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Genetic dissection of the control of normal differentiation in myeloid leukemic cells

(Fc and C3 rosettes/immune phagocytosis/lysozyme/macrophages and granulocytes/chromosomes)

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ABSTRACT **Normal myeloid precursors and MGI^+D^+ myeloid leukemic cells can be induced to differentiate to mature cells by the normal protein inducer MGI. The sequence of differentiation is the induction of C3 and Fc rosettes, C3 and Fc immune phagocytosis (IP), synthesis and secretion of lysozyme, and formation of mature macrophages and granulocytes. Mutant clones of myeloid leukemic cells have been isolated with differences in the time of induction of C3 and Fc rosettes and C3 and Fc IP, in which lysozyme was induced without going through the stage of Fc or C3 IP, and with differences in inducibility by MGI to mature macrophages or granulocytes. Only one out of five MGI^-D^- clones gave rise to MGI^+D^+ mutants. The ability to obtain mutants from this clone was associated with its special chromosome constitution, and these mutants showed a change in their ability for cap formation by concanavalin A. The steroid inducer dexamethasone can induce in MGI^+D^+ clones differentiation to macrophages but not to granulocytes. Differentiation by steroid inducer in different clones occurred either with or without induction of Fc rosettes and Fc IP, and induction of C3 rosettes was not always associated with induction of C3 IP. The use of mutants that differ in their competence to be induced by MGI or steroid inducer has shown that there are separate controls for the induction of C3 and Fc rosettes, C3 and Fc IP, lysozyme, macrophages, and granulocytes.**

An *in vitro* system to study the control mechanisms involved in induction of differentiation in normal (1–7) and leukemic (6–14) myeloid cells has been described. The use of this system has shown that normal cells and some clones of mouse myeloid leukemic cells can be induced by purified macrophage and granulocyte inducer (MGI) protein (7, 15) to undergo differentiation to mature macrophages and granulocytes. The formation of mature cells was preceded by the induction of Fc and C3 rosettes (9, 12) and the synthesis and secretion of lysozyme (13). Clones of myeloid leukemic cells that can be induced by MGI (MGI^+) for differentiation-associated markers, including mature cells (D^+) (8), will be referred to as MGI^+D^+. Another clone type was inducible by MGI for Fc and C3 rosettes and lysozyme but not mature cells (MGI^+D^-); a third type could not be induced by MGI for any of these properties (MGI^-D^-) (9, 12, 13). The protein inducer (2–4) that we now call MGI has also been referred to as colony stimulating factor (16) or activity (17). MGI^+D^+ myeloid leukemic clones can also be induced for some stages of differentiation by compounds such as cytosine arabinoside, actinomycin D, and 5′-iododeoxyuridine (9, 18) and certain steroid inducers (SI) such as dexamethasone, prednisolone, and estradiol (9, 12, 19). Some MGI^-D^- clones that were not inducible by MGI could be induced for some properties by SI and there appear to be different cellular sites for inducibility by MGI and SI (12). The MGI^+D^+ and MGI^+D^- clones used in previous experiments were from a different myeloid leukemia than the MGI^-D^- clones. We have now isolated MGI^+D^+ mutants from an MGI^-D^- clone (no. 7), determined the relationship between chromosome constitution and mutability of MGI^-D^- clones, and isolated a new MGI^+D^- mutant from an MGI^+D^+ clone (no. 11). The different mutants have been used to further dissect the controls that regulate induction of normal differentiation in myeloid leukemic cells.

MATERIALS AND METHODS

Cells and Cell Cultures. MGI^+D^+ clones 11 and 12 and MGI^+D^- clones 2, 5, and 13 (8) were from a cell line of myeloid leukemia originating in an SL mouse (20); MGI^-D^- clones 1, 6, 7, 8, and 10 were from five independently arising myeloid leukemias in x-irradiated SJL/J mice (21). MGI^+D^+ mutants 7-M5 and 7-M9 were obtained from MGI^-D^- clone 7 by selection of colonies that showed migration in agar in the presence of MGI (8). MGI^+D^- mutant 11-M14 was derived from MGI^+D^+ clone 11 by selection of a clone that did not show migration in agar. Normal myeloid precursors from SL mouse bone marrow were obtained by isolating the non-rosette-forming cells after C3 rosette formation and Ficoll-Hypaque density centrifugation (14). Cells were cultured in Eagle's medium with a 4-fold concentration of amino acids and vitamins (H-21, Grand Island Biological Co., Grand Island, NY) and 10% inactivated fetal calf serum. Induction of differentiation was tested by incubation with 25% conditioned medium, from lungs of mice injected with 5 μg of *Salmonella typhimurium* endotoxin, that contains MGI (10), or with 1 μM dexamethasone (Sigma Chemical Co., St. Louis, MO) (19).

Assays for Properties of Cells. Fc and C3 rosettes were assayed by a modification (9) of the method of Bianco *et al.* (22). Immune phagocytosis (IP) was assayed by incubating cells with Fc or C3 rosettes for 60 min at 37° centrifuging for 5 min at 500 × *g*, and dispersing the pellet in 0.1 ml of distilled water for 10 sec to lyse the unphagocytosed erythrocytes, followed by addition of 0.1 ml of Eagle's medium at twice the usual concentration. The percentage of cells with one or more ingested erythrocytes was determined on a May-Grunwald Giemsa stained smear prepared with a Shandon-Elliot cytocentrifuge. Lysozyme activity in cell extracts and in the growth medium (secreted) was assayed (23) by the decrease in turbidity (at 540 nm) of a suspension of *Micrococcus lysodeikticus* as described (13). Differentiation to mature cells was determined in colonies

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Abbreviations: MGI, protein inducer of differentiation in normal and leukemic myeloid cells; MGI^+ cells, cells inducible by MGI for some differentiation-associated properties; D^+ cells, cells that can be induced to differentiate to mature granulocytes and/or macrophages; SI, steroid inducer that can induce some differentiation-associated properties in myeloid leukemic cells; IP, immune phagocytosis; Con A, concanavalin A.

Table 1. Induction of differentiation by MGI and SI in MGI$^+$D$^+$ clone 11, its MGI$^+$D$^-$ mutant 11-M14, and MGI$^+$D$^-$ clone 5

Type of clone	Clone no.	Inducer	Inducibility for* Rosettes, % Fc	Rosettes, % C3	Immune phagocytosis, % Fc	Immune phagocytosis, % C3	Lysozyme, μg equiv./ 5 × 10^6 cells	% colonies with mature Macrophages	% colonies with mature Granulocytes
		None	0.3	3.2	0	0	0	0	0
MGI$^+$D$^+$	11	MGI	32.3	56.4	24.1	25.2	8.5	100†	50†
		SI	0.2	25.1	0	6.8	1.0	80†	0
		None	0.2	1.6	0	0	0	0	0
MGI$^+$D$^-$	11-M14	MGI	3.6	12.3	1.3	8.3	0.3	0	0
		SI	0.2	3.4	0	0	0	0	0
		None	0.1	2.1	0	0	0	0	0
MGI$^+$D$^-$	5‡	MGI	26.1	40.8	0	0	2.1	0	0
		SI	0.1	10.2	0	0	0	0	0

* Rosette formation, immune phagocytosis, and lysozyme were determined 4 days after seeding with 25% lung conditioned medium that contained MGI or with 1 μM dexamethasone (SI). Mature macrophages and granulocytes were determined 12 days after seeding cells in agar with 10% lung conditioned medium or 1 μM dexamethasone. Mature macrophages were found at 3 days after incubation with MGI and 9 days after incubation with SI.

† After incubation of clone 11 with MGI, the macrophage colonies contained 80–100% mature macrophages and colonies with mature granulocytes contained 90–95% macrophages and 5–10% granulocytes. With SI the macrophage colonies contained 20–40% mature macrophages.

‡ Similar results were obtained with MGI$^+$D$^-$ clones 2 and 13.

formed in agar (2, 3). Cells with macrophage morphology and phagocytosis of agar will be referred to as mature macrophages. Cells were tested for cap formation with purified fluorescein-labeled concanavalin A (Con A; Miles-Yeda, Rehovot) with or without 0.1 μg of vinblastine per ml as described (24).

RESULTS

Sequence of Differentiation in Normal and Clone 11 MGI$^+$D$^+$ Myeloid Leukemic Cells. Incubation of normal myeloid precursors from mouse bone marrow or MGI$^+$D$^+$ clone 11 myeloid leukemic cells with conditioned medium containing MGI resulted in the same sequence of differentiation. The first change observed was the induction of C3 and Fc rosettes, followed by the synthesis and secretion of lysozyme, the formation of mature macrophages, and mature granulocytes (14). We have now used an additional differentiation-associated marker, immune phagocytosis (IP) of erythrocytes coated with antibody (Fc IP) or with antibody and complement (C3 IP) (25, 26). C3 and Fc IP, but not phagocytosis of uncoated erythrocytes, were induced 4 hr after C3 or Fc rosettes and before the production of lysozyme in both the normal and clone 11 MGI$^+$D$^+$ myeloid leukemic cells (Fig. 1). Although C3 and Fc rosettes and IP were induced at the same time in the normal and clone 11 MGI$^+$D$^+$ leukemic cells, lysozyme and granulocytes appeared earlier in the normal cells.

Control of Fc and C3 Rosettes and IP. In contrast to the inducibility by MGI of all the differentiation markers in MGI$^+$D$^+$ clones 11 and 12, these clones were induced by the steroid inducer (SI) dexamethasone for C3 rosettes, C3 IP, lysozyme, and mature macrophages, but not for Fc rosettes, Fc IP, or mature granulocytes (Table 1 and Fig. 2). A MGI$^+$D$^-$ mutant (11-M14) derived from MGI$^+$D$^+$ clone 11, like other MGI$^+$D$^-$ clones (Table 1), was induced by SI only for C3 rosettes without induction of any of the other properties, and a MGI$^-$D$^-$ clone (no. 6) was induced by SI only for Fc rosettes. In MGI$^-$D$^-$ clone 7 and its MGI$^+$D$^+$ mutants, SI induced both C3 and Fc rosettes and IP (Table 2); in the mutant 7-M9, C3

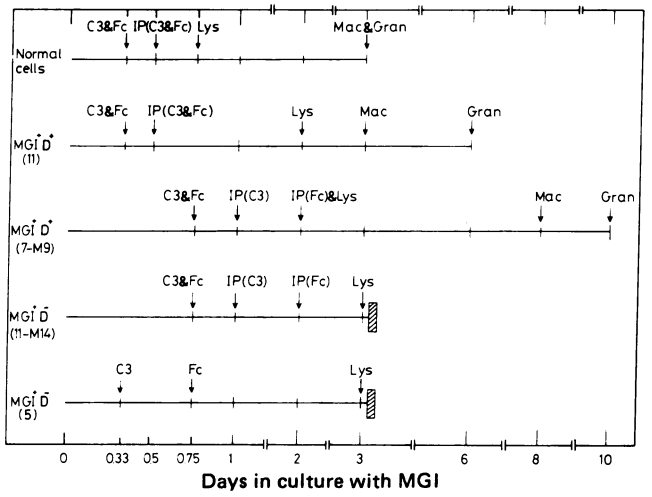

FIG. 1. Sequence and time of induction of different markers of normal myeloid differentiation by MGI. C3 and Fc, cells with a rosette; IP (C3 and Fc), cells with immune phagocytosis; Lys, lysozyme; Mac, mature macrophages; Gran, mature granulocytes; ▨, no further differentiation. MGI$^+$D$^-$ clones 2 and 13 gave results similar to those with clone 5. The times of induction with MGI are the times when induction was first detected.

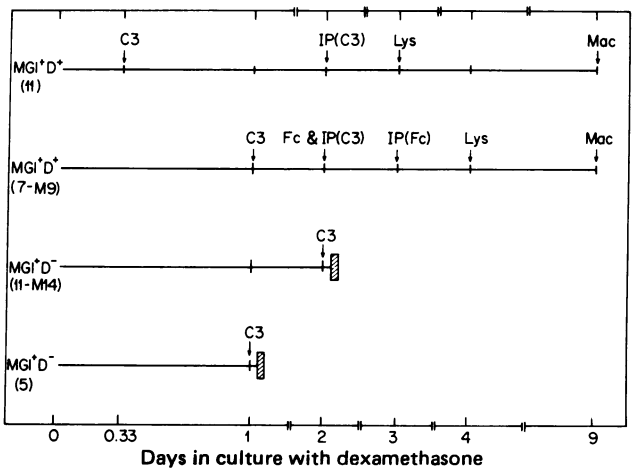

FIG. 2. Sequence and time of induction of different markers of normal myeloid differentiation by the steroid inducer (SI) dexamethasone. Symbols as in Fig. 1. The times of induction with SI are the times when induction was first detected.

Table 2. Induction of differentiation by MGI and SI in MGI⁻D⁻ clone 7 and its MGI⁺D⁺ mutant clones 7-M5 and 7-M9*

Type of clone	Clone no.	Inducer	Inducibility for: Rosettes, % Fc	Rosettes, % C3	Immune phagocytosis, % Fc	Immune phagocytosis, % C3	Lysozyme, μg equiv./ 5 × 10⁶ cells	% colonies with mature: Macrophages	% colonies with mature: Granulocytes
MGI⁻D⁻	7	None	2.3	8.6	0	0	0	0	0
		MGI	2.8	9.1	0	0	0	0	0
		SI	13.2	42.3	0.2	10.1	0	0	0
MGI⁺D⁺	7-M5	None	1.5	8.0	0	0	0	0	0
		MGI	5.2	24.2	1.1	10.2	1.8	100†	20†
		SI	15.4	39.1	0.6	12.2	0	80†	0
MGI⁺D⁺	7-M9	None	2.0	18.1	0	0	0	0	0
		MGI	20.1	45.6	3.5	22.1	3.6	100†	50†
		SI	18.2	40.6	2.8	16.4	0.3	100†	0

* The mutant 7-M5 is a spontaneous mutant and 7-M9 is a mutant obtained after treatment of clone 7 with nitrosoguanidine. These two clones are representative of the MGI⁺D⁺ mutants selected from clone 7. The induced properties were tested as in the footnote of Table 1.

† After incubation with MGI, the macrophage colonies contained 60–95% mature macrophages and the colonies that contained granulocytes had 90–95% mature macrophages and 1–5% mature granulocytes. With SI, macrophage colonies contained 40–60% mature macrophages.

rosettes were induced before Fc rosettes (Fig. 2). Normal myeloid cells did not differentiate or grow in the absence of MGI (11, 14), and there was also no induction of differentiation or growth by adding only SI to these normal cells.

Differences in the control of C3 and Fc rosettes and IP in different clones were also found with MGI (Fig. 1). Although in normal cells and MGI⁺D⁺ clones 11 and 12, C3 and Fc rosettes and C3 and Fc IP were induced together, in MGI⁺D⁻ mutant 11-M14 and MGI⁺D⁺ mutants 7-M5 and 7-M9, C3 and Fc rosettes were induced together but C3 IP was induced before Fc IP (Figs. 1 and 3). In MGI⁺D⁻ clones 2, 5, and 13, C3 rosettes were induced before Fc rosettes and there was no induction of C3 or Fc IP (Fig. 1 and Table 1).

Control of Lysozyme Production. Synthesis and secretion of lysozyme were induced in MGI⁺D⁺ clones 11 and 12 (13), 7-M5, and 7-M9 by MGI (Tables 1 and 2; Fig. 4) and SI (Tables 1 and 2). The study of other clones has shown that there was no induction of lysozyme by SI in clones that were induced only for C3 rosettes (MGI⁺D⁻ clones 2, 5, 13, and 11-M14), only for Fc rosettes (MGI⁻D⁻ clone 6), or for both C3 and Fc rosettes and C3 and Fc IP (MGI⁻D⁻ clone 7) (Tables 1 and 2). There was also induction of lysozyme by SI in MGI⁺D⁺ clones 11 and 12 that were not induced by SI for Fc rosettes or Fc IP.

MGI⁺D⁻ clones 2, 5, and 13, which were induced by SI only for C3 rosettes, were induced by MGI for C3 and Fc rosettes

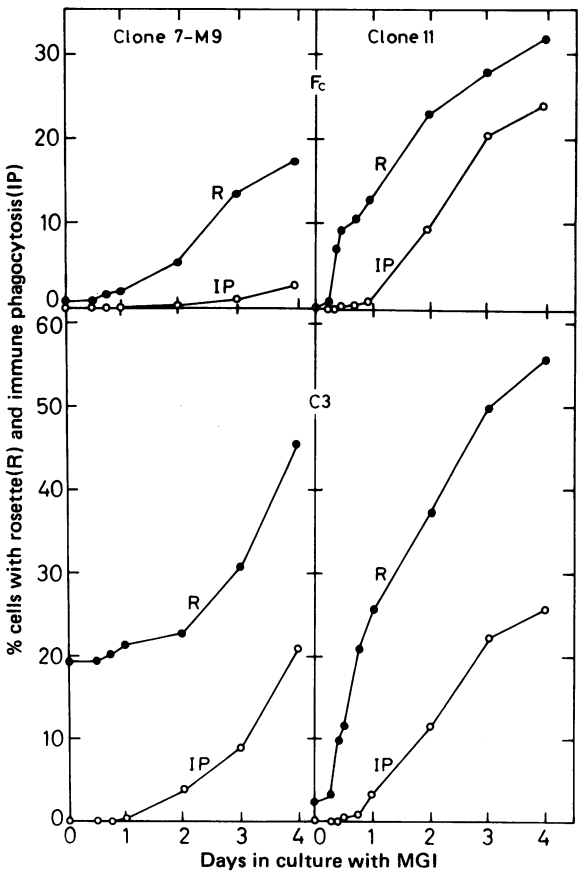

FIG. 3. Time of induction of Fc and C3 rosettes and immune phagocytosis by MGI in MGI⁺D⁺ clone 11 and MGI⁺D⁺ mutant 7-M9 derived from MGI⁻D⁻ clone 7. R, cells with a rosette; IP, cells with immune phagocytosis.

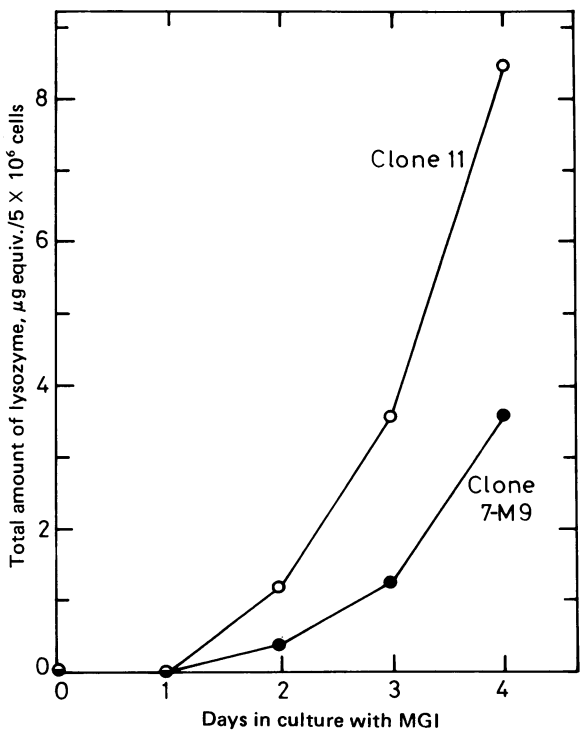

FIG. 4. Time of lysozyme induction by MGI in MGI⁺D⁺ clone 11 and MGI⁺D⁺ mutant 7-M9 derived from MGI⁻D⁻ clone 7. In both clones, 60–75% of the lysozyme was extracellular.

Table 3. Frequency of cells with and without a Con A-induced cap

Type of clone	Clone no.	% Con A cap-forming cells: Without vinblastine (F receptors)	% Con A cap-forming cells: Only with vinblastine (A receptors)	% cells without a cap even with vinblastine (O receptors)
MGI$^+$D$^+$	11, 12	60	37	3
MGI$^+$D$^-$	11-M14	20	30	50
MGI$^+$D$^-$	2, 5, 13	7	83	10
MGI$^-$D$^-$	7	45	12	43
MGI$^+$D$^+$	7-M5	50	21	29
MGI$^+$D$^+$	7-M9	90	2	8
MGI$^-$D$^-$	1, 6, 8	0	50	50
MGI$^-$D$^-$	10	0	0	100
Normal myeloid precursors		35	59	6

and lysozyme (Table 1). However, in contrast to normal cells and MGI$^+$D$^+$ clones where induction of IP precedes the production of lysozyme, in these MGI$^+$D$^-$ clones there was an induction of lysozyme without the induction of IP (Table 1). The data with MGI have also shown that in some MGI$^+$D$^+$ clones (11 and 12) lysozyme was induced after C3 and Fc IP, but in MGI$^+$D$^+$ clone 7-M9, lysozyme was induced at the same time as Fc IP (Figs. 1, 3, and 4). These results indicate that the induction of synthesis and secretion of lysozyme by MGI or SI is controlled independently of the induction of C3 and Fc rosettes and IP.

Control of Differentiation to Macrophages and Granulocytes. MGI$^+$D$^+$ clones 11 and 12 (8, 10), 7-M5, and 7-M9 (Table 2) were induced by MGI to form mature macrophages and granulocytes, but were induced by SI to form only macrophages (Tables 1 and 2). Clones 11 and 12 were induced by SI to form macrophages without Fc rosettes (19) or Fc IP (Table 1); clones 7-M5 and 7-M9 were induced by SI for all the differentiation associated properties except mature granulocytes (Table 2). Clones 11, 7-M5, and 7-M9 were induced by MGI for 95–98% mature macrophages and 2–5% mature granulocytes; clone 12 was induced for 40–45% macrophages and 55–60% granulocytes.

Cap Formation by Con A. After incubation with fluorescent Con A, myeloid leukemic cells can form a cap without vinblastine by receptors referred to as "free" (F) (27) or in the presence of vinblastine by receptors "anchored" (A) to tubulin (27) or the cells cannot form a cap even after the addition of vinblastine and these receptors may be "anchored" to structures other than tubulin (24) (O receptors). We have shown that with a similar total number of Con A receptors the ability of clones to be induced to differentiate by MGI was associated with the frequency of cells with F and O receptors (24). The results with all the cell types tested, including the new mutants, show that in normal myeloid precursors and all MGI$^+$D$^+$ clones, the frequency of cells with F receptors was more than 30% (35–90%) and higher than the frequency of cells with O receptors. All the D$^-$ clones compared to the D$^+$ clones had a lower frequency of cells with F, a higher frequency of cells with O, or both (Table 3).

Chromosome Constitution and Induction of Differentiation Mutants. Five MGI$^-$D$^-$ clones derived from five independently arising myeloid leukemias were tested for their ability to produce MGI$^+$ mutants spontaneously or after

Table 4. Induction of differentiation mutants*

Type of clone	Clone no.	No treatment, no. of mutants/ 10^4 colonies	Nitrosoguanidine, 1 μg/ml: Cloning efficiency after 20 hr, % of control	Nitrosoguanidine, 1 μg/ml: Cloning efficiency after 5 days, % of control	Nitrosoguanidine, 1 μg/ml: No. of mutants/ 10^4 colonies
MGI$^-$D$^-$	1	0	6	80	0
	6	0	10	95	0
	8	0	2	90	0
	10	0	20	100	0
	7	52†	5	85	180†
MGI$^+$D$^+$	11	10	0.1	4	890

* Mutants were detected by the formation of colonies in agar with or without cell migration after incubation with 5% lung conditioned medium (8). Cells were seeded for colony formation in agar without a previous treatment with nitrosoguanidine at 500–1000 cells per 50-mm petri dish. In the experiments with nitrosoguanidine, the cells were treated for 20 hr with 1 μg of the mutagen per ml, washed, grown in liquid medium for 5 days to allow expression of the mutations (28), and then seeded in agar at 10^3–10^5 cells per petri dish.

† The mutants without nitrosoguanidine were obtained from clone 7 that had been in culture 3 months longer than the time at which mutants were obtained with the mutagen. At this earlier time, spontaneous mutants were not detected.

treatment with *N*-methyl-*N*-nitro-*N*-nitrosoguanidine, as measured by ability of the cells to migrate in agar in the presence of MGI (8). MGI$^+$ colonies were obtained from MGI$^-$ clone 7, but there were no such colonies from any of the other 4 MGI$^-$ clones out of a total of 4×10^4 colonies scored (Table 4). Clone 7 showed 180 MGI$^+$ colonies per 10^4 colonies after treatment with nitrosoguanidine at the time when there were no spontaneous mutants. After further culture of this clone for 3 months, there were 52 spontaneous MGI$^+$ colonies per 10^4 colonies. Twenty of the nitrosoguanidine-induced and five of the spontaneous MGI$^+$ colonies were isolated and found to be stable after 6 months in culture. Clone 7-M5 is an example of a spontaneous and 7-M9 of a nitrosoguanidine-induced mutant. Both could be induced for all the properties found with MGI$^+$D$^+$ clones (Table 2), including induction of the normal requirement for MGI for cell viability and growth (11). The ability of MGI$^-$ clone 7 to produce MGI$^-$ mutants was associated with a difference in its chromosome constitution. This clone contained one normal and two abnormal chromosomes no. 12, whereas the other four MGI$^-$ clones had two normal chromosomes no. 12 (29). The mutants derived from clone 7 had lost either the one normal or one of the abnormal chromosomes no. 12 (29).

MGI$^+$D$^+$ clone 11 produced 10 spontaneous and 890 mutagen-induced colonies per 10^4 colonies that did not migrate in agar in the presence of MGI. The 10 spontaneous and 19 out of 20 nitrosoguanidine-induced variants tested reverted back to MGI$^+$D$^+$; this was already detected after about 1 week of culture of the isolated colonies. The one stable clone (11-M14) was MGI$^+$D$^-$ (Table 1); compared to the parental clone 11 (29), this mutant clone had lost the abnormal chromosome no. 12 found in clone 11 and one chromosome no. 4 was translocated onto an additional chromosome no. 15.

DISCUSSION

The present experiments used mutants of myeloid leukemic cells that differ in the timing and degree of inducibility of differentiation-associated properties by two different inducers,

the normal regulatory protein MGI and a steroid inducer, SI, dexamethasone. The results have shown that there are separate controls for the induction of C3 rosettes, Fc rosettes, C3 IP, Fc IP, lysozyme, mature macrophages, and mature granulocytes. Differences in inducibility of these properties by MGI and SI support our suggestion that MGI and SI have different cellular sites for induction (12). The finding of clones with induction of C3 IP without induction of Fc IP substantiates antibody blocking data (30) on the independence of these functions. In contrast to MGI^+ myeloid leukemic cells that can be induced to differentiate by the normal macrophage and granulocyte regulator MGI, Friend erythroleukemic cells (31) have lost the ability to be induced to differentiate by the normal erythroid regulator, erythropoietin (32). These cells can be induced to partly differentiate by various compounds, and there also appear to be different cellular sites for induction (33) and a difference in the type of induction (34) by different compounds. Erythroleukemic cells, like the MGI^- clone 7 of myeloid leukemic cells, may be able to give rise to mutants that can be induced to differentiate by the normal regulatory protein. It will be of interest whether, as in clone 7, the formation of such mutants is associated with a special chromosome constitution that allows the loss of genes that can suppress induction of differentiation by the normal regulatory protein (29).

The results obtained with different mutants have extended our previous data (24) regarding the association between the competence of a clone to be induced for mature cells by MGI and the relative frequencies of cells with "free" and specifically "anchored" surface Con A receptors. The inducing protein MGI, like other peptide hormones (35), presumably has receptors on the cell surface. It will be of interest to determine whether this association also applies to receptors for MGI. The results have, therefore, shown that appropriate mutants and different inducers can be used to dissect the controls that regulate the normal differentiation of myeloid leukemic cells.

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