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Tumor Necrosis Factor Changes Sensitivity of Differentiation of Mouse Leukemia M1 Cells by Lipopolysaccharide

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A clone of mouse leukemia M1 cells was induced to differentiate by lipopolysaccharide (LPS) (LPS-sensitive clone) while another clone of the same cells was resistant (LPS-resistant clone). LPS and lipid A preparations from *Pseudomonas diminuta* and *Pseudomonas vesicularis* were as active as *Escherichia coli* LPS in the induction of differentiation of the LPS-sensitive clone. Synthetic lipid A precursor Ia (compound 406), which has no interleukin 1 (IL-1)-inducing activity toward monocytes, had strong differentiation-inducing activity toward the LPS-sensitive clone. The combined treatment of the LPS-sensitive clone with LPS and recombinant tumor necrosis factor (rTNF) did not further increase the degree of differentiation induced by LPS alone. By contrast, the LPS-resistant clone was markedly induced to differentiate by LPS in the presence of rTNF. Combined treatment of the LPS-resistant clone with LPS and other cytokines such as recombinant IL-1α, recombinant granulocyte colony-stimulating factor, and interferon-γ was not effective in inducing marked synergistic differentiation. These results raise the possibility that rTNF changes the sensitivity of M1 cells to induction of differentiation by LPS.

Key words: Differentiation — Tumor necrosis factor — Lipid A

LPS is known to induce a wide variety of biological changes, including production of cytokines by macrophages,[1] formation of antibody by B lymphocytes,[2] and cytotoxicity.[3] Some leukemia cells such as mouse myeloid leukemia M1 cells[4] and rat myelomonocytic leukemia c-WRT-7 cells[5] are induced to differentiate by LPS, but other cells are not. In the present study, we examined the synergistic effects of LPS and cytokines using two clones of M1 cells: one clone is induced to differentiate by LPS while the other clone is not. We found that the LPS-resistant clone could be induced to differentiate by LPS when rTNF is simultaneously present. The relation between the structure of LPS and lipid A and their differentiation-inducing activity was also studied.

MATERIALS AND METHODS

Materials RA, Ara-C, and DEX were purchased from Sigma Chemical Co. 1α, 25$(OH)_2D_3$ was kindly donated by Chugai Pharmaceutical Co. Human rTNF (1.1×10^8 U/mg) was kindly provided by Fujisawa Pharmaceutical Co. Human rIL-1α (10^8 U/mg) was obtained from Genzyme Corporation. Mouse IFN-α, β was purchased from Japan Chemical Research Co. Ltd. ^{125}I-TNF (700 Ci/mmol) was from Amersham. Polyclonal antibody for mouse rTNF was kindly provided by the Suntory Institute for Biomedical Research.

LPS and lipid A preparations LPS and free lipid A were prepared from *E. coli* F515, *P. diminuta* JCM 2788 and *P. vesicularis* JCM 1477 as previously described.[6,7] Purified lipid A components, A3 and A2 fractions, were isolated from free lipid A of *P. diminuta* and *P. vesicularis* by preparative thin-layer chromatography as previously described.[6] The synthetic *E. coli*-type lipid A, compound 506, was kindly provided by Daiichi Pure Chemicals Co., Ltd.

Assay for differentiation-inducing activity Leukemia cells (1×10^5 cells/ml) were treated with the sample solution containing various concentrations of LPS, lipid A, or rTNF for 2 days. Differentiation-inducing activity was monitored by measuring the ability of the cells to reduce NBT as described previously.[8]

TNF-receptor binding assay LPS-sensitive and resistant clones (2×10^5 cells) were incubated with 10 μg/ml of LPS for various lengths of time in MEM medium. The cells were washed once with MEM medium by centrifugation, then incubated with 2×10^5 dpm of ^{125}I-TNF (specific activity, 700 Ci/mmol) for 2 h at 37°C. Thereafter, the medium was removed, the cells were washed three times by centrifugation with MEM medium

The abbreviations used are: Ara-c, 1-β-D-arabinofuranosylcytosine; 1α,25$(OH)_2D_3$, 1α,25-dihydroxyvitamin D_3; DEX, dexamethasone; rG-CSF, recombinant granulocyte-colony-stimulating factor; rIL-1α, recombinant interleukin 1α; mIFNα, β, mouse interferon α, β; LPS, lipopolysaccharide; NBT, nitroblue tetrazolium; rTNF, recombinant tumor necrosis factor.

and dissolved in 2% sodium dodecyl sulfate, and the radioactivity bound to the cells was counted in a γ counter. Non-specific binding determined in the presence of an excess (100 nmol) of unlabeled rTNF was subtracted from the total binding to calculate the specific binding.

Other methods The biological activity of TNF was determined by a cell lytic L929 assay.[9, 10] To prepare Western blots, samples were subjected to SDS-PAGE by the method of Laemmli[11] in a 10% polyacrylamide slab gel, the proteins were transferred to a nitrocellulose membrane, and the TNF was visualized using a rabbit anti mouse rTNF antibody followed by horseradish peroxidase-conjugated goat antiserum to rabbit IgG.

RESULTS

LPS-sensitive and resistant clones A clone of mouse leukemia M1 cells, LPS-sensitive clone, was induced to differentiate by treatment with *E. coli* F515 LPS. Fig. 1 shows the effects of various concentrations of LPS on the LPS-sensitive clone. The differentiation-inducing activity of LPS increased with increasing concentration of LPS and about 50% of the cells were induced to differentiate by treatment with 10 μg/ml of LPS for 2 days. Addition of LPS at more than 10 μg/ml was cytotoxic to M1 cells as judged by trypan blue staining. An LPS concentration of 10 μg/ml or less was used in the present study. rTNF by itself did not induce marked differentiation in the LPS-sensitive clone. Addition of various concentrations of rTNF in combination with LPS did not significantly affect the degree of differentiation induced by LPS alone.

Fig. 2 shows the effects of low-molecular-weight differentiation inducers, which are known to induce marked differentiation in M1 cells, on the LPS-sensitive M1 cell clone. As is evident from this figure, DEX, 1α,25-$(OH)_2D_3$, and Ara-C had synergistic or additive effects with rTNF. Similar effects were observed when various concentrations of DEX, 1α,25-$(OH)_2D_3$, and Ara-C were used in combination with rTNF. LPS had the strongest differentiation-inducing activity on the LPS-sensitive clone among the low-molecular-weight inducers

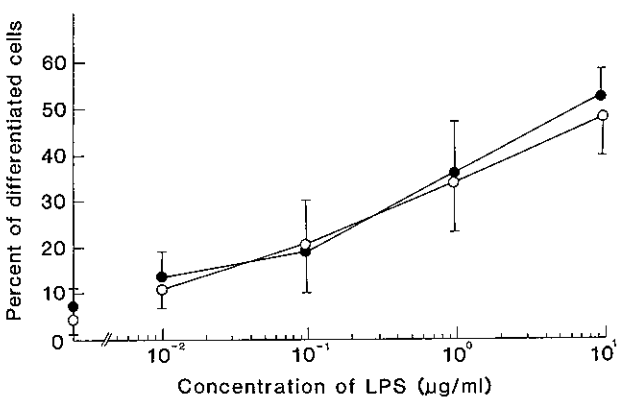


Fig. 1. Effects of the concentrations of *E. coli* LPS in the presence or absence of rTNF on the differentiation of the LPS-sensitive clone of M1 cells. The clone was cultured for 2 days with the indicated concentrations of LPS in the presence (●) or absence (○) of 170 U/ml of rTNF. Differentiation-inducing activities of LPS or its combination with rTNF were measured by NBT staining as described in the text. Each value is the mean±SD of 6 determinations. The differences between values obtained for the presence and absence of rTNF at various LPS concentrations were not statistically significant.

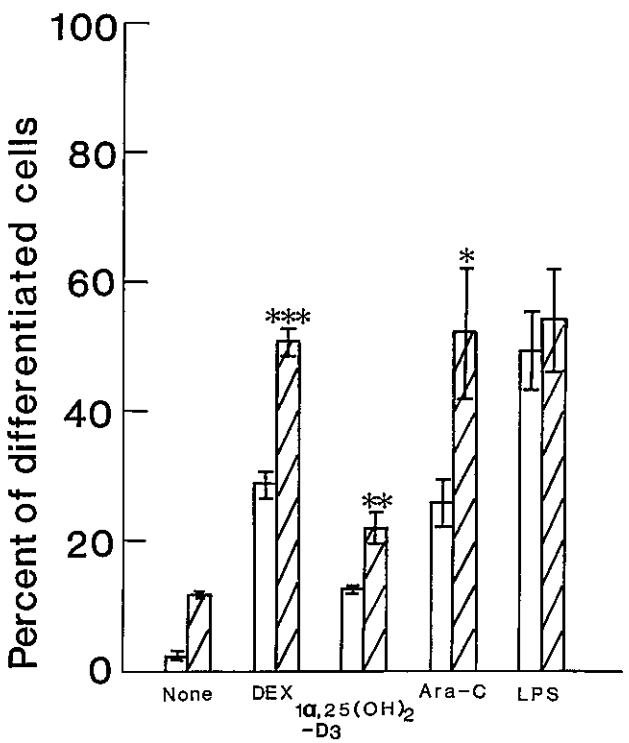


Fig. 2. Differentiation of an LPS-sensitive clone of M1 cells induced by low-molecular-weight inducers. The clone was cultured for 2 days with various inducers alone (open bars) or in combination (hatched bars) with 170 U/ml of rTNF. Differentiation-inducing activity of low-molecular-weight inducers or their combination with rTNF was measured by NBT staining as described in the text. Each value is the mean±SD of triplicate determinations. Concentrations of low-molecular-weight inducers were DEX, 10^{-6} *M*; 1α,25$(OH)_2D_3$, 10^{-9} *M*; Ara-C, 10^{-7} *M*; LPS, 10 μg/ml. The differences between values obtained for low-molecular-weight inducers alone and in combination with LPS were significant at $P<0.001$ (***), $P<0.01$ (**), and $P<0.05$ (*).

and the combination treatment of the cells with LPS and rTNF did not further increase the degree of differentiation induced by LPS alone.

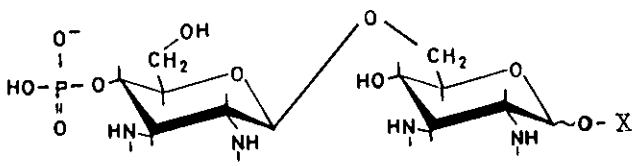
Table I. Effects of LPS and Lipid A on the Differentiation of an LPS-sensitive M1 Cell Clone

Treatment	NBT-reducing activity (%)
None	8.2±3.5
E. coli F515	
LPS	58.8±8.7
Lipid A	57.7±14.6
P. diminuta	
LPS	65.5±7.8
Lipid A	43.4±6.3
A3 fraction	35.1±2.0
A2 fraction	6.1±3.3
P. vesicularis	
LPS	59.1±6.0
Lipid A	43.1±4.2
A3 fraction	35.1±2.0
A2 fraction	3.1±0.7
Synthetic compound	
LA-14-PP (406)	44.6±3.7
LA-15-PP (506)	35.6±2.3
LA-16-PP (516)	15.2±3.7
LA-15-PP (504)	17.2±0.5
LA-16-PP (514)	6.2±0.2

The LPS-sensitive clone of M1 cells was cultured in the presence of 10 μg/ml of LPS or lipid A preparations for 2 days and differentiation-inducing activity was measured by NBT staining. Each value is the mean±SD of triplicate determinations.

Next, we examined which part of the structure of LPS or what structure of lipid A is required to induce differentiation in M1 cells. Table I shows the differentiation-inducing activity of various LPS and lipid A preparations on the LPS-sensitive clone. Lipid A from *E. coli* LPS was as active as *E. coli* LPS. LPS and lipid A preparations from *P. diminuta* and *P. vesicularis*, which are composed of 2 mol of 2,3-diamino-2,3-dideoxy-D-glucose and 1 mol of nonglycosidic phosphate as the backbone components and fatty acids as shown in Table II, had similar differentiation-inducing activity to *E. coli* type LPS and lipid A. To examine the influence of the fatty acyl moiety

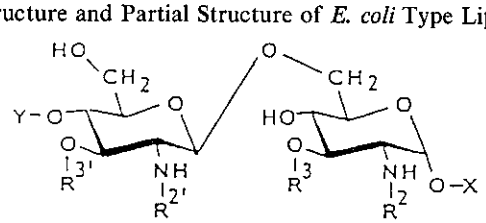
Table II. Chemical Structure of Lipid A Preparations from *P. vesicularis* and *P. diminuta*



Fraction	Amide-bound	Ester-bound
P. vesicularis		
A3 fraction	C12-OH, C14-OH, C14-5,9OH	C12-OH, C14
P. diminuta		
A3 fraction	C12-OH, C13-OH, C14-OH, C14-5,9OH	C12-OH, C14, C14:1

Abbreviations: C14, tetradecanoyl; C14:1, tetradecenoyl; C12-OH, 3-hydroxydodecanoyl; C13-OH, 3-hydroxytridecanoyl; C14-OH, 3-hydroxytetradecanoyl; C14-5,9OH, 5,9-dihydroxytetradecanoyl.

Table III. Chemical Structure and Partial Structure of *E. coli* Type Lipid A



Compound	R3′	R2′	R3	R2	Y	X
406	C14-OH	C14-OH	C14-OH	C14-OH	P	P
506	C14-O-(C14)	C14-O-(C12)	C14-OH	C14-OH	P	P
516	C14-O-(C14)	C14-O-(C12)	C14-OH	C14-O-(C16)	P	P
504	C14-O-(C14)	C14-O-(C12)	C14-OH	C14-OH	P	H
514	C14-O-(C14)	C14-O-(C12)	C14-OH	C14-O-(C16)	P	H

Abbreviations: P, $PO(OH)_2$; C14, tetradecanoyl; C14-OH, (R)-3-hydroxytetradecanoyl; C14-O-(C12), (R)-3-decanoyloxytetradecanoyl; C14-O-(C14), (R)-3-tetradexanoyloxytetradecanoyl; C14-O-(C16), (R)-3-hexadecanoyloxytetradecanoyl.

of *P. diminuta* and *P. vesicularis* lipid A preparations, the differentiation-inducing activities of two components of lipid A preparations of both strains were measured. The major components of both strains, referred to as A3 fraction, had a strong differentiation-inducing activity. By contrast, the A2 fractions of both strains, having incomplete acyl residues, exhibited practically no differentiation-inducing activity. The results suggest that the structure of the hydrophobic part, including amide-linked acyloxyacly groups of the lipid A molecule plays some role in inducing differentiation of the LPS-sensitive clone.

It is remarkable that synthetic lipid A precursor Ia (compound 406), lacking nonhydroxylated fatty acids, had a strong differentiation-inducing activity toward the LPS-sensitive clone of M1 cells. The activity of compound 516 is weaker than that of compound 506; the former has a 3-hexadecanoyloxytetradecanoyl residue instead of the 3-hydroxytetradecanoyl residue of the latter (Table III). Similarly, compound 514 had a weaker differentiation-inducing activity than compound 504; the former also has a 3-hexadecanoyloxytetradecanoyl residue instead of the 3-hydroxytetradecanoyl residue. When compounds 506 and 516 were compared with 504 and 514, respectively, the latter, lacking a mol of phosphate, had weaker activity than the former, indicating the contribution of the phosphate group.

Effect of LPS on the LPS-resistant clone Fig. 3 shows the effect of LPS concentration on the LPS-resistant clone. The LPS-resistant clone of M1 cells was not significantly induced to differentiate by treatment with either LPS or rTNF alone. However, when the LPS-resistant clone was treated with the combination of LPS and rTNF, the cells were remarkably induced to differentiate. Even at 10^{-2} μg/ml of LPS, addition of 170 U/ml of rTNF induced marked differentiation. Therefore, LPS and rTNF seem to act synergistically in inducing differentiation of the LPS-resistant clone.

Fig. 4 shows the effect of rTNF concentration on the differentiation on the LPS-resistant clone. Treatment of LPS-resistant clone with rTNF alone even at 170 U/ml induced almost no differentiation. When 10 μg/ml of LPS was present, 1.7 U/ml of rTNF had a synergistic effect. The synergistic effect of rTNF and LPS was much greater when the rTNF concentration was increased from 1.7 to 170 U/ml. Combined treatment of the LPS-resistant clone with 10 μg/ml of LPS plus 170 U/ml of rTNF had practically no effect on cell viability as measured by trypan blue staining, but cell growth was inhibited by 59.4±7.7% (mean±SD of triplicate determinations) as measured by counting the number of cells. Treatment of the LPS-resistant clone with 10 μg/ml LPS inhibited cell growth by 20.8±5.0% (mean±SD of triplicate determinations).

Combined treatment of 10 μg/ml of *E. coli* lipid A with rTNF induced differentiation of the LPS-resistant clone; 36.5±2.5% of the cells were induced to differentiate by treatment with 10 μg/ml lipid A plus 170 U/ml rTNF for 2 days, while 8.5±0.8% were induced to differentiate by lipid A alone as judged by NBT staining

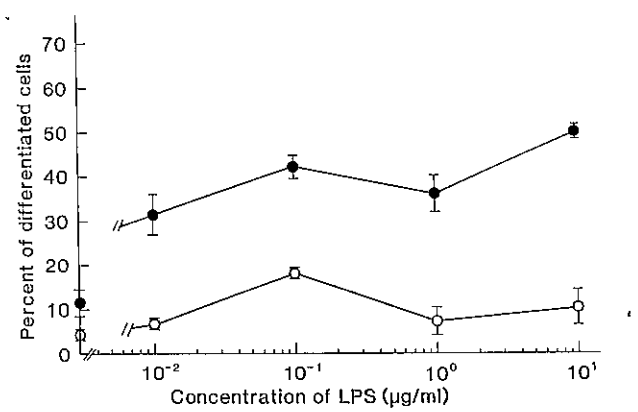


Fig. 3. Effects of the concentration of *E. coli* LPS in the presence or absence of rTNF on the differentiation of the LPS-resistant clone of M1 cells. The LPS-resistant clone was cultured for 2 days with the indicated concentrations of LPS in the presence (●) or absence (○) of 170 U/ml of rTNF. Differentiation-inducing activities of LPS or its combination with rTNF were measured by NBT staining as described in the text. Each value is the mean±SD of triplicate determinations.

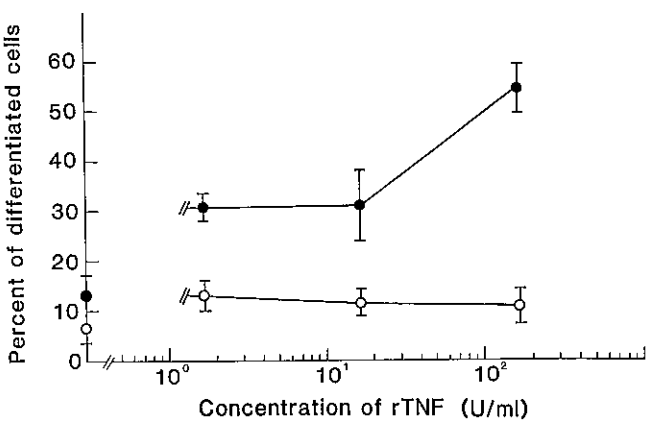


Fig. 4. Effects of the concentrations of rTNF in the presence or absence of *E. coli* LPS on the differentiation of an LPS-resistant clone of M1 cells. The LPS-resistant clone was cultured for 2 days with the indicated concentrations of rTNF in the presence (●) or absence (○) of 10 μg/ml of LPS. Differentiation-inducing activities of LPS or its combination with rTNF were measured by NBT staining as described in the text. Each value is the mean±SD of triplicate determinations.

(the value is the mean±SD of triplicate determinations). Synthetic lipid A (compound 406) also induced differentiation of LPS-resistant clone when combined with rTNF; 31.0±3.7% of the cells were induced to differentiate by combined treatment with 10 μg/ml of compound 406 with rTNF as measured by NBT staining, whereas 15.5±2.0% became stainable with compound 406 alone (mean±SD of triplicate determinations). These results indicate that the differentiation-inducing activity of LPS in combination with rTNF in the case of the LPS-resistant clone is due to the lipid A part of LPS, as was found for the LPS-sensitive clone of M1 cells.

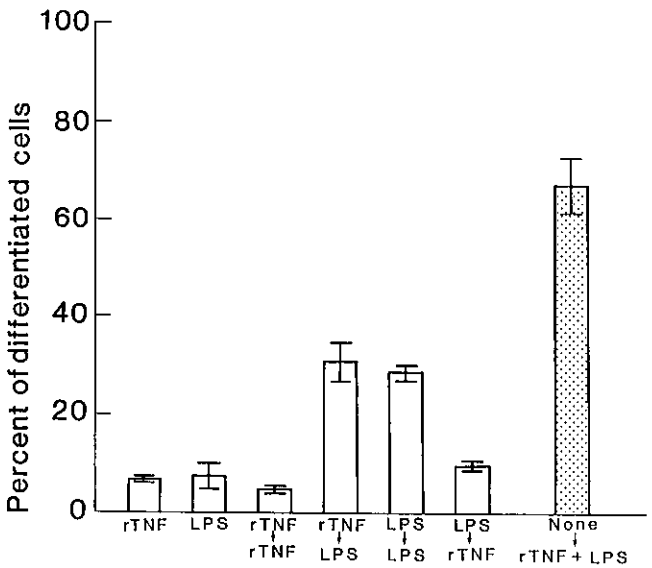


Fig. 5. Effects of pretreatment with *E. coli* LPS or rTNF on the LPS-resistant clone of M1 cells. The clone was pretreated with LPS or rTNF for 1 day, washed, and then cultured with LPS or rTNF for 2 additional days as indicated in the figure. The concentrations of LPS and rTNF used were 10 μg/ml and 170 U/ml, respectively.

Fig. 5 shows the effect of pretreatment with LPS and rTNF on an LPS-resistant clone of M1 cells. Pretreatment with either rTNF or LPS, followed by subsequent treatment with rTNF caused almost no differentiation of the cells. However, pretreatment with either rTNF or LPS, followed by treatment with LPS induced differentiation in about 30% of the cells and these values were significantly higher than those obtained by pretreatment with either rTNF or LPS, followed by treatment with rTNF ($P<0.001$). Simultaneous treatment with LPS and rTNF was most effective in inducing differentiation of the cells and the value of differentiated cells obtained was significantly higher than the values obtained with various combinations of pretreatment and subsequent treatment with LPS or rTNF alone ($P<0.001$).

Fig. 6 shows the effects of combination of LPS and several cytokines such as rIL-1α, mIFN-α,β, and rG-CSF on the differentiation of the LPS-resistant clone of M1 cells. These cytokines alone or combined treatment with rTNF and rIL-1α were ineffective in inducing differentiation of the LPS-resistant clone. Combined treatment with rIL-1α plus LPS, or rTNF plus rIL-1α also had no effect on the induction of differentiation. Combined treat-

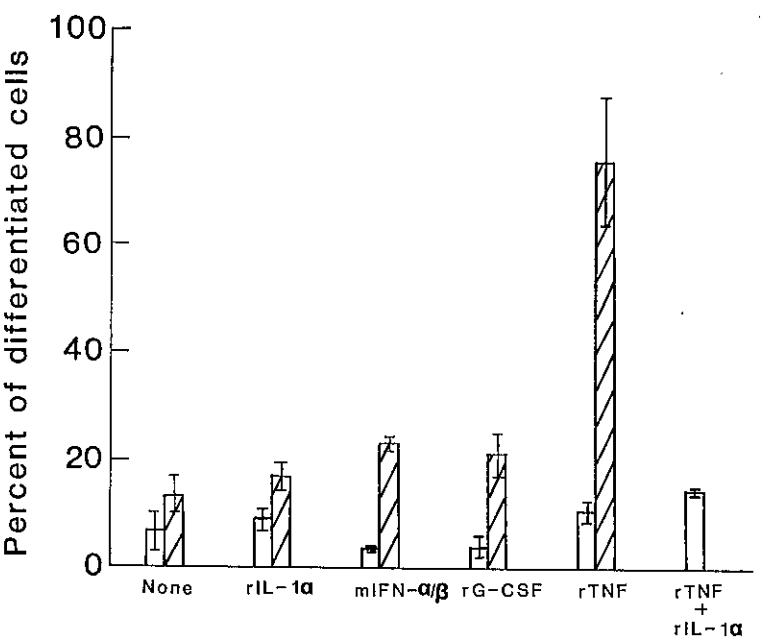


Fig. 6. Effects of combination of LPS and cytokines such as rIL-1α, mIFN-α, β, and rG-CSF on the differentiation of the LPS-resistant clone of M1 cells. The clone was cultured for 2 days with various cytokines alone (open bars) or in combination (hatched bars) with 10 μg/ml of LPS. Differentiation-inducing activities of LPS or its combination with rTNF were measured by NBT staining as described in the text. Each value is the mean±SD of triplicate determinations. The concentrations of rIL-1α, mIFN-α, β, rG-CSF, and rTNF used were 0.28 U/ml, 100 U/ml, 0.1 ng/ml, and 170 U/ml, respectively.

ment with LPS plus either mIFN-α, β or rG-CSF was not effective in inducing marked synergistic differentiation. The strongest differentiation-inducing activity on the LPS-resistant clone was obtained by combining rTNF and LPS.

TNF-receptors and TNF-production Since the LPS-sensitive clone is insensitive to rTNF and the LPS-resistant clone is sensitive to rTNF in the presence of LPS, the numbers of TNF receptors on the LPS-sensitive and resistant clones of M1 cells were compared. The amounts of ^{125}I-TNF bound to LPS-sensitive and resistant clones were practically identical and were not changed by LPS treatment for at least 24 h (results not shown). Even after 72 h, the amounts of TNF-receptors of both clones were practically the same.

Another possibility to explain the difference in sensitivity to induction of differentiation by LPS is that the amounts of TNF, if any, produced by the clones are different. Therefore, the amounts of TNF produced in the cytosol and membrane fractions and in the conditioned media of both clones, before and after treatment with LPS, were measured. However, we could not detect TNF activity in these fractions by L929 lytic assay (results not shown). Moreover, TNF could not be detected in these fractions by Western blotting and immunoprecipitation, or by enzyme immunoassay on a microtiter plate using anti mouse rTNF antibody (results not shown).

DISCUSSION

The two clones of M1 cells used in the present study showed different sensitivity to LPS. The LPS-sensitive clone was induced to differentiate by LPS while the LPS-resistant clone could not be induced by either LPS alone or rTNF alone, but combined treatment of the clone with LPS and rTNF did induce marked differentiation. This means that the LPS-resistant clone became sensitive to LPS in the presence of rTNF. That is to say, rTNF induced sensitivity of the LPS-resistant clone to LPS.

The mechanism of induction of differentiation in the LPS-sensitive and resistant clones by LPS is interesting. Although rTNF had no significant effect on the degree of differentiation induced by LPS in the LPS-sensitive clone, but was remarkably effective in inducing differentiation in the LPS-resistant clone when applied simultaneously with LPS, we found no significant difference in the numbers of TNF-receptors between LPS-sensitive and resistant clones of M1 cells. We also measured the amounts of TNF produced in the conditioned media and in the cytosol of both LPS-sensitive and resistant clones and found that TNF could not be detected in the fractions of these clones by either L929 lytic assay, Western blotting, immunoprecipitation, or ELISA on microplates using anti mouse rTNF antibody. Moreover, addition of anti mouse rTNF antibody to the LPS-sensitive clone of M1 cells did not inhibit the induction of differentiation by LPS. It is, therefore, unlikely that TNF produced in the cells by LPS affects the induction of differentiation. Other cytokines examined, including rIL-1α, mIFN-α, β, rG-CSF, and rIFN-β, were not effective in inducing marked differentiation in the LPS-sensitive clone in combination with LPS. Considering these results, we suggest the following possible two explanations for the difference in sensitivity to LPS and to rTNF between LPS-sensitive and resistant clones. 1) rTNF may bind similarly to TNF-receptors in both clones. Binding of LPS to the LPS-receptors[12] may result in differentiation in the LPS-sensitive clone. In the case of the LPS-resistant clone, binding of LPS to the LPS-receptor is not sufficient to induce differentiation, but simultaneous binding of LPS and rTNF to each receptor may change the structure of the LPS-receptor sufficiently to permit LPS to induce differentiation by an unknown mechanism. 2) The number of LPS receptors, if any, are different in LPS-sensitive and resistant clones. When rTNF binds to TNF-receptor of LPS-resistant clone, LPS-receptor appeared at the surface of the cells, and then LPS can bind to its receptor, leading to differentiation. However, knowledge of the LPS receptor is not sufficient to evaluate this possibility at the present stage of investigation and precise determination of the number of LPS-receptors is practically impossible because of nonspecific binding of LPS to the plasma membranes of cells. Further work is needed to test these hypotheses.

LPS-resistant clones of M1 cells,[4] and rat myelomonocytic leukemia (cWRT-7) cells[5] have been reported. Weiss and Sachs[4] demonstrated that LPS indirectly induces differentiation of an LPS-sensitive clone of M1 cells by inducing colony-stimulating factor. Onozaki *et al.*[13] reported that IL-1 by itself did not induce differentiation of M1 cells but combined treatment with IL-1 and LPS synergistically induced their differentiation into macrophage-like cells. Onozaki *et al.*[14] also demonstrated that incubation of M1 cells with either LPS or TNF induces production of IFN-β and this IFN-β is probably responsible for the induction of differentiation of M1 cells by IL-1 and TNF or by LPS. In the present study, rIL-1α alone or in combination with LPS or rTNF was not effective in inducing differentiation in either LPS-sensitive or resistant clones. We cannot explain the reason for this discrepancy at present.

It is quite unexpected that synthetic lipid A (compound 406) has differentiation-inducing activity toward the LPS-sensitive clone of M1 cells. Compound 406 is not able to induce IL-1 production in human mononuclear

cells[15] and is about 0.01 times as pyrogenic as compound 506. Therefore, the minimal lipid A structure required for differentiation-inducing activity toward M1 cells is different from that required for IL-1 production. Since compound 406 has little toxicity to animals, it should be a useful differentiation inducer for leukemic cells.

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