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Prolongation of Survival Time of Mice Inoculated with Myeloid Leukemia Cells by Inducers of Normal Differentiation[1]

Yoshio Honma,[2] Takashi Kasukabe, Junko Okabe, and Motoo Hozumi

Department of Chemotherapy, Saitama Cancer Center Research Institute, Ina-machi, Kitaadachi-gun, Saitama-362, Japan

ABSTRACT

Studies were made on the effects of inducers on the leukemogenicity of sensitive mouse myeloid leukemia cells (M1) that could be induced to undergo cell differentiation into mature granulocytes and macrophages *in vitro* by incubation with inducers (certain proteins, bacterial lipopolysaccharides, or glucocorticoids) and of resistant M1 cells that could not be induced to differentiate into mature cells. Inducers of cell differentiation significantly enhanced the survival times of mice inoculated with sensitive cells but scarcely affected the survival times of mice inoculated with resistant cells. Some mice inoculated with the sensitive cells and treated with lipopolysaccharide did not develop leukemia. The sensitive and resistant clone cells contained similar common tumor-related surface antigens. Treatment with lipopolysaccharide was also effective in athymic nude mice inoculated with the sensitive M1 cells. Lipopolysaccharide or glucocorticoid significantly stimulated differentiation of the sensitive cells cultured in a diffusion chamber *in vivo* but had little effect on differentiation of resistant cells. These results suggest the possibility of treating, with partial success, leukemia *in vivo* with differentiation inducers.

INTRODUCTION

Treatment with various inducers *in vitro* causes mouse myeloid leukemia (M1) cells to differentiate into forms that are functionally and morphologically similar to macrophages and granulocytes (14, 16, 27). Syngeneic mice inoculated with untreated M1 cells all died of leukemia, but on treatment with an inducer *in vitro* the M1 cells lost their leukemogenicity (17). We previously showed that resistant M1 cells that could not be induced to differentiate into mature cells were much more leukemogenic than were sensitive cells that could be induced to undergo cell differentiation (11). These findings, which suggest that leukemogenicity of M1 cells in syngeneic mice is related to *in vivo* inducibility of differentiation of the cells, may have significant implications for leukemia therapy.

The present experiments were undertaken to clarify 3 questions. (*a*) Is the leukemogenicity of sensitive cells affected by treatment with inducers of cell differentiation? (*b*) Do resistant and sensitive cells differ in immunogenicity? (*c*) Can the possible decrease of leukemogenicity caused by inducers be separated from enhancement of T-lymphocyte-mediated immune responses?

MATERIALS AND METHODS

Animals. Inbred SL mice were maintained as reported previously (11). Athymic nude mice with a BALB/c genetic background were supplied by CLEA Japan Inc., Tokyo, Japan, and housed under specific-pathogen-free conditions using Clean Racks (Sanki Kogyo, Tokyo, Japan). Five-week-old female mice were used for experiments.

Cells and Cell Culture. The cells used were 3 clones of the M1 cell line isolated from a spontaneous myeloid leukemia in an SL mouse (13). The cells were cultured in suspension in 6-cm Falcon plastic dishes in Eagle's minimum essential medium with twice the normal concentrations of amino acids and vitamins and supplemented with 10% heat-inactivated calf serum. The cells were cultured at 37° in a humid atmosphere at 5% CO_2 in air.

Administration of LPS[3] and Dexamethasone. Preparations of LPS (Boivin type) from *Salmonella typhimurium, Salmonella abortus-equi,* and *Escherichia coli* 026:B6 were obtained from Difco Laboratories, Detroit, Mich. LPS was dissolved in sterilized PBS, and 0.2 ml of the solution was injected i.p. into mice twice a week. Dexamethasone was purchased from Sigma Chemical Co., St. Louis, Mo. Stock steroid solution (20 mg/ml) was prepared in absolute ethanol. Mice were given i.p injections of 0.2 ml of PBS containing 1 μl of the steroid solution (20 μg/mouse) 3 times a week. The first dose of LPS or dexamethasone was injected 3 hr after tumor challenge.

Test of Phagocytosis. Phagocytic activity was assayed as reported previously (10). Cells were inoculated at a concentration of 4 to 5 × 10^5 cells/ml into 2 ml of culture medium and incubated with 0.1 ml of LPS solution diluted with PBS. After 48 hr, the cells were washed and incubated for 4 hr with a suspension of polystyrene latex particles (2 μl/ml of serum-free culture medium). Then the cells were washed thoroughly 4 times with PBS, and the percentage of phagocytic cells was calculated.

Culture and Morphological Examination of Cells in Diffusion Chambers. The chambers were made as described previously (11), and 0.1 ml of cell suspension (5 × 10^4 cells) was placed in each. Two chambers were implanted into the peritoneal cavity of each mouse. The cells were collected by the Ficoll-pronase method for chamber processing (2). Cell types were determined from their morphology after staining with May-Grünwald-Giemsa.

Antiserum. M1 cells were incubated with mitomycin C, 25 μg/ml, at 37° for 30 min. Then they were washed 3 times with PBS and resuspended in PBS to give 5 × 10^7 cells/ml. Syngeneic SL mice were given s.c. injections of 0.2 ml of cell

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[2] To whom requests for reprints should be addressed.

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[3] The abbreviations used are: LPS, lipopolysaccharide; PBS, phosphate-buffered saline (138 mM sodium chloride-2.7 mM potassium chloride-8 mM dibasic sodium phosphate-1.5 mM monobasic potassium phosphate, pH 7.4).

suspension (10^7 cells) 5 times at 10-day intervals. Then untreated viable M1 cells were injected once, and serum was collected 10 days later. The sera from 10 mice were combined and were absorbed with calf serum immunoadsorbent, prepared as described by Avrameas and Ternynck (1).

Immune Adherence Test. Cells were washed 3 times with special gelatin-Veronal buffer, consisting of 137 mM NaCl, 5.7 mM KCl, 0.15 mM $CaCl_2$, 0.5 mM $MgCl_2$, 3.1 mM Veronal (5,5-diethylbarbituric acid), 1.8 mM sodium Veronal, 5.6 mM glucose, and 0.1% gelatin, pH 7.5. Guinea pig serum was absorbed 3 times with packed M1 cells as described previously (29) and was used as the complement preparation. The immune adherence test was carried out on washed cells as described by Nishioka *et al.* (23). The percentage of cells with rosettes was calculated.

Statistical Analysis. The results were evaluated by an unpaired 2-tailed *t* test and were considered significant if the *p* value was less than 5%.

RESULTS

Enhancement of Differentiation of M1 Cells *in Vivo* by LPS or Glucocorticoid. The differentiation-stimulating activities of mouse serum on normal mouse bone marrow cells and M1 cells were enhanced by injection with LPS, and LPS itself induced differentiation of M1 cells (6, 9, 19, 21, 31). The effects of LPS from *S. typhimurium* on induction of phagocytic activity, a typical marker of differentiated cells, in DR3, DS1-1, and DS4 clone cells are shown in Chart 1. After treatment of the cells with LPS, 0.25 μg/ml, for 2 days, about 45% of the sensitive DS4 cells were phagocytic, whereas only 7% of the less sensitive DS1-1 cells were phagocytic. None of the resistant DR3 cells were phagocytic, even after treatment with LPS, 10 μg/ml.

We have found that, in diffusion chambers in syngeneic SL mice without any additional manipulation or injection of inducer, resistant M1 cells remained undifferentiated, whereas sensitive M1 cells differentiated into mature granulocytes and macrophages (11). Therefore, we injected LPS i.p. into syngeneic SL mice to determine whether it would enhance differentiation of sensitive cells in the diffusion chambers. As shown in Chart 2, their differentiation was significantly enhanced; only 5% of the cells in the diffusion chambers were myeloblastic in LPS-treated mice, whereas 55% were myeloblastic in the controls. LPS treatment also slightly increased the numbers of resistant M1 cells in intermediate stages of differentiation in the diffusion chambers. Morphological differentiation of the sensitive M1 cells, but not the resistant ones, was also markedly enhanced by injection of dexamethasone. These results indicate that injection of an inducer of differentiation of M1 cells *in vitro* can also enhance differentiation of the cells *in vivo*.

Prolongation of Survival Times of Mice Inoculated with M1 Cells by LPS Treatment. The effects of LPS on the survival times of mice inoculated with these cell clones were examined. For this, syngeneic mice inoculated with 10^5 cells of these clones were given injections of 25 μg of LPS from *S. typhimurium* twice a week. The first dose was injected 3 hr after tumor challenge. As expected, LPS significantly increased the survival times of mice given the sensitive M1 cells, but not that of the mice given the resistant cells (Chart 3). The mean survival time of untreated mice inoculated with DS4 cells was about 39 days, whereas that of those treated with LPS was about 60 days. Chart 4 shows that the effect of LPS was dose dependent. LPS was not toxic to SL mice at a dose of 100 μg or less, but it had a lethal effect at 200 μg or more. About 95% of the mice inoculated with 10^5 highly sensitive DS4 cells developed leukemia, even when injected with high doses of LPS. The most striking effect of LPS was seen in mice inoculated with the less sensitive cells. Mice treated with 100 μg of LPS survived for more than 50 days, whereas untreated mice died within 27 days after injection of DS1-1 cells. Since the resistant cells were less sensitive than the sensitive cells to the effect of LPS on induction of differentiation, these *in vivo* results are compatible with *in vitro* findings on inducibility of differentiation of the cells.

The minimum cell numbers of clones DS4, DS1-1, and DR3 required to induce leukemia in syngeneic SL mice on i.p. injection were 3 to 6 × 10^3, 2 to 5 × 10^2, and 20 to 50 cells, respectively. The survival time depended on the cell number, and mice inoculated with subthreshold number of the cells

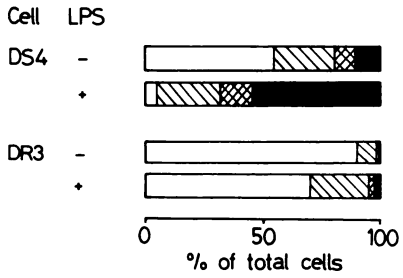

Chart 2. Stimulation by LPS of morphological differentiation of M1 cells in 6 days on culture in a diffusion chamber *in vivo*. Mice were given i.p. injections of 25 μg LPS 2 and 4 days after implanting the diffusion chambers. □, myeloblasts; ▨, cells in an intermediate stage of differentiation; ▩, mature granulocytes; ■, mature macrophages.

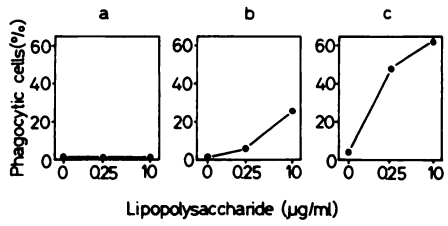

Chart 1. *In vitro* induction by LPS of phagocytosis in various clones of M1 cells. Phagocytosis was determined 2 days after seeding the cells with LPS from *S. typhimurium*. *a*, resistant (DR3) cells; *b*, less sensitive (DS1-1) cells; *c*, highly sensitive (DS4) cells.

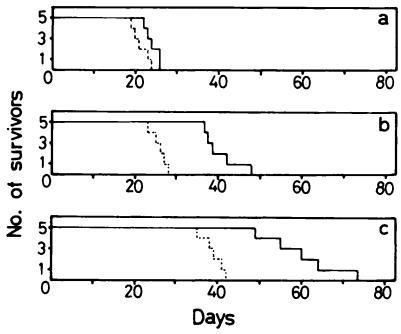

Chart 3. Leukemogenicity of sensitive and resistant M1 cells in SL mice given injections of LPS. Mice were given i.p. injections of 10^5 cells. ——, mice treated twice a week with 25 μg LPS from *S. typhimurium*; - - - -, untreated mice; *a*, DR3 cells; *b*, DS1-1 cells; *c*, DS4 cells.

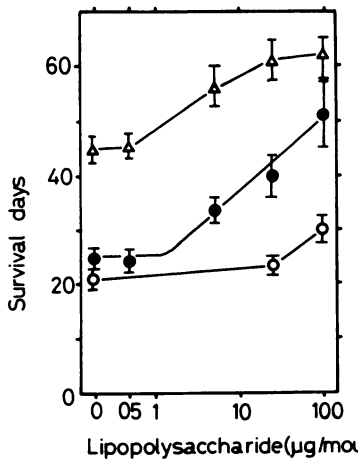

Chart 4. Effects of various doses of LPS on survival of mice inoculated with 10^5 M1 cells. Each *point* represents the mean for 10 to 35 mice; *bars*, S.D. The survival time of mice still surviving on Day 120 was taken as 120 days. △, DS4 cells; ●, DS1-1 cells; ○, DR3 cells. Mice were treated with LPS twice a week.

were followed for 150 days. A longer follow-up is difficult since spontaneous myeloid leukemia frequently develops in aged SL mice (over 1 year). LPS retarded development of leukemia in mice inoculated with 1.5×10^4 to 1.5×10^6 DS4 cells (Chart 5). After prolonged LPS treatment of mice inoculated with 1.5×10^4 cells, the mean survival time was about 73 days, and 4 of 20 mice did not develop leukemia, whereas the mean survival time of untreated mice was about 48 days and all of the mice died of leukemia. Two of 35 mice inoculated with 1 to 1.5×10^5 cells and treated with LPS did not develop leukemia. However, all of the mice inoculated with 10^6 cells died of leukemia. These results indicate that LPS significantly prolonged the survival time of mice with the sensitive M1 cells. The survival time of mice with the sensitive cells also prolonged by LPS from *S. typhimurium, S. abortus-equi,* or *E. coli*. A single injection of LPS into mice on Day 0 did not affect the survival days, and LPS treatment was effective only when it was started within at least 7 days after inoculation of leukemia cells (Table 1).

Prolongation of Survival Time by Glucocorticoids. Since glucocorticoid hormones induce differentiation of M1 cells *in vitro* (12), we examined their effect on the survival times of mice inoculated with sensitive M1 cells (Table 2). The survival times were prolonged by treatment with dexamethasone, (20 μg/mouse) but they were shortened by a high dose (400 μg/mouse). Similar results were obtained on s.c. injection of prednisolone in corn oil into mice twice a week. These results suggest that, at an optimal dose, glucocorticoid prolongs the survival times of syngeneic SL mice inoculated with sensitive M1 cells.

Detection of Surface Antigens of Cells by Immune Adherence. The surface antigens of resistant DR3 cells and sensitive DS4 cells were determined by the immune adherence test with syngeneic antiserum against DR3 cells. The immune adherence titers of sensitive cells were similar to those of resistant cells (Chart 6). Antibodies to DR3 cells were completely absorbed with DS4 cells, and the immune adherence reactions were completely negative with normal mouse serum. The surface antigens in sensitive and resistant cells seemed to be qualitatively similar, judging from results of an immune adherence test with antiserum from SL mice immunized with sensitive DS4 cells (data not shown). Therefore, these 2 clone cells, DR3 and DS4, have common antigens and similar surface antigen contents. The other clone, DS1-1, also had a similar surface antigen content.

Survival Times in Immunodeficient Mice. When newborn syngeneic SL mice were given i.p. injections of 5×10^3 cells from cultures of sensitive and resistant M1 cells, mice inoculated with the sensitive DS4 cells survived longer than those given injections of the resistant DR3 cells (Table 3). For confirmation of leukemogenicity in immunodeficient animals, survival of athymic nude BALB/c mice inoculated with the leukemia cells was examined. Results showed that the nude mice with the sensitive cells survived longer than did those with the resistant cells.

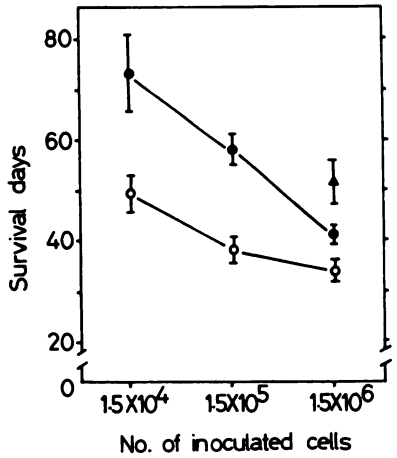

Chart 5. Effect of LPS treatment on survival of SL mice inoculated with various numbers of DS4 cells. Each *point* represents the mean for 10 to 35 mice; *bars*, S.D. The survival time of mice still surviving on Day 120 was taken as 120 days. ○, untreated mice; ●, mice treated with 25 μg LPS twice a week; ▲, mice treated with 100 μg LPS twice a week.

Table 1

Effect of LPS on survival times of SL mice inoculated with M1 cells

Treatment of LPS[a]	Source of LPS	Survival days[b]
Control		41.8 ± 3.3[c]
From Day 14	*S. typhimurium*	42.8 ± 4.8
From Day 7	*S. typhimurium*	59.7 ± 5.7[d]
From Day 0	*S. typhimurium*	60.4 ± 6.0[d]
Day 0 only[e]	*S. typhimurium*	41.0 ± 1.4
From Day 0	*S. abortus-equi*	63.8 ± 8.7[d]
From Day 0	*E. coli*	62.0 ± 5.4[d]

[a] Mice were given i.p. injections of 25 μg LPS starting on the indicated day after inoculation of DS4 cells.
[b] Each mouse was given an i.p. injection of 10^5 DS4 cells.
[c] Mean ± S.D.
[d] Significantly different from control ($p < 0.01$).
[e] Mice were given injections of LPS 3 hr after tumor challenge.

Table 2

Increased survival time of SL mice inoculated with sensitive M1 cells on treatment with dexamethasone

	Survival days[a]	No. of mice
Control	31.5 ± 2.9[b]	7
Treated[c]	43.7 ± 4.0[d]	8

[a] Each mouse was given an i.p. injection of 3×10^5 DS4 cells.
[b] Mean ± S.D.
[c] Mice were given i.p. injections of 0.2 ml of PBS containing 20 μg of dexamethasone.
[d] Significantly different from control ($p < 0.01$).

Prolongation of Survival Times of Athymic Nude Mice with M1 Cells by LPS. LPS is reported to be an immunopotentiator in mice and to inhibit growth of some tumors (3, 5, 22, 24–26). Therefore, we examined its effect on the growth of M1 cells in athymic nude mice. Table 4 shows that LPS increased survival times of athymic nude mice inoculated with M1 cells. This finding suggests that the inhibitory effect of LPS is not directly involved in T-lymphocyte-mediated immune responses. The effect of LPS in nude mice seemed to be rather greater than that in normal syngeneic SL mice, possibly due in part to the difference in genetic background of the athymic nude mice.

DISCUSSION

The resistant M1 cell clone used in the present experiments originated from a sensitive M1 cell clone. They showed no detectable differences from the sensitive clone in cell morphology, *in vitro* proliferation rate, or agglutinabilities with various lectins (11). They also showed no detectable difference from the sensitive clone in tumor-related surface antigens. However, they did differ in survival time in immunodeficient mice. Thus, this difference in survival time does not seem to be due to differences in the immunological properties described above.

Injection of LPS, a potent inducer of differentiation of the cells, greatly prolonged the survival time of mice inoculated with sensitive M1 cells and only slightly prolonged that of resistant cells. This finding is consistent with the effects of LPS on induction of differentiation of sensitive and resistant cells *in vivo* or *in vitro*, suggesting that the effect of LPS in stimulating cell differentiation *in vivo* may be associated with increase in survival time. However, LPS has been shown to have both direct and indirect antitumor effects (3, 5, 22, 24–26, 28). T-lymphocyte-mediated immune responses may not be directly involved in the effect of LPS on the survival time, because LPS also prolonged the survival times of athymic nude mice inoculated with M1 cells. LPS treatment stimulates the antitumor activity of macrophages (7, 8, 26, 30). Moreover, it was recently found that the gene for control of the tumoricidal capacity of macrophages is either closely linked with, or identical to, the LPS-responsive gene (26). Leukocytes are important in production of a differentiation-stimulating factor for M1 cells (15), and one of the many effects of LPS on macrophages is to enhance production of this factor.[4] Therefore, development of cytotoxic macrophages may be associated with increase in production of inducer for differentiation of M1 cells. The inducer also stimulates bone marrow colony formation (6, 9, 27). It has been suggested that the inducer plays a role in the control of granulopoiesis and macrophage production *in vivo* (21). Butler *et al.* (5) postulated that one antitumor effect of LPS may be mediated by the bone marrow colony-stimulating factor. Glucocorticoid, which induces differentiation of M1 cells, also prolonged the survival time of mice with sensitive M1 cells, although glucocorticoid is known to be an immunosuppressive agent (18). These results suggest that the effect of LPS on the survival time is partly attributable to its effect in stimulating differentiation of the cells *in vivo* (Chart 2).

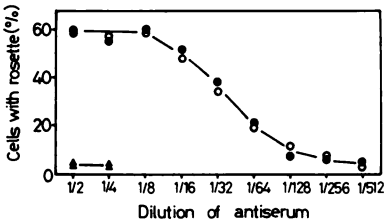

Chart 6. Immune adherence reactivity of sensitive (DS4) and resistant (DR3) M1 cells with syngeneic anti-DR3 cell serum. ○, DS4 cells; ●, DR3 cells; △, DS4 cells with anti-DR3 cell serum previously absorbed with DS4 cells; ▲, DR3 cells with anti-DR3 cell serum previously absorbed with DS4 cells.

Table 3

Leukemogenicity of M1 cells in immunodeficient mice

	Cell clone	No. of cells inoculated/mouse	Survival days[a]	No. of mice
Newborn SL mice	DS4	5×10^3	32.8 ± 2.3[b]	7
	DR3	5×10^3	22.7 ± 3.8	9
Athymic nude mice	DS4	3×10^4	51.0 ± 6.3	10
	DR3	3×10^4	23.6 ± 2.1	10
	DS4	10^5	41.6 ± 6.1	12
	DR3	10^5	22.0 ± 2.0	12

[a] All the mice developed leukemia and died.
[b] Mean ± S.D.

Table 4

Effect of LPS on survival time of athymic nude mice inoculated with M1 cells

	Survival days[a]	No. of mice
Control	36.8 ± 6.3[b]	15
Treated[c]	56.4 ± 7.5[d]	13

[a] Mice were given i.p. injections of 2×10^5 DS4 cells.
[b] Mean ± S.D.
[c] Mice were given i.p. injections of 25 μg LPS from *S. typhimurium*.
[d] $p < 0.01$.

When mice were inoculated with 10^4 sensitive M1 cells, untreated animals all died of leukemia, but some of those treated with LPS survived. However, LPS treatment alone is not always effective in mice bearing M1 cells and a high dose of LPS had a lethal effect, especially in tumor-bearing animals (4). Dexamethasone, which also induces differentiation, was also effective at a certain dose in increasing the survival times of mice bearing sensitive M1 cells, but a high dose caused considerable side effects, such as immunosuppression. More effective therapy for leukemia with fewer side effects might be achieved by combination therapy with LPS, glucocorticoid, and some other inducers. Alternatively, other inducers with fewer side effects might be more effective in inhibiting the leukemogenicity of M1 cells by inducing their differentiation. Moreover, in order to develop adequate leukemia therapy based on induction of differentiation, we must identify suitable drugs able to induce 100% of the population to differentiate, because if any leukemia cells survive at all, the disease cannot be cured.

Previous studies with Friend erythroleukemia cells indicated that the time course of development of erythroleukemia could be altered by treatment with active differentiation-inducing agents, although none of the compounds tested significantly increased overall survival (20). The previous and the present experiments suggest that cancer therapy might be achieved by inducing phenotypic reversion and normal differentiation of malignant cells. Further experiments on this possibility should contribute to establishment of new forms of cancer therapy.

[4] M. Hozumi, unpublished data.

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