INDUCTION OF IN VITRO PROLIFERATION
AND MATURATION OF HUMAN ANEUPLOID
MYELOGENOUS LEUKEMIC CELLS*

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Maturation and development of mammalian hematopoietic cells are influenced by a variety of chemicals and physiological mediators. These influences can be studied by culturing immature hematopoietic cells in vitro and observing the induction of mature cellular characteristics. Culture medium conditioned with activated lymphocytes, prepared by incubating lymphocytes with mitogen or allogeneic antigens, has been analyzed as a source of lymphocyte mediators for cell differentiation. This conditioned medium induced the development of normal mouse prothymocytes into mature T cells (1), human B lymphocytes into plasma cells (2), and maturation of erythrocytes (5). It also has activity for induction of differentiation of malignant hematopoietic cells. Fu et al. (3–4) showed T lymphocyte-mediated development of plasma cells from B lymphocytes from chronic lymphocytic leukemia (CLL)[1] patients, and Chiao et al. (6) showed the development of pre-T lymphocytes from acute lymphocytic leukemia (ALL) patients into mature T cells. We recently demonstrated that activated T lymphocyte-conditioned medium can induce maturation of the human promyelocytic leukemic HL-60 cell line along the monocytic cell pathway (7). We now report that supplementation with lymphocyte-conditioned medium can induce terminal maturation of aneuploid leukemic cells from patients with acute myelogenous leukemia. Cell cycle analysis has demonstrated the relationship of cellular proliferation to induced cellular maturation.

Materials and Methods

Patient Identification. Patient R.I., a 21-yr-old male, had acute myelogenous leukemia. The leukocyte count was $10,900/mm^3$; hemoglobin, 11.9 g/deciliter; and platelets, $47,000/mm^3$. There were 60% monoblasts, 6% myelocytes, 5% metamyelocytes, 26% lymphocytes, and 3% monocytes, and a bone marrow examination was consistent with a diagnosis of M5B according to French, American, British (FAB) classification. He received daunorubicin, cytosine arabinoside, and thioguanine, without achieving a remission, failed to respond to further chemotherapy, and died 6 mo later.

Patient A.N. was a 20-yr-old female, who presented with a platelet count of $40,000/mm^3$,

and she was treated with prednisone for presumed idiopathic thrombocytopenic purpura, without response. Subsequently, a bone marrow aspiration established the diagnosis of acute nonlymphocytic leukemia (M2). The leukocyte differential count included 1% nucleated erythrocytes, 71% blasts, 10% neutrophils, 1% bands, 15% lymphocytes, and 2% monocytes. She was treated with adriamycin and cytosine arabinoside and then daunorubicin in combination with cytosine arabinoside and thioguanine, and achieved remission 6 mo later. She relapsed after 3 mo, failed further therapy, and died 3 mo later.

Patient M.B. was a 75-yr-old male who presented with a leukocyte count of 5,000/mm^3; hemoglobin, 10.6 g/deciliter; and platelets, 55,000/mm^3. A bone marrow aspiration was hypercellular, with 10% erythroid precursors, 15% blasts, 23% promyelocytes, 18% myelocytes, 17% polymorphonuclear leukocytes, 16% monocytes, and 1% lymphocytes, consistent with an accelerated phase of chronic myelogenous leukemia. Cytogenic study revealed normal karyotype without evidence of the Philadelphia chromosome. His leukocyte count subsequently rose to >400,000, with 1% nucleated erythrocytes, 66% blasts, 10% promyelocytes, 4% myelocytes, 8% bands, 1% eosinophils, and 10% neutrophils. The morphology according to FAB was M2. The patient was treated with a continuous infusion of cytosine arabinoside without response. He developed pulmonary insufficiency, renal failure, and died 1 mo later.

Membrane Marker Determinations. Spontaneous rosetting of T lymphocytes with sheep erythrocytes (SRBC) and detection of surface membrane immunoglobulins by immunofluorescence using rabbit IgG-F(ab')$_2$ reagent were performed as described (6, 8). Membrane receptors for complement components were detected by a rosette method, using indicator erythrocytes coated with rabbit IgM antibodies and sensitized with mouse serum as a source of complement (6). Phagocytosis was studied by incubating cells with 0.8-μm latex particles (7). Cells engulfing many particles were scored as positive.

Isolation of Immature Leukemic Cells. Peripheral blood or bone marrow leukocytes were isolated from heparinized samples from leukemic patients by Ficoll-Hypaque gradient centrifugation (9). Immature cell-enriched preparations were obtained by first removing adherent and phagocytic cells using the carbonyl iron method (10). Cells bearing receptors for complement or IgG and T cells were removed by centrifugation after the formation of respective rosettes (6). Cells attached to the plastic or glass surface after vigorous pipetting were reported as adherent cells. To collect adherent cells, cold 1X trypsin-EDTA (Flow Laboratories, Inc., Rockville, MD) was added to flasks and incubated at 4°C for 3-30 min. Cells were collected after pipetting and washed with 20% fetal calf serum (FCS) supplemented RPMI 1640 medium.

Leukemic Cell Cultrues and Conditioned Media. Immature cell preparations were cultured at 0.25-0.5 × 10^6 cells/ml in RPMI 1640 medium supplemented with 2 mM of glutamine, 1% antibiotics, and 15% heated FCS. Lymphocyte conditioned medium (CM) or control medium was added at a final concentration of 30%. CM was prepared from normal human peripheral blood lymphocytes (PBL) using the method described elsewhere (6, 7). PBL from multiple donors were cultured at 1 × 10^6 cells/ml in RPMI 1640 medium with 1% phytohemagglutinin M (PHA) (Gibco, Grand Island Biological Co., Grand Island, NY) and 1-5% autologous plasma. The culture supernatants from 3-d cultures were used as unseparated lymphocyte-conditioned medium. Control medium was prepared by adding 1% PHA to PBL from a single donor that had already been incubated for 3 d.

The cultures were maintained at 37°C in a 5% CO$_2$ humidified atmosphere, and the medium was changed every 2-4 d after the initiation of cultures. Cell viability was determined by the trypan blue exclusion method. To test cell markers and functions, cultured cells were washed with 4°C RPMI 1640 medium, incubated in RPMI 1640 at 37°C for 1 h, and washed again with 37°C RPMI 1640. To determine the cell type providing the activity in unseparated CM, CM were also prepared from isolated SRBC rosetting T lymphocytes, T lymphocyte-depleted PBL preparations, and monocyte-depleted PBL preparations. Monocytes were removed by the carbonyl iron method (10). Myeloperoxidase staining (7) showed that these preparations and the T lymphocyte preparations had <0.1% monocytes. Each CM was prepared using the same culture conditions described for unseparated CM.

Cell Morphology. Smears of cultured lymphocytes were made using a Shandon Elliott cytospin centrifuge (Shandon Southern Instruments Inc., Sewickley, PA). Cell morphology was examined with May-Grunwald Giemsa stain. Staining for nonspecific esterase using α-napthyl

acetate or naphthol ASD chloroacetate was carried out according to the method of Yam et al. (12). Morphology of adherent cells was examined after their adherence to a cover glass placed in the culture containers.

Flow Cytometry of DNA and RNA.   The cells were stained for DNA and RNA with acridine orange according to the method described by Darzynkiewicz et al. (13) and Traganos et al. (14). This staining procedure has been shown to be stoichiometric for DNA and RNA (15). Double-stranded RNA is denatured in the presence of EDTA. Native double helical DNA intercalates the dye and fluoresces orthochromatically green. Single-stranded RNA forms a different complex with acridine orange and fluoresces red (16). The DNA and RNA measurements permit identification of live and dead cells, and the number of cells in $G_{0/1}$, S, and G_{2M} phases of cell cycle. The RNA content of cells in $G_{0/1}$ is expressed as RNA index (RI), which is the mean RNA content of $G_{0/1}$ cells of the sample times 10, divided by the median RNA content of control G_0 human lymphocytes (15). To determine DNA stemlines, the ratio of DNA $G_{0/1}$ leukemic cells to DNA of G_0 normal lymphocytes used as a control is calculated.

A computer-interfaced research cytofluorograph (model FC 200; Ortho Instruments, Westwood, MA) was used to obtain simultaneous measurements of the fluoresence of individual cells in two separate wavelength bands (F_{530}, in a band of 515–575 nm; and F_{600}, in a band from 600–650 nm) (15). Green pulse-width, i.e., the time the nucleus took to pass through the illuminating beam, was also recorded and used to distinguish single cells from cell doublets and other cell aggregates (15). The three cell features were measured per sample and stored as correlated measurements in a Nova 1220 minicomputer (Data General, Southwood, MA) and on magnetic disks. Data analysis was performed with a Tektronix 4010-1 graphic terminal (Tektronix, Beaverton, OR) using computer programs developed by T. Sharpless (16).

Autoradiography.   Cell labeling and autoradiography were performed according to the method of Nishikori et al. (17). Cells in FCS-supplemented medium were labeled with 1 Ci/ml tritiated thymidine (15 Ci/mM, New England Nuclear, Boston, MA) at 37°C for 1 h. Autoradiographs were developed from cytocentrifuge-prepared slides using Kodak NTB emulsion (Eastman Kodak Co., Rochester, NY) and a 7-d exposure time.

Results

CM-induced Development of Adherent Cells.   Table I shows that the isolated cell preparations from leukemic patients lacked SRBC rosetting capacity, membrane immunoglobulins, IgG or complement receptors, and phagocytic capacity. These preparations were enriched with blasts and promyelocytes. Adherent cells developed 2–24 h after incubation with CM and were one of the earliest indications of CM-induced maturation. Cultures with control medium or lacking CM showed either very few or no adherent cells. The majority of these adherent cells were macrophage-like

TABLE I

Membrane Markers and Characteristics of Isolated Immature Cells from Myelogenous Leukemia Patients

Patient	Diagnosis	FAB morphology	Cell source	Cell preparation	Blast	Membrane markers and characteristics (%)					
						SRBC rosette	IgG receptors	Complement receptors	Membrane immunoglobins	Phagocytic cells	Adherent cells
					%						
M.B.	Myeloproliferative disorder with a blast crisis	M2	Marrow	Unseparated	29	3	1	23	1	10	Present
				Isolated	62	0	2	0	0	0	Absent
A.N.	AML	M2	Blood	Unseparated	62	1.5	1	10.5	0.2	10	Present
				Isolated	85	0	4	0	0	0	Absent
R.J.	ANNL	M5B	Marrow	Unseparated	65	7	0	8	1	11	Present
				Isolated	93	0.5	0	0	0	0	Absent

TABLE II

CM-induced Development of Adherent Macrophage-like Cells and Their Properties

Patient	Culture period	Differential counts of adherent cells				Cellular markers	
		Blast	Promye-locyte	Mono-cyte-like	Macro-phage-like	Comple-ment re-ceptors	Phago-cytic cells
	d		%			%	
M.B.	2	2	12	0	86	38	40
	5	0	3	10	87	58	62
A.N.	1	3	15	15	67	42	45
	5	0	5	10	85	48	48
R.I.	2	4	6	8	82	40	65
	4	0	2	5	93	62	78

TABLE III

Differential Count Analysis and the Detection of Monocytes and Macrophage-like Cells in Culture Suspensions of CM-supplemented Cultures of Leukemic Cells

Patient and FAB	Culture period	Culture condition	Blast	Promye-locyte	Myelo-cyte	Granulo-cyte	Lympho-cyte	Mono-cyte-like	Macro-phage-like	Comple-ment re-ceptor-bearing cells	Phago-cytic cells
	d										
M.B.	0	(Before culture)	62	30	5	2	0	1	0	0	0
(M2)	1	+CM	60	38	0	1	0	0	1		
		+Control medium	58	39	0	3	0	0	0		
	2	+CM	27	44	0	0	0	7	22	20	22
		+Control medium	58	38	0	3	0	0	1	0	1
	4	+CM	44	30	0	0	0	11	15	25	28
		+Control medium	50	39	4	7	0	0	0	0	0
A.N.	0	(Before culture)	98	2	0	0	0	0	0	0	0
(M2)	2	+CM	48	39	0	0	0	0	13	14	10
		+Control medium	45	50	3	1	1	0	0	1	1
	4	+CM	0	0	0	0	0	44	56	43	68
		+Control medium	40	48	2	1	1	4	4	1	1
R.I.	0	(Before culture)	89	1	0	6	4	0	0	0	0
(M5B)	2	+CM	65	15	0	6	1	3	15	0	0
		+Control medium	70	18	0	17	1	0	0	0	0
	4	+CM	2	0	0	0	0	36	62	35	60
		+Control medium	43	45	0	7	0	0	5	1	0

after 5 d in culture (Table II). They were positive for α-napthyl esterase and negative for naphthol ASD chloroacetate stains, indicating that they were monocytic cells. Table II also shows that cells with membrane complement receptors and phagocytic capacity were detected among the adherent cells. The sum of the percentages of macrophage-like cells and cells bearing complement receptors exceeded 100%, suggesting that macrophage-like cells possessed the receptors. By the same analysis, it can be seen that these cells also had phagocytic capacity.

Changes in Cell Morphology and Function in Culture Suspensions. Cells in culture suspensions underwent morphological changes, and some cell aggregates were detected. Table III shows 29%, 13%, and 18% of the cells in CM cultures of M.B., A.N., and R.I. were monocyte- and macrophage-like after 2 d, compared with 0% in the control cultures and initial culture preparations. As the proportion of monocyte- and macrophage-like cells increased with time, the proportion of immature cells decreased.
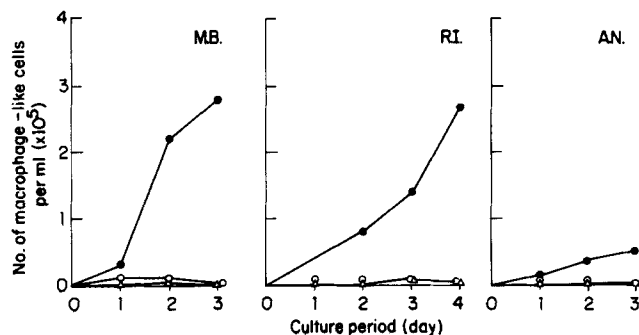
Fig. 1. Increase of absolute number of macrophage-like cells in CM-supplemented cultures of leukemic cells of patients M.B., R.I., and A.N. ● indicates the number of macrophage-like cells per ml in CM-supplemented cultures; ○ indicates the number of macrophage-like cells per ml in control cultures lacking CM; △ indicates the number of macrophage-like cells per ml in cultures supplemented with a control medium prepared by adding 1% PHA to PBL from a single donor that had been incubated for 3 d.

CM cultures of A.N. and R.I., which initially had 98% and 89% blasts, had 0% and 2% of these cells and 100% and 98% of mature cells by day 4.

Control cultures of M.B., A.N., and R.I. were terminated on days 8, 6, and 5, because of cell death. The CM-supplemented M.B., A.N., and R.I. cultures were maintained for 35, 9, and 11 d.

The absolute number of monocyte- and macrophage-like cells was determined by multiplying cell type proportions by cell number per unit volume. Fig. 1 depicts the increase in absolute number of macrophage-like cells in CM cultures of M.B., A.N., and R.I. from initiation of the cultures to the first medium change. The number of macrophage-like cells increased from 0 to ~2.7 × 10^5 cells/ml in the CM culture of M.B. by day 3. No macrophage-like cells were detected in control cultures. The total viable cell number in CM cultures increased from 0.5 × 10^6 cells/ml on day 1 to 1.2 × 10^6 cells/ml on day 4. In cultures lacking CM, the cell number increased from 0.5 × 10^6 cells/ml on day 1 to 1.1 × 10^6 on day 3, and decreased to 0.8 × 10^6 on day 4. Fig. 1 shows that the increase of macrophage-like cells was similar in A.N. and R.I. cultures. The development of mature cells and increase in number of these cells in CM cultures strongly indicates lymphocyte CM-induced development from immature cells.

The development of monocyte- and macrophage-like cells in CM cultures was coupled with reactivity with α-napthyl acetate, which is characteristic of monocytic cells, and a decrease in the number of cells with the myeloid esterase marker, naphthol ASD chloroacetate. In contrast, the number of cells with myeloid markers in control cultures remained high. These observations suggest that CM induces cellular development along the monocytic cell pathway.

The proportion of cells with complement receptors and phagocytic capacity increased in CM cultures. Approximately 25%, 43%, and 35% of the cells in M.B., A.N., and R.I. cultures showed complement receptors by day 4 (Table III). Complement receptor cells, detected around day 2 to 3, appeared later than adherent cells. Smears of cells rosetting with complement receptor indicator erythrocytes were prepared. It was determined that they were monocyte- or macrophage-like cells. Table III also
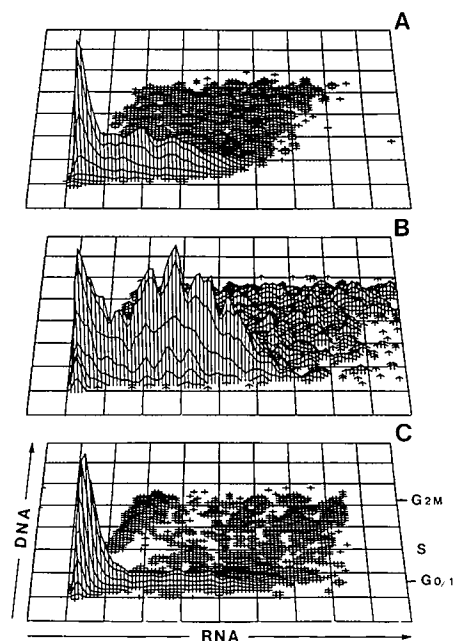
Fig. 2.   DNA-RNA histograms of flow cytometric measurements of leukemic cells of patient M.B. after 2 d in culture. Frame B shows CM cultures had a higher proportion of cells in S phase than did cultures supplemented with control medium in frame A. Most of the adherent cells obtained from CM cultures (frame C) had low RNA content, and the proportion of these cells with low RNA was greater than that of the cells in culture suspensions (frame B) or the cells supplemented with control medium (frame A). These measurements show that the adherent cell population in frame C had the lowest proportion of S phase cells.

TABLE IV

Flow Cytometric Measurement of Aneuploid DNA Content, Reduced RNA Content, and Cell Cycle Phases of CM-induced Mature Cells

Patient	Culture period	Culture condition	RNA index (R.I.)	DNA content	Cell cycle phases		
					$G_{0/1}$	S	G_2M
	d			C		%	
M.B.	0	(Marrow biopsy)	17.3	2.4	76	19	5
	2	With CM	24.9	2.4	59	31	10
		With control medium	18.1	2.4	75	18	7
		Adherent cells	12.8	2.4	82	9	9
	6	With CM	9.9	2.4	69	24	7
		With control medium	22.5	2.4	86	11	3
R.I.	0	(Marrow biopsy)	21.3	2.2	91	8	1
	2	With CM	13.5	2.2	78	16	6
		With control medium	18.7	2.2	92	5	3
	5	With CM	13.5	2.2	84	10	6
		With control medium	(Cells had died)				

shows that 28%, 68%, and 60% of the cells in M.B., A.N., and R.I. cultures were phagocytic by day 4. No increase of complement receptors or phagocytic cells was seen in cultures with control medium or lacking CM.

Reduction of RNA Content and Aneuploid DNA Content.   Flow cytometric measurement of cells during the maturation induction period showed an appearance of cell populations having lower cellular RNA content than the cells not treated with CM. Early changes in RNA content in M.B. cells are shown in the histogram of Fig. 2, and quantitative data are presented in Table IV. After 2 d in culture, cell suspensions of CM cultures had a higher proportion of cells with higher RNA content (R.I. 24.9, Table IV) than was found in cultures lacking CM (R.I. 18.1). This might be because of the presence in the CM cultures of a higher proportion of proliferating immature cells in S phase (31%) than in cultures lacking CM (18%). Adherent cells in CM cultures had a significantly lower RNA content (R.I. 12.8) than did the cells in culture suspensions (R.I. 24.9). Approximately 75% of the $G_{0/1}$ adherent cells had low RNA content. Because the sum of the proportion of low RNA adherent cells (75%) and the proportion of adherent cells with macrophage-like morphology (86%, Table I) was >100%, this would indicate that the macrophage-like cells had low RNA content. These adherent cell populations showed cells in the cell cycle, as indicated by cells in S and G_2M with a low RNA content, fulfilling the criteria for viable cells because the cells maintained their capacity to be stained with acridine orange. Dead and dying cells regularly lose DNA or the capacity of the DNA to be stained, which results in lower green fluorescence.

Analysis of fresh and cultured cells by flow cytometry from patients M.B., A.N., and R.I. revealed hyperdiploid DNA stemlines. Fig. 3 shows that the majority of M.B. cells had 2.4 DNA content (C). Most of these cells had a large $G_{0/1}$ RNA content. Similar analysis revealed that almost all cells from R.I. and A.N. were 2.2 C hyperdiploid. Cultured cells from these leukemic patients were also measured for DNA content. Table IV shows that the same hyperdiploid stemline was detected in these cells as was seen in the fresh patient cells. Table IV shows further that almost all the adherent cells from CM-induced M.B. cultures were 2.4 C, as were the undiffer-
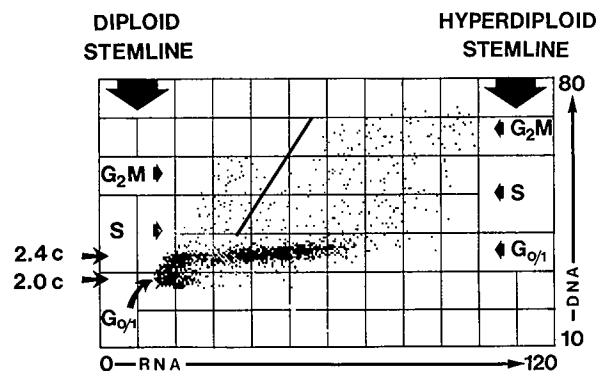

FIG. 3. Scatterplot of simultaneous flow cytometric measurements of DNA and RNA showing hyperdiploid DNA content of patient mononuclear cells before culturing. Cells with diploid DNA content (2.0 C) proliferating from $G_{0/1}$ to S and G_2M phases of the cell cyle are shown on the left side of the plot. Almost all MB cells were found in the 2.4 C hyperdiploid position and most of them were in $G_{0/1}$ phase with high RNA content. These aneuploid cells in $G_{0/1}$, S, and G_2M are shown on the right side of the plot, separated from the diploid cells by an oblique line. Each dot on the scatterplot represents the electronic signal of the red RNA and green DNA fluorescence of a single cell passing through the laser beam during measurement. The details of the DNA and RNA staining and fluorescence are described in the Materials and Methods section.

Table V

Inductive Activity for the Maturation of Leukemia Cells Using CM Prepared from T Lymphocytes

	PHA-stimulated allogeneic lymphocytes (unseparated PBL)	PHA-stimulated allogeneic T lymphocytes	PHA-stimulated allogeneic lymphocyte preparations of monocytes	PHA-stimulated allogeneic non-T lymphocyte preparations	Medium conditioned with unseparated PBL from a single donor with 1% PHA added at end of culture
Day 2, R.I. culture					
Adherent cells	+	+	+	−	−
Morphology (%)					
Blast and promyelocyte	80	85	83	90	88
Monocyte and macrophage-like	18	12	14	0	0
Intermediate*	2	3	3	10	12
Day 4, R.I. culture					
Adherent cells	+	+	+	−	−
Morphology (%)					
Blast and promyelocyte	2	15	15	85	88
Monocyte and macrophage-like	98	85	85	2	5
Intermediate*	0	0	0	13	7
Complement receptors	65	48	53	1	1
Day 4, M.B. culture	+	+	+	−	−
Adherent cells					
Morphology (%)					
Blast and promyelocyte	74	63	66	93	89
Monocyte and macrophage-like	26	33	34	3	0
Intermediate*	0	4	0	4	11
Complement receptors (%)	25	28	28	1	0
Day 6, M.B. culture					
Adherent cells	+	+	+	−	−
Morphology (%)					
Blast and promyelocyte	70	75	74	94	94
Monocyte and macrophage-like	30	25	26	2	0
Intermediate*	0	0	0	4	6
Complement receptors	27	20	19	0	0

* Mixture of myelocytes, granulocytes, and lymphocytes.

entiated cells. Because morphological and marker analysis of these adherent cells showed that ~86% of the cells were macrophage-like, this would indicate that these macrophage-like cells were hyperdiploid. A similar conclusion could be derived from the analysis of R.I. and A.N. cells. Nearly all of these cells cultured with CM for 4–5 d maintained their 2.2 C DNA stemline, while morphologically they were monocyte- and macrophage-like and acquired mature characteristics. These results indicate that CM-induced maturation of leukemic cells to monocyte- and macrophage-like cells may be independent of the DNA abnormality.

Cellular Proliferation. Cultures supplemented with CM showed a larger proportion of proliferating cells than cultures lacking CM. Table IV depicts CM-stimulated cell proliferation as shown by cell cycle phases. 2-d M.B. cultures supplemented with CM had 31% cells in the replicating S phase, an increase from 19% in the initial preparations. This indicates that the stimulatory activity for proliferation was not due to the presence of PHA, but was most likely attributable to activated lymphocyte products. On day 6, the proportion of cells in S phase in CM cultures was 24% and remained higher than the control cultures. A decrease of cells in S phase in control cultures was reflected in a steady decline in cell viability and cell counts beginning from day 3, resulting in their termination by day 8. CM cultures had a cell viability >94% and were continued for 35 d. At this point, almost all cells were macrophage-like, and cell proliferation had ceased. During the culture period, adherent cells developed continuously. This was shown by removing cells in suspensions and transferring them to a new flask. Development of macrophage-like adherent cells always ensued. From days 7 to 20, ~24% of the cells were in S phase, while ~20% of the cells in culture suspensions were monocyte- and macrophage-like, and 80% were blast and promyelocyte-like cells. Analysis of autoradiographs of cellular incorporation of [³H]thymidine after 12 d in culture showed ~20% radiolabeled blast-like cells and no labeled macrophage-like cells, excluding proliferation as their source. These observations indicate that CM supports proliferation of the immature cells, and the differentiated mature cells developed after cessation of cell replication. Proliferation and maturation of immature cells were concurrent events in the CM cultures.

From days 20 to 30, proportions of blasts and promyelocytes decreased, while monocyte- and macrophage-like cells increased, leading to a predominance of macrophage-like cells. Cell counts and viability decreased, and cell proliferation ceased. CM-induced proliferation was also seen in A.N. and R.I. cultures (Table V).

T Lymphocytes as Source of Maturation Inducer. To determine the cell types involved in producing the maturation activity in unseparated CM, conditioned media from different cell types were prepared. The CM were tested with leukemic cells for the development of adherent cells, morphological changes, and increase in cells with complement receptors. Table V shows that, whereas T lymphocyte CM and monocyte-depleted lymphocyte CM induced maturation, non-T lymphocyte CM and CM from unstimulated lymphocytes did not. These results suggest that T lymphocytes are the cell type most responsible for production of the maturation activity in CM and that the inducer stems from activated T lymphocytes.

Discussion

In this paper, we report induced proliferation and maturation by an activated T lymphocyte product in primary cultures of human leukemic cells. We cultured immature leukemic cells isolated from patient leukocyte preparations after the depletion of cells showing mature cellular functions and membrane characteristics. The use of these immature cells provided a distinct advantage because the presence of mature cells in culture could be attributed to induction.

The development of mature cells as a result of induced maturation was shown by multiple criteria. These included development of adherent cells with macrophage-like morphology. These cells as well as the morphologically mature monocytes and macrophages that appeared in the culture suspensions were shown to attain membrane

complement receptors, phagocytic capacity, and reactivity with α-napthyl acetate. Quantitation of cellular RNA content showed that mature cells with low RNA content developed during the induction period, correlating with other criteria. There was an increase in the proportion as well as number of monocyte- and macrophage-like cells. With the evidence showing that the macrophage-like cells did not proliferate, our observations would indicate induced differentiation leading to the development of mature cells. A number of recent papers have reported the development of macrophages from cells from leukemic patients after in vitro incubation with phorbol esters (18–20). Our observations of the properties of the macrophage-like cells are in line with the characteristics of macrophages described in these reports.

Cellular RNA content was quantitated in this study by precise measurement of RNA in individual cells. Andreeff et al. (15) showed that immature cells from AML patients had high RNA content, whereas maturing cells from CML patients had low RNA content. Our recent report (7) showed that macrophages had low RNA content when they were induced to differentiate from the leukemic HL-60 cell line that had high RNA content. In the present study, cells with decreased RNA content were found in CM-induced cultures. These cells were most evident among the adherent cells with macrophage-like morphology and acquired characteristics of mature cells.

In all three patients, hyperdiploid DNA stemlines were found in all cells throughout the culture period by DNA-RNA flow cytometry. It was found that macrophage-like cells with mature functional and marker features had the same aneuploid stemline as the undifferentiated cells. This is crucial evidence for the leukemic origin of the differentiated cells and excludes the origin of differentiated cells from normal cells in the cultures. These analyses indicate that leukemic cells can be induced to differentiate to mature cells, and the in vitro induced maturation may be independent of the DNA abnormality. Evidence showing that differentiated cells share the same genetic defect as the undifferentiated leukemic cells has not been well established. Descriptions of aneuploid leukemic cell differentiation have mainly derived from work with cell lines or from chromosomal defects detected in the undifferentiated cells (3, 4, 7, 21).

Because a higher rate of proliferation was found in CM-supplemented cultures than in those lacking CM, interaction of CM with the cells must have resulted in the stimulation of some cells from the resting phase into the replicating phase. The long-lived cultures of patient M.B. provided an opportunity for analysis of the relationship of cell replication to differentiation. From the onset of induction, there was simultaneous proliferation of immature cells and development of mature cells. These two events must be intertwined. At the outset of the culture period, there were more immature cells than mature cells. Mature cells later became predominant as the proliferating population of immature cells was reduced. This indicated a shift from an initial state of simultaneous replication and differentiation to a state characterized mainly by differentiation leading to termination of the cultures. A difference in the balance between proliferation and differentiation might help to explain why the M.B. cultures lasted longer than the other cultures. The other cultures were found to have fewer proliferating S phase cells (16%, Table V) than M.B. cultures (31%), and their proliferation also ended earlier. The continuous development of mature cells seen in M.B. cultures would suggest that proliferation may precede cellular maturation that takes place after cell replication has ceased. This is in accordance with the recent study of CM-induced maturation of human leukemic HL-60 promyelocytes in which

proliferation was shown to precede differentiation, and interaction between CM and cells resulted in suppression of DNA replication before the cells expressed mature functional markers (7).

Estimates made using culture suspensions would indicate ~15–30% of the leukemic cells became mature after 2 d in culture. The actual proportions of mature cells might be higher because there was continuous development of an adherent cell population comprised mainly of maturing cells. However, this may not necessarily indicate maturation of every immature cell. Weinberg's recent observations (22) of phorbol ester-induced maturation of HL-60 leukemic cells showed that the induced macrophage-like cells were capable of killing the undifferentiated parent HL-60 cells. This would indicate that the process of differentiation and the capacity of the maturing cells to eliminate tumor cells may both control the undifferentiated cell population. It is likely that the differentiation process precedes and initiates the cytotoxic process. Whether the induced macrophage-like cells in our studies of patient leukemic cells may have the capacity to control the growth of undifferentiated cells is currently being studied in our laboratory.

Summary

Human leukemic cells were induced to proliferate and mature to macrophage-like cells in primary cultures supplemented with conditioned medium (CM) from phyto-hemaggglutinin and alloantigen-stimulated normal T lymphocytes. Blast and pro-myelocyte-enriched preparations, isolated after depletion of adherent phagocytic cells and lymphoid cells from samples of myelogenous leukemia patients, were suspended in liquid cultures with 30% CM. Cell cycle analysis was performed throughout the course of induced cellular maturation. Within 24 h of exposure to CM, cells with macrophage-like morphology were identified among the developing adherent cells. Approximately 15–30% of the cells in culture suspensions also developed macrophage-like morphology and esterase reactivity with α-napthyl acetate after incubation for 2 d. The number of these nonproliferating cells increased and became predominant in the later culture period. Flow cytometric measurement of DNA content showed that these mature cells had the same aneuploid stemline as the undifferentiated leukemic cells, indicating that genetically abnormal leukemic cells can be induced to differentiate. Reduction in the total RNA content of the macrophage-like cells was also determined by flow cytometry. Reduction in RNA and development of adherent cells served as early markers of maturation, in addition to the later acquisition of complement receptors and phagocytic capacity. Cell cycle analysis showed that CM stimulated the proliferation of immature cells. This initial proliferation may precede intertwined events of proliferation and concurrent maturation of immature cells. Later in the culture period, cellular proliferation decreased, leading to termination of the cultures.

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