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Different Blocks in the Differentiation of Myeloid Leukemic Cells

(surface receptors for erythrocytes sensitized with antibody and with antibody and complement/rosettes with sensitized erythrocytes/macrophages/granulocytes)

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ABSTRACT **Some clones of mouse myeloid leukemic cells (D^+) can be induced to undergo cell differentiation to mature macrophages and granulocytes, and other clones (D^-) could not be induced to differentiate to mature cells. Normal mature macrophages and granulocytes have surface receptors that form rosettes with erythrocytes coated with specific immunoglobulin or immunoglobulin-complement. The D^+ clones were induced to form receptors by prednisolone, cytosine-arabinoside, 5-iododeoxyuridine, actinomycin D, or serum from mice injected with endotoxin. All these compounds thus induced a common change in the cell surface membrane. The induction of receptors required protein synthesis, and receptors were formed before the appearance of mature cells. There were two types of D^- clones. One type was induced by these compounds to form receptors, although with a lower inducibility than D^+ clones; in the other type there was no induction of receptors. The results indicate that there are different blocks in the differentiation of myeloid leukemic cells. Some leukemic cells (IR^+D^+) can be induced to form receptors and to differentiate to mature cells; others (IR^+D^-) can form receptors but not mature cells; and a third type (IR^-D^-) could not be induced to form receptors or mature cells.**

Malignant cells blocked in various stages of cell differentiation can be of value in elucidating the mechanism of differentiation and the blocks that can occur during carcinogenesis. We have shown that some, but not all, undifferentiated mammalian myeloid leukemic cells can be induced to undergo differentiation to mature macrophages and granulocytes (1, 2) and had found two types of clones. One type (D^+) could be induced to undergo cell differentiation, and the other type (D^-) could not be induced to differentiate to mature cells (3–7).

Normal mature macrophages and granulocytes have surface receptors for immunoglobulin and immunoglobulin–complement that can be detected by the formation of rosettes with sensitized sheep erythrocytes (8, 9). The present studies were undertaken to determine the inducibility of these receptors on the surface of D^+ and D^- myeloid leukemic cells and the relationship of these receptors to the formation of mature cells. Cell differentiation to mature granulocytes and macrophages can be induced in D^+ leukemic cells by serum from mice injected with bacterial endotoxin (4). We have also found that differentiation of D^+ cells to mature macrophages can be induced by cytosine-arabinoside, actinomycin D, 5-iododeoxyuridine, or the steroid hormone prednisolone. In the present experiments we have, therefore, studied the induction of surface receptors on D^+ and D^- clones by these different compounds.

Abbreviations: D^+, clones of myeloid leukemic cells that can be induced to undergo differentiation to mature cells; D^-, clones that are inducible; IR, immunoglobulin and immunoglobulin-complement receptors on the cell surface; EA, erythrocytes sensitized with antibody; EAC, erythrocytes sensitized with antibody and complement; ara-C, cytosine-arabinoside; IdU, 5-iododeoxyuridine.

* This is publication no. VII in a series on "Control of normal differentiation of myeloid leukemic cells."

MATERIALS AND METHODS

Cells. Ten clones of myeloid leukemic cells were used. Three D^+(nos. 9, 11, and 21) and three D^-(nos. 5, 13, and 16) clones were isolated and clone-purified three times (3) from a myeloid leukemic cell line growing in culture obtained from an SL mouse with myeloid leukemia (10). The other four clones were four myeloid leukemic cell lines (1–4) (Sukster, Fibach, and Sachs, to be published) derived from four independently arising myeloid leukemias in SJL/J mice (11). The cells of all 10 lines produced leukemia after inoculation into adult animals and grew in suspension in liquid medium as myeloblasts. Cells were cultured in Eagle's medium with a 4-fold concentration of amino acids and vitamins and 10% (v/v) inactivated (56° for 30 min) horse or fetal-calf serum. The same results were obtained with both types of serum. In order to obtain the same concentration of cells for determinations of the percent of cells with receptors at 1, 2, 3, and 4 days after seeding, cells were seeded at 2.4, 1.2, 0.6, and 0.3×10^6 cells, respectively, in 5 ml of medium per 50-mm petri dish. The cells seeded at these four concentrations had the same growth rate. Normal mature macrophages and granulocytes were collected from the peritoneal cavity of mice 4 days after intraperitoneal injection of thioglycollate medium and 4 hr after intraperitoneal injection of 1% glycogen solution, respectively. The cells were washed three times with phosphate-buffered saline, pH 7.2, and suspended in Eagle's medium without bicarbonate, whose pH was adjusted to 7.0 with NaOH, before they were tested for surface receptors.

Sensitization of Sheep Erythrocytes with Antibody and Complement. The erythrocytes were sensitized as described (12). Sheep erythrocytes stored at 4° in Alsevers solution were washed three times with phosphate-buffered saline and suspended in phosphate-buffered saline to give a 0.5% (v/v) suspension. An equal volume of 1:1500 dilution of rabbit antiserum directed against sheep erythrocytes was added, and the mixture was incubated for 30 min at 37°. The erythrocytes sensitized with antibody (EA) were washed three times with phosphate-buffered saline and suspended at 0.5% (v/v) in Eagle's medium without bicarbonate, pH 7.0. Erythrocytes sensitized with antibody and complement (EAC) were ob-

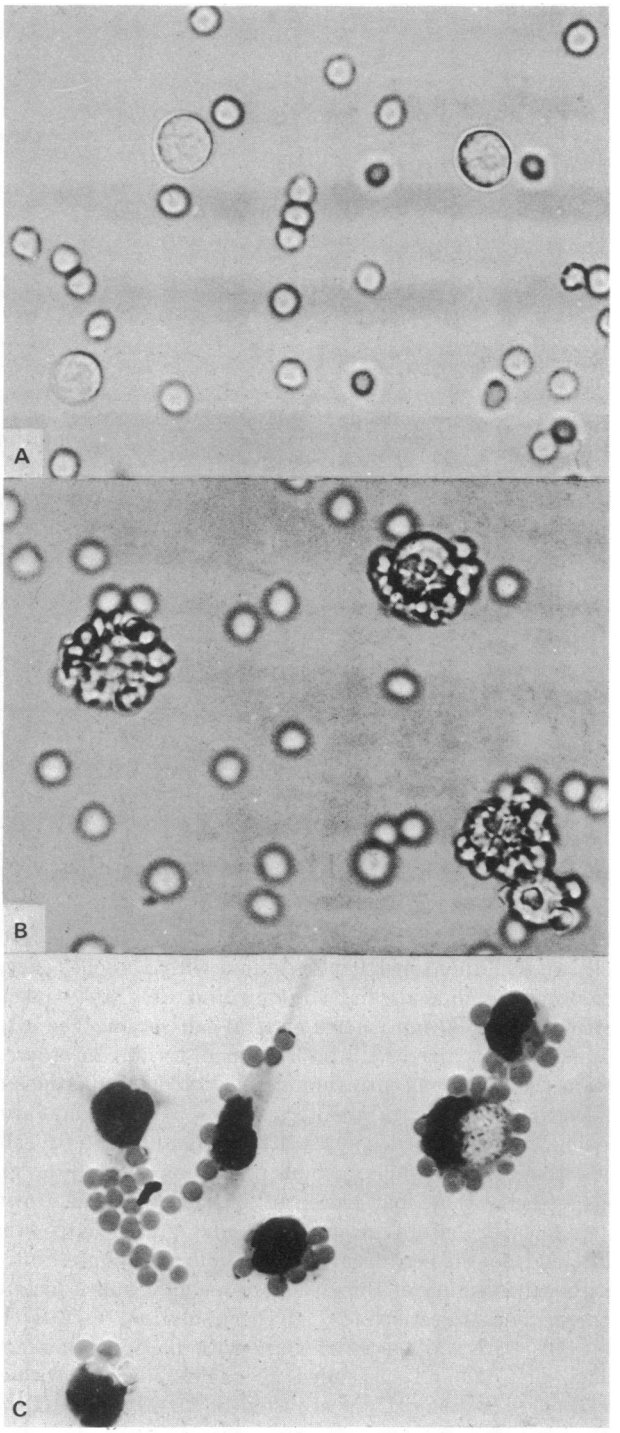

FIG. 1. Rosette formation by D^+ cells. (*A*) Untreated cells, ×680. (*B*) Rosettes induced by treatment with endotoxin serum, ×680. (*C*) Rosettes on cells attached to a petri dish after treatment with endotoxin serum; cells stained with May–Grunwald Giemsa, ×1038.

tained by incubating equal volumes of EA cells and a 1:10 dilution of fresh normal mouse serum for 30 min at 37°. The cells were then washed three times with phosphate-buffered saline and suspended in the same medium used for the EA cells.

Assay for Surface Receptors by Rosette Formation. EA or EAC cells (10^8) were mixed with 10^6 of the cells tested for receptors in 1 ml of medium in a 10-mm diameter plastic tube. The cells were centrifuged for 3 min at 500 × *g* (13) and incubated for 30 min at 37° without resuspending the pellet. The cells were then gently dispersed, and the percent of cells with EA or EAC rosettes was determined in a hemocytometer (12). A cell with at least 10 attached erythrocytes was counted as having a rosette, and 1000 cells were counted for each point. There were no rosettes with unsensitized sheep erythrocytes, and the formation of EA rosettes was inhibited by IgG. When the frequency of rosette-forming cells was higher than 10%, the standard deviation was about ±10%. When the frequency was lower than 10%, the standard deviation was ±20–30%. D^+ cells can be induced to attach to the surface of a petri dish (4). The attached cells were tested for receptors by addition of EA or EAC cells to the petri dish, incubation for 30 min at 37°, washing with phosphate-buffered saline, and staining with May–Grunwald Giemsa.

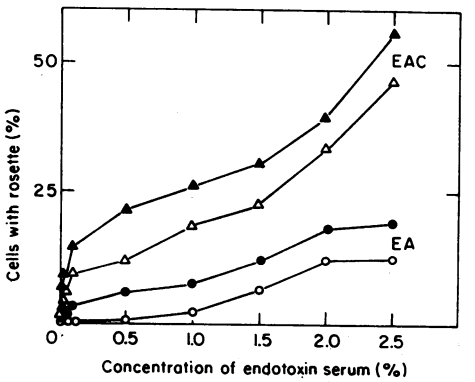

FIG. 2. Induction of EAC and EA receptors in D^+ and D^- cells by different concentrations of endotoxin serum. The percent of cells with rosettes was determined 4 days after the cells were seeded with endotoxin serum. ● and ▲, D^+ clone no. 21; ○ and △, D^- clone no. 5.

Compounds Used for Induction of Receptors. Serum from mice injected with endotoxin (endotoxin serum) was obtained 3 hr after intravenous injection of 5 μg of *Salmonella typhimurium* endotoxin (4). Cytosine-arabinoside and prednisolone were obtained from Sigma Chemical Co., and actinomycin D and 5-iododeoxyuridine were from Cal Biochem, Inc. Prednisolone was dissolved at 1 mg/ml in absolute ethanol and added at 1 μM, so that the final ethanol concentration was 0.05%.

RESULTS

Induction of Surface Receptors on D^+ and D^- Cells. Normal mature macrophages and granulocytes have two types of surface receptors that can be measured by the formation of rosettes with sheep erythrocytes. One type is detected with erythrocytes sensitized with antibody (EA) and the other type with erythrocytes sensitized with antibody and complement (EAC) (8, 9). Normal mouse peritoneal granulocytes and macrophages contained about 50–75% cells with EAC and 25–40% cells with EA receptors.

In control cultures tested at 0–4 days after seeding, there were 0.1–4% cells with EAC and 0–0.4% cells with EA in the D^+ clone no. 21. The D^- clone no. 5 had 0–2.5% cells with EAC and 0–0.1% cells with EA. The frequency of cells with receptors was increased in these D^+ and D^- clones by incubation with serum from mice injected with endotoxin

(endotoxin serum) (Figs. 1 and 2) but not by incubation with normal mouse serum. At the optimum concentration of endotoxin serum (2.5%), the frequency of cells with receptors was about 50% with EAC and about 15% with EA in both the D^+ and D^- clones (Fig. 2). The lowest concentration of endotoxin serum that induced the formation of receptors (0.05%) was also about the lowest concentration required for the differentiation of D^+ cells to mature granulocytes and macrophages and for the cloning of normal bone marrow cells *in vitro* (4).

An increase in the number of cells with receptors was first observed after 8–10 hr, and reached an optimum at 4 days after treatment. There was a more rapid initial increase in the D^+ than in the D^- clone (Fig. 3). D^+ cells can be induced to attach to the petri dish by treatment with endotoxin serum (4). About 80% of these attached cells contained EAC, and 60% contained EA receptors. Like the receptors found on normal mature macrophages and granulocytes (8), rosette formation with EAC was inhibited by 10 mM Na_3H EDTA. D^+ and D^- cells treated with endotoxin serum had the same growth rate, and the induction of receptors was observed before the appearance of mature differentiated D^+ cells. Incubation of cells with 1–100 μg/ml of purified trypsin (Worthington Biochemical Co.) for 30 min at 37° did not induce the formation of receptors.

Induction of receptors in D^+ and D^- cells was inhibited by cycloheximide (Table 1). D^+ and D^- cells washed after incubation with endotoxin serum for 24 hr showed a much lower frequency of cells with receptors at 4 days than cells incubated with endotoxin serum for 4 days. Cells washed after 6 hr of incubation showed the same low frequency of cells with receptors as the untreated cells at 4 days (Table 2). These results indicate that the induction of receptors requires protein synthesis and the presence of endotoxin serum for more than 6 hr.

Induction of Surface Receptors by Various Compounds. In addition to the induction of mature macrophages and granulocytes by endotoxin serum (4), the formation of mature macrophages can be induced in D^+ cells by cytosine-arabinoside (ara-C), actinomycin D, 5-iododeoxyuridine (IdU), and the steroid hormone prednisolone (to be published). All these compounds induced the formation of EAC and EA receptors, except prednisolone, which only induced EAC. The results with the optimum concentration are shown in Table 3. There was always a higher induction of receptors in D^+ than in D^- cells except with ara-C, which induced about the same frequency of EAC receptors in the D^+ and D^- clones (Table 3). Treatment with the optimum concentration of ara-C, actinomycin D, or IdU completely inhibited cell multiplication, and there was no apparent cell lysis during the 4 days of the experiments. At the optimum concentration of prednisolone and endotoxin serum, the cells multiplied at the same rate as the untreated control cultures, and there was also no apparent cell lysis. The induction of receptors, therefore, occurred both in the multiplying and nonmultiplying cultures. Experiments with ara-C and prednisolone have also shown that, as with endotoxin serum, induction of receptors was inhibited by cycloheximide and required the presence of these compounds for more than 6 hr.

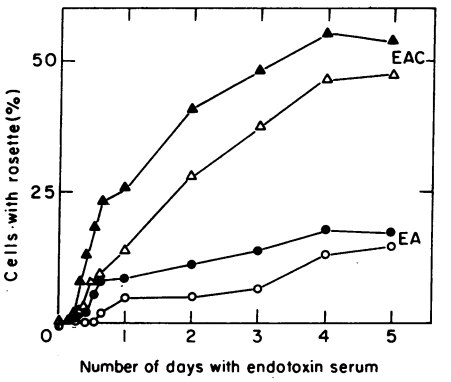

FIG. 3. Induction of EAC and EA receptors in D^+ and D^- cells at different times after the addition of 2.5% endotoxin serum. ● and ▲, D^+ clone no. 21; ○ and △, D^- clone no. 5.

D^- Clones That Could Not Be Induced to Form Surface Receptors. Three D^+ clones that could be induced to differentiate to form mature macrophages and granulocytes and seven D^- clones that could not be induced to produce these mature cells were tested for the induction of receptors. The results with endotoxin serum indicate (Table 4) that all three D^+ clones could be induced to form EAC and EA receptors. However, of the seven D^- clones, three were induced to form both types of receptors, and in four clones there was no induction of either EAC or EA. Similar results were obtained when induction was tested with ara-C or actinomycin D.

TABLE 1. *Inhibition of the induction of receptors by cycloheximide**

Clone no.	Clone type	Type of receptor	Cells with rosette (%) No treatment	Endotoxin serum, 2.5%	Endotoxin serum + cycloheximide, 5.6 μg/ml
21	D^+	EA	0.2 ± 0.1	7.4 ± 1.2	1.1 ± 0.2
5	D^-	EA	0	3.7 ± 0.8	0.5 ± 0.1
21	D^+	EAC	1.4 ± 0.1	23.5 ± 2.5	2.3 ± 0.2
5	D^-	EAC	0.5 ± 0.1	10.7 ± 2.0	1.0 ± 0.1

* Endotoxin serum was added at the time of seeding, and the percent of cells with rosettes was determined 16 hr after addition of endotoxin serum.

TABLE 2. *Effect of removal of endotoxin serum on the induction of receptors**

Clone no.	Clone type	Type of receptor	Cells with rosette (%) No treatment	Time with endotoxin serum 6 hr†	24 hr†	4 days
21	D^+	EA	0.1	0.2	4.6	18.6
5	D^-	EA	0	0.1	1.0	14.2
21	D^+	EAC	3.3	2.5	18.2	50.3
5	D^-	EAC	2.4	2.5	16.1	45.6

* The percent of cells with rosettes was determined 4 days after seeding.

† The cells were washed after 6 and 24 hr of incubation with endotoxin serum and then cultured in medium without this serum for 4 and 3 days, respectively.

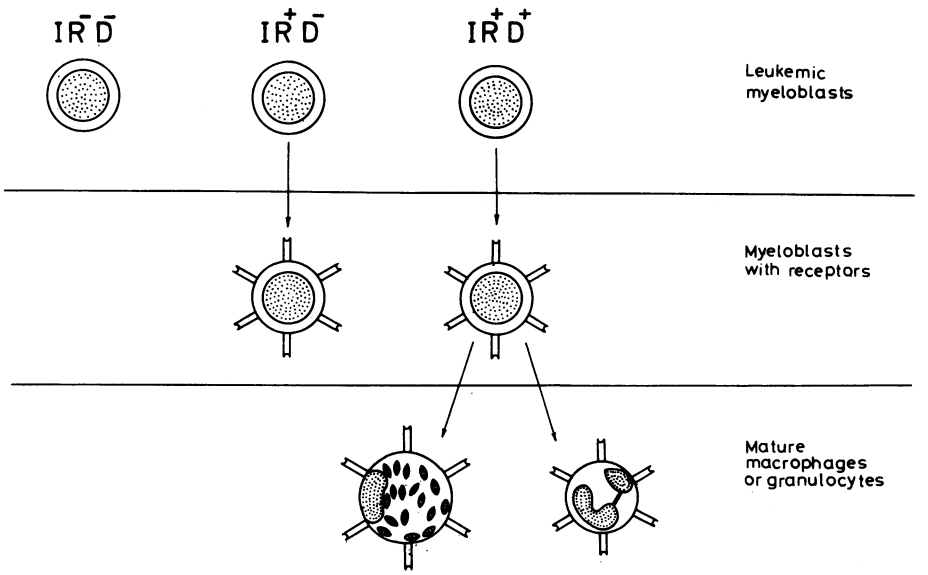

FIG. 4. Model of the blocks in cell differentiation in myeloid leukemic cells. IR, immunoglobulin and immunoglobulin–complement receptors; D, differentiation to mature macrophages and granulocytes.

DISCUSSION

The existence of D^+ and D^- clones has made it possible to test the relationship between the induction of cell differentiation in myeloid leukemic cells and the formation of receptors that are found on the surface of normal mature macrophages and granulocytes. Our results have shown that in D^+ leukemic cells, compounds that induced differentiation to mature cells induced the formation of these receptors and that there was an induction of receptors before the appearance of mature cells. As in the case of normal mature macrophages and granulocytes, the frequency of cells with EAC was higher than the frequency with EA receptors. Rosette formation with EAC in the leukemic cells was inhibited by EDTA. This result indicates that receptors induced in the myeloid leukemic cells are like those on normal mature macrophages and granulocytes and different from those on B lymphocytes (8). Rosette formation with EA was inhibited by IgG, and it will be of interest to determine the IgG subclass specificity of these receptors (14). The receptors could not be unmasked by treating myeloid leukemic cells with trypsin. Induction required protein synthesis and the presence of the inducing compound for more than 6 hr.

The formation of receptors was induced in D^+ cells by serum from mice injected with endotoxin, by cytosine-arabinoside, actinomycin D, 5-iododeoxyuridine, and the steroid hormone prednisolone. This steroid can also induce alkaline phosphatase activity in some, but not all, transformed fibroblasts (15). All these compounds induced the formation of EAC and EA, except prednisolone, which only induced EAC. These compounds also induce the attachment of D^+ cells to the surface of a petri dish (to be published), so that they all induced a common change in the cell surface membrane.

Some D^- clones could be induced to form receptors, although they showed a lower inducibility than D^+ cells, whereas in other D^- clones there was no induction of receptors by any of these compounds. The results show that there are different blocks in the differentiation of myeloid leukemic cells (Fig. 4). Some clones (IR^-D^-) (IR for immunoglobulin and immunoglobulin–complement receptors) could not be induced to form receptors or to differentiate to mature cells; other clones (IR^+D^-) could be induced to form receptors but not mature cells; and a third type of clone (IR^+D^+) could be induced to form receptors and to undergo differentiation to mature cells. This finding indicates that, starting with an undifferentiated blast cell, there are at least two stages in differentiation. The first involves the formation of immunoglobulin and immunoglobulin–complement receptors on the cell surface and the second the formation of mature differentiated cells. Undifferentiated teratoma cells have a surface antigen common to cleavage-stage embryonic cells (16). It will be of interest to determine the normal cells in the embryonic differentiation of white blood cells that correspond to the leukemic blast cells.

TABLE 3. *Induction of receptors by different compounds**

Clone no.	Clone type	Type of receptor	Cells with rosette (%)				
			No treatment	Ara-C†	Act. D†	IdU†	Prednisolone†
21	D^+	EA	0.4	4.0	1.3	2.9	0.4
5	D^-	EA	0.1	0.6	0.4	0.8	0.2
21	D^+	EAC	3.8	23.5	21.5	27.4	16.9
5	D^-	EAC	2.5	21.0	8.9	9.2	10.6

* The percent of cells with rosettes was determined 4 days after seeding with ara-C, actinomycin D (act. D), IdU, or prednisolone.
† Concentrations in μg/ml: ara-C, 0.1; actinomycin D, 0.005; IdU, 0.5; prednisolone, 0.4.

TABLE 4. *Induction of receptors by endotoxin serum* in D^+ and D^- clones*

Clone no.	Cell type	Cells with rosette (%)					
		EA			EAC		
		At time of seeding	1 day	5 days	At time of seeding	1 day	5 days
9	D^+	0	7.0	17.6	0.1	26.2	48.0
11		0	5.5	18.5	0.1	30.0	54.0
21		0	7.2	17.5	0.2	28.3	53.8
5	D^-	0	3.5	15.0	0	16.2	46.0
13		0	4.0	16.0	0	18.2	48.2
16		0	4.3	17.5	0	17.0	46.5
1	D^-	0	0	0	0	0	0
2		0	0	0	0	0	0
3		0	0	0	0	0	0
4		0	0	0	0	0	0

* 2.5% endotoxin serum.

Note Added in Proof. We have now found that the formation of rosettes by EA receptors on the IR^+ myeloid leukemic cells and on mature normal cells was inhibited (90% inhibition with 200 μg/ml) by mouse IgG2a but not by IgG2b, IgG1, IgA, or IgM. This result indicates that the EA receptors induced on the leukemic cells have the same IgG sublcass specificity as the EA receptors on normal cells.

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