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Indirect induction of differentiation in myeloid leukemic cells by lipid A

(lipopolysaccharide/genetic susceptibility/macrophages/MGI protein)

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ABSTRACT **Normal myeloid and MGI^+D^+ clones of myeloid leukemic cells can be induced for Fc and complement component 3 rosettes, lysozme, and mature macrophages and granulocytes by a protein with macrophage- and granulocyte-inducing (MGI) activity, whereas MGI^+D^- clones can be induced by this protein for rosettes and lysozme but not mature cells. Lipopolysaccharides (LPS) from different bacteria induced the appearance of rosettes, lysozyme, and macrophages in some MGI^+D^+ clones but did not induce any of these changes in MGI^+D^- clones. Lipid A gave the same results as LPS. Incubation of MGI^+D^+ cells with LPS also induced an MGI activity detectable in the culture medium. This activity behaved like MGI in inducing (*i*) rosettes, lysozyme, and mature cells in MGI^+D^+ leukemic cells including a clone resistant to LPS, (*ii*) rosettes and lysozyme in MGI^+D^- leukemic cells, and (*iii*) differentiation of normal myeloid cells to mature macrophages and granulocytes. This activity was induced in MGI^+D^+ cells by LPS before the induction of rosettes or lysozyme. The results indicate that the lipid A portion of LPS indirectly induces differentiation of MGI^+D^+ myeloid leukemic cells by inducing MGI protein. It is suggested that induction of specific regulatory proteins may be a more general mechanism for the induction of differentiation by surface-acting compounds.**

The normal macrophage- and granulocyte-inducing protein (1–4), which we now call MGI (5, 6), can induce the differentiation of normal myeloid precursors (1–4, 7) and of some MGI^+ clones of myeloid leukemic cells (6, 8–11) to mature macrophages and granulocytes. Certain MGI^+ clones (MGI^+D^+) can be induced by MGI for Fc and complement component 3 (C3) rosettes (10, 12), immune phagocytosis (13), lysozyme synthesis and secretion (14), and mature cells (6, 8, 9), whereas other clones (MGI^+D^-) can be induced for rosettes and lysozme but not for mature cells (10, 14). The inducing activity (1–4) that we now call MGI has also been referred to as "colony-stimulating" factor (15) or activity (16). Some of the stages of differentiation can be induced in MGI^+D^+ clones by certain steroid hormones (10, 17) and compounds such as cytosine arabinoside, actinomycin D, 5-iododeoxyuridine, and dimethyl sulfoxide (10, 14, 18).

Bacterial lipopolysaccharides (LPS) are presumably surface-acting compounds that can induce various changes including stimulation of cell multiplication and antibody formation by B lymphocytes (19, 20), production of pyrogens, and cytotoxicity (21–23). It has been shown that the active part of LPS in inducing these changes is lipid A (19–23). The present experiments were undertaken to determine whether LPS from different bacteria can induce differentiation of myeloid leukemic cells, whether the active part of the molecule is lipid A, and whether induction of differentiation by this presumably surface-acting compound is direct or is indirect by inducing the production of MGI in the myeloid leukemic cells.

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MATERIALS AND METHODS

Cells and Cell Cultures. MGI^+D^+ clones 9, 11, 12, and 21 and MGI^+D^- clones 2, 5, and 13 (9, 10) were obtained from a cell line of myeloid leukemia originating in an SL mouse (24). All the clones grew in suspension as myeloblasts or promyelocytes and produced leukemia after inoculation into isologous adult mice. Normal mature macrophages were collected from the peritoneal cavity of SL mice at 3 days after intraperitoneal injection of 1 sodium caseinate (Difco). The cells were cultured in tissue culture petri dishes (Nunc, Denmark) 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 (56° for 30 min) horse serum. Unless otherwise stated, the leukemic cells were seeded for the experiments at 3×10^5 cells per 50-mm petri dish. Counts of the number of cells in the cultures at different times after seeding showed a reproducibility of ±15%. Normal mouse bone marrow cells were tested for the formation of colonies with macrophages or granulocytes in agar as described (1–3).

Assays for Fc and C3 Rosettes, Lysozyme, and Mature Cells. Unless otherwise state, these assays were run with cells grown in liquid medium. Fc rosettes with sheep erythrocytes coated with antibody and C3 rosettes with sheep erythrocytes coated with antibody and complement were assayed after incubation for 30 min at 37° by a modification (10) of the method of Bianco *et al.* (25). For cells counted in suspension, the percentage of cells with 10 or more attached erythrocytes was determined by counting about 1000 cells per sample in a hemocytometer. For cells counted for rosettes when attached to the petri dish, cells with three or more attached erythrocytes were counted as a rosette. The results generally showed a standard deviation of ±15% when the percentage of rosette-forming cells was higher than 10%. With lower percentages the standard deviation was generally ±25%. Lysozyme activity in cell extracts and in the growth medium (secreted) was assayed according to Gordon *et al.* (26) by the decrease in turbidity (at 540 nm) of a suspension of *Micrococcus lysodeikticus* as described (14), with hen egg-white lysozyme (Miles Laboratory, Kankakee, IL) as a standard. The standard deviation of the

Abbreviations: MGI, protein inducer of differentiation in normal and leukemic myeloid cells; MGI^+ cells, cells that are inducible by MGI for some differentiation-associated properties; D^+ cells, cells that can be induced to differentiate to mature granulocytes and/or macrophages; C3, third component of complement; LPS, lipopolysaccharide.

Table 1. Induction of Fc and C3 rosettes, lysozyme, and macrophages by LPS in different clones of myeloid leukemic cells

Clone type	Clone	% cells with rosette: Fc −LPS	Fc +LPS	C3 −LPS	C3 +LPS	Lysozyme, μg equiv/ 5 × 10⁶ cells +LPS	% macrophages +LPS
MGI⁺D⁺	11	0.3	51	1.6	100	20.9	100
	21	0.4	10	1.0	35	3.3	10
	9	0.1	7	0.4	15	0.9	5
	12	0.0	0.1	0.6	3.5	<0.05	<0.01
MGI⁺D⁻	13	0.4	0.6	1.5	1.6	0	0
	5	0.1	0.3	0.7	0.6	0	0

Rosettes, lysozyme, and macrophages were determined 4 days after seeding with or without LPS (10 μg/ml) from *S. typhimurium*. There was no lysozyme or macrophages in any of the clones without LPS. The percentage of cells with rosettes was tested in cells attached to the petri dish in clone 11 and, in the other clones, in attached cells, if present, and in the nonattached cells.

lysozyme assays was generally up to ±20%. Cells were assayed for the ability to form mature macrophages or granulocytes in liquid medium or in agar (1–3). Cells exhibiting macrophage morphology and phagocytosis will be referred to as mature macrophages.

Assays for MGI Activity. Conditioned media were assayed for MGI with normal and leukemic myeloid cells. In the assay with normal cells, normal bone marrow cells were assayed in agar for the ability to form colonies with differentiation to macrophages and granulocytes (1–3). In the assay with myeloid leukemic cells, the conditioned media were assayed in liquid medium on MGI⁺D⁺ clone 11, MGI⁺D⁻ clone 2, and MGI⁺D⁺ clone 11RLPS derived from MGI⁺D⁺ clone 11. Clone 11RLPS was obtained after growth of clone 11 for about 2 months in mass culture with LPS at 1 μg/ml, cloning in the presence of the same concentration of LPS, and continued culture of an isolated clone in the presence of LPS at 1 μg/ml. Clone 11RLPS was not induced to differentiate by LPS, but it could still be induced to differentiate, like its parental MGI⁺D⁺ clone 11, for rosettes, lysozyme, and mature cells by conditioned medium from mouse lungs containing MGI (13).

Sources of LPS and Lipid A. LPS from *Salmonella typhimurium*, *S. enteritidis*, *S. minnesota* 9700, *Escherichia coli* 026:B6, and *E. coli* 055:B5 were obtained from Difco. LPS from *S. minnesota* R595, which contains lipid A and 3-deoxy-D-mannooctulosonic acid and lacks the O chains and most of the core, was kindly provided by Otto Lüderitz (Max Plank Institute fur Immunbiologie, Freiburg, West Germany) and lipid A isolated from an *E. coli* Re LPS was kindly supplied by Chris Galanos (Max Plank Institute für Immunbiologie, Freiburg, West Germany).

RESULTS

Induction of Fc and C3 Rosettes, Lysozyme, and Macrophages in Myeloid Leukemic Cells after Treatment with LPS. Four MGI⁺D⁺ and three MGI⁺D⁻ myeloid leukemic clones were tested for their ability to be induced to differentiate by LPS. MGI⁺D⁺ clones, but none of the MGI⁺D⁻ clones, were induced for Fc and C3 rosettes, the production and secretion of lysozyme, and the formation of macrophages. Of the four MGI⁺D⁺ clones tested, the highest degree of induction was obtained with clone 11 and the lowest with clone 12. With clone 11, 100% of the cells were induced to form C3 rosettes and mature macrophages (Table 1) and about 90% of the induced lysozyme was extracellular. No induction of differentiation to intermediate or mature granulocytes was obtained in any of the MGI⁺D⁺ clones even after 15 days' incubation with LPS. After incubation of clone 11 with LPS (10 μg/ml), induction of C3 and Fc rosettes was first detected at about 12 hr and lysozyme, at 3 days. Lysozyme was then produced at a linear rate for 3 days and then at a decreased rate (Fig. 1). With different concentrations of LPS, induction of lysozyme in clone 11 was detected after 4 days' incubation with LPS (2×10^{-2} μg/ml).

In the absence of LPS, all the clones had a similar growth rate. Counts of the number of cells at various days after seeding indicated that with LPS at 10 μg/ml the 100% induction of macrophage differentiation in clone 11 was associated with an apparently complete inhibition of cell multiplication (Fig. 2). Therefore, in this respect these differentiated cells behaved like normal macrophages. In MGI⁺D⁺ clones 21 and 12 and MGI⁺D⁻ clones 5 and 13, there was no decrease in the total number of cells after 3 days in culture with LPS. However, at this time MGI⁺D⁺ clone 9 showed 70% less cells than did the untreated control and MGI⁺D⁻ clone 2 gave about 95% cell lysis after 1 day. The few remaining cells in clone 2 did not show any differentiation-associated properties.

Induction by LPS from Different Bacteria and by Lipid A. The induction of differentiation in MGI⁺D⁺ clones by LPS was similar with LPS obtained from *S. typhimurium*, *S. minnesota*, *S. enteritidis*, *E. coli* 026:B6, and *E. coli* 055:B5 and with LPS from *S. minnesota* R595 which contains lipid A and 3-deoxy-D-mannooctulosonic acid and lacks the O chains and

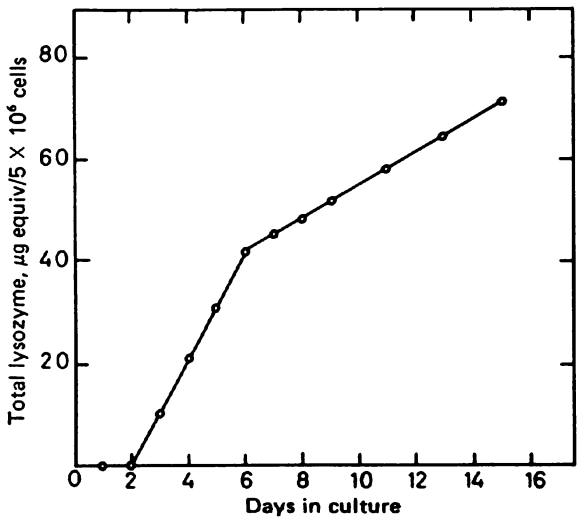

FIG. 1. Total amount of lysozme produced by MGI⁺D⁺ clone 11 at different times in culture with LPS (10 μg/ml) from *S. typhimurium*.

Table 2. Induction of Fc and C3 rosettes and lysozyme in MGI$^+$D$^+$ clone 11 by LPS from different bacteria and by lipid A

Origin of LPS and lipid A	% cells with rosette		Lysozyme, μg equiv/ 5×10^6 cells
	Fc	C3	
S. typhimurium	61	100	20.9
S. enteritidis	50	100	20.8
S. minnesota 9700	45	100	18.3
E. coli 026:B6	50	100	20.0
E. coli 055:B5	50	100	20.3
S. minnesota R595	75	100	21.9
Lipid A from *E. coli*	48	100	24.1

Rosettes and lysozyme were determined 4 days after seeding with LPS or lipid A at 10 μg/ml.

most of the core. Lipid A gave results similar to those with LPS (Table 2). These results indicate, that the differentiation of MGI$^+$D$^+$ clone 11 can be induced by LPS from different bacteria and that lipid A is as active as LPS.

Induction of MGI Activity in MGI$^+$D$^+$ Myeloid Leukemic Cells Treated with LPS or Lipid A. In order to determine whether incubation with LPS induces the production of MGI activity and whether this precedes the induction of Fc and C3 rosettes or lysozyme, conditioned medium from MGI$^+$D$^+$ clone 11 cells incubated with LPS was assayed for the presence of MGI. Conditioned medium harvested after 24 hr of incubation of clone 11 with LPS (10 μg/ml) induced colonies with normal bone marrow cells, of which 30% contained mature macrophages and 70%, mature granulocytes. There was no such colony formation after incubation of normal bone marrow cells with LPS or lipid A at 10 μg/ml. This conditioned medium from LPS- or lipid A-treated clone 11 cells was also able to induce Fc and C3 rosettes and lysozyme without cytotoxicity in MGI$^+$D$^-$ clone 2 (which showed a high cytotoxicity and no induction with LPS) and rosettes, lysozyme, and macrophages with MGI$^+$D$^+$ clone 11RLPS (resistant to LPS and which was not induced for any of these properties by LPS or lipid A). There was no induction of any of these properties by conditioned medium from clone 11 not treated with LPS or lipid A. Results obtained with serum-free conditioned medium obtained after incubation of clone 11 with LPS showed that the inducing activity in this conditioned medium for Fc and C3 rosettes and lysozyme was trypsin-sensitive (Table 3). Normal macrophages can be induced for a MGI activity by LPS (27, 28). This inducing activity from normal peritoneal macrophages was also trypsin-sensitive. These results indicate that the inducing activity in the conditioned medium from clone 11 was not LPS or lipid A but was a trypsin-sensitive activity that behaves like MGI.

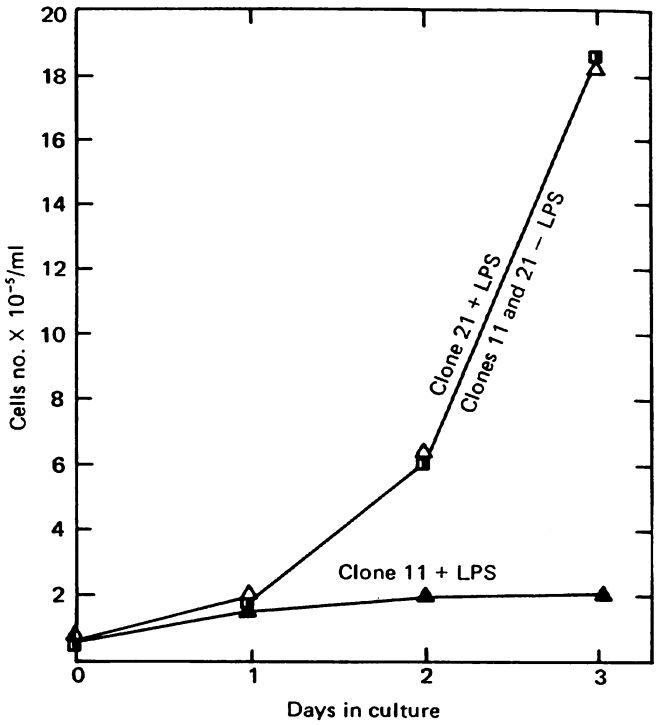

FIG. 2. Number of cells of MGI$^+$D$^+$ clone 11 and MGI$^+$D$^+$ clone 21 cultured with or without LPS (10 μg/ml) from *S. typhimurium*.

To determine the time of production of this MGI activity in relation to the time of induction of Fc and C3 rosettes or lysozyme, clone 11 was incubated with LPS (10 μg/ml) and the conditioned medium was tested for MGI activity by its ability to induce lysozyme production in MGI$^+$D$^+$ clone 11RLPS. The results (Fig. 3) indicate that MGI activity in the medium was already detectable at 6 hr after addition of LPS, whereas the earliest induction detectable with any of the other properties was the induction of rosettes at 12 hr. This indicates that MGI production was detected before any of the other differentiation-associated properties tested.

DISCUSSION

MGI$^+$D$^+$ clones of myeloid leukemic cells can be induced for Fc and C3 rosettes, lysozyme, and mature macrophages and granulocytes by the differentiation-inducing MGI protein

Table 3. Trypsin sensitivity of MGI induced by LPS in MGI$^+$D$^+$ clone 11 and normal peritoneal macrophages

CM induced by LPS from:	Induction in MGI$^+$D$^+$ clone 11								
	% cells with rosette						Lysozyme, μg equiv/5 × 10^6 cells		
	Fc			C3					
	Control	CM	Trypsinized CM	Control	CM	Trypsinized CM	Control	CM	Trypsinized CM
MGI$^+$D$^+$ clone 11	0.1	20.0	0.1	0.8	89.0	2.6	0	6.9	0
Normal macrophages	0.1	21.7	2.0	0.4	95.0	6.1	0	8.4	0

Rosettes and lysozyme were determined 4 days after seeding. To prepare serum-free conditioned medium (CM), at 1 day after seeding with LPS (10 μg/ml) from *S. typhimurium* MGI$^+$D$^+$ clone 11 and normal peritoneal macrophages were washed and the cells were cultured for 3 more days in medium without serum or LPS. The CMs were incubated with trypsin (Worthington Biochemical Corp. NJ) at 400 μg/ml for 2 hr at 37° and then soybean trypsin inhibitor (Sigma Chemical Corp,. St. Louis, MO) at 400 μg/ml was added before testing (at a final concentration of 10% CM). The activity of the CM was not destroyed when trypsin and soybean trypsin inhibitor were added to the CM at the same time.

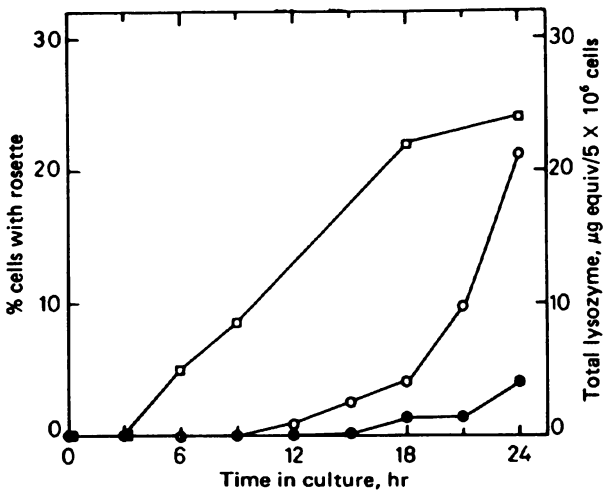

FIG. 3. Time of induction of MGI activity (□) and Fc (●) and C3 (○) rosettes after seeding 1.5 × 10^6 cells per ml of MGI$^+$D$^+$ clone 11 with LPS (10 μg/ml) from *S. typhimurium*. The presence of MGI activity in the medium of the treated cells was tested by the ability of the undiluted conditioned medium to induce lysozyme in MGI$^+$D$^+$ clone 11RLPS. This clone was not inducible for lysozyme by additions of LPS at 10 μg/ml.

obtained from cultured fibroblasts (8) or from lungs or serum of mice injected with LPS (10–13). The present experiments have shown that some MGI$^+$D$^+$ clones, but not MGI$^+$D$^-$ clones, can be induced to differentiate to mature macrophages by the presumably cell-surface-acting LPS from different bacteria. Lipid A was as active as LPS. The results have also shown that treatment of MGI$^+$D$^+$ myeloid leukemic cells with LPS or lipid A induces an activity in the conditioned medium that behaves like MGI. LPS and lipid A can be distinguished from MGI by differences in induction with normal myeloid cells, with MGI$^+$D$^-$ clones, and with MGI$^+$D$^+$ clone 11RLPS that is resistant to induction of differentiation by LPS. As expected, the inducing activity of the protein MGI, but not LPS or lipid A, is sensitive to trypsin (Table 4). It has been shown that purified MGI from fibroblasts has a molecular weight of about 68,000 (5, 29, 30) and that purified MGI from lungs has a molecular weight of about 23,000 (31). It will be of interest to determine the size and other chemical properties of the MGI produced by MGI$^+$D$^+$ cells and whether the lower molecular weight MGI is derived from the higher molecular weight MGI.

Studies on the time of induction of detectable MGI by LPS in MGI$^+$D$^+$ cells have indicated that induction of MGI was detected before the induction of Fc and C3 rosettes or lysozyme. The results therefore suggest that induction of differentiation in MGI$^+$D$^+$ myeloid leukemic cells by LPS and lipid A is due to an indirect mechanism and that differentiation in these cells was induced by MGI. It will be of interest to determine which of the other compounds (10, 17, 18) that can induce differentiation-associated properties in MGI$^+$D$^+$ leukemic cells act directly and which, like LPS or lipid A, may induce differentiation indirectly by inducing the production of MGI. MGI$^+$D$^+$ cells differ from MGI$^+$D$^-$ cells in their chromosome constitution (11, 32). This indicates that induction of differentiation by LPS and lipid A may be associated with a special genotype. The induction of MGI in leukemic cells of the appropriate genotype by LPS or lipid A, and possibly other compounds, may thus be a useful approach to the therapy of these leukemias.

In addition to LPS and lipid A, there are other presumably surface-acting compounds, such as lectins, that can induce cellular changes in various types of cells (33–35). It can be suggested that these other compounds may also induce these changes indirectly. The induction of specific regulatory molecules by certain cell types, including proteins that can induce specific cell differentiation, may therefore be a more general mechanism to explain the action of these surface-acting compounds. This mechanism may play a significant role in determining the induction of specific cell behavior during development by surface-acting molecules (33, 36, 37).

Table 4. Differences in the inducing activity of LPS and MGI

Inducing activity	LPS	MGI
Differentiation of MGI$^+$D$^+$ clones 9,11,12,21	+	+
Differentiation of MGI$^+$D$^+$ clone 11RLPS*	−	+
Differentiation of MGI$^+$D$^-$ clones 2,5,13	−	+
Colonies with differentiation from normal myeloid cells	−	+
Sensitivity to trypsin	−	+

* MGI$^+$D$^+$ clone 11RLPS was derived from MGI$^+$D$^+$ clone 11 by continued growth in the presence of LPS.

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