Biochemical and Biophysical Research Communications **267,** 124–128 (2000)
doi:10.1006/bbrc.1999.1917, available online at http://www.idealibrary.com on IDEAL®

Erythromycin Suppresses Nuclear Factor-κB and Activator Protein-1 Activation in Human Bronchial Epithelial Cells

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Received November 19, 1999

Erythromycin (EM), and related 14-member macrolide antibiotics, has attracted attention for its effectiveness in airway diseases including diffuse panbronchiolitis and sinobronchial syndrome. However, its molecular mechanisms remain unknown. We evaluated the effects of EM on activation of several transcription factors, including nuclear factor-κB (NF-κB) and activator protein-1 (AP-1) in human bronchial epithelial cell line BET-1A, which are known to regulate the expression of many proinflammatory cytokines and chemokines such as interleukin-8 (IL-8). BET-1A cells were cultured with hormonally defined Ham's F12 medium, and were stimulated by phorbol myristate acetate (PMA). EM suppressed mRNA expression as well as the release of IL-8 at therapeutic and noncytotoxic concentrations (% inhibition of IL-8 protein release: 42.2 ± 5.5%, at 10^{-6} M). Furthermore, electrophoretic mobility shift assays revealed that EM inhibited the activations of NF-κB and AP-1 induced by PMA in BET-1A cells. These data indicate that EM has inhibitory effects not only on the mRNA expression and release of IL-8, but also on the activation of transcription factors NF-κB and AP-1. Our findings support the concept that the recruitment of neutrophils in airway diseases may be regulated by NF-κB and AP-1.

Key Words: **IL-8; NF-κB; erythromycin/AP-1.**

Erythromycin (EM), a macrolide antibiotic, has been reported to be effective for the treatment of chronic airway diseases such as diffuse panbronchiolitis (DPB), bronchial asthma, and chronic sinusitis (1–3). However, its molecular mechanism remains unknown. DPB, which is prevalent in Japan and is also characterized by chronic airway inflammation with infiltration of neutrophils in respiratory bronchioles, has been successfully treated with low-dose, long-term administration of EM (4). At this dosage, EM cannot be expected to act as an antibacterial agent, which suggests that EM has some anti-inflammatory actions. Kadota and associates showed a significant reduction of neutrophils as well as a neutrophil chemotactic activity in bronchoalveolar lavage fluid after EM was administered to the patients with DPB (2). It is possible that EM modulates airway inflammatory responses by decreasing the local chemokine release and thus reducing the recruitment of inflammatory cells such as neutrophils.

Interleukin-8 (IL-8), a neutrophil chemoattractant peptide, is one of the CXC chemokines which is known to be released by monocytes (5), macrophages (6), fibroblasts (7) and epithelial cells (8). Previous studies in our laboratory have demonstrated that the release of IL-8 from bronchial epithelial cells was increased after the stimulus of IL-1α, which was inhibited by macrolide antibiotics (9). Although several studies demonstrated that IL-8 plays a pivotal role in neutrophilic airway inflammations (10–12), the molecular regulatory mechanisms involved in the induction of the chemokine and the recruitment of neutrophils are not well understood. Expression of several genes, including IL-6, IL-8 and tumor necrosis factor (TNFα), is controlled by a number of DNA-binding proteins which interact with specific sequence motifs in the promoter region of the gene (13). IL-8 expression is elicited by stimuli that cause translocation of the nuclear transcription factor NF-κB to the nucleus (14).

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0006-291X/00 $35.00

In the experiments described in this report, we examined how EM has an inhibitory effect on the neutrophilic inflammation in airway diseases like DPB by studying the activation of transcription factors such as NF-κB and AP-1 in relation to the production of IL-8. Since EM is effective for the patients with DPB in a long-term treatment (4), we studied the effect of EM on the activation of transcription factors not only for 24 h but also for 72 h preincubation with bronchial epithelial cells.

MATERIALS AND METHODS

Culture of bronchial epithelial cells. Human bronchial epithelial cell line BET-1A (a kind gift from Drs. J. F. Lechner and C. C. Harris, National Cancer Institute, Bethesda, MD) was cultured by the method reported previously (15). Briefly, the cells were plated onto collagen-coated 24-well flat-bottom tissue culture plates (Koken, Tokyo, Japan) at the density of 5×10^4 cells/well in hormonally defined Ham's F12 medium (HD-F12) as reported. HD-F12 contained 1% penicillin-streptomycin, 5 μg/ml insulin (Life Technologies, Grand Island, NY), 5 μg/ml transferrin (Life Technologies), 25 ng/ml epidermal growth factor (Collaborative Research, Lexington, MA), 15 μg/ml endothelial cell growth supplement (Collaborative Research), 2×10^{-10} M triiodothyronin (Life Technologies), and 10^{-7} M hydrocortisone (Life Technologies). The cells were incubated in a humidified atmosphere at 37°C and 5% CO_2. The medium was changed at day 1 and subsequently every 2 days. For the evaluation of effects of EM (a kind gift from Dr. S. Omura, Kitasato Institute, Tokyo), the cells were incubated with various concentrations of EM before the stimulation by phorbol myristate acetate (PMA). The number of the cells was counted by a standard hemacytometer, and cell viability was assessed by trypan blue dye exclusion method.

Northern blot analysis for IL-8 mRNA expression in BET-1A. Northern blot analysis was performed by the method described previously (16). Briefly, total cellular RNA was extracted by the method of Chomczynski and Sacchi (17) and electrophoresed on formaldehyde-denatured agarose gel (15 μg/lane) followed by capillary transfer onto Biodyne nylon membrane (Pall Corporation, East Hills, NY). RNA integrity and equivalency of loading were routinely evaluated by ethidium bromide fluorescence. Blots were baked, prehybridized and hybridized with a ^{32}P-5′-end-labeled oligonucleotide probe specific for human IL-8 and β-actin. The probes used in this study were reported previously (18, 19). Blots were stringently washed after hybridization and exposed to X-ray film (Kodak, Rochester, NY).

Electrophoretic mobility shift assay. After the cells were washed with PBS, the nuclear proteins were isolated by the method reported previously (20) with modifications. In brief, 2 to 3×10^6 cells were harvested with the addition of trypsin-EDTA solution (Life Technologies), rinsed in Tris-buffered saline, resuspended in lysis buffer (10 mM Hepes, 10 mM KCl, 0.1 mM EGTA, 0.1 mM EDTA, 1 mM DTT), 0.5 mM PMSF and incubated on ice for 15 min. Nonidet P-40 (10%) was added to lyse the cells, and then the cells were centrifuged for 6 min at 4°C at 600*g*. The nuclear pellet was resuspended in extraction buffer (20 mM Hepes, 50 mM KCl, 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF) and vortexed for 15 min at 12,000 rpm at 4°C. The supernatant was collected, divided into aliquots, and stored at −70°C. Protein concentration was determined by the Bradford dye-binding procedure(Bio-Rad Protein Assay, Richmond, CA), standardized with BSA. For the detection of NF-κB DNA binding, Gel Shift Assay System obtained from Promega (Madison, WI) was used. The sequence of the oligonucleotides containing a tandem repeat of the consensus sequence for NF-κB DNA binding site was as follows: 5′-AGTTGAGGGGACTTTCCCAGGC-3′, 3′-TCAACTCCCCTGAAAGGGTCCG-5′. The specific dsDNA for AP-1 was as follows; 5′-CGCTTGATGAGTCAGCCGGAA-3′, 3′-GCGAACTACTCAGTCGGCCTT-5′. The specific dsDNA for CREB was as follows; 5′-AGAGATTGCCTGACGTCAGAGAGCTAG-3′, 3′-TCTCTAACGGACTGCAGTCTCTCGATC-5′. Synthetic double stranded oligonucleotides were labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase as recommended by the manufacturer. The DNA binding reaction was conducted at room temperature for 20 min in a volume of 25 μl. The reaction mixture contained 10 μg nuclear extract, 10 mM Tris (pH 7.5), 1 mM EDTA, 100 mM NaCl, 1 mM DTT, 1 mM EGTA, 4% (v/v) glycerol, 0.08 mg/ml sonicated salmon sperm DNA, and ^{32}P-labeled double-stranded oligonucleotides at 0.7 fmol/μg nuclear extract. After incubation, the samples were loaded onto a 4% polyacrylamide gel (polyacrylamide:bis (30%:0.8% w/v), 2.5% glycerol in 0.5× Tris-borate-EDTA) and run at 120 V for 2 h. Each gel was then dried and subjected to autoradiography.

For supershift studies, 2 μl of anti-p65, anti-p50 (Santa Cruz Biotech., Inc., Santa Cruz, CA), or control antisera was added to the reaction mixture containing the NF-κB oligonucleotide. Binding of the antibody to the appropriate transcription factor was indicated by a supershift in the EMSA.

Cytokine assay. Specific immunoreactivity for IL-8 in culture supernatants was measured by ELISA kits (R&D Systems, Minneapolis, MN). Each sample was assayed in duplicates as recommended by the manufacturer.

Statistical analysis. The results were analyzed by Student's *t* test for comparison between the two groups and by nonparametric equivalents of ANOVA for multiple comparison as reported (9, 21).

RESULTS

IL-8 production by PMA and its inhibition by EM. BET-1A cells spontaneously released IL-8 protein into the extracellular medium as assessed by ELISA. Addition of exogenous PMA (10^{-7}) to these cells for 24 h induced a significant release of IL-8 ($P < 0.01$, ANOVA). PMA was administered for 24 h after the cells were incubated with different concentrations of EM, and IL-8 proteins in the supernatants were measured by ELISA. EM inhibited the release of IL-8 protein in a dose-dependent fashion (Fig. 1, % inhibition: $20.5 \pm 4.5\%$ with EM 10^{-7}, $42.2 \pm 5.5\%$ with EM 10^{-6}, $59.1 \pm 4.8\%$ with EM 10^{-5}, $P < 0.01$ compared to PMA alone, ANOVA). EM at these concentrations for 24 h incubation had no significant cytotoxicity to BET-1A cells as assessed by trypan blue dye exclusion.

Changes in IL-8 mRNA levels of BET-1A cells by EM. Total cellular RNA was extracted after BET-1A cells were incubated with different concentrations of EM followed by the stimulation of PMA. As shown in Fig. 2, IL-8 mRNA was increased by the addition of PMA, which was inhibited by the preincubation with EM in a dose-dependent fashion.

Electrophoretic mobility shift assay (EMSA) for the detection of transcription factors. BET-1A cells were treated with EM at the concentration of 10^{-6} M for 24 h or 72 h before the addition of 10^{-7} M of PMA and the nuclear extracts were isolated 4 h after PMA treatment for EMSA as described under Materials and Methods. The specificity of the NF-κB binding was ascertained by the supershift of the bands with the antibody to p50

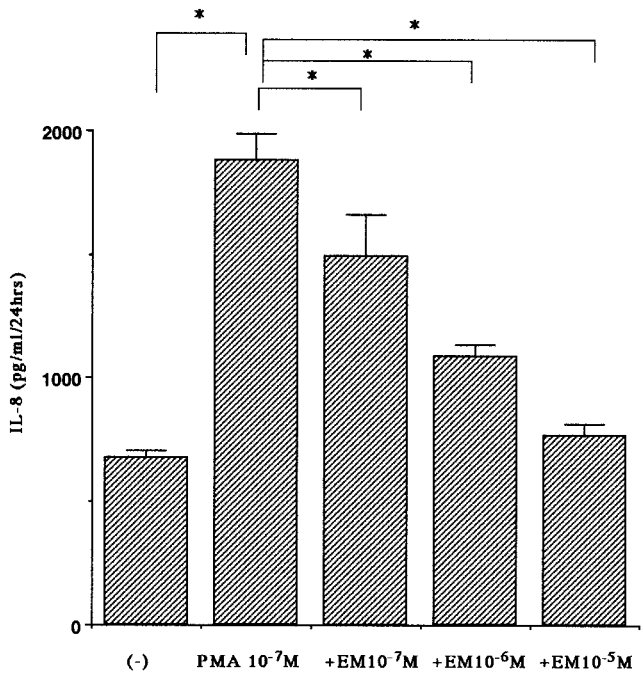

FIG. 1. Dose-dependent effect of EM on IL-8 release. BET-1A cells stimulated by PMA were preincubated with different concentrations of EM, and the supernatants were harvested after 24 h for IL-8 measurements. *P value < 0.01 (ANOVA). The data were shown as mean ± SEM.

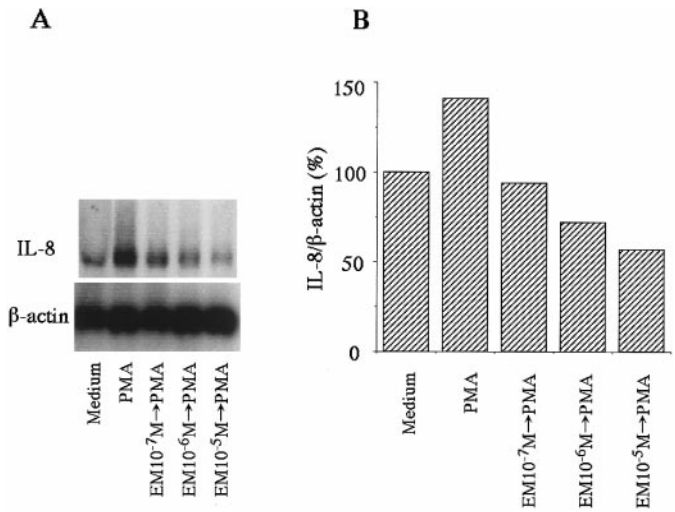

FIG. 2. Effect of EM on IL-8 mRNA levels in BET-1A cells *in vitro*. BET-1A cells were pretreated with different concentrations of EM before the stimulation of PMA. Total cellular RNA was extracted and Northern blot analysis was performed to evaluate the changes in IL-8 mRNA levels. (A) IL-8 mRNA level increased after the stimulation of PMA, which was inhibited by the preincubation with EM in a dose-dependent fashion. (B) Measurements of densitometric signals of IL-8 corrected by β-actin transcripts were shown.

as well as the reduced intensity of the signals with excess amounts of cold DNA NF-κB probe (Fig. 3). The activation of NF-κB induced by PMA was inhibited by the preincubation with EM, especially at 72 h. The activation of AP-1 was also inhibited by the preincubation with EM at 24 h and 72 h, while cyclic AMP-response element binding protein (CREB) activation in BET-1A was not changed by the addition of EM (Fig. 4). None of the transcription factors were affected by EM when added simultaneously with PMA (data not shown).

DISCUSSION

EM has been shown to be effective in patients with DPB below the concentration at which EM acts as an antibiotics (4). This fact suggests that EM may be effective for patients with DPB by a mechanism other than anti-bacterial activity. One of the possible mechanisms is an anti-inflammatory action. We previously reported that EM and clarithromycin as well as dexamethasone inhibited mRNA expression of IL-6 by normal bronchial epithelial cells (22). Moreover, we demonstrated that those macrolide antibiotics uniquely suppressed mRNA expression and release of IL-8 in airway epithelium, while other antibiotics including aminobenzyl penicillin, cefazolin, tetracycline and josamycin did not change IL-8 mRNA (9). These observations led us to study the effects of EM on transcription factors such as NF-κB and AP-1, which may influence the expression of IL-8 mRNA by interacting the promoter region of the gene.

In the present report, we for the first time found that EM inhibited the activation of both NF-κB and AP-1

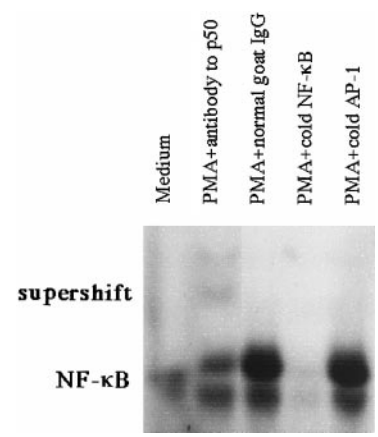

FIG. 3. EMSA analysis for NF-κB binding in BET-1A cells *in vitro*. The cells were pretreated with 10^{-6} M of EM for 24 h before the stimulation of PMA and the nuclear extracts were isolated for EMSA assays as described under Materials and Methods. NF-κB was activated by the addition of PMA. The specificity of the binding was ascertained by the supershift of the band with the antibody to p50 (lane 2), as well as the reduced intensity of the signals with excess amount of cold NF-κB probe (lane 4) compared with control antisera (lane 3). Addition of excess cold AP-1 probe (lane 5) showed no effect.

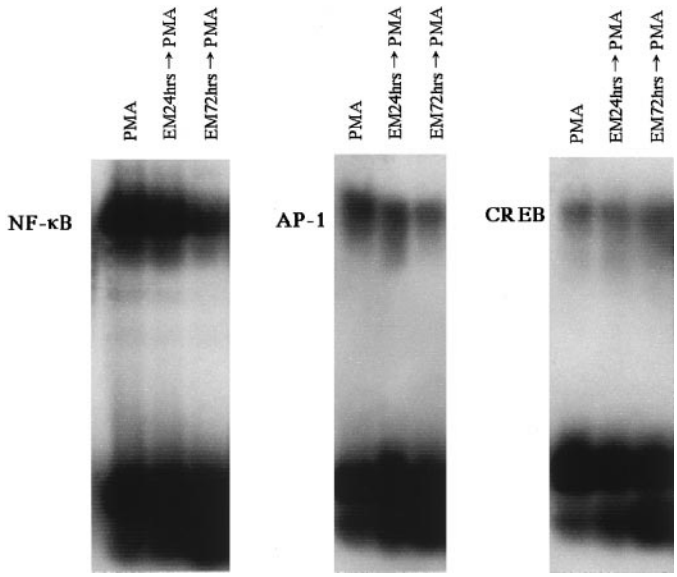

FIG. 4. Effects of EM on the activation of transcription factors in BET-1A cells as assessed by EMSA. The cells were pretreated with 10^{-6} M EM for 24 and 72 h before the stimulation of PMA and the nuclear extracts were isolated for EMSA assays as described under Materials and Methods. The activation of NF-κB induced by PMA was inhibited by the preincubation with EM, especially at 72 h. The activation of AP-1 was also inhibited by the preincubation with EM at 24 and 72 h, while CREB activation in BET-1A was not changed by the addition of EM.

when EM was pretreated. EM did not inhibit the activations of NF-κB and AP-1 when added simultaneously with PMA, but it did suppress AP-1 binding activity when the cells were treated 24 and 72 h before PMA. Interestingly, NF-κB activation was suppressed when EM was added 72 h before PMA, whereas the activation of CREB was not affected at both time points. It took longer time for EM to inhibit the activation of NF-κB than to inhibit that of AP-1. We do not know the exact reason for this. However, these findings may have relevance to the clinical effectiveness of EM, which is used at a low-dose and for a long-term period to the patients with DPB or other chronic airway inflammations.

The genes that are regulated by NF-κB include several cytokines which participate in leukocyte recruitment and activation, including IL-6, IL-8, TNFα and monocyte-chemotactic protein-1 (MCP-1) (13). IL-8 gene contains binding sites for several transcription factors including AP-1, NF-κB and NF-IL-6 (14). Mukaida and associates reported that the transcription of IL-8 gene requires the coordinated activation of these transcription factors, depending on the cell type and stimulating agents (23, 24). NF-κB is the most crucial factor for IL-8 gene transcription although NF-IL-6 and AP-1 are required for the maximal expression. Our studies with EMSA showed that EM at a therapeutic concentration suppressed the activation of both NF-κB and AP-1, which suggests that the mRNA expression of IL-8 may be regulated by these transcription factors.

EM also has a motilin-like stimulating activity on gastrointestinal smooth muscles (25). Therefore, anti-inflammatory activity of EM via inhibiting the transcription factors reported here may be the third bioactivity of this macrolide antibiotic. Characterization of the chemical structure responsible for its potential would be important to pursue, and further investigation for the molecular mechanism would be necessary for a possible new type of an anti-inflammatory agent.

ACKNOWLEDGMENTS

We thank Takako Kobayashi for her excellent technical support. This work was supported in part by the Diffuse Lung Disease Research Committee, Japan Ministry of Health and Welfare, and the Manabe Medical Foundation.

REFERENCES

1. Kudoh, S., Uetake, T., Hagiwara, K., Hirayama, M., Hus, L. H., Kimura, H., and Sugiyama, Y. (1987) *Jpn. J. Thorac. Dis.* **25,** 632–642.
2. Kadota, J., Sakito, O., Kohno, S., Sawa, H., Mukae, H., Oda, H., Kawakami, K., Fukushima, K., Hiratani, K., and Hara, K. (1993) *Am. Rev. Respir. Dis.* **147,** 153–159.
3. Miyatake, H., Taki, F., Taniguchi, H., Suzuki, R., Takagi, K., and Satake, T. (1991) *Chest* **99,** 670–673.
4. Nagai, H., Shishido, H., Yoneda, R., Yamaguchi, E., Tamura, A., and Kurashima, A. (1991) *Respiration* **58,** 145–149.
5. Yoshimura, T., Matsushima, K., Oppenheim, J. J., and Leonard, E. J. (1987) *J. Immunol.* **139,** 788–793.
6. Carre, P. C., Mortenson, R. L., King, T. E., Jr., Noble, P. W., Sable, C. L., and Riches, D. W. (1991) *J. Clin. Invest.* **88,** 1802–1810.
7. Rolfe, M. W., Kunkel, S. L., Standiford, T. J., Chensue, S. W., Allen, R. M., Evanoff, H. L., Phan, S. H., and Strieter, R. M. (1991) *Am. J. Respir. Cell Mol. Biol.* **5,** 493–501.
8. Churg, A. (1996) *Am. J. Respir. Crit. Care Med.* **154,** 1124–1140.
9. Takizawa, H., Desaki, M., Ohtoshi, T., Kawasaki, S., Kohyama, T., Sato, M., Tanaka, M., Kasama, T., Kobayashi, K., Nakajima, J., and Ito, K. (1997) *Am. J. Respir. Crit. Care Med.* **156,** 266–271.
10. Chang, M. M., Wu, R., Plopper, C. G., and Hyde, D. M. (1998) *Am. J. Physiol.* **275,** L524–532.
11. Grau, G. E., Mili, N., Lou, J. N., Morel, D. R., Ricou, B., Lucas, R., and Suter, P. M. (1996) *Lab. Invest.* **74,** 761–770.
12. Yamamoto, T., Kajikawa, O., Martin, T. R., Sharar, S. R., Harlan, and J. M., Winn, R. K. (1998) *J. Immunol.* **161,** 5704–5709.
13. Barnes, P. J., and Karin, M. (1997) *N. Engl. J. Med.* **336,** 1066–1071.
14. Mukaida, N., Okamoto, S., Ishikawa, Y., and Matsushima, K. (1994) *J. Leukocyte Biol.* **56,** 554–558.
15. Takizawa, H., Desaki, M., Ohtoshi, T., Kawasaki, S., Kohyama, T., Sato, M., Nakajima, J., Yanagisawa, M., and Ito, K. (1998) *Eur. Respir. J.* **12,** 57–63.
16. Nakano, J., Takizawa, H., Ohtoshi, T., Shoji, S., Yamaguchi, M., Ishii, A., Yanagisawa, M., and Ito, K. (1994) *Clin. Exp. Allergy* **24,** 330–336.
17. Chomczynski, P., and Sacchi, N. (1987) *Anal. Biochem.* **162,** 156–159.

18. Kasama, T., Strieter, R. M., Lukacs, N. W., Burdick, M. D., and Kunke, S. L. (1994) *J. Immunol.* **152,** 3559–3569.
19. Takizawa, H., Ohtoshi, T., Kawasaki, S., Kohyama, T., Desaki, M., Kasama, T., Kobayashi, K., Nakahara, K., Yamamoto, K., Matsushima, K., and Kudoh, S. (1999) *J. Immunol.* **162,** 4705–4711.
20. Carter, A. B., Monick, M. M., and Hunninghake, G. W. (1998) *Am. J. Respir. Cell Mol. Biol.* **18,** 384–391.
21. Ohtoshi, T., Takizawa, H., Okazaki, H., Kawasaki, S., Takeuchi, N., Ohta, K., and Ito, K. (1998) *J. Allergy Clin. Immunol.* **101,** 778–785.
22. Takizawa, H., Desaki, M., Ohtoshi, T., Kikutani, T., Okazaki, H., Sato, M., Akiyama, N., Shoji, S., Hiramatsu, K., and Ito, K. (1995) *Biochem. Biophys. Res. Commun.* **210,** 781–786.
23. Mukaida, N., Mahe, Y., and Matsushima, K. (1990) *J. Biol. Chem.* **265,** 21128–21133.
24. Matsusaka, T., Fujikawa, K., Nishio, Y., Mukaida, N., Matsushima, K., Kishimoto, T., and Akira, S. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 10193–10197.
25. Kondo, Y., Torii, K., Itoh, Z., and Omura, S. (1988) *Biochem. Biophys. Res. Commun.* **150,** 877–882.