of chromosome 1 (1p3200 → cen) (*20*). However, this appears to be proximal to the most common breakpoint of chromosome 1 in neuroblastomas (*16*), and no example of N-*ras* translocation in a human neuroblastoma has been identified thus far.

It is known that sequences from the short arm of chromosome 2 other than N-*myc* are amplified in neuroblastomas (*7*). However, the fact that N-*myc* is amplified may be particularly important since there is evidence that it may be a proto-oncogene. First, it bears partial homology with a known proto-oncogene, c-*myc*. Second, it appears to be a rather conserved sequence. We have used N-*myc* hybridization to analyze Eco RI-digested DNA from almost 100 different individuals and have not detected a single case with polymorphism. Furthermore, a band of different size but similar intensity was detected when this probe was hybridized under stringent conditions to DNA's from mouse, rat, and hamster, which suggests conservation through recent evolution (*6, 21*). Third, N-*myc* amplification is associated with increased levels of mRNA transcripts of N-*myc* in these tumors (*7, 22*). Our results demonstrate clinical prognostic significance of N-*myc* amplification, and they suggest an important role for this sequence in human neuroblastoma.

Note added in proof: In a recent report (*23*), ten retinoblastomas and a retinoblastoma cell line were studied for amplification and mRNA expression of N-*myc*. Genomic amplification of N-*myc* was identified in two of the ten tumors and in the Y79 cell line, but all 11 had increased levels of N-*myc*–related mRNA compared to control cultures. These results confirm the probable importance of N-*myc* in retinoblastoma and expand the tumors with which N-*myc* activation is associated to include both neuroblastoma and retinoblastoma.

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Human Monocytic Cell Lines Derived from Cord Leukocytes by Co-cultivation with Irradiated CM-S Cells

Abstract. *The CM-S cell line was established from the bone marrow of a child with congenital hypoplastic anemia and resembles its monocyte-macrophage lineage. Lethally x-irradiated CM-S cells from various passages and clones, representing different stages in the progression of the transformed growth phenotype, were tested for their ability to affect the survival and proliferation of normal human cord or adult blood leukocytes in co-culture. One clone, CM-SM, which is tumorigenic in athymic mice, consistently immortalized umbilical cord mononuclear cells but did not immortalize adult peripheral blood leukocytes. Six autonomous monocyte-like diploid cell lines were obtained and all were found to be of cord origin. Three lines were tumorigenic in athymic mice. Attempts to immortalize human leukocytes with cell-free supernatants from CM-S cells were unsuccessful.*

The autonomous cell line CM-S was derived from the bone marrow of a male child suffering from congenital hypoplastic anemia, a rare childhood defect of erythropoiesis (*1*). The cells grow in suspension culture, where most appear morphologically undifferentiated but express some of the enzyme and antigen markers characteristic of precursor monocytic and granulocytic cells (*2*). When incubated in culture with factors present in conditioned medium or with various chemical compounds, CM-S cells can undergo marked morphological and

functional changes (*3*). For example, in response to the tumor promoter 12-*O*-tetradecanoyl phorbol-13-acetate (TPA), many of the cells become adherent, stop dividing, differentiate, and acquire morphological and functional properties of the monocyte-macrophage lineage. These include enhanced synthesis of lysozomal enzymes, lysozyme production, Fc and C3 rosette formation, and the capacity to phagocytose membrane-bound, immunoglobulin G (IgG)–coated erythrocytes, inert particles, or bacilli. CM-S cells produce and release into the supernatant a number of biologically active polypeptides that mediate the growth and differentiation of other cells or of CM-S cells themselves (*4, 5*). The polypeptides include interleukin 1; colony-stimulating factor, a species-specific growth factor capable of stimulating CM-S replication and of inducing formation of colonies of monocyte-granulocyte precursors from fresh human bone marrow seeded in soft agar; and another protein factor (molecular weight $\leq$ 10,000) that apparently retains only autostimulatory activity.

The CM-S cell line was established approximately 4 years ago. The cells retained an euploid karyotype in continuous culture for over 120 passages, did not form colonies in agar, and did not produce tumors in athymic mice. Eventually, however, the cells gradually acquired chromosome abnormalities and showed an increased capacity to grow in liquid culture and to form colonies in agar and tumors in nude mice (*6*). These aneuploid CM-S cells still retain most of the characteristics of their monocyte precursors. A monocyte-macrophage inducible clone (CM-SM) (*7*) was isolated in agar from line CM-S at the 160th passage. Karyological analysis of CM-SM cells revealed a stable karyotype [47,XY,+8,inv-dup(4)(p13 → p15)] in > 95 percent of cells examined in metaphase. CM-SM cells grow well in agar and liquid cultures, and an inoculum of 10^7 cells can form tumors in athymic mice after 2 to 3 months.

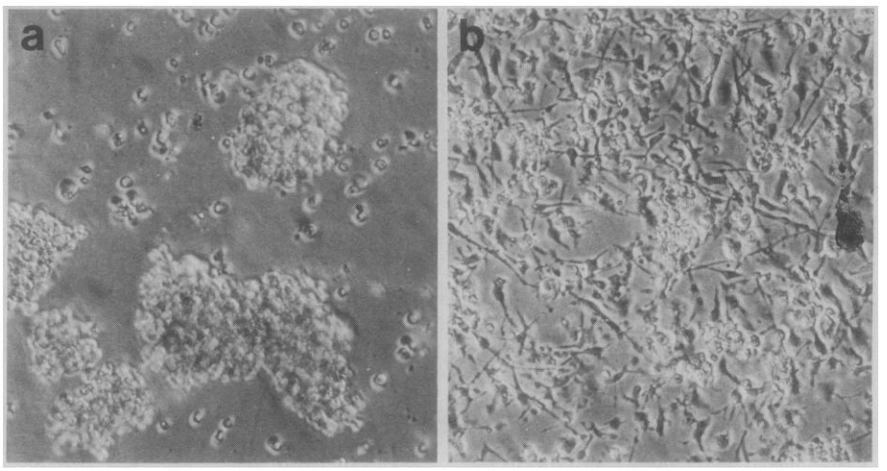

Fig. 1. Morphology of CM-15 cells after 2 days in culture in the absence (a) or presence (b) of TPA (10^{-7} *M*) (phase contrast, ×400).

We used different uncloned euploid and aneuploid variants of the CM-S line, representing different stages in the progression of the transformed phenotype, and the clone CM-SM in an attempt to immortalize human umbilical cord and adult blood leukocytes in co-culture. Lethally irradiated CM-SM cells consistently immortalized normal human cord blood leukocytes (CBL's) in co-culture, giving rise to continuous monocyte-like cell lines of cord leukocyte origin. Late aneuploid CM-S cells immortalized CBL's with low frequency, while early euploid CM-S cells were never successful. Immortalization of human adult leukocytes was not achieved.

Cells of the uncloned CM-S line and clone CM-SM were cultured in Dulbecco's modified Eagle's medium supplemented with 20 percent heat-inactivated fetal calf serum, 10 percent trypticase soy broth, and antibiotics in tightly closed plastic flasks at 37°C. The cells were seeded at a density of about 10^6 cells per milliliter and split 1:3 every 3 days. Exponentially growing cells were lethally x-irradiated (7500 R), centrifuged, and resuspended in fresh medium at 6×10^6 cells per milliliter. Blood was obtained from fresh umbilical cords from normal female adults at term. Mononuclear leukocytes were separated by Ficoll-Hypaque gradient centrifugation

Table 1. Characteristics of immortalized cell lines derived from co-culture of x-irradiated CM-SM cells and mononuclear leukocytes from female infant CBL's. Uncloned, aneuploid CM-S cells at the 125th passage [karyotype 46,XY,inv-dup(4)(p13 → p15)] or cloned CM-SM cells were x-irradiated (7500 R) and mixed at a density of 6×10^6 cells per milliliter with equal numbers of CBL's obtained from Ficoll-Hypaque centrifugation. Cells were centrifuged (700*g* for 10 minutes at 20°C), kept in a pellet for 1 hour at 37°C, resuspended, and plated in two T-30 flasks. Medium was changed twice a week. Growing cultures were divided at 1:2 dilution after 4 weeks. Cultures initially required their own conditioned medium and then became autonomous, providing they were seeded at high density ($\geq 10^6$ cells per milliliter).

Line*	Months after establishment	Origin	Growth properties†			Differentiated phenotype‡	
			Untreated				
			Doubling time (hours)	Saturation density ($\times 10^6$ cells per milliliter)	Saturation density with TPA ($\times 10^6$ cells per milliliter)	Not treated with TPA	Treated with TPA (10^{-7} *M*)
CM-RR	26	Rx-CM-S aneuploid × CBL's	30	7.0 ± 0.8	2.4 ± 0.4	–	+
CM-15	24	Rx-CM-SM × CBL's	30	6.4 ± 1.2	2.8 ± 0.5	–	+
CM-18	21	Rx-CM-SM × CBL's	48	3.2 ± 0.4	2.0 ± 0.3	±	+
CM-41	16	Rx-CM-SM × CBL's	32	6.5 ± 1.1	2.4 ± 0.4	–	+
CM-G4	10	Rx-CM-SM × CBL's	40	3.8 ± 0.5	3.2 ± 0.3	–	±
CB-2	10	Rx-CM-SM × CBL's	36	4.6 ± 0.4	3.0 ± 0.5	–	±

*Karyotype of each line, 46,XX. †Doubling time was measured from the linear portion of the growth curve. Saturation densities are means ± standard errors for three experiments. ‡Criteria for monocyte-macrophage differentiation included positive nonspecific esterase activity, adherency, Fc-IgG and C3B rosette formation, lysozyme production, and immunoerythrophagocytosis.

Table 2. Co-cultivation of female infant CBL's with x-irradiated CM-S cells. Various concentrations of x-irradiated CM-S cells (34th to 90th passage or 197th to 214th passage) or CM-SM cells were mixed with 10^6 CBL's in a 15-ml tube, centrifuged and kept in a pellet for 1 hour at 37°C, gently resuspended, and plated in microtiter plates (2×10^5 cells per 0.1 ml per well). Cells were fed (1:2 dilution) every 4 days. Foci of growing cells were observed after 2 to 3 weeks in culture. The number and size of these foci progressively increased thereafter. Cell immortalization was proved by repeated cell divisions. Results are means (± standard errors) for three different experiments.

Cultivation	Number of cultures positive for immortalization	Minimum number of CM-S cells necessary for CBL transformation per well
CBL's mixed with uncloned euploid CM-S cells	0 of 192	
CBL's mixed with uncloned aneuploid CM-S cells	11 (±5) of 192	$5 (\pm 2) \times 10^5$
CBL's mixed with cloned CM-SM cells	81 (±18) of 96	$2.5 (\pm 1.3) \times 10^4$
CM-S cells alone (euploid or aneuploid)	0 of 96	
CM-SM cells alone	0 of 48	
CBL's alone	0 of 96	

(400*g* at 20°C for 30 minutes), washed, and resuspended at 6×10^6 cells per milliliter. Equal concentrations of irradiated CM-SM (or CM-S) cells and leukocytes were mixed in plastic tubes or wells, centrifuged (700*g* at 20°C for 10 minutes), and kept in a pellet for 1 hour at 37°C (*8*). The cells were then resuspended in their own supernatant medium, cultured in plastic flasks, and fed (1:2 dilution) once a week.

In control irradiated CM-SM and CM-S cultures no evidence of proliferation was seen. Control leukocyte cultures showed a transient proliferation of mononuclear cells, but all cells died within 3 weeks. One cell line of cord origin was established in 16 attempts with an aneuploid mass culture of CM-S cells (125th passage). In seven different experiments five continuous cell lines were established with CM-SM cells as donor. In these co-cultures, foci of round mononuclear cells were nearly always observed after 2 to 3 weeks; these then rapidly increased in size and cell number. Proliferating cells were initially mostly adherent cells; later, however, round mononuclear cells detached and continued to grow in suspension as single cells or small clusters. For growth these cultures initially required their own conditioned medium (1:2 or 1:3 dilution with fresh medium), but eventually (after five to ten passages) they became independent, providing they were seeded at high densities ($\leq 10^6$ cells per milliliter). Continuous growth of the cells was confirmed by repeated cell dilutions. Karyological analyses, performed on 3- to 4-month-old cultures, showed that these cells had a normal diploid karyotype with female sex chromosomes in virtually all counted metaphases. These lines are thus clearly derived from the cord leukocytes and not from the male CM-SM cells. Table 1 reports the growth properties of these six transformed CBL lines. Efforts to transform adult or

Table 3. Characteristics of the CM-SM and CBL cell lines. Cell properties were analyzed by standard procedures (*3, 7, 15*).

Properties, reagents	CM-SM cells	CBL lines					
		CB-2	CM-RR	CM-15	CM-41	CM-G4	CM-18
ASD chloroacetate esterase	−	−	−	−	−	−	−
Myeloperoxidase	−	−	−	−	−	−	−
Acid phosphatase	+	+	+	+	+	+	+
α-Naphthyl-acetate-esterase (NaF-sensitive)	+	+	+	+	+	+	+
Cytochrome oxidase	+	+	+	+	+	+	+
Terminal deoxynucleotidyl transferase	−	−	−	−	−	−	−
Lysozyme	10 to 60%	5 to 30%	5 to 10%	0 to 5%	5 to 30%	0	20 to 60%
Ig (all classes)	−	−	−	−	−	−	−
HLA-ABC	100%	100%	100%	100%	100%	100%	100%
HLA-DR	100%	100%	100%	100%	100%	100%	100%
MoU-26	5%	25%	5%	10%	20%	30%	20%
MoU-50	30%	30%	15%	20%	30%	30%	30%
MLR-2	35%	50%	30%	30%	40%	20%	40%
OK-M1	45%	50%	15%	20%	50%	20%	60%
OK-T3,T4,T6,T10	−	−	−	−	−	−	−
OK-T9	30%	30%	10%	15%	15%	10%	25%
Lyt 3	−	−	−	−	−	−	−
Rosetting							
EAC3B, IgG-EA	5 to 40%	5 to 30%	0 to 5%	0 to 5%	5 to 30%	0 to 5%	5 to 40%
Sheep and ox erythrocytes	−	−	−	−	−	−	−
Immunoerythrophagocytosis (C3B-E, IgG-E)	+	+	−	+	+	−	+
Growth factor production*							
GM–colony-stimulating factor	+	+	+	+	+	+	+
Autostimulatory factor	+	+	+	+	+	+	+
Growth in athymic mice†	2 of 5	3 of 5	2 of 5	0 of 3	0 of 3	1 of 5	0 of 3

*Growth factor production was assayed by clonogenic assays (*5, 13*). †Number of mice with tumors among mice inoculated subcutaneously with 10^7 cells after 2 to 3 months.

CBL's with cell-free supernatants (as is, or concentrated 100 times) were always unsuccessful.

The minimum number of CM-SM or CM-S cells necessary to achieve transformation of a fixed number of CBL's was determined in microtiter plates. The results of several repeated experiments are summarized in Table 2. The data indicate that the frequency of CBL immortalization was significantly higher with CM-SM cells than with uncloned CM-S cells and that the minimum number of CM-SM cells required was significantly lower than that of uncloned aneuploid CM-S cells. It is worth noting that the immortalizing capacity of these cells in vitro parallels their tumorigenic capacity in nude mice. Table 3 shows the morphological, cytochemical, and functional properties of the six CBL lines, all of which had a strikingly similar monocyte-like phenotype. Most of the cells in these mass cultures were positive for a number of enzymes usually associated with normal human precursor cells of this lineage. All lines were positive for histocompatibility antigens (HLA's) ABC and DR, and a large fraction of the cells reacted with four hybridoma monoclonal antibodies specific for the macrophage lineage: OK-M1 (Ortho), which predominantly detects late macrophages; MoU-26 and MoU-50 (*9*), which detect early monocyte precursors; and MLR-2 (*10*), which also detects early myeloid precursors. The cells do not express deoxynucleotidyl transferase, do not synthesize surface or cytoplasmic immunoglobulins of any class, do not express Lyt-3 human antigen detected by monoclonal antibody, do not form spontaneous rosettes with untreated or neuraminidase-treated sheep or ox erythrocytes, and, in general, do not react with monoclonal antibodies of the OK-T series (*11*), with known specificity for human T-lymphocyte subsets. Some reactivity was consistently observed with OK-T9 antibody, but this antibody reacts with cells of various hemopoietic lineages (*12*).

Most cells of line CM-18 grow spontaneously in adherency; the other five lines (CM-RR, CB-2, CM-15, CM-41, and CM-G4) normally grow in suspension. On being treated with TPA, cells of three of these lines (CM-RR, CM-15, and CM-41) become adherent (Fig. 1) and rapidly differentiate to resemble macrophages, as determined by enzyme cytochemical and functional studies (increased activity of nonspecific esterases, cytochrome oxidase, and acid phosphatase; synthesis of lysozyme; and capacity to phagocytose membrane-bound, IgG-coated erythrocytes). All six CBL lines produce and release growth factors autostimulatory for CM-S cells and active in promoting colony formation of monocyte-granulocyte precursors from normal human bone marrow in the clonogenic assay in agar. Colony-stimulating factor from other sources, such as human placental conditioned medium (*13*), stimulates the growth of CM-S cells and the CBL lines (*5*). Athymic mice inoculated with 10^7 CB-2, CM-RR, or CM-G4 cells developed tumors after 2 to 3 months.

There are several possible explanations for the immortalization of CBL by x-irradiated CM-S cells. It seems unlikely that these lines are hybrid, since (i) all six have a diploid-euploid karyotype as analyzed by high-resolution chromosome banding techniques, (ii) all are of cord origin as shown by the sex chromosomes, and (iii) all differ from one another and from CM-S by HLA-ABC and HLA-DR typing. If somatic cell hybrids were formed after cell-cell contact, the hybrids would have had to rapidly and specifically lose almost the entire genetic material of the "donor" CM-S cell in order to regain the observed pattern of the "recipient" CBL.

Another possibility is that soluble growth factors released by the CM-S cells favored the proliferation and eventually selected a subset of monocyte precursors from the cord cells, which then predominated in the culture. Our observations are consistent with this possibility. Conditioned medium from uncloned CM-S cells, CM-SM cells, or human placenta was necessary for initiation and early replication of the CBL lines. The lines, however, became autonomous if they were seeded at high cell densities. At lower densities, doubling times and saturation densities decreased until, at a seeding density of 1×10^5 to 3×10^5 cells per milliliter, the cells did not divide unless conditioned medium was added. This suggests that the CBL lines had begun to produce their own growth factors, providing an autologous stimulus for their life maintenance and self-renewal. This model, however, does not explain why early euploid CM-S cells or cell-free supernatants containing high titers of colony-stimulating factor consistently failed to immortalize CBL's. Cell contact seems to be required in addition to growth factors to achieve continuous leukocyte replication. Transforming factors [as described in other cell systems (*14*)] produced by the irradiated CM-S cells could be involved. The development of the transforming phenotype in the course of CM-S passages might correspond to a progressively increased capacity of these cells to produce such factors.

It is also possible that cell contact favored transmission of some genetic material from CM-S to CBL cells. CM-SM cells should provide a convenient cell system for identifying the molecular basis for such transformation.

The technique of co-culturing irradiated CM-SM cells with normal human CBL's could be used to generate a large panel of human promonocytic cell lines for studies of monocyte heterogeneity and function.

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