Novel Peripheral Blood-derived Human Cell Lines with Properties of Megakaryocytes

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ABSTRACT For 18 mo, we derived 18 cell lines from 11 donors with various clinical profiles ranging from normal to leukemic. Suspension cultures were initiated with 1×10^6 mononuclear blood cells/ml of nutrient medium containing 10% human serum and 10% lectin-stimulated human lymphocyte conditioned medium. The cultures were monitored weekly by morphological analyses of Wright-Giemsa-stained cell preparations. All successful cultures showed a significant decline in viability during the first 3–4 wk with rare "lymphoid" cells observed in mitosis. Within the next 2 wk, the proliferating cells gave rise to a rapidly expanding population of mononuclear cells. As the cultures expanded, cell morphology became heterogeneous with respect to cell size and nuclear ploidy, with an accumulation of giant multinuclear cells that were suggestive of megakaryocytes. Even though the cells did not have the classical morphology of mature platelet-forming megakaryocytes, 90% of the cells within a cell line were positive by direct or indirect immunofluorescence for the platelet membrane glycoproteins IIb and IIIa; for surface markers HLA-Dr and B2-microglobulin; for intracellular platelet-derived growth factor and platelet factor IV; and for membrane affinity or binding with serum platelet-derived growth factor and platelet factor IV. These results suggest that a blood precursor cell, most likely a primitive megakaryoblast, was isolated from the peripheral blood and was provided with an optimal culture environment for sustained growth. These cells did not mature to a more differentiated stage, perhaps owing to regulatory factor deficiencies in this in vitro system. The remarkable frequency of obtaining cell lines with megakaryocyte properties from normal peripheral blood and the capacity of some normal donors to repeatedly yield these cell lines make this cell culture system indeed unique by being selective for putative megakaryocyte precursors.

Most human hemopoietic cell lines derived from peripheral blood have been obtained from patients with leukemia or lymphoma—Burkitt's lymphoma (DAUDI), T cell leukemia (MOLT-3), chronic myelogenous leukemia (K562), acute promyelocytic leukemia (HL60), and erythroleukemia (HEL). All cell lines have been well characterized (1–5). From normal donors, only B cell lines have been spontaneously obtained and express Epstein-Barr virus (EBV)[1] antigens. B cell lines can also be obtained by the transformation of fresh blood cells with EBV. T cell lines from the peripheral blood can be maintained but are dependent upon the continuous presence of T cell growth factor (6). We report the phenomenon of having obtained 18 cell lines from 11 donors including not only normal donors but patients with leukemia and preneoplastic disorders. The cell lines were negative for classical markers of lymphocytes and myeloid cells but spontaneously expressed properties of megakaryocytes. The cells had surface antigens detected by immunofluorescence using antibodies against glycoprotein (Gp) IIb, GpIIIa, platelet-derived growth factor (PDGF), platelet factor IV (PF4), and other platelet-related proteins. These cell lines displayed properties unlike those of the well-characterized cell lines derived from human blood (1–5) and may provide the opportunity to investigate cells in continuous culture that are closely related to or the same as primitive megakaryoblasts.

[1] *Abbreviations used in this paper:* EBNA and EBV, Epstein-Barr nuclear antigen and Epstein-Barr virus, respectively; Gp, glycoprotein; HCM, lectin-stimulated human lymphocyte conditioned medium; HS, human serum; PDGF, platelet-derived growth factor; PF4, platelet factor IV; PPP, platelet-poor plasma.

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MATERIALS AND METHODS

Materials: All sterile tissue culture plastics were obtained from Falcon Labware, Div. of Becton, Dickinson & Co. (Oxnard, CA), culture medium RPMI 1640 from Gibco Laboratories (Grand Island, NY), Ficoll-Paque from Pharmacia Fine Chemicals Div. (Uppsala, Sweden), and Wright-Giemsa stain from EM Science (Gibbstown, NJ). BSA and histochemistry kits were from Sigma Chemical Co. (St. Louis, MO); No. 90 for esterases (α-naphthyl acetate and naphthol AS-D chloroacetate), No. 395 for periodic acid-Schiff, and No. 390 for leukocyte peroxidase. Human serum (HS) was obtained from normal donors who had received no recent medications including aspirin and steroids. The serum was collected from coagulated blood and was heat inactivated at 56°C for 30 min. Platelet-poor plasma (PPP) was prepared from normal blood donors (7) and lectin-stimulated human lymphocyte conditioned medium (HCM) was harvested from lectin-stimulated lymphocytes (6, 8, 9) and was a gift of Dr. D. Zagury (University of Paris).

Antibodies: All antibodies for flow cytometry were from Becton-Dickinson & Co. except for OKT9 and OKT10 which were from Ortho Pharmaceutical (Raritan, NJ). The immunized rabbit sera against fibrinogen, fibronectin, factor VIII-Ag, and the non-immunized rabbit control serum were obtained from DAKO (Westbury, NJ). Fluorescein-conjugated secondary antibodies were goat anti-mouse and goat anti-rabbit (Cappel Laboratories, Inc., Cochranville, PA) Rabbit antiserum against PDGF was generously supplied by Dr. H. Antoniades (Harvard University). The β-thromboglobulin IgG and PF4 IgG and $F(ab)_2$ preparations were a gift of Dr. S. Niewiarowski (Temple University). Monospecific antisera against GpIIb and GpIIIa and absorbed with thrombasthenia platelets (10) were a gift of Dr. L. Leung (Cornell Medical Center); monoclonal antibodies B2.12 and B59.2 against GpIIIa (11) were from Dr. P. Thiagarajan (Jefferson Medical College).

Blood Mononuclear Cell Preparations: Heparinized blood was diluted 1:2 with RPMI, layered onto Ficoll-Paque, and centrifuged for 30 min at 280 *g*. The light-density cell fraction was washed twice and resuspended in RPMI containing 10% (vol/vol) of heat-inactivated human serum (RPMI-10% HS).

Cytocentrifuge Preparations: Fresh blood mononuclear cells obtained from the Ficoll gradients and cultured cells were washed twice with RPMI and resuspended in PBS that contained 5% BSA. We deposited aliquots containing 5×10^4 cells onto alcohol-washed glass microscope slides by using a cytocentrifuge (Shandon Southern Instruments Inc., Sewickley, PA) at 1,000 rpm for 5 min. Wright-Giemsa differential stain was used for morphological evaluation. For cytochemical reactions, the cytocentrifuge preparations were stained for esterases, periodic acid-Schiff, and leukocyte peroxidase following manufacturer's directions.

Cell Culture Methods: Suspension cultures were initiated by placing fresh mononuclear blood cells into culture flasks (25 cm^2) at a final cell concentration of 1×10^7 per 10 ml of nutrient medium RPMI-10% HS supplemented with 10% (vol/vol) of a fivefold concentrate of HCM. Control cultures received no HCM. The cultures were kept at 37°C and 5% CO_2 in a humidified incubator. At weekly intervals, 0.1 ml of cells from the culture flasks was cytocentrifuged, stained with Wright-Giemsa stain, and examined for cells in mitosis. Established cell lines were maintained by the continuous process of splitting the cultures and refeeding with fresh nutrient medium. Cells were pelleted and resuspended at a final concentration of 5×10^5/ml of RPMI-10% HS, RPMI-10% fetal bovine serum, or RPMI-10% PPP. Cultures were split when the cell density reached 2×10^6/ml as determined by trypan blue exclusion test for viability.

Immunofluorescence Techniques: For flow cytometry, cells were incubated with a fluorescein-conjugated primary antibody for 45 min at 4°C. For indirect staining, the primary antibody was followed by incubation for 45 min at 4°C with a fluorescein-conjugated secondary antibody. Control cells were stained with either IgG or IgM. After analysis on the CYTOFLUOGRAF (Ortho Pharmaceutical), percentage of positive values was corrected for the background contributed by the control cells. For fluorescent microscopy and indirect immunofluorescence labeling of the cell membrane, cultured cells were washed and the pellet was resuspended in 50 μl of the primary antibody for 2 h at room temperature. Non-immunized rabbit IgG or RPMI-10% BSA was used as a control for the primary monospecific antisera and monoclonal antibodies, respectively. The cells were then washed twice in PBS and incubated in fluorescein-conjugated goat anti-rabbit IgG for 1 h at room temperature. After being washed two times in PBS, the cells were mounted on a glass slide with FA mount (Difco Laboratories Inc., Detroit, MI) and examined with a Zeiss fluorescence microscope. Immunofluorescence for cytoplasmic staining of fixed cells was done after cytocentrifuge cell preparations were fixed in cold methanol for 20 min. All primary antisera were diluted 1:20–50 and incubated with the cells for 1.5–4 h at room temperature in a humidified chamber. Following two 10-min washes with PBS, fluorescein-conjugated antibody diluted 1:40 in PBS was added for 1 h. After another 10-min PBS wash and staining with 0.01% methyl green in PBS, the cells were examined.

DNA Analysis: Cells were pelleted and resuspended in 10 ml of propidium iodide in hypotonic citrate solution (0.05 mg/ml in 0.1% sodium citrate; osmolarity of 5 mOsmol) to stain DNA[13] and stored at 4°C until analyzed. Each stained sample was incubated with RNAase (bovine pancreas, 50 μg/ml; Calbiochem-Behring Corp., La Jolla, CA) for 30 min at room temperature prior to flow cytometry studies in order to remove RNA-associated propidium iodide staining. The stained cell suspensions were filtered through a 105-μm monofilament nylon mesh (Small Parts, Inc., Miami, FL) to remove any large debris immediately before analysis. The flow cytometer was an EPICS V flow cytofluorometer (Coulter Electronics Inc., Hialeah, FL) equipped with a Spectra-Physics 164-05 argon ion laser (Spectra-Physics Inc., Mountain View, CA). A 100-μm-diam flow tip was used. The cells in suspension were excited, with the laser adjusted to deliver 1 W at 488 nm, with a flow rate of approximately 1,000 cells/s. Interference (510 nm) and long wavelength pass (515 nm) filters were used to block scattered laser light from detection by the photomultipliers. The red fluorescence was passed through a 610-nm long wavelength pass filter before detection by a photomultiplier. Red fluorescence signals were collected for each cell with the Coulter MDADS minicomputer, using logarithmic converters so that the wide range of fluorescence intensities would all be on scale. The number of cells in each DNA peak was determined by taking the integral from one peak nadir to the next. Data were transferred to a Data General Eclipse computer for one parameter graphic display on a Tektronix 4662 plotter (12, 13).

Cytogenetic Analysis: Karyotypes were obtained from bone marrow aspirates, peripheral blood, and established cell cultures using a variety of synchronization and banding techniques (14–16). Most cells showing aneuploidy, polyploidy, or structural anomalies were karyotyped to assess the extent and nature of the anomalies.

Electron Microscopy: Cells were washed twice with cold 0.2 M phosphate buffer (pH 7.4), pelleted, and fixed in cold 1.25% glutaraldehyde for 30 min. After a buffer wash, the pellet was postfixed for 30 min in 1% osmium tetraoxide, washed with buffer, and stained with 0.5% uranyl acetate for 30 min. Alcohol dehydration was followed by propylene oxide and epoxy infiltration overnight and then embedded for 48 h. Samples were thin-sectioned, stained with uranyl acetate and lead citrate, and viewed on a Hitachi HU-124 transmission electron microscope.

RESULTS

Cell Line Emergence

18 cell lines were obtained from 11 donors with blood profiles ranging from normal to leukemic (Table I). No common feature of the donors appeared to be correlated with the probability of having successful cultures yielding cell lines. Clinical extremes included severe anemia and the presence of young blood cells normally confined to the bone marrow (S-124, B-403, P-320), moderate anemia with increased circulating blood platelets (M-408, W-803), and no anemia or evidence of any abnormalities in the peripheral blood (all remaining cell lines). At the time of culture initiation, the source of human serum used to supplement the nutrient growth medium was not a contributing factor. Originally, all cultures were supplemented with serum from an anemic donor; therefore, this serum was believed to be partially responsible for establishment of the cell lines. Subsequently, cell lines were obtained by using numerous normal sera as well as serum from the donor with iron-deficiency anemia. Two independent cell lines were obtained from the same mononuclear cell preparation (O-918) when the cultures were initiated with either normal or anemic serum. Furthermore, exogenous growth factor (HCM) was not a strict requirement since one control or nonsupplemented culture (M-408) was successful. The HCM-supplemented culture from this donor did not produce a cell line. However, HCM was required for the other cell lines to emerge.

Emergence of the cell lines followed a distinctive pattern by occurring between 30–40 d after culture initiation. Exam-

ination of stained cytocentrifuge preparations allowed for the early detection of successful cultures by the appearance of a population of actively proliferating "lymphoid" cells. Weekly monitoring of the cultures by morphological evaluation showed a rapid increase in the number of lymphoid cells which resulted in culture expansion by diluting the cells with fresh RPMI-10% HS. Culture doubling time varied somewhat with each cell line, but culture splitting was routinely done each 3–4 d after a 2.5–5-fold increase in total number of cells. The cell lines did not require serum for growth. When parallel flasks containing cells were resuspended and grown for 1 mo in RPMI-10% HS or RPMI-10% PPP from the HS donor, no difference in growth was seen between the two flasks at each culture split.

TABLE I

Disease Status and Frequency of Cell Lines Obtained from Each Peripheral Blood Donor

Donor designation	Clinical status	No. of cell lines/ No. of attempts
	Neoplastic	
S-1214	Acute lymphoblastic leukemia	2/3
B-403	Acute nonlymphocytic leukemia	2/2
P-320	Acute megakaryoblastic leukemia	2/3
	Preneoplastic	
O-418	Polycythemia vera	2/2
G-617	Essential thrombocythemia	1/1
D-221	Essential thrombocythemia	1/1
	Nonneoplastic	
M-408	Anemia	1/4
W-803	Anemia	2/3
E-518	Normal	1/1
C-306	Normal	2/2
S-111	Normal	2/2

Cell Line Morphology

All cell lines have characteristically displayed unusual morphology from the time of emergence to over 18 mo in continuous culture and over 65 culture passages. The cultures grew as single cells and in large clumps (Fig. 1 *a*) which most often were macroscopically visible in the culture flask. The clumps were easily disrupted mechanically into single cells. Size heterogeneity and pleomorphism due to cytoplasmic extensions (Fig. 1, *b* and *c*) gave the cells a distinctive appearance. The membrane spicules and processes frequently involved only a portion of the total cell surface (Fig. 1 *d*). After cytocentrifugation and staining, giant cells were easily recognized and had multiple nuclei or nuclear lobes (Fig. 2 *a*). The polarized cytoplasm elongation was often associated with spicule formation (Fig. 2, *b* and *c*) which resulted in fragmentation and shedding of numerous extracellular particles (Fig. 2 *d*).

Electron Microscopy

The ultrastructure features of the predominant small cell from the cultures as viewed by transmission electron microscopy were consistent with the morphology of undifferentiated

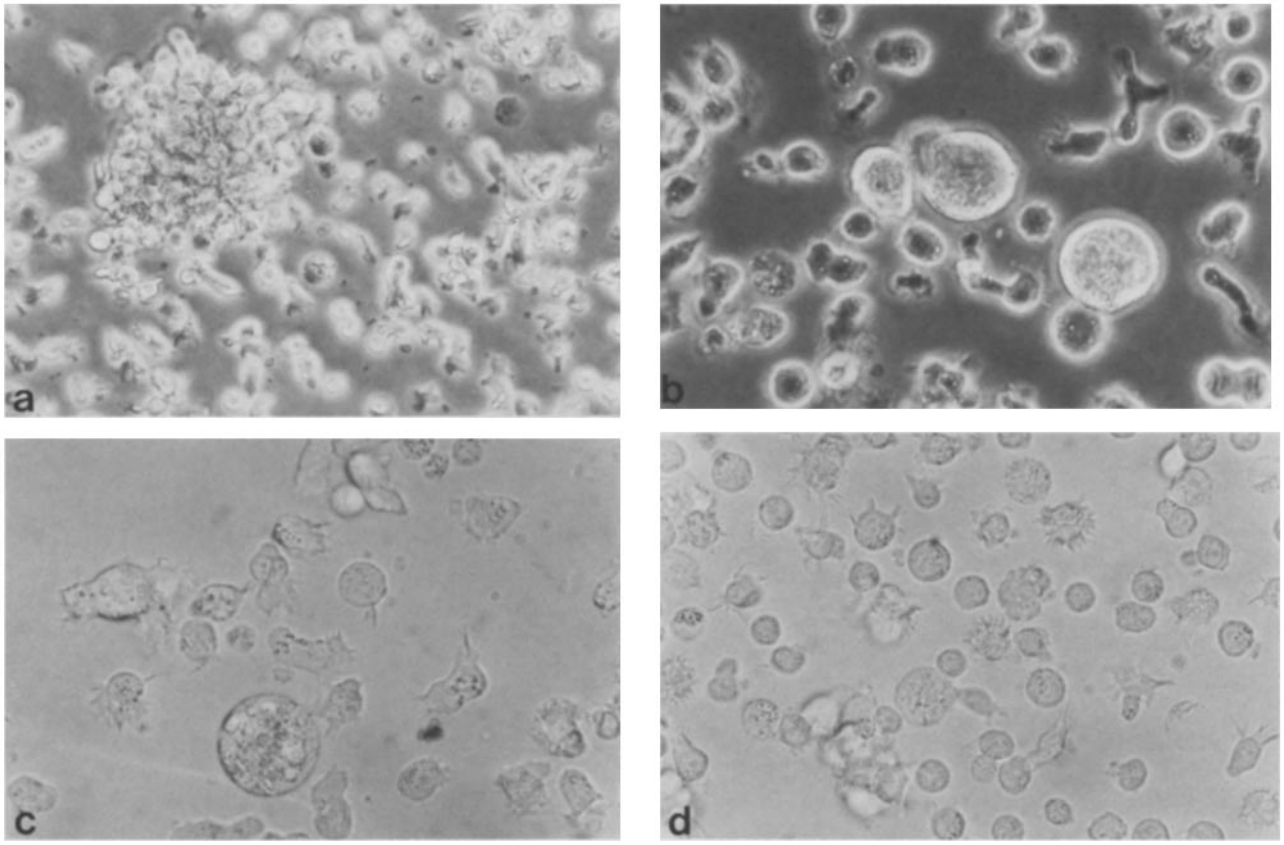

FIGURE 1 Viable cell morphology during culturing. (*a*) Characteristic cell aggregate seen in all these cell lines. × 120. (*b–d*) Cell size heterogeneity and cytoplasmic polymorphism as evidenced by pseudopodia and spicules. × 460.

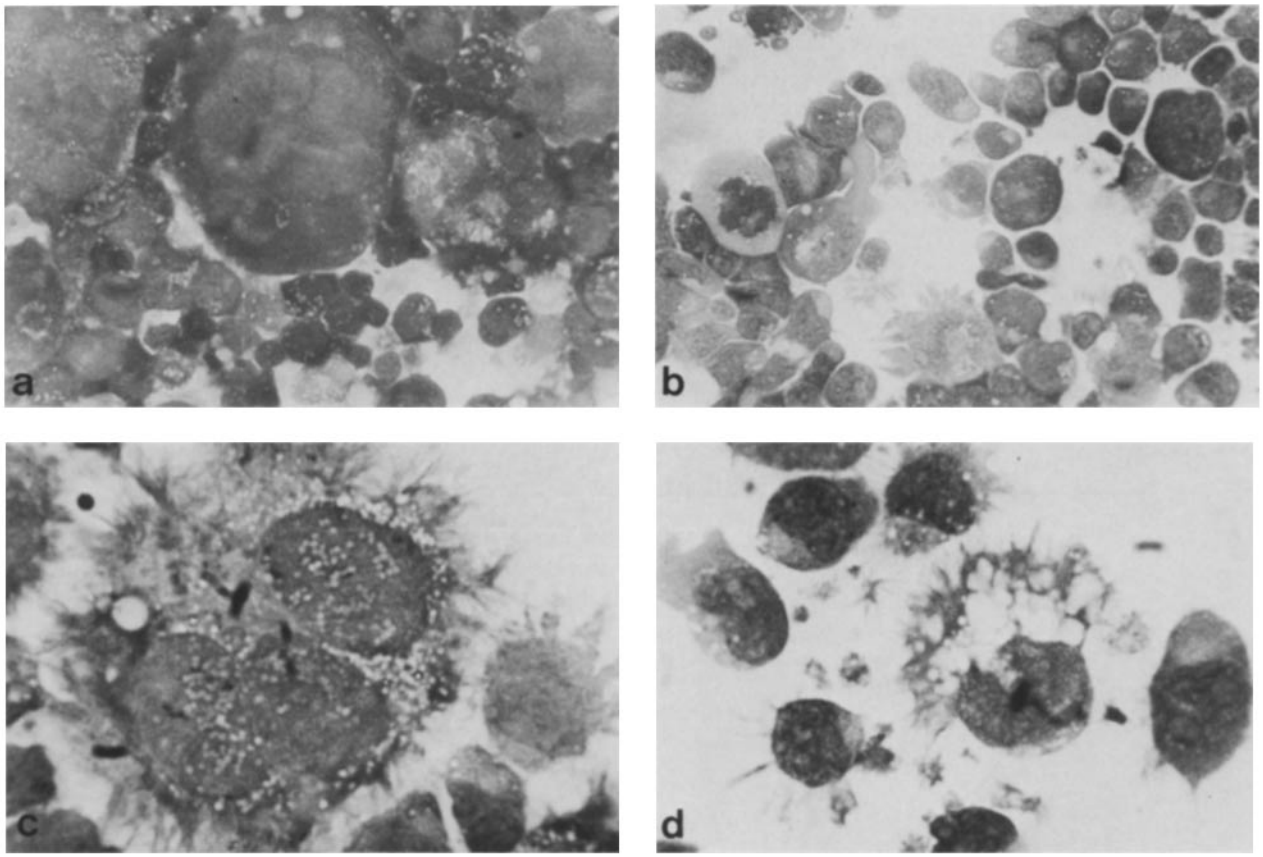

FIGURE 2 Cytocentrifuged cultured cells after staining with Wright-Giemsa. (a) Giant cells with multiple nuclei are in striking contrast to the dominant diploid cells. × 460. (*b*) Cytoplasmic shedding from small cells. × 460. (c) Multilobed cell and adjacent small cell with cytoplasmic spicules. × 1,150. (*d*) Cytoplasmic fragmentation and release of small extracellular particles. × 1,150.

cells. There was a high nucleus to cytoplasm ratio, a single nucleus containing prominent and sometimes multiple nucleoli, and the cytoplasm showed sparse organellar development with few small mitochondria, some strands of endoplasmic reticulum, and few free ribosomes. A Golgi system was present but was not well developed. One of the larger cells (two to three times the size of the small cell) (Fig. 3*a*) had multiple nuclei or lobes with nucleoli and had a centrally located large Golgi area. Granular endoplasmic reticulum and a moderate number of mitochondria and ribosomes were present. The cells showed extensive cytoplasmic projections. The Golgi area of this cell as seen at a higher magnification (Fig. 3*b*) was packed with vesicles and longitudinal sections of the Golgi system. In Fig. 3*c*, granular endoplasmic reticulum, free ribosomes, and polyribosomes were demonstrated in the cytoplasm of a large cell. Microfilaments were visualized in the cytoplasmic projections and extracellular particles with an apparent loss of ribosomes (Fig. 3*c*).

EBV and Histochemical Reactions

One of the first assays used to identify the lineage of the cell lines was the Epstein-Barr nuclear antigen (EBNA) (17) because the probability existed that EBV-positive peripheral blood B lymphocytes had spontaneously transformed into permanent cell lines. One cell line (M-408) had 70% cells positive for EBNA. However, the other cell lines were negative for EBNA after repeated testing. M-408 was the only cell line that did not require HCM and was also the only one positive for EBNA. Histochemical reactions were negative for leukocytic peroxidase and the chloroacetic esterase. α-Naphtyl acetate esterase was positive and was partially resistant to NaF inhibition. The periodic acid-Schiff staining pattern was variable, showing both a diffuse pattern as described for megakaryocytes (18) and a stippled, particulate reaction which was characteristic of blood platelets.

DNA Analysis

Morphological nuclear ploidy was verified by flow cytometry analysis of the cell lines that had been reacted with a DNA stain. S-1214 (Fig. 4) had a pattern of ploidy in which the 2N and 4N populations accounted for 95% of the total cells. Polyploidies of 8N and greater (4%) were characteristic of most of the cell lines.

Cytogenetic Analyses

All cell lines tested showed random aneuploidy and/or polyploidy. The polyploid cells were predominantly tetraploid cells. The preneoplastic patient (O-418) showed no structural abnormalities or marker chromosomes in the fresh peripheral blood and bone marrow. The chromosomal pattern after 12 and 42 culture passages (4- and 8-mo culture duration) remained essentially unchanged. The three cell lines derived from leukemia patients had structural and numerical abnormalities, some of which were present in the fresh blood/bone marrow preparations. In one of the cell lines (S-415), the pattern after 4 and 10 mo in continuous culture remained atypical. Extensive sequential cytogenetic analyses of all the

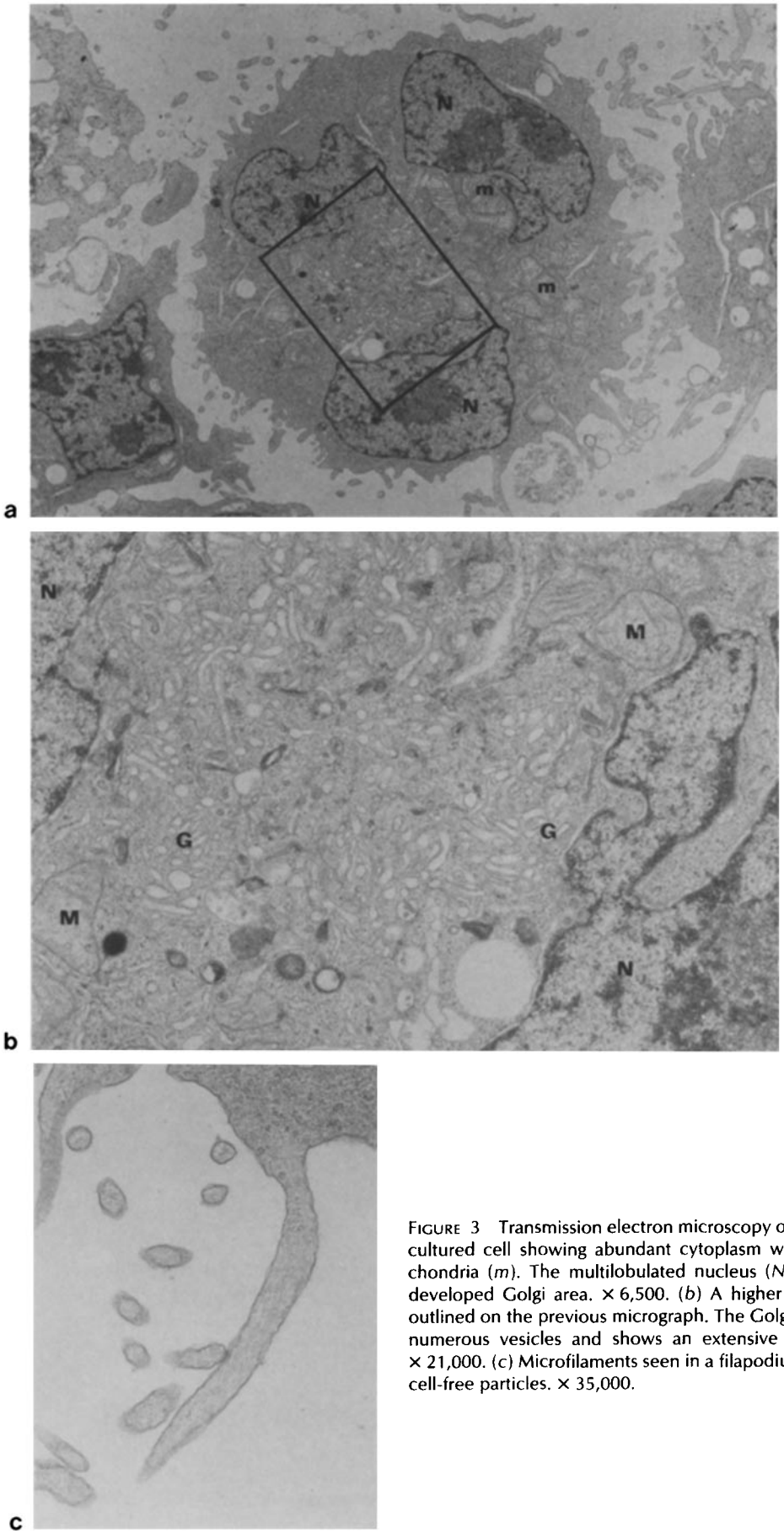

FIGURE 3 Transmission electron microscopy of the cell lines. (*a*) A cultured cell showing abundant cytoplasm with numerous mitochondria (*m*). The multilobulated nucleus (*N*) surrounds a well-developed Golgi area. × 6,500. (*b*) A higher view of the region outlined on the previous micrograph. The Golgi (*G*) is packed with numerous vesicles and shows an extensive membrane system. × 21,000. (*c*) Microfilaments seen in a filapodium and in numerous cell-free particles. × 35,000.

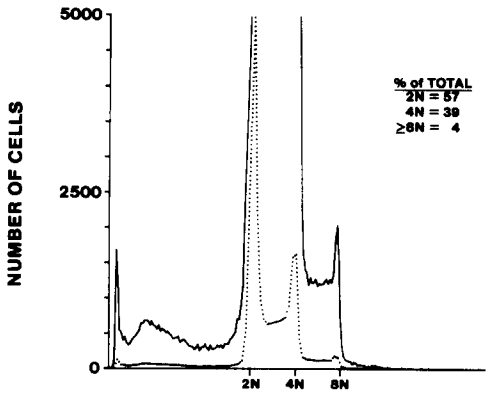

FIGURE 4 DNA distribution obtained by flow cytometry of unfixed culture cells stained with propidium iodide. The dotted curve represents data plotted at full scale for all cells measured (200,000 cells). The solid curve is a 16 × amplification of the dotted curve.

TABLE II

Indirect Immunofluorescence of Cell Lines Using Antibodies against Platelet-related Proteins

Platelet antigens detected by:	Membrane	Cytoplasm
	% cells	*% cells*
Monospecific antisera		
Fibrinogen	<10+	Negative
Fibronectin	Negative	<20+
B-Thromboglobulin	Negative	<10 weakly+
Factor VIII-Ag	<10+	<10 weakly+
PF4	>90+	10–80+
PDGF	>90+	10–20+
GpIIb	>80+	ND
GpIIIa	>80+	ND
Monoclonal antibody		
GpIIIa	>80+	ND

Membrane labeling was performed on cell suspensions and cytoplasmic reactivity was detected on fixed cells as described in Materials and Methods. Positive control cells were normal blood mononuclear preparations containing platelets, and negative control cells were REA—a pre-B lymphoblastic cell line established in this laboratory and grown in RPMI-10% HS. Bone marrow smears containing megakaryocytes and platelets were also a positive control for the cytoplasmic reactions. *ND*, Not determined.

cell lines are currently underway and will be reported in a subsequent publication.

Flow Cytometry

Surface reactivity of the cell lines as analyzed by flow cytometry was negative with standard monoclonal antibodies against membrane antigens found on human T lymphocytes (Leu-4, Leu-5), immature lymphocytes (cAlla), monocytes (Leu-M3), and B lymphocytes (light chains). The cell lines were positive for several membrane markers having no strict specificity for cell lineage. The transferrin receptor (OKT9), a marker associated with cell proliferation, was detected on 75% of the cells. HLA-Dr and B_2 microglobulin were on 95% of the cells. This pattern of reactivity has been constant after 1 yr in culture and over 50 passages.

Immunofluorescence by Fluorescent Microscopy

Cytoplasmic and cell surface reactivities that used antisera for numerous platelet-secreted proteins (Table II) showed weak expression of fibrinogen, fibronectin, β-thromboglobulin, and Factor VIII-Ag. Very strong membrane positivity for PF4, PDGF, and GpIIIa was found on >80% of the cells. GpIIb and GpIIIa were detected by monospecific antisera, and GpIIIa by monoclonal antibodies B59 and B2.12. The PF4 membrane reaction was positive on most of the viable cells (Fig. 5, *a* and *b*). A similar pattern was observed for PDGF. Cytoplasmic staining with PF4 (Fig. 5*c*) showed a diffuse pattern in the multilobed cell. Membrane spicules, as demonstrated by the application of PDGF antibodies to air-dried, methanol-fixed cells (Fig. 6, *a* and *b*), emphasized the cytoplasmic concentration at one end of the cell and the branching, very fine filopodia. Conventional cytoplasmic staining for PDGF (Fig. 6*c*) did not detect this phenomenon, but rather was suggestive of the gradual diffusion of the immunofluorescence-labeled protein into the nonreactive cytoplasmic extension.

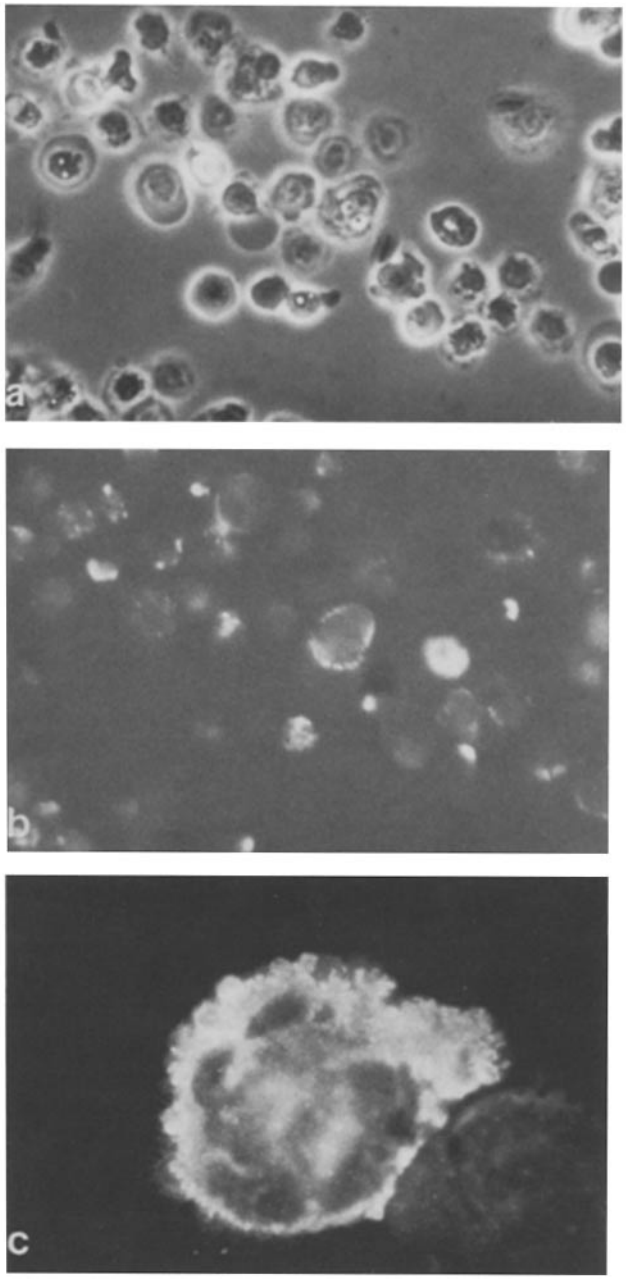

FIGURE 5 Cultured cells which exhibit a positive PF4 reaction by indirect immunofluorescence. (a) Phase-contrast microscope view of *b*. × 460. (c) A cell with diffuse cytoplasmic PF4 adjacent to a negative cell. × 1,150.

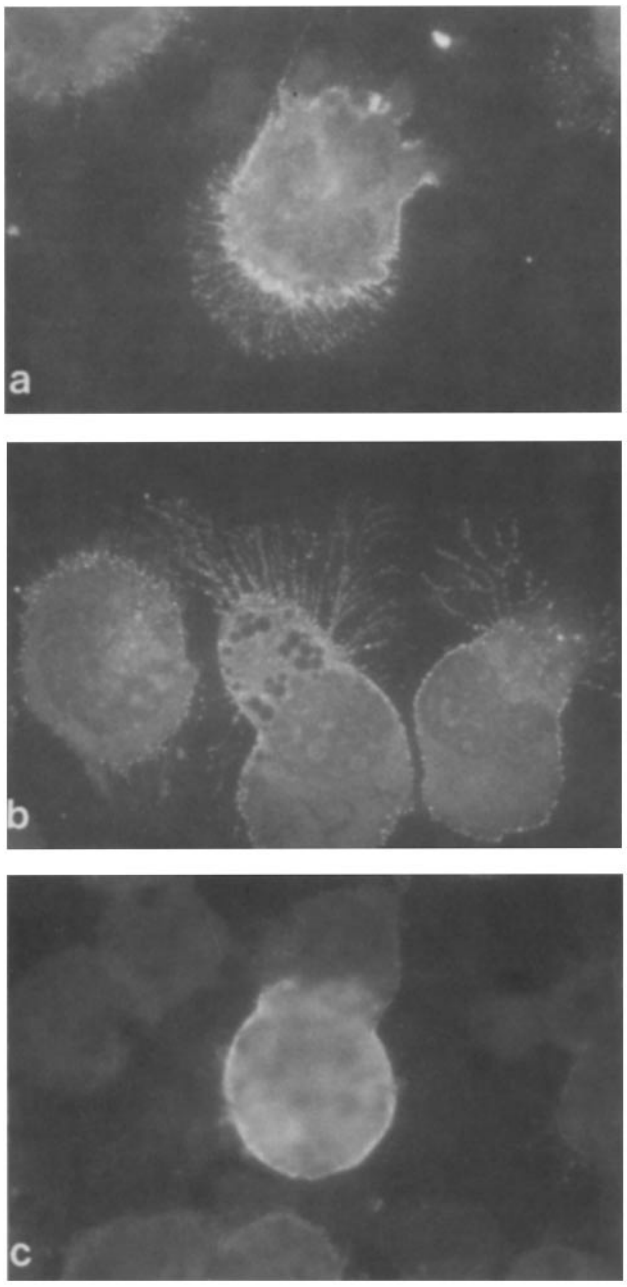

FIGURE 6 Indirect immunofluorescence pattern of PDGF reactivity. (*a* and *b*) Cells were fixed to detect membrane staining. × 1,150. (*c*) A cell with immunofluorescence-positive PDGF located in the cytoplasm. × 1,150.

HS and PPP Cultures

When cells cultured in RPMI-10% HS were compared with those grown in RPMI-10% PPP, the membrane PF4 and PDGF were not detected on the PPP-cultured cells (Table III). This led us to investigate the effects of culturing cells in RPMI-1% BSA to eliminate these membrane positivities and then to observe the effect of supplementing the serum-deprived cultures briefly with HS before harvesting for cell analysis. PDGF and PF4 positivities were significantly reduced or completely abolished in RPMI-1% BSA but were restored after a 10-min exposure to HS (Table III). The GpIIb and IIIa reactions were not affected by these culture conditions. Cell lines HEL, HL60, K562, and REA (a pre-B cell) were grown in RPMI-10% HS and were negative controls for the PF4 and PDGF markers.

DISCUSSION

Cell lines with properties of megakaryocytes and platelets have been obtained at an exceptionally high frequency from the peripheral blood of normal donors and patients with various blood diseases. Although we do not know the identity of the cell in the peripheral blood that gave rise to these cell lines, the circulating progenitor cell was among the mononuclear cells obtained from standard Ficoll gradients and put into culture. Traditionally, these preparations are considered to consist of T and B lymphocytes and monocytes. However, the presence of circulating stem cells (19) and the well-documented existence of megakaryocytes in the peripheral blood (20, 21) increase the differential potential of the morphologically homogenous population of small mononuclear cells in the Ficoll preparations. Exogenous growth factors and mitogens contained in the HCM supplement of the culture medium appear to have created a selective in vitro environment by stimulating directly or indirectly through accessory cells the cell line precursor. This stimulation or activation may, on occasion, occur spontaneously as seen with the cell line M-408 which had not received HCM. Early culture events were crucial to the success of the progenitor cell to survive and to become established. Apparently, an optimal microenvironment was created by the interactions among the mononuclear cells either as cell-to-cell contacts or by the production of regulatory factors. Any interference with these primary events was purposely avoided. Cells were not fed with either fresh nutrient medium or HCM after culture initiation but were allowed to remain in the original flask until either cell line emergence or culture failure was noted. Our "hands off" approach to these cultures may well have been the reason for their success. This requirement for a specific cellular microenvironment may account for the paradox in our obtaining cell lines from peripheral blood with no apparent proliferative activity of the lymphocytes, monocytes, and unidentified precursor cells. In this study, most cell preparations obtained from leukemic donors failed to yield cell lines. This might be related to a proliferating population that rapidly utilized necessary regulatory or stimulatory factors which then were not available to the megakaryocyte progenitor. These leukemic cell preparations were usually deficient in accessory cells such

TABLE III

Various Culture Conditions Distinguish between Membrane Affinity for Serum Platelet Proteins and Stable Membrane Markers

	Platelet proteins		
Culture conditions	PDGF	PF4	GpIIIa
Continuous			
RPMI-10% HS	>90%	>90%	>80%
7 d			
RPMI-10% PPP	Negative	Negative	>80%
24 h			
RPMI-1% BSA	Negative	Negative	ND
RPMI-1% BSA + HS*	>80%	>90%	ND

Indirect immunofluorescence was done on unfixed cells obtained from cultures that had been deprived of human serum by cell washing and replacement with RPMI-10% PPP or RPMI-1% BSA. *ND*, Not determined.
* Cells grown in RPMI-10% BSA for 24 h were incubated for 10 min after the addition of 10% HS and before harvesting for immunofluorescence analyses.

as T and B lymphocytes and monocytes. Furthermore, the source of cells for our in vitro studies was peripheral blood and not bone marrow. As with the leukemic cell preparations, bone marrow contains a high proportion of immature proliferating cells and a small number of accessory T lymphocytes.

The distinctive morphology and unusual behavior in culture were the first indication these cell lines may be of megakaryocytic origin. Human cell lines of such giant cells, progressive polyploidy and characteristic cytoplasmic projections with shedding have not been previously described. Furthermore, the new cell lines did not express a phenotype consistent with or specific for any other known hemopoietic cell lineage other than megakaryocytes. The histochemistry profile consisted of α-naphthyl acetate positivity partially resistant to NaF and is associated only with megakaryocytes and platelets. Monocytes are α-naphthyl acetate positive but sensitive to NaF. The negative chloroacetate esterase and leukocyte peroxidase reactions were not consistent with a committed myeloid origin of the cell lines. Flow cytometry did not detect lymphocyte or monocyte membrane markers, but did show that the cells were expressing HLA-Dr, B_2 microglobulin, and the transferrin antigens. These antigens are not specific for any one cell lineage and therefore are not sufficient for identification of these cells as megakaryocytes.

Mature megakaryocyte identification is classically dependent upon the distinctive ultrastructure of alpha granules and demarcation membranes as well as the presence of platelet secretory proteins and platelet membrane glycoproteins. The cell lines did not show late differentiation morphology or alpha granule formation as seen by transmission electron microscopy. Apparent maturation of the primitive cells containing sparse organelles did occur, as evidenced by large cells having acquired morphology consistent with differentiation, namely, an extensive membrane system and cytoplasmic projections containing microfilaments. The identifying markers of the cell lines were the platelet-associated proteins PF4 and PDGF and platelet membrane glycoproteins IIb and IIIa. The cells were synthesizing PDGF as determined by the in vivo protein labeling with subsequent identification of labeled PDGF by immunoprecipitation (22). This correlates with the intracellular PDGF seen by immunofluorescence. Cytoplasmic PF4 was also detected by immunofluorescence. Therefore, PDGF and PF4 are early synthesized proteins that are detectable before the formation of alpha granules where these two proteins are stored in platelets.

The role of PF4 in such a primitive cell is of interest. The function of PF4 in the platelet is to bind heparin and neutralize the anticoagulant activity of heparin (23). However, it is doubtful this antiheparin activity of PF4 is of physiological importance due to low concentrations of heparin in the blood (23). It has been suggested that PF4 may be involved in regulation of cell growth by reacting with heparin sulfate which occurs on the membrane of many cells (24). Perhaps the primary function of the early synthesized PF4 and PDGF is to regulate growth and differentiation of the primitive megakaryoblast.

PDGF is a growth factor for connective tissue cells. There is speculation that some connective tissue neoplasms result from the synthesis and secretion of PDGF by cells that are normally responsive to PDGF through specific receptors (25). This "paracrine" production of PDGF could generate a self-sustaining, automitogenic cell mass (25). This scheme would give partial explanation for the phenomenon of obtaining a putative primitive megakaryoblast cell line from normal donors. The megakaryocyte is the prime candidate among the other possible blood cell lineages that would normally have the capacity to be self-sustaining through such a mechanism.

The establishment of megakaryocytic cell lines that are easily manipulated as suspension cultures would have important implications in regard to the mechanism of megakaryocytic differentiation and ultimately platelet formation. Unlike most cell lines that have been immortalized to express a single function and phenotype, these cell lines spontaneously show the capacity for maturation. The use of inducers of cell differentiation may potentially offer one the opportunity to study the regulatory mechanisms that are involved in the maturation of diploid cells to platelet-forming megakaryocytes. Furthermore, these cell lines may provide important insight into the role of the megakaryocyte as a possible reservoir of retroviral activity (26, 27). This concept has become particularly pertinent in view of the recent demonstration that the simian sarcoma virus *onc* gene, v-sis, is derived from the gene (or genes) encoding a platelet-derived growth factor (28, 29).

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