell lines were obtained, and each cell line was designated as "K-21", "K-22", "K-23-L", and "K-23-M". After the cell line was established, each medium change was done once every 3-5 days. After 3 months of culture, RPMI-1640 supplemented with 10% FCS was used to maintain the lines.

Cytological and cytochemical observations

Smears from the cultured cells were stained with Wright-Giemsa for light microscopic examination. Both cover slip smears and cytospin smears were observed.

For transmission electron microscopic (TEM) examination, the cells were doubly fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) and 1% OsO_4 in the same buffer, dehydrated in an ethanol series and then embedded in Epon 812[17]. The thin sections cut with a glass knife on poter-Blum MT-B ultra-microtome were stained with 2% uranyl acetate and Raynolds' lead citrate solutions[18], then examined with a Hitachi HU-12A electron microscope at 75KV.

For scanning electron microscopic (SEM) examination, the cells were fixed with glutaraldehyde and OsO_4, dried with critical point dryer HCP-1, coated with Au-Pd alloy in an ion coater IB-2 and then observed in a Hitachi HHS-2R scanning electron microscope.

For cytochemical study, smears were subjected to peroxidase staining (Inagaki, 1976)[19], α-naphthyl butyrate esterase staining (Li, 1973)[20], naphthol

AS-D chloroacetate esterase staining (Yam, 1971)[21], and acid phosphatase staining (Shibata, 1978)[22].

C_3 and Fc 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 described previously (Ueki, 1975)[23].

Detection of immunoglobulin and Epstein-Barr viral nuclear antigen (EBNA)

Rabbit antisera against human IgG, IgM and C_3 were purchased from Medical and Biological Laboratory, Japan.

The serum determined anti-EBNA positive was obtained from Dr. Sasai, Department of Pediatrics, Kawasaki Medical School, and fresh serum was aspirated from normal person for supporting C_3. Rabbit antisera against IgG and IgM were used to detect cytoplasmic immunoglobulins, and the others were used to detect EBNA.

Direct immunofluorescence technics were attempted.

Assay of lysozyme activity

The lysoplate method (Osserman,1966)[24] was used for assay of lysozyme activity. Culture supernatants and cell lysates were used, as samples and lysozyme of egg white (Sigma Chemical Co., USA) as control.

Chromosomal analysis

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

Terminal deoxyribonucleotidyl transferase (TdT) assay

For the qualitative assay the indirect immunofluorescence technic was employed using rabbit anti-TdT serum and goat anti-rabbit IgG purchased from Bethesda Research Laboratories, Inc., USA. For the quantitative assay, the method of Koziner (1977)[25] combined with that by Marcus (1976)[26] was used.

Detection of phagocytic activity

As for phagocytized particles, latex particles coated with human IgG (Hyland Co., USA), polystylene latex particles (Dow Chemical Co., USA), colloid iron sulfate (Dainihonseiyaku Co., Japan), horse radish peroxidase (Sigma, USA), IgG or IgM-C_3 complex conjugated sheep red cells, and glutaraldehyde treated sheep red cells (Rabinovitch, 1967)[27] were used. Examined cells were re-

suspended in RPMI-1640 containing 0-40% FCS and incubated for 1-24 h (usually 4 h), then cover slip smears of them were observed. Later on, improved Neubauer hemocytometer with bright line (Kayagaki Irikakogyo Co. Ltd., Japan) was used for counting.

Induction of cell differentiation

As the inducer, 10-50 volume % ascites of patient in the terminal stage of breast cancer obtained from Prof. Tsuneaki Senoo, Department of Surgery, Kawasaki Medical School, 0.75-1.5% dimethyl sulfoxide, 0.4-10 μg/ml of lipopolysaccharide, 2×10^{-6} M prostaglandin E, 1-100 μg/ml of dexamethasone, 10ng/ml of actinomycin D, 1 μg/ml of concanavalin A, 2.0 IU of erythropoietin, 2 mM sodium butyrate, or arginine deficient medium with 5-10% FCS were used. Erythropoietin was obtained from Dr. Takaji Miyamae and Dr. Makoto Kawakita, Department of Medicine, Kumamoto University Medical School, Japan. Sometimes inducers of more than double conbination were used.

Cells were harvested and adjusted to 2.5×10^5 cells/ml suspended in the medium which contained inducer. Two ml of each medium with cells was poured into 35 mm Petri dishes. Each medium containing inducer was changed every fourth day, except for the arginine deficient medium, which was changed every second day.

As the indices of cell differentiation, morphological change, secretion of lysozyme, stainability of α-naphthyl butyrate esterase, phagocytic activity, hemoglobin reaction against diaminobenzidine[28] were examined. These examinations were made every second day for 14 days for each inducer.

RESULTS

Cytological and cytochemical observation

Fig. 1 shows the cultured cells observed by a phase contrast inverted microscope of K-21 and K-22 and K-23-M cells. As seen here, the cells of each cell line were very similar to each other. These cells were observed forming clumps, or being attached to glass surface, or being suspended as individual cells. When they became attached to glass surface, they tended to change to fibroblast-like shape or dendritic type of macrophage-like shape. A single cell size of each cell line was similar and approximately 5-25 μm.

On soft agar these cells showed a locomotive mobility.

The morphological characteristic of lymphoblastoid cell line is the "hand-mirror" shape cell with slender pseudopodia located at one end (the uropod); sometimes they have a strong tendency to form clumps with the uropods pointing away from the center. That characteristic was found in all of these cell lines. But in an early period such as within 90th culture day, there were some

irregular "hand-mirror" shape cells with multiple pseudopodia. That tendency was also seen by SEM (Fig. 2). Even if in the later period a small number of multiple-psuedopodia cells were observed.

By the observation of cover slip smears stained with Wright-Giemsa, a similar morphology was presented as that observed by inverted microscope

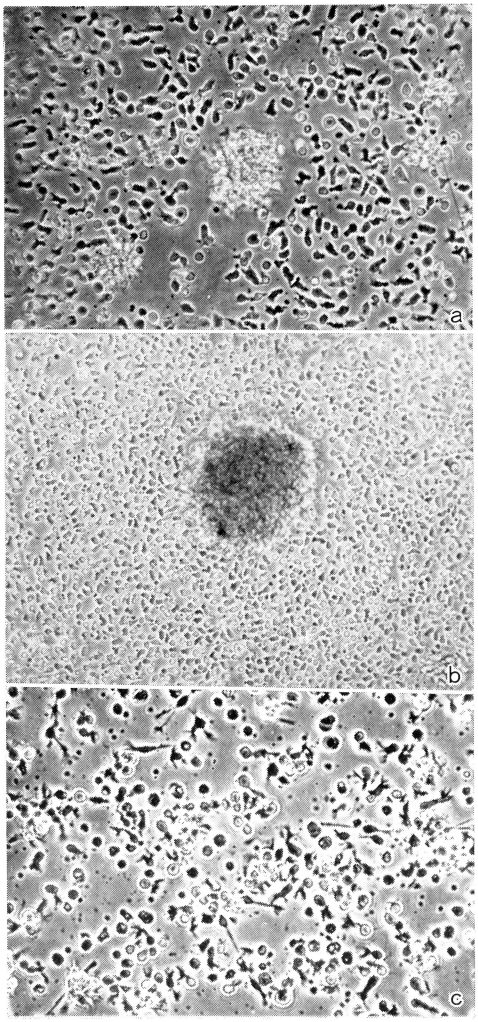


Fig. 1. a K-21 3 months of culture. ×200
b K-22 3 months of culture. ×100
c K-23-M 9 months of culture. ×200
(phase contrast inverted microscopic exam.)

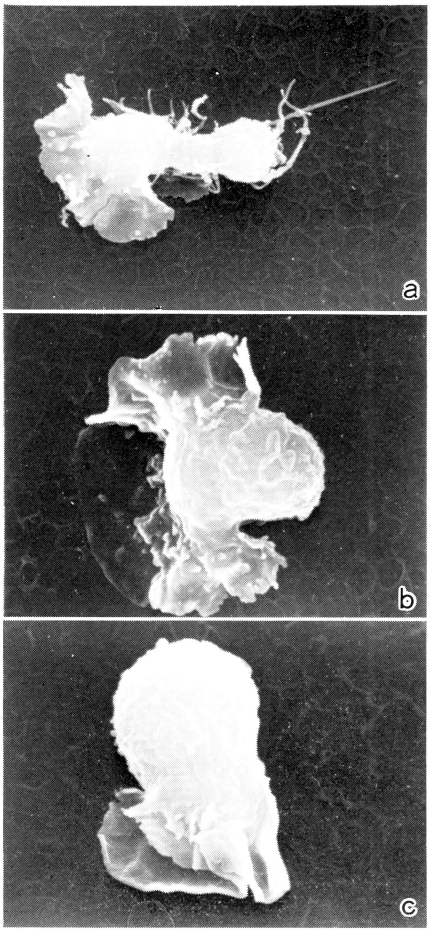


Fig. 2. a K-21 51st culture day. ×2500
b K-22 42nd culture day. ×4000
c K-22 42nd culture day. ×4000
(SEM)

(Fig. 3a, 3b, 3c & 4). Fig. 4a and Fig. 4b show the multiple-pseudopodia cells. Almost all line cells had azure granules. Occasionally the cells with lobulated nucleus (Fig. 4c) or hairy cell leukemia like cells (Fig. 4d) could be seen.

Before these cell lines had been established, there were also some common findings. Namely, within about 10th culture day there were leukemia cells in the smears of K-21 and K-22. Some leukemic cells had Auer rod in their cytoplasm. As for K-23-M, there were differentiated cells than those in the blood of the patient, such as neutrophils or basophils like a blood picture of the patient in chronic phase of chronic myelocytic leukemia.

After around the 5th culture day, most cultured cells were undergoing degeneration, and at 10-20th culture day most of them had eosinophilic granules. These granules were not the same as those seen in eosinophils.

On cytochemical examination, by about the 40th culture day, most cells of each line were peroxidase and α-naphthyl butyrate esterase positive. Thereafter, the stainability of peroxidase became negative, except for K-23-M cells which showed a strong stainability of peroxidase (Fig. 5a) until the 75th culture day, after which it disappeared. The α-naphthyl butyrate esterase staining sometimes became diffusely positive (Fig. 5b), and sometimes disappeared completeletely. Usually these line cells were stained weakly positive in a dot. For those cells that were positive, treatment with the stain containing 0.04 M NaF after being treated with the 0.04 M NaF solution for 1 min. showed negative stainability. As to naphthol AS-D chloroacetate esterase, within the 70th culture day about 50% of K-23-M cells were diffusely, weakly positive (Fig. 5c), but thereafter became negative. The other line cells were negative after the 40th culture day ; before that the examination was not done. Acid phosphatase was consistently positive in most cells of these lines, but stainability changed from diffusely strong positive to finely granular positive.

Fc and C_3 receptors

In K-21 cells, 62% of cells had C_3 receptor and 12% of them had Fc receptor on the 106th culture day. In K-22 cells C_3 receptor was 25% and Fc receptor was 2% on the 97th culture day. As to K-23-M cells, the surface markers were examined several times. C_3 receptor was always detected in over 80% of them, but Fc receptor fluctuated in a wide range, from 2% at 8 months of culture to 85-97% (Fig. 6) at 9 months, to 24-38% at 11 months of cultutre.

Cytoplasmic immunoglobulins

K-21 and K-23 were IgG weakly positive and IgM negative. In contrast, K-22 was both IgG and IgM positive.

EBNA

K-21 and K-23 were EBNA negative. In contrast, K-22 was positive (Fig. 7).

Lysozome activity

After cell lines were established, lysozyme activity of both cell lysates and culture supernatants was always negative in each cell line.

Chromosomal analysis

Every cell line was diploid and Ph^1 chromosome negative.

TdT assay

TdT proved always to be negative

Phagocytic activity

Before treated sheep red cells (SRC) were used, it was arduous to determine phagocytic activity. At first latex particles coated with human IgG were used, and around the 80th culture day, all of K-21 cells and K-22 cells showed a strong phagocytic activity after 4 h incubation with these particles. Next, polystyrene latex particles were used, and the same result was obtained. It remained a question whether a simple attachment of latex particles to the cell surface membrane was seen. The same question was present when colloid iron sulfate or horse radish peroxidase were used. When opsonized SRC were used, true phagocytic activity was demonstrated (Fig. 8a, 8b).

The percentage of phagocytosis in the nucleated cells (%phagocytosis) varied. Sometimes there were no cells phagocytizing opsonized SRC. The average %phagocytosis by K-21 was about 5%, by K-22 about 1%, and by K-23-M about 25%. %phagocytosis by K-23-M cells varied, sometimes rising over 50%.

There are three possible explanations for this variability : first, that the phagocytic activity of these cells was unstable, second, that opsonization of SRC was unstable, and third, that the counting method was wrong. Indeed, most phagocytizing cells were seen at the appropriate place near the tail of smear. Then glutaraldehyde treated SRC (GRC) were used as the phagocytized particle and improved Neubauer hemocytometer was used for counting. GRC are stable for more than 6 months. Fig. 8c, 8d and 8e show the GRC phagocytosis of K-23-M cells.

Even when GRC and hemocytometer were used, the fluctuation of %phagocytosis continued (K-23-M fluctuated from 10% to 70%). It became clear that phagocytic activity of these cells was unstable.

As illustrated in Fig. 8e, many lymphoblastoid cells were observed undergoing a marked phagocytosis like that seen only in the monocyte or macrophage.

For the final confirmation of phagocytosis, TEM was conducted. As seen

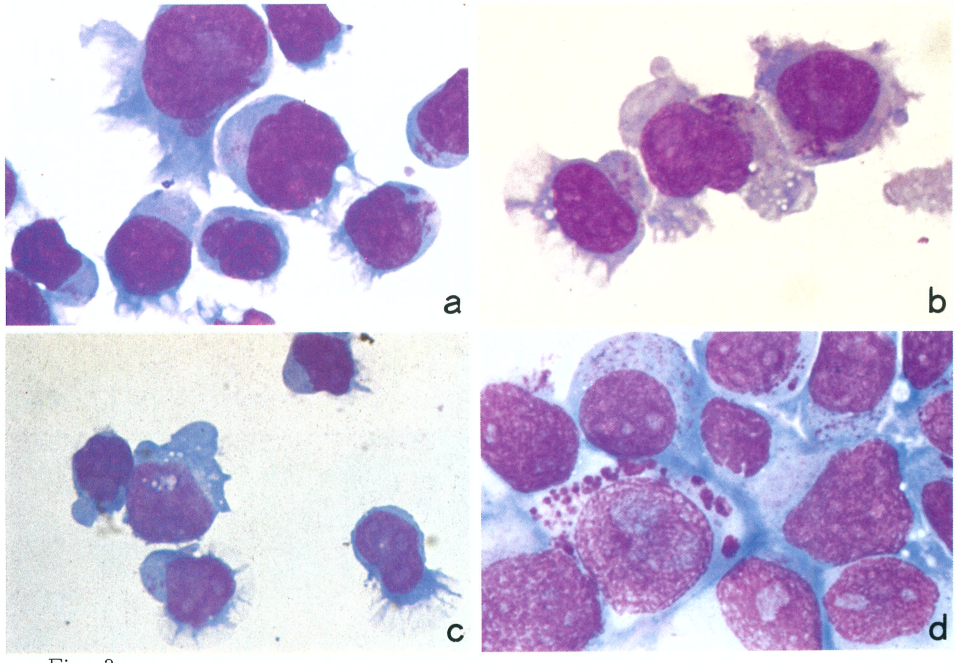


Fig. 3.

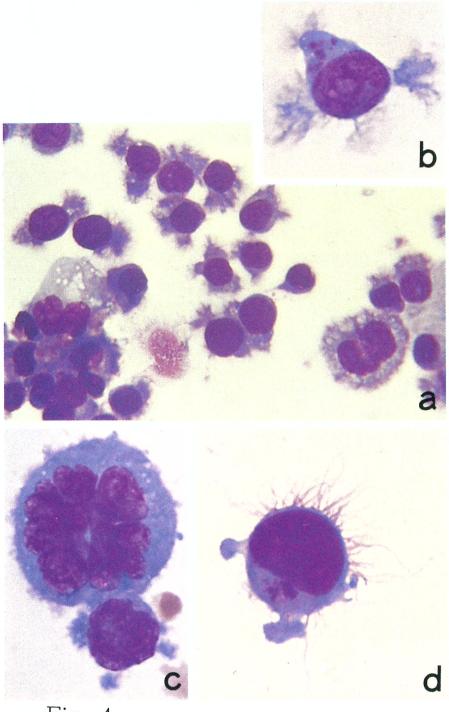


Fig. 4.

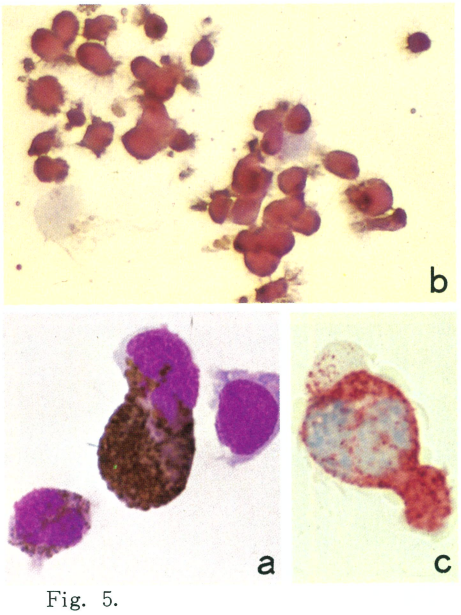
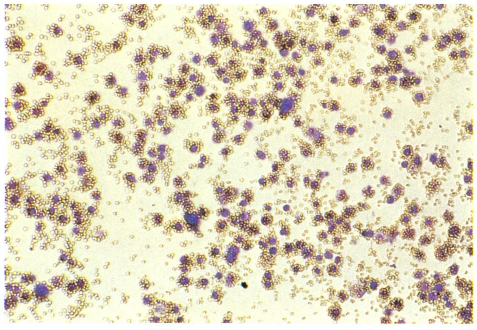


Fig. 5.



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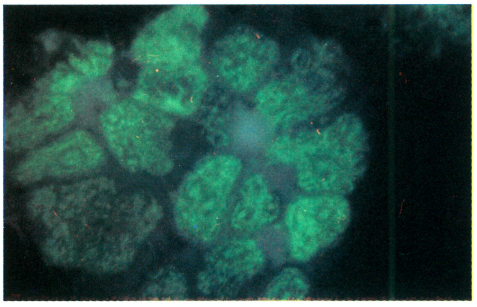

Fig. 7.

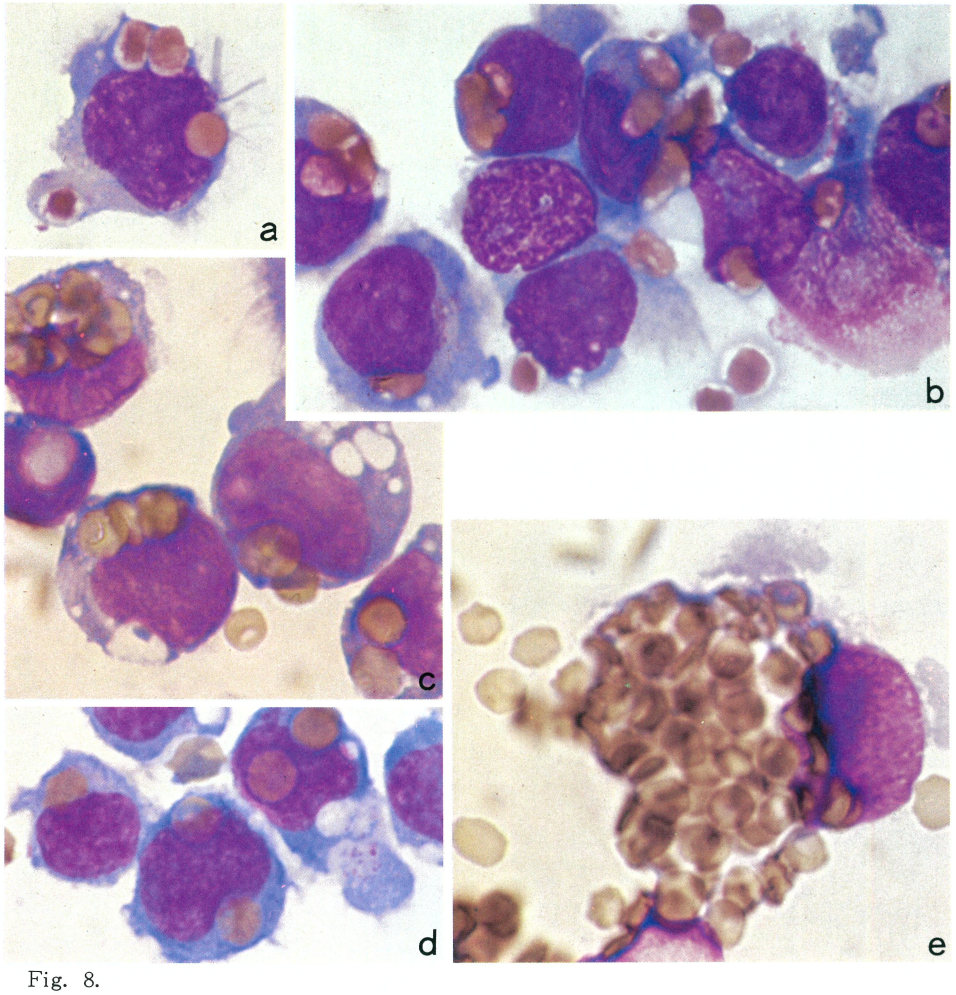

Fig. 8.

Fig. 3. a K-21 3 months of culture. ×1000
b K-22 4 months of culture. ×1000
c K-23-L 15 months of culture. ×1000
cover slip smears
d K-22 4 months of culture. ×1000
cytospin smear
(Wright-Giemsa staining)

Fig. 4. a K-22 67th culture day. × 400
b K-23-M 64th culture day. × 900
c K-21 (lobulation) 107th culture day. ×1000
d K-22 (hairly cell leukemia like cell) 88th culture day. ×1000
(Wright-Giemsa staining)

Fig. 5. a K-23 17th culture day. peroxidase staining ×1000
b K-22 75th culture day. α-naphthyl butyrate esterase staining. ×400
c K-23 34th culture day. naphthol AS-D chloroacetate esterase staining. ×1000

Fig. 6. K-23 Fc receptor. 9 months of culture. Hematoxylin staining. ×100

Fig. 7. K-22 EBNA positive. 6.5 months of culture. Immunofluorescent technic. ×1000

Fig. 8. a K-21 phagocytosis of IgG coated SRBC. 4 months of culture
b K-23-M phagocytosis of IgM-C_3 complex conjugated SRBC. 7 months of culture
c K-23-M GRC phagocytosis. 5.5 months of culture
d K-23-M GRC phagocytosis. 7 months of culture
e K-23-M GRC phagocytosis. 7 months of culture
(Wright-Giemsa staining. ×1500)

in Fig. 9, GRC were present within the cell membrane.

It is clear that lymphoblastoid cells have some properties possessed only by the monocyte or macrophage.

The GRC phagocytosis of K-23-M cells will be reported in another paper.

When GRC were used %phagocytosis of K-21, K-22 and K-23-L cells never exceeded 10%.

Induction of cell differentiation

No tendency to differentiate could be found. Sometimes %phagocytosis increased to 70%, but it is doubtful whether the inducer (actinomycin D or concanavalin A) was the cause of increased %phagocytosis. After 3 months of experimentation the inducers used in this experiment did not induce differentiation of these line cells.

DISCUSSION

All four cell lines were lymphoblastoid cell lines (LCL) as defined by Nilsson[5].

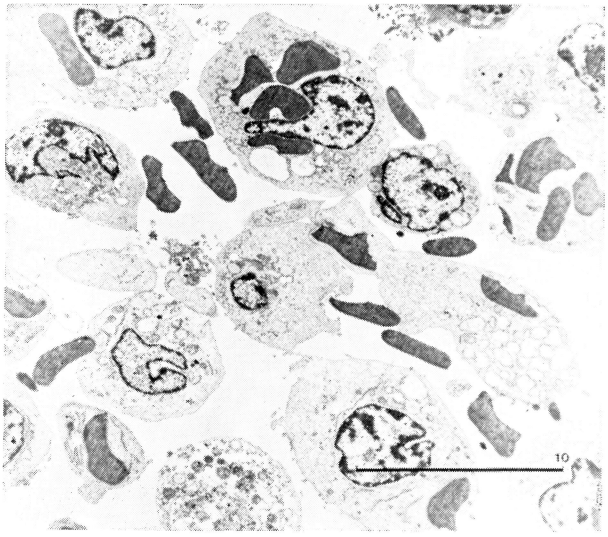

Fig. 9. K-23-M GRC phagocytosis. 6.5 months of culture (TEM)

Several experimental approaches to LCL have been reported, e.g. morphology (Moore et al., 1968[29] ; Nilsson and Pontén, 1975[5]), immunoglobulin production (Tanigaki et al., 1966[2] ; Finegold et al., 1967[30] ; Nilsson, 1971[31]), presence of Epstein-Barr viral nuclear antigen (Nilsson et al., 1971[4] ; Klein et al., 1974[32]), agglutination with concanavalin A (Glimelius et al., 1975[33]), β_2-microglobulin production (Nilsson et al., 1974[34]), chromosome analysis (Jarvis et al., 1974[35] ; Zech et al., 1976[36]) and surface receptors Shevach et al., 1972[37] ; Huber et al., 1976[38]) etc.

As the result of these experiments, it is beyond doubt that LCL has many B-lymphocytic properties. Recently LCL has been regarded as a model for B-lymphocyte differentiation[39]. However, even in these reports some monocytic properties are described[5,29], such as "LCL has slight phagocytic activity", "LCL has locomotive mobility" or "The morphological characteristic of LCL is pleomorphism. Sometimes it takes a pear-shaped appearance with pseudopodia, and when it becomes attached to glass surface, it acquires a fibroblast-like shape" etc. These are also common findings of LCL.

Since Tanigaki's report in 1966,[2] almost all workers have been interested in LCL as a B-lymphocytic cell line, but this is only one aspect of LCL and the other aspects of LCL must be considered.

The most remarkable point of this report is the phagocytic activity of K-23-M cells. No previous reports describe such strong phagocytic activity of LCL as is shown in Fig. 8e. It is difficult to explain this phenomenon if

LCL is considered just a B-lymphocyte. It is known that GRC can not even attach to the surface of a lymphocyte[40].

The pseudopodia cell is one of the morphological characteristics[5] of LCL, but it is more similar to monocyte or myelocytic leukemia cell than to the lymphocyte when observed by phase contrast inverted microscope or SEM[41]. Moreover, the presence of many multiple-pseudopodia cells at an early period of culture shows the possibility that the morphological change does not proceed from non-pseudopodia cell to pseudopodia cell but rather from multiple-pseudopodia cell to single pseudopodia cell. This may also support the idea the pseudopodia cell is derived from the monocyte. However, a few multiple-pseudopodia cells can be seen in Mono-1 cultured for 7 years.

In passing, these pseudopodia can not be observed by cytospin smears (Fig. 3d). When morphological change is observed it is important to know which smear was used, cytospin one or cover slip one.

Incidentally, as the characteristics of morphological change in an earlier period, pseudoeosinophils can be observed in the process of cell degeneration. This phenomenon has been observed since 1967 by Clarkson et al.[42] It is also one of the common findings when myelocytic cells are cultured.

It is difficult to explain the meaning of α-naphthyl butyrate esterase positive in LCL. Almost all positive cells were stained weakly in a dot, like the lymphocyte described by Willcox et al.[43] Sometimes the cells were stained diffusely, moderately positive (Fig. 5b). Moreover, such a stainability was blocked by NaF. The staining method used here was reported by Li et al.[20] They described "when α-naphthyl butyrate was used (as substrate at pH 6.0-6.3), enzyme activity was strong only in monocytes and very weak or not demonstrable in lymphocytes, megakaryocytes, plasma cells, granulocytes and primitive myeloblasts. Addition of approximately 0.04 M NaF to the incubation medium completely abolished the staining in monocytes, megakaryocytes, platelets and plasma cells but not in the granulocytes or lymphocytes". Therefore, the stainability of α-naphthyl butyrate esterase may be considered one of the monocytic properties. Isozyme assay of non-specific esterase should be recommended.

Finally, in regard to the high density of Fc receptors, there have been some lines previously established such as U-61-M[38] or Mono-1[12]. A high density of Fc receptors is considered a specific characteristic of the monocyte or macrophage[44]. Although there was much variability, it is an important finding that some LCLs had a high density of Fc receptors.

In spite of the fact that some of these monocytic properties are known, almost all researchers believe that LCL is derived from the B-lymphocyte.

Furthermore, some researchers designated them as "B-cell normal line"[45]. It is clear that all LCL have many specific properties of the the B-lymphocyte, but it is also clear that some of these cells have some specific properties of the monocyte or macrophage as well. Perhaps, K-23-M will be useful for the elucidation of the onset mechanism of phagocytic activity of human hematopoietic cells. Reconsideration should be given to the non-lymphocytic properties of LCL, so that this may become a more useful cell line to understand hematopoietic cells.

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