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Control of Normal Differentiation of Myeloid Leukemic Cells to Macrophages and Granulocytes

(mice/chromosome number/differentiation-inducing protein)

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ABSTRACT **Cells from a myeloid leukemic line in culture can be induced by the differentiation-inducing protein MGI to form colonies with normal differentiation to mature macrophages and granulocytes. This line consisted of clones that can be induced to undergo normal cell differentiation (D^+ clones) and clones (D^- clones) that were not inducible. D^+ clones were able to undergo differentiation to both macrophages and granulocytes. Normal differentiation was induced even in clones that were no longer diploid. D^+ clones can segregate some D^- progeny, and D^- clones can segregate some D^+ progeny. This, therefore, provides a system for studies on the genetic and chemical control of cell differentiation in leukemic cells.**

We have shown that the differentiation of different types of hematopoietic cells can be studied in tissue culture (1–5). Various cells release a protein inducer, MGI, that is required for the formation of colonies *in vitro* with mature, differentiated macrophages and granulocytes from single normal undifferentiated hematopoietic cells (3–9). We have also shown that undifferentiated cells from human patients with acute myeloid leukemia can be induced to differentiate to mature granulocytes *in vitro* (10), and that induction of differentiation in a mouse myeloid leukemic line in culture (11) can be induced by MGI (12). The present studies with this line of mouse leukemic cells were undertaken to determine whether the leukemic line consists of clones that can be made to differentiate (D^+) and clones that cannot be made to differentiate (D^-), whether D^+ clones can segregate D^- progeny and vice versa, and whether normal differentiation to macrophages and granulocytes can be induced in leukemic cells that are no longer diploid.

MATERIALS AND METHODS

Cells and Cell Cloning. The cell line used in the present studies was established *in vitro* from a myeloid leukemia of SL mice (11) and kindly supplied by Dr. Y. Ichikawa. The cells grow in suspension in liquid medium as myeloblasts. Subcultures were made every 4–5 days, by seeding 5×10^5 cells per 50-mm petri dish in 5 ml of Eagle's medium with a 4-fold concentration of amino acids and vitamins (EM) and with 10% inactivated (56° for 30 min) horse serum. For cloning (3), the leukemic cells were seeded in 1.7 ml of soft agar (0.33%) on a harder agar base (0.5)% in 50-mm petri dishes in EM with 20% horse serum. 10–12 Days after seeding, the colonies were examined with an inverted microscope. For the identification of cell types, colonies were smeared on a glass slide and stained with May–Grunwald Giemsa. Clones were isolated from plates containing no more than three colonies at 10–12 days after seeding 20 cells. Colonies were isolated from the agar with a fine pasteur pipette; the cells were suspended in 2.5 ml of EM with 10% serum, pipetted to obtain a cell suspension, and either directly recloned in agar or seeded for mass culture in 35-mm and then in 50-mm petri dishes. To obtain three-times purified clones, colonies were isolated from agar, and grown in mass culture for 14 days after each recloning.

Preparation of Conditioned Medium (CM) Containing MGI. Serum-containing CM was prepared from mouse-embryo secondary cell cultures. 1×10^6 Cells were seeded per 50-mm petri dish in 5 ml of EM with 10% horse serum. CM was harvested 2 days after seeding, centrifuged at $1000 \times g$ for 10 min, filtered through a 0.45-μm Millipore filter,and stored at −20°. Serum-free CM from the mouse fibroblast line E1 was prepared and MGI was purified as described (8). Unless otherwise stated, CM was added to the lower agar layer at a 15% concentration.

Chromosome Preparations. To obtain chromosome preparations from colonies in agar, 1 ml of EM with 0.5 μg of colcemide (Ciba) and agar at final concentration of 0.33% was added to plates with 12-day-old colonies. After overnight incubation at 37°, colonies were isolated from the agar and suspended separately or pooled in 0.5–1.0 ml of hypotonic solution (75 mM KCl). After 20–30 min at room temperature (24°), freshly prepared acetic acid–alcohol fixative (1 part glacial acetic acid and 3 parts methyl alcohol) was added, and the cell suspension was mixed gently. After three more changes of the fixative, the cells were dropped on a glass slide, air dried, and stained with Giemsa. Chromosome preparations from mass culture were obtained by addition of 0.3 μg/ml of colcemide to petri dishes containing 2×10^6 cells per ml at 2 days after seeding. After 3 hr of incubation at 37°, the cells were centrifuged, suspended in the hypotonic solution, incubated for 15 min at 37°, and fixed with acetic acid–alcohol (four changes). The cells were dropped on a glass slide, air dried, and stained. Results on chromosome numbers in Table 2 and Fig. 3 are based on 50 cells per clone.

RESULTS

Differentiation in the uncloned line

As in previous experiments (12), when cells from the uncloned line were seeded in soft agar without CM containing MGI,

Abbreviations: MGI, inducer required for development of macrophage and granulocyte colonies; CM, conditioned medium containing MGI; EM, Eagle's medium with a 4-fold concentration of amino acids and vitamins.

about 2% formed colonies of undifferentiated blast cells. Addition of CM increased the cloning efficiency to 15%. About 60% of the colonies were compact and contained only undifferentiated blast cells (D^- colonies); about 40% (D^+ colonies) contained a dispersed periphery with cells in various stages of differentiation (Fig. 1). Depending on the batch of CM, the differentiation was to mature macrophages, or to both mature macrophages and granulocytes. Since the serum-containing CM from secondary embryo cells gave larger colonies and a better degree of differentiation than the serum-free CM from the line E1, either before or after purification of MGI (12), this serum-containing CM was used in all subsequent studies.

Isolation of D^+ and D^- clones

For determination of the heritability of D^+ and D^-, cells were cloned in agar with CM, 20 colonies of each type were isolated, and the type of colony was determined after 14 days of growth in mass culture. Counts of 100 progeny colonies per clone showed that the progeny of 20 D^+ colonies were nearly all D^+, and the progeny of 20 D^- colonies were nearly all D^-.

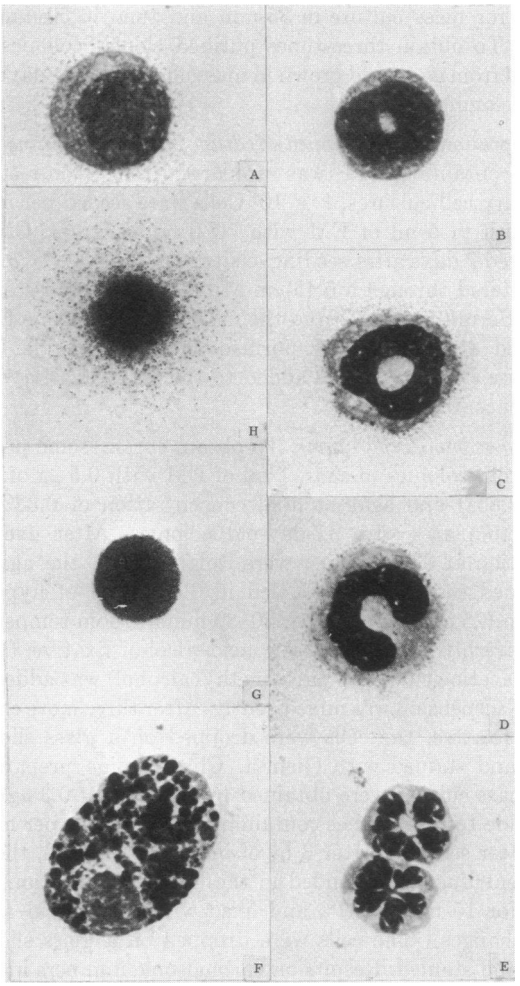

FIG. 1. Induction of cell differentiation in myeloid leukemic cells to mature macrophages and granulocytes. *A* = undifferentiated blast cell. *B–E*, stages in the differentiation to mature granulocytes. *F*, mature macrophage. *G*, D^- colony. *H*, D^+ colony. *A–F* are stained with May-Grunwald Giemsa, ×970. *G* and *H* are unstained, ×24.

4 D^+ and 4 D^- colonies were then recloned three times. Results on differentiation with different concentrations of CM are shown in Table 1. These results indicate that there was no differentiation in any of the clones without the addition of CM, and that within the number of colonies tested, all the D^+ clones gave D^+ progeny and all the D^- clones gave D^- progeny. D^+ clones at 10, 12, 14, and 16 days after seeding in agar with 15% CM contained about 10, 30, 60, and 90% differentiated cells, respectively.

Differentiation in the D^+ colonies was induced either by addition of CM at the time when the cells were seeded for cloning, or by addition of CM in 0.33% agar to 10-day-old colonies formed without CM. The growth curves of D^+ and D^- clones in mass culture (Fig. 2) indicate that, although various clones showed differences in their growth curve, there was no correlation between the growth curve and colony type.

Differentiation of D^+ clones to macrophages and granulocytes

With some batches of CM the D^+ colonies contained only macrophages, while with other batches they contained both macrophages and granulocytes. The second type of CM was tested with 3 D^+ clones, each of which had been recloned three times. The results have shown that such purified clones at 12 days after seeding produced 45% progeny colonies with both macrophages and granulocytes, in addition to 15% with only granulocytes and 40% with only macrophages. The granulocyte colonies contained cells in various stage of differentiation to mature neutrophil granulocytes (Fig. 1). No cell division was observed in the mature macrophages and granulocytes.

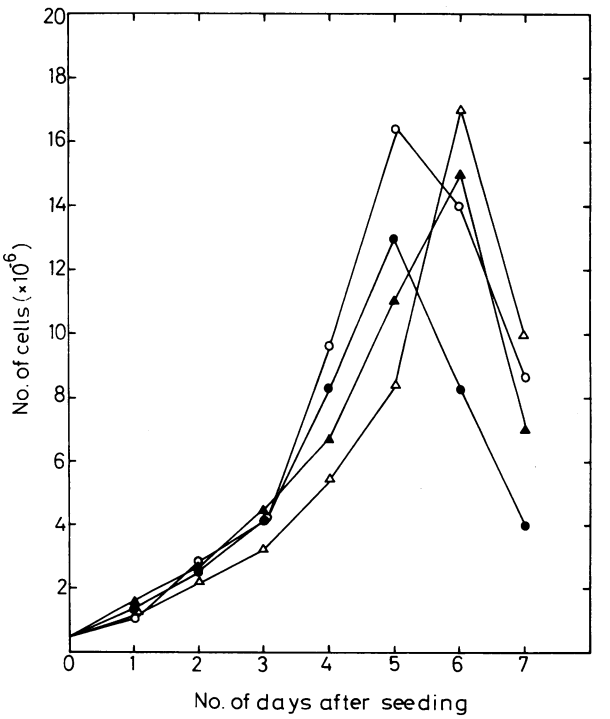

FIG. 2. Growth curves of D^+ and D^- clones in mass culture. 500 Cells were seeded per 50-mm petri dish in 5 ml of medium with 10% horse serum. The medium was not changed during the 7 days of culture. ○, clone 1; △, clone 3; ●, clone 5; ▲, clone 8. Clones 1 and 3 are D^+, clones 5 and 8 are D^-.

TABLE 1. *Formation of D⁺ and D⁻ colonies with different concentrations of CM*

CM (%)	Uncloned		Clone no. 1		3		5		8	
	D⁺	D⁻	D⁺	D⁻	D⁺	D⁻	D⁺	D⁻	D⁺	D⁻
0	0	20	0	23	0	21	0	15	0	35
12.5	39	62	160	0	119	0	0	120	0	262
25	43	35	80	0	85	0	0	50	0	148
50	22	15	53	0	32	0	0	22	0	95

500 Cells were seeded per 50-mm petri dish with different concentrations of CM containing MGI. The results from the 4 clones are representative of the 8 clones tested.

Segregation in D⁺ and D⁻ clones

4 D⁺ and 4 D⁻ clones were tested about 40 cell generations after seeding for the third clone isolation, to determine whether D⁺ clones can segregate D⁻ progeny and vice versa. 4000 Cells from each clone were seeded in 8 petri dishes with CM. The results show (Table 2) that 3 of the D⁺ clones gave 0.4, 0.6, and 0.8% D⁻ progeny and 2 of the D⁻ clones gave 0.25, and 1.2% D⁺ progeny. Isolation of three revertant colonies of each type showed, that D⁺ gave D⁺ and D⁻ gave D⁻ progeny.

Chromosome numbers in D⁺ and D⁻ clones

The chromosome numbers in 8 clones were counted, in cells grown in mass culture and in colonies grown in agar. The same modal chromosome number was found under both conditions. The results of such a comparison in 1 D⁺ and 1 D⁻ clone are shown in Fig. 3*A* and *B*. The same chromosome numbers as those in the pooled colonies in agar (Fig. 3*A*) were also found in single isolated colonies. All the clones studied showed a clear modal chromosome number (Fig. 3). The modal number in the 8 clones (Table 2) were 40, 41, or 42 for D⁺ and 40 or 41 for D⁻. One abnormal subtelocentric or one large telocentric chromosome was observed in the 2 D⁺ clones and in 1 of the D⁻ clones with a modal number of 40. The chromosome counts have shown that differentiation can occur in D⁺ clones that are no longer diploid.

DISCUSSION

A tissue culture line of mouse myeloid leukemic cells can be induced (11, 12) to form colonies with normal, differentiation to mature macrophages and granulocytes by CM that contains MGI (12). The present results have shown that this line consisted of clones that can be induced to undergo differentiation (D⁺ clones) and clones (D⁻ clones) that are not inducible. About 90% differentiated cells can be induced in D⁺ clones at 16 days after seeding in agar. As in the case of colonies obtained from normal cells, no cell division was observed in the mature macrophages and granulocytes obtained from the myeloid leukemic cells. Treatment with MGI may thus be of potential value in the therapy of leukemia.

Induction of colony formation with differentiated cells from undifferentiated normal cells requires the protein MGI and a low molecular weight cofactor, both of which are found in CM. Adenine or adenine-containing nucleotides can substitute for the cofactor (8, 9). MGI induced differentiation in the uncloned line of leukemic cells without addition of the cofactor or its substitutes. The leukemic cells also produced a cofactor that can activate MGI to induce the formation of differentiated colonies from normal cells (12).

TABLE 2. *Chromosome numbers and segregation of D⁺ and D⁻ clones*

Clone no.	Parental clones: Modal no. of chromosomes	Abnormal chromosomes	Clone type	No. and type of progeny colonies: D⁺	D⁻
1	40	1 ST*	D⁺	835	0
2	40	1 LT*	D⁺	957	6
3	41	None	D⁺	625	5
4	42	None	D⁺	721	3
5	40	1 LT	D⁻	0	751
6	41	None	D⁻	0	1030
7	40	None	D⁻	3	1202
8	41	None	D⁻	10	851

For determination of the type of progeny, 4000 cells per clone were seeded in eight 50-mm petri dishes with 15% CM. The modal chromosome numbers were found in at least 75% of the cells.

* ST, subtelocentric chromosome; LT, abnormal large telocentric chromosome.

Experiments with different batches of CM have now shown that some batches induce differentiation in D⁺ clones only

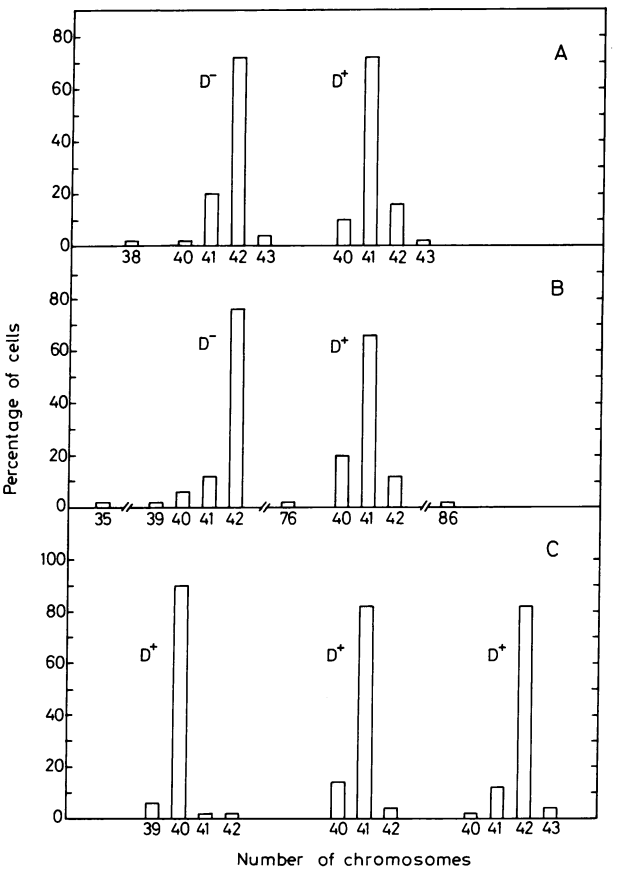

FIG. 3. Chromosome numbers in D⁺ and D⁻ clones. *A*, a D⁺ and a D⁻ clone growing in agar. 12 Colonies were pooled from each clone. *B*, the same clones as in *A*, growing in mass culture. *C*, D⁺ clones with modal numbers of 40, 41, and 42 (clones 2, 3, and 4) in mass culture.

to macrophages, whereas, other batches induce differentiation to both macrophages and granulocytes. This result makes it possible to determine the requirements for differentiation to one or both cell types in the progeny of a single cell, and whether there are different requirements for cofactors.

It will be of interest to determine whether the difference between D^+ and D^- cells is due to a difference in a surface membrane or intracellular receptor for MGI. It will also be of interest to determine the relationship between induction of cell differentiation and the possible presence of viral genes (13, 14) and the nature of the block in myeloid leukemic cells in which differentiation was induced only to immature granulocytes (15).

The study of chromosome numbers has shown that there were the same modal chromosome numbers in cells grown in mass culture and in colonies grown in agar. The D^+ clones had a modal number of 40, 41, or 42, and the clones with a modal number of 40 had one abnormal chromosome. These results have shown that normal differentiation was induced in D^+ clones that were no longer diploid. The D^- clones had a modal number of 40 or 41. The use of banding techniques (16, 17) for identification of chromosomes may further clarify the chromosomal changes that are compatible with the induction of normal cell differentiation and whether, as in some other types of tumor cells (18–20) there are identifiable differences between the chromosomes of normal, D^+, and D^- cells.

The study of progeny clones has shown that D^+ clones can segregate some D^- progeny and that D^- clones can segregate some D^+ progeny. This, therefore, provides an experimental system for studies on the genetic and chemical control of hematopoietic cell differentiation in leukemic cells.

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