Defect in the Induction of Nitric Oxide Synthase by Lipopolysaccharide (LPS) in an LPS-Resistant Mutant of a Murine Macrophage-Like Cell Line, J774.1

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Nitric oxide ($\cdot\text{NO}$)-generating activity was examined in a lipopolysaccharide (LPS)-resistant mutant of a murine macrophage-like cell line, J774.1, treated with LPS or LPS and interferon- γ (IFN- γ). This mutant, an LPS1916 cell line, showed no NO_2^- accumulation in the culture medium, and no expression of NOS activity in the cell extract, $\cdot\text{NO}$ synthase (NOS(II)) protein or NOS(II) mRNA on treatment with up to 10^4 ng/ml LPS, although the parental cell line, JA-4, showed significant $\cdot\text{NO}$ production. The addition of 10 U/ml IFN- γ , together with more than 1 ng/ml LPS to JA-4 cells, increased $\cdot\text{NO}$ production remarkably, while IFN- γ did not reverse the defect of $\cdot\text{NO}$ production in LPS1916 cells when they were treated with less than 10 ng/ml LPS; however, it induced $\cdot\text{NO}$ production by the mutant cells with more than 100 ng/ml LPS. Analysis of NOS activity, NOS(II) protein and NOS(II) mRNA revealed that LPS1916 cells are not devoid of the NOS(II) gene, but are rather defective in transcription of the gene in response to LPS, and this defect is partly reversed by IFN- γ with higher LPS doses at more than 100 ng/ml. In addition, the delay of NOS(II) mRNA induction in LPS1916 cells, compared to that in JA-4 cells, treated with LPS+IFN- γ seems to suggest some additional inducer(s) of NOS(II) transcription, followed by LPS signaling.

Key words nitric oxide synthase (NOS(II), iNOS); lipopolysaccharide (LPS); macrophage; J774.1; LPS-resistant mutant; interferon- γ (IFN- γ)

Lipopolysaccharide (LPS) causes macrophages to release reactive oxygen intermediates (ROIs) such as superoxide (O_2^-), hydrogen peroxide (H_2O_2) and nitric oxide ($\cdot\text{NO}$),^{1,2} which are known as effector molecules for the antimicrobial or antitumor activity of activated macrophages.^{3–5} However, it is not easy to study the biological functions of $\cdot\text{NO}$ and O_2^- separately in activated macrophages because LPS usually causes the macrophages to produce both $\cdot\text{NO}$ and O_2^- simultaneously.

In the course of our studies, LPS1916, an LPS-resistant mutant of a macrophage-like cell line, J774.1,¹ was proven to show altered LPS-responses; however, while JA-4, the parental cell line and a subline of J774.1, was highly responsive to LPS and was induced by treatment with 1–10 ng/ml LPS to produce O_2^- upon stimulation with a phorbol ester (PMA), the mutant cells did not produce O_2^- on treatment with up to 10^4 ng/ml LPS. However, this defect of LPS1916 cells was partly reversed by IFN- γ , and the mutant produced as much O_2^- as JA-4 cells on stimulation with PMA. The defective induction of the $\cdot\text{NO}$ -generating activity of the mutant cells has been unclear, although our preliminary study showed a marked decrease in $\cdot\text{NO}$ production in this mutant after LPS-treatment.^{6,7} In the present paper, we investigated the $\cdot\text{NO}$ synthetic pathways of this mutant precisely by analyzing NO_2^- accumulation, inducible nitric oxide synthase (NOS(II)) expression, NOS activity in cell lysates and NOS(II) mRNA expression in LPS1916 cells and JA-4 cells.

MATERIALS AND METHODS

Materials L-Arginine, L-citrulline, L-valine, N^G -hydroxy-L-arginine, β -NADPH, FAD, FMN, dithiothreitol (DTT) and lipopolysaccharide (LPS; *E. coli*, O55: B5, chro-

matographically purified) were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). (6R)-5,6,7,8-Tetrahydro-L-biopterin (H_4B) was obtained from Alexis (San Diego, CA, U.S.A.), and N^G -monomethyl-L-arginine (L-NMMA) was from Wako Pure Chemicals (Osaka, Japan). ^{125}I -Labeled protein A was prepared by the iodination of protein A (Sigma) with [^{125}I]NaI (NEN, Du Pont, Lincoln Park, NJ, U.S.A.) according to the chloramine-T method.⁸ L-[U- ^{14}C]Arginine was obtained from Amersham International, PLC (Little Chalfont, England). Recombinant mouse interferon- γ (IFN- γ) was kindly donated by Toray Industries, Inc. (Tokyo).

Cell Culture The JA-4 and LPS1916 cell lines were maintained by culturing in Ham's F12 medium (Dainippon Pharmaceutical Co., Osaka), supplemented with 50 U/ml penicillin, 50 $\mu\text{g}/\text{ml}$ streptomycin (Flow Laboratories, Irving, Scotland) and 10% (v/v) heat-inactivated fetal bovine serum (GIBCO BRL, Life Technologies, Inc., Grand Island, NY, U.S.A.) on plastic petri dishes (Falcon[®], #1001, Beckton Dickinson) in a CO_2 incubator (under 5% CO_2 –95% humidified air) at 37 °C and passaged twice a week.

Induction of NOS(II) in Macrophages As reported previously,⁹ the cells were seeded at 4×10^5 cells/ml onto 60 mm-petri dishes (Corning) in 5 ml of the fresh culture medium and then incubated at 37 °C overnight. Then, the medium was replaced with 5 ml of fresh medium containing LPS with or without IFN- γ . The cells were incubated further at 37 °C for an appropriate time, then the culture medium was collected to assay NO_2^- . Simultaneously, the cells were extracted with 50 μl of the lysis buffer, comprising 0.1% Triton X-100, 0.1 mM EDTA, 1% aprotinin in 20 mM HEPES–NaOH buffer, pH 7.5. This cell lysate was centrifuged at 10000 rpm for 1 min at 4 °C (Tomy, model MRX-150, Tokyo), and 40 μl of the resulting supernatant was collected

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into a new microfuge tube. The final cell extract was stored at -80°C until use.

Alternatively, the cells were seeded onto a 24-well clustered flat bottomed plate (Costar) at 2×10^5 cells/0.5 ml/well, then treated with LPS and/or IFN- γ as described above. The culture supernatant was collected and assayed for NO_2^- .

SDS-PAGE and Immunoblotting As described previously,⁹⁾ 100 μg aliquots of the cell extracts were boiled in an SDS-sample buffer, then electrophoresed with Fairbank's system. The proteins in the gels were electro-transferred onto a polyvinylidene fluoride (PVDF) membrane (Immobilon-P[®]; Millipore Co., Bedford, MA, U.S.A.) with a Mini-Slab Blotting System[®] (Bio-rad, Hercules, CA, U.S.A.). Then, the blotted membrane was blocked with milk casein (Snow Brand Milk Products Co., Sapporo), and the proteins on the membrane were reacted with a monoclonal anti-macrophage NOS(II) antibody (Transduction Laboratories, Lexington, KY, U.S.A.), then with rabbit anti-mouse IgG (Zymed Laboratories, Inc., San Francisco, CA, U.S.A.), and finally with ^{125}I -labeled protein A. After being air-dried, this membrane was placed on an imaging plate (Fuji Photo Film Co., Tokyo), and the intensity of the band corresponding to NOS(II) with a molecular weight of 130 kDa was quantitated with a BAS2000 bioimage analyzer (Fuji Photo Film Co.).

NOS Activity in Cell-free Extracts *in Vitro* Analysis of NOS activity in cell-free extracts *in vitro* was performed as described previously.⁹⁾ In brief, the 50 μl reaction mixture comprised 100 μM β -NADPH, 10 μM H_4B , 10 μM FAD, 10 μM FMN, 100 μM DTT, 100 μM L-[U- ^{14}C]arginine and 50 mM L-valine in 50 mM HEPES-NaOH buffer, pH 7.5, and 100 μg of a cell extract of LPS1916 or JA-4 cells as an enzyme source. The mixture without the enzyme was preincubated at 37°C for 3 min, then the enzyme in 20 μl of the lysis buffer was added to start the reaction, followed by incubation at 37°C for 10 min. The reaction was terminated by the addition of 200 μl of ice-cold 85% ethanol.

RT-PCR Analysis of NOS(II) mRNA mRNA was isolated from a cell pellet with a micro mRNA purification kit (Quick Prep[®] Micro mRNA Purification Kit; Pharmacia Biotech, Uppsala, Sweden). The final amount of RNA was determined by the absorbance at 260 nm. One hundred

nanograms of the final RNA samples were reverse-transcribed into cDNA by the RT-PCR method using oligo-d(T)₁₆ as the reverse transcriptase primer, then amplified by the PCR primers for the sequence of cDNA of mouse NOS(II) and β -actin using an RT-PCR kit (Gene Amp[®] RNA PCR Core Kit; Perkin Elmer Co., Langen, Germany). The sense primer for NOS(II) was 5'-CCAAGAATTCACCTACTTGGACATTACGACCCC-3', and the antisense primer was 5'-G-AAAGGATCCAAGGGAGCAATGCCCGTACCAGGCC-3'. The sense primer for β -actin was 5'-GATGGTGGGTATGGGTCAGAAGGA-3', and the antisense primer was 5'-G-CTCATTGCCGATAGTGATGACCT-3'.¹⁰⁾ The PCR reaction was performed under the following conditions: 35 cycles of denaturation at 94°C for 30 min, annealing at 60°C for 1 min and extension at 72°C for 1.5 min. The PCR products were run on a 2% agarose gel and visualized by ethidium bromide staining. The bands in the gel were photographed and their intensity was analyzed with an NIH image[®] using a Macintosh computer.

Assaying of NO_2^- Assaying of NO_2^- was performed using Griess Romijn reagent.¹¹⁾

Protein Assays Protein concentrations were determined according to Bradford¹²⁾ with bovine serum albumin as a standard.

RESULTS

Nitric Oxide Production Induced by LPS and/or IFN- γ

First, the time course of NO_2^- accumulation was examined in the culture medium of LPS1916 cells treated at 37°C with 100 ng/ml LPS or 100 ng/ml LPS+10 U/ml IFN- γ (Fig. 1). Treatment with LPS alone induced no NO_2^- accumulation in the culture medium of LPS1916 cells, while it induced a small but significant accumulation in JA-4 cells during incubation longer than 12 h (Fig. 1A). The addition of IFN- γ and LPS to both cell lines induced NO_2^- accumulation 6 h after incubation; however, the accumulation in the culture medium of LPS1916 cells was apparently less than in that of JA-4 cells (Fig. 1B). As for the LPS-doses, LPS did not induce NO_2^- production in LPS1916 cells up to 10^4 ng/ml, although it significantly induced that in JA-4 cells at more than 100

Fig. 1. Time Course of NO_2^- Accumulation

JA-4 (closed symbols) and LPS1916 (open symbols) cells were treated with nothing (●, ○), 100 ng/ml LPS (▲, △) (Fig. 1A), or 100 ng/ml LPS+10 U/ml IFN- γ (■, □) (Fig. 1B) at 37°C for up to 24 h. NO_2^- in the culture medium was assayed as described in the text. The results are the means $\pm$ S.E. for two independent experiments with duplicate samples.

Fig. 2. Effects of the LPS and IFN- γ Concentrations on NO_2^- Accumulation in the Culture Medium of the LPS1916 and JA-4 Cell Lines

A) JA-4 (closed symbols) and LPS1916 (open symbols) cells were treated with various concentrations of LPS in the absence ($\bullet$, $\circ$) or presence ($\blacktriangle$, $\triangle$) of 10 U/ml IFN- γ at 37 °C for 8 h. B) JA-4 (closed symbols) and LPS1916 (open symbols) cells were treated with various concentrations of IFN- γ in the absence ($\bullet$, $\circ$) or presence ($\blacktriangle$, $\triangle$) of 100 ng/ml LPS at 37 °C for 8 h. NO_2^- was assayed as described in the text, and the results are the means $\pm$ S.E. for three independent experiments with duplicate samples.

Fig. 3. Effect of the LPS Concentration on Induction of the NOS(II) Protein in the LPS1916 and JA-4 Cell Lines

JA-4 (closed symbols) and LPS1916 (open symbols) cells were treated with various concentrations of LPS in the absence ($\bullet$, $\circ$) or presence ($\blacktriangle$, $\triangle$) of 10 U/ml IFN- γ at 37 °C for 8 h. The photographs indicate the bands corresponding to the NOS(II) protein of 130 kDa with a BAS2000 bioimage analyzer in a typical experiment, and the amounts of NOS(II) were calculated from the intensities of the bands using a standard curve for known amounts of the NOS(II) protein from an extract of the RAW264.7 macrophage-like cell line treated with LPS+IFN- γ at 37 °C for 8 h.⁹⁾

ng/ml (Fig. 2A). The addition of 10 U/ml IFN- γ together with less than 10 ng/ml LPS induced no release of NO_2^- by LPS1916 cells, but an increase in LPS to more than 100 ng/ml induced it significantly. In JA-4 cells, the addition of IFN- γ with LPS induced NO_2^- accumulation with more than 1 ng/ml LPS in a dose-dependent manner. IFN- γ alone induced no release of NO_2^- in either of the cell lines at concentrations up to 10^3 U/ml (Fig. 2B). However, the simultaneous addition of 100 ng/ml LPS with IFN- γ induced NO_2^- release by these cell lines at more than 1 U/ml IFN- γ . The level of NO_2^- release was always higher in JA-4 cells than in LPS1916 cells under the experimental conditions in Fig. 2. These results suggest that the LPS1916 cell line is defective

in the induction of $\cdot\text{NO}$ production by LPS alone, but this defect is partly recovered by the simultaneous addition of IFN- γ .

Induction of NOS(II) As for the induction of the NOS(II) protein in LPS1916 cells, the amounts of NOS(II) were estimated in cells treated at 37 °C for 8 h with LPS or LPS+IFN- γ by immunoblot analysis with an anti-murine macrophage NOS(II) antibody. The results showed a dose-dependent increase in the NOS(II) protein in response to LPS only in the presence of IFN- γ (Fig. 3), as in the case of NO_2^- release (Fig. 1). NOS activity was also examined with LPS1916 cell lysates *in vitro*, and the results were similar to those of NO_2^- release (Fig. 2A) and the amount of NOS(II)

protein (Fig. 3), showing that this mutant responded, in terms of NOS activity induced, to LPS only in the presence of IFN- γ (Fig. 4).

Induction of NOS(II) mRNA Since the above results suggest that the defect in $\cdot$ NO-generation in the mutant cells

Fig. 4. Effect of the LPS Concentration on the NOS Activity in the Cell Lysates

JA-4 (closed symbols) and LPS1916 (open symbols) cells were treated with various concentrations of LPS in the absence (●, ○) or presence (▲, △) of 10 U/ml IFN- γ at 37 °C for 8 h, then the cell lysates were prepared and assayed as described in the text. The results are the means for a typical example of repeated experiments.

is due to a defect in NOS(II) protein induction in response to LPS alone, but that this defect is partly reversed in the presence of IFN- γ and LPS, we next examined NOS(II) mRNA induction in the mutant cells by RT-PCR analysis (Fig. 5). The quantity of NOS(II) mRNA was estimated from the intensity of the band corresponding to NOS(II) mRNA of 456 bp in agarose gels. LPS alone did not induce NOS(II) mRNA in LPS1916 cells throughout the experiments, but LPS+IFN- γ induced a considerable amount of NOS(II) mRNA at 6 h, which then gradually decreased with time until 12 h. In contrast, in JA-4 cells, LPS alone induced a slow but time-dependent increase in NOS(II) mRNA, while LPS+IFN- γ induced a rapid and much higher increase in the mRNA at 3 h, which then rapidly decreased until 6 h and then reached the basal level at 12 h (Fig.5). These results suggest that the defect in the induction of NOS(II) protein in LPS1916 cells treated with LPS alone is due to a failure of NOS(II) gene expression, and the partial recovery of the defect by IFN- γ -treatment might be explained by restoration of the transcription of the NOS(II) gene.

We next examined the LPS dose-dependence of NOS(II) mRNA induction in JA-4 and LPS1916 cells treated with or without 10 U/ml IFN- γ at 37 °C for 6 h. LPS alone induced no NOS(II) mRNA in LPS1916 cells under the experimental conditions, although it induced a slight but significant level of the mRNA in JA-4 cells at more than 10 ng/ml. However, the addition of 10 U/ml IFN- γ significantly induced NOS(II)

Fig. 5. Induction of NOS(II) mRNA

A) Time course of NOS(II) mRNA expression in the JA-4 (closed symbols) and LPS1916 (open symbols) cell lines treated with nothing (●, ○), 100 ng/ml LPS (▲, △), or 100 ng/ml LPS+10 U/ml IFN- γ (■, □) at 37 °C for up to 12 h. B) Effect of the LPS concentration on NOS(II) mRNA expression in both cell lines treated with up to 103 ng/ml LPS in the presence or absence of 10 U/ml IFN- γ at 37 °C for 6 h. The symbols are the same as in A) except for no addition of LPS or IFN- γ . The photographs show the PCR products corresponding to the NOS(II) (456 bp) and β -actin (630 bp) bands. The amounts of NOS(II) mRNA in A) and B) are shown as % values relative to β -actin, individually. The results are for a typical example of repeated experiments.

mRNA in LPS1916 cells in the presence of more than 10 ng/ml LPS, while in JA-4 cells, the amount of the mRNA increased with LPS doses of more than 1 ng/ml.

These results suggest that the defect in $\cdot\text{NO}$ generation in LPS1916 cells treated with LPS was not due to absence of the NOS(II) gene but to a defect in transcription of the NOS(II) gene.

DISCUSSION

In this study, we examined the defect in $\cdot\text{NO}$ production by LPS1916 cells after LPS-treatment, and we suggest that the mutant lacks LPS-responsiveness in the induction of $\cdot\text{NO}$ -producing activity due to a defect in the induction of NOS(II) mRNA. We also showed that the defect in LPS1916 cells was partly reversed by the addition of IFN- γ together with LPS, suggesting that LPS1916 cells are not devoid of the NOS(II) gene but are altered by mutation to express the NOS(II) gene in response to LPS. Based on extensive studies on NO_2^- accumulation, NOS(II) protein, NOS activity *in vitro* and NOS(II) mRNA, we conclude that NOS(II) expression is regulated mainly at the transcriptional level in these cell lines, as has been shown in many other reports.^{4,13,14} However, the reported extent of NO_2^- production (Fig. 2A), amounts of NOS(II) protein (Fig. 3), NOS(II) activities *in vitro* (Fig. 4) and the levels of NOS(II) mRNA (Fig. 5) in JA-4 and LPS1916 cells in response to LPS and/or IFN- γ was not always consistent. This may be because NOS(II) activity is not only regulated at the transcriptional levels but also at such post-transcriptional levels as dimer formation or cellular NADPH levels, as we pointed out previously.⁹ It is also characteristic that the induction of NOS(II) mRNA in LPS1916 cells is delayed compared to that in JA-4 cells treated with LPS+IFN- γ (Fig. 5A). This might be due to the delay of LPS-signaling in LPS1916 cells, because the responses of the mutant cells to IFN- γ were not much different from those of JA-4 cells.⁶ It is therefore feasible that the induction in LPS1916 cells may require some additional inducers of NOS(II) transcription, following LPS signalings. We are currently searching for such regulatory factors using these cell lines.

On the other hand, this mutant produces O_2^- upon stimulation with PMA after activation with IFN- γ alone or with LPS+IFN- γ but not with LPS alone, while the JA-4 cell line produces much higher amounts of O_2^- after activation with IFN- γ alone, LPS alone and LPS+IFN- γ .^{1,6,7} The present studies on the profiles of $\cdot\text{NO}$ production and their mechanisms in LPS1916 and JA-4 cells seem to support the idea that O_2^- generation corresponds to an inhibition of the bacterial growth in these cells infected with *Legionella pneumophila*⁶ and *Listeria monocytogenes*⁷ after treatment with

LPS, but $\cdot\text{NO}$ does not seem to be involved in this inhibition, as judged from the results of our preliminary experiments involving an NOS inhibitor, L-NMMA.^{6,7} However, we have no information as to whether this mutant shows defense against infection by other pathogens, such as *Leishmania major*, which has been shown to be sensitive to $\cdot\text{NO}$.^{5,10,15} Because the production of $\cdot\text{NO}$ and O_2^- by JA-4 and LPS1916 cells may be individually controlled by varying the LPS dose and the addition of IFN- γ , the crucial roles of $\cdot\text{NO}$ and O_2^- , not only in the microbicidal activity but also in the regulation of other functions of activated macrophages, will be clarified by using these cell lines.

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