BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **232,** 183–187 (1997)
ARTICLE NO. RC976182

Superoxide Scavenging Activity of Erythromycin–Iron Complex

Hiroyuki Muranaka,* Moritaka Suga,* Keizo Sato,* Kazuko Nakagawa,* Takaaki Akaike,† Tatsuya Okamoto,*,† Hiroshi Maeda,† and Masayuki Ando*

**First Department of Internal Medicine and †Department of Microbiology, Kumamoto University School of Medicine, Kumamoto 860, Japan*

Received January 13, 1997

We evaluated superoxide ($O_2^{\cdot-}$) scavenging activity of erythromycin (EM) and of EM-iron complex by means of electron spin resonance spectroscopy, luminol-dependent chemiluminescence assay, and cytochrome *c* reduction assay. The EM-iron complex was produced by mixing EM with equal molar iron chloride and was stable in neutral buffer. The EM-iron complex reduced the amount of $O_2^{\cdot-}$ produced by xanthine oxidase/hypoxanthine without inhibiting the enzyme activity. It also reduced the amount of $O_2^{\cdot-}$ release from phorbor ester-stimulated human neutrophils and alveolar macrophages. EM alone showed few such effects. The scavenging activity of the complex was equal to that of L-ascorbic acid. These results *in vitro* suggest a possibility that the $O_2^{\cdot-}$-scavenging effect of EM-iron complex contributes to the anti-inflammatory action of EM used in treating chronic inflammatory lung disease independent of its antimicrobial activity.

The macrolide antibiotic, erythromycin (EM) is widely used. Its efficacy when given at a low dose and for long periods in treating patients with chronic lower respiratory tract inflammation appears to be independent of its antimicrobial action [1, 2]. Several studies have reported that EM does [1-4] or dose not [5] regulate neutrophil functions, reduces mucous secretion [6] or modulates cytokine release from bronchial epithelial cells [7, 8], and promotes monocyte-to-macrophage differentiation [9]. Thus, EM may inhibit inflammation by multiple mechanisms.

Toxicity of oxygen-derived free radicals has been suggested as a major cause of inflammatory tissue injury [10-12]. Antioxidants have demonstrated significant protective effects against oxidative stress in various diseases [11-14]. Antiarthritic drugs, such as D-penicillamine, or captopril containing thiol groups are known as radical scavengers [15, 16].

Superoxide ($O_2^{\cdot-}$) is the precursor of a series of highly toxic oxidants [10-13]. Medicinal compound such as cimetidine and pyrimine with metal have been shown to possess $O_2^{\cdot-}$ scavenging activity [17-19]. The purpose of this study was to determine the $O_2^{\cdot-}$ scavenging activity of EM and EM-iron complex in cell-free and human whole-cell systems.

MATERIALS AND METHODS

Chemicals. EM was donated by Dainippon Pharmaceutical Co. Ltd., Osaka, Japan. 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO, purity > 99%) was purchased from Dojindo Laboratories (Kumamoto, Japan), 3,5-Dimethoxy-4-hydroxycinnamic acid (sinapinic acid), Cu, Zn-superoxide dismutase (SOD), luminol, cytochrome *c*: type IV from Sigma Chemical Co., (St. Louis, MO), dimethyl sulfoxide (DMSO), phorbol myristate acetate (PMA) from Nakarai Tesque Inc., (Kyoto, Japan), trifluoroacetic acid (TFA) from Applied Biosystems Division (Foster City, USA), and xanthine oxidase (XO), hypoxanthine (HPX) from Boehringer-Mannheim GmbH (Mannheim, Germany).

Preparation of the cells. Human peripheral blood polymorphonuclear leukocyte (PMN) were isolated by Ficoll-Hypaque density centrifugation followed by dextran sedimentation. Cells were suspended in Krebs-Ringer phosphate (KRP) at 1×10^7 cells/ml. Human alveolar macrophages (AM) were obtained by bronchoalveolar lavage (BAL) from normal volunteers using 200 ml of sterile saline. Lavage fluid specimens were filtered through sterile gauze and centrifuged at $500 \times g$ for 10 min at 4 °C. The pellets were washed three times with KRP. The AM suspensions were > 95% pure (neutrophils < 1%) and had > 97% viability based on Giemsa staining, nonspecific esterase staining, and trypan blue dye exclusion. AM were resuspended in KRP at 1×10^7 cells/ml.

Production of EM–iron complex. EM powder or iron chloride was dissolved in DMSO, deionized water and then diluted with KRP. EM-iron complex was produced by mixing the solutions of EM and iron chloride at various molar ratios. The spectrum of EM, iron chloride and EM-iron complex were obtained by a Hitachi double wavelength spectrophotometer (Hitachi Ltd., Tokyo, Japan) at pH 6.8. Maximum concentration of DMSO in experiments was 2.5%. DMSO itself did not affect $O_2^{\cdot-}$ assay in any system at the concentration (data not shown).

Mass spectrometry. Experiments were carried out by matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS). The mass spectrometer used was a time-of-flight mass spectrometer

0006-291X/97 $25.00

(KOMPACT MALDI III; Shimadzu corp., Kyoto, Japan) operated in the positive ion and reflector mode. This instrument was equipped with a drift length of 0.7 m and a nitrogen laser at 337 nm. EM powder or iron chloride was dissolved in 100% ethanol and diluted with deionized water at a concentration of 20 mg/ml in 20% ethanol. The matrix used was sinapinic acid dissolved at a concentration of 10 mg/ml in 0.1% TFA. It was placed over 0.5 μl of the samples mounted on a stainless steel target. It was dried in a cold air stream before loading into mass spectrometer [20, 21]. The ion peaks were calibrated with insulin, BSA, and substance-P in the calibration file of the software.

Thin layer chromatography (TLC). Each aliquot (15 μl) of the EM and EM-iron complex solutions was applied to the TLC plate; it was developed with n-hexane/chloroform/methanol (26:5:1, v/v). The spots of EM and its reaction products were detected under UV radiation.

Superoxide production. Superoxide was produced by the XO/HPX system, and by PMA-stimulated PMN or AM. The reaction mixture of XO/HPX system contained 0.2 U/ml of XO, 360 μM HPX, and 0.5 mM diethylenetriamine-pentaacetic acid (DTPA) in 10 mM sodium phosphate buffer at pH 7.3. The scavenging activities of EM, EM-iron complex, SOD, ascorbic acid, and epigallocatechin were estimated by adding them into the system at various concentrations. The XO activity was assayed by monitoring uric acid production as an increase in absorbance at 290 nm on a spectrophotometer for 3 min at room temperature. The cells were suspended at 1-5 $\times$ 10^6 cells/ml with 0.5 μg/ml of PMA, 45 mM DMPO and 500 μM DTPA in 200 μl of KRP, with or without EM or EM-iron complex. Every buffer and solution of reaction mixtures used was treated with Chelex 100 resin (Bio-Rad Laboratories, Richmond, CA) before use to remove trace metals.

Electron spin resonance spectroscopy (ESR) spin trapping. The $O_2^{\cdot-}$ was assayed by ESR spin trapping with DMPO and the $O_2^{\cdot-}$ scavenging activity was calculated from the relative intensity peak height of the DMPO-OOH ESR signal [22]. The reacting mixtures were immediately transferred into quartz ESR flat cells (effective volume 160 μl). Then ESR spectra were recorded (JES-RE1X, JEOL Ltd., Akishima, Japan) at room temperature under the condition indicated in figure legends. The effect of EM or EM-iron complex, at various doses, on the $O_2^{\cdot-}$ release from PMA-stimulated PMN was observed by the signal intensity of DMPO-OOH adducts after reaction proceeded for 33 min. Computer simulation for experimental spectra was performed with the ESS-20 ESR simulation program (Labotec Co. LTD, Tokyo, Japan) described previously [19]. After recording the ESR spectra, the signal intensities of DMPO-OOH adducts were normalized against the signal intensity of a manganese oxide marker. Absolute concentrations of the spin adducts of free radicals were determined by a double integration of the ESR spectrum; 1.0 μM of Tempol solution was used for the primary standard of ESR absorption according to the method previously described [23].

Chemiluminescence (CL) assay. The CL response induced by the $O_2^{\cdot-}$ released from PMN was measured by a six-channel luminometer equipped with a data-analyzing computer (Model LB 9505C, Laboratorium Berthold AG, Wildbed, Germany). The reaction was initiated by addition of 1 μg/ml PMA to 8 $\times$ 10^5 PMN in KRP containing 2 μg/ml luminol. The CL response was continuously recorded for 60 min at 26 °C.

Cytochrome c reduction assay. The ferricytochrome *c* reduction rate of $O_2^{\cdot-}$ released from PMN was measured as reported previously [24] at 540 and 550 nm using a Hitachi 557 double wavelength spectrometer equipped with a thermostat-controlled cell. The reaction mixture contained 1 $\times$ 10^6 PMN, 80 μM ferricytochrome *c* and 0.5 μg/ml PMA in KRP with or without 17.0 μM EM or EM-iron complex. After incubation at 37 °C for 10 min, spontaneous $O_2^{\cdot-}$ release was measured for 5 min. PMA was added to the reaction mixture and ferricytochrome *c* reduction was observed for 20 min. Ferricytochrome *c* reduction was completely terminated by addition of 20 μg of SOD. The amount of reduced cytochrome *c* was calculated using a molar absorption coefficient of 19.1 $\times$ 10^3. The initial rate of $O_2^{\cdot-}$ release was expressed as nanomoles of cytochrome *c* reduced/min/10^6 cells.

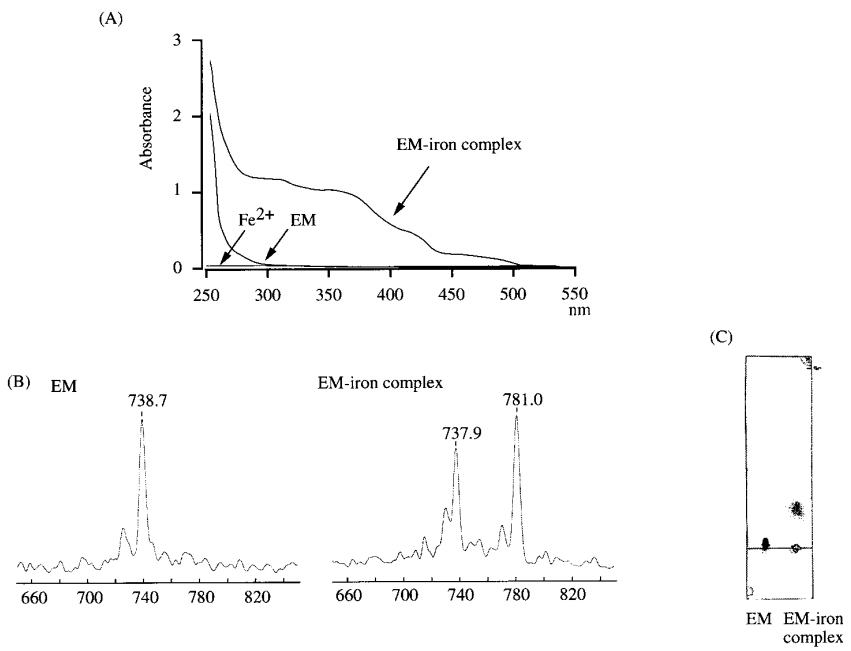

FIG. 1. Production of EM-iron complex. EM-iron complex was produced by mixing 681 μM EM and 340.5 μM iron chloride at room temperature. The spectrum of EM-iron complex had peaks at 300 nm and 350 nm and was distinct from the spectra of EM and iron chloride at pH 6.8 (A). The production of EM-iron complex was confirmed by MALDI-MS (B) and TLC (C).

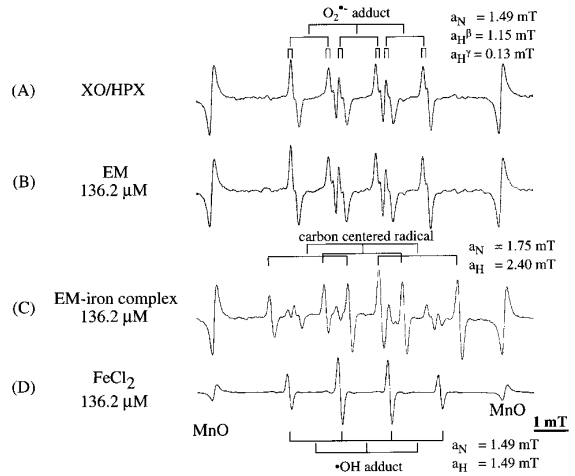

FIG. 2. ESR spin trapping for various radicals generated in XO/HPX reaction mixtures. The reaction mixture contained 0.2 U/ml XO, and 360 μM HPX in 10 mM sodium phosphate buffer at pH 7.3. (A) DMPO-OOH adducts were produced by $O_2^{\bullet-}$ from the XO/HPX system and after addition of EM (B) or EM-iron complex (C). DMPO-OH adducts were produced by addition of iron chloride (D). ESR spectra were recorded at room temperature under the following conditions; modulation frequency, 100 kHz; modulation amplitude, 0.079 mT; scanning field, 336.2 ± 5 mT; receiver gain, (A)-(C) 500, (D) 125; response time, 0.3 sec; sweep time, 2 min; microwave power, 20 mW; and microwave frequency, 9.421 GHz.

Statistical analysis. We repeated each type of experiment at least three times and confirmed that similar data were obtained respectively. The results, obtained in triplicate, are expressed as mean values ± SD.

RESULTS

Production of EM–Iron Complex

The spectra of EM, the EM-iron complex and iron chloride at pH 6.8 are shown in Fig. 1A. The uv/vis spectrum of EM-iron complex was distinct from that of EM or iron chloride. The binding ratio of EM and iron chloride was confirmed to be 1:1 based on the maximum synthesis of the complex at a molar ratio 1:1. The MALDI-MS spectrum of EM (MW; 734.2) showed a single component of 738.7 Da (ΔD = +4.5) and that of EM-iron complex showed two components of 737.9 Da (ΔD = +3.7) and 781.0 Da (ΔD = −9.0) (Fig. 1B). These data and TLC (Fig. 1C) confirmed the formation of EM-iron complex.

$O_2^{\bullet-}$ Scavenging Effect of EM and EM–Iron Complex in the XO/HPX System

ESR spin trapping indicated that DMPO-OOH (a_N = 1.49 mT, a_H^β = 1.15 mT, a_H^γ = 0.13 mT) adducts were produced by the XO/HPX system and DMPO-OH (a_N = 1.49 mT, a_H = 1.49 mT) adducts were formed by reaction with iron-chloride (Fig. 2). EM did not change the control signal. However, 136.2 μM of EM-iron complex decreased DMPO-OOH signals significantly and produced another carbon centered (a_N = 1.75 mT, a_H = 2.4 mT) signal (Fig. 2C). EM-iron complex appeared to scavenge $O_2^{\bullet-}$ in a concentration-dependent manner but EM itself did not (Fig. 3). The concentration of EM-iron complex resulting in reduction of $[O_2^{\bullet-}]$ to 50% of initial values (IC_{50}) was 30 μM. To estimate the relative scavenging activity of EM-iron complex, the IC_{50} values of known $O_2^{\bullet-}$ scavengers were determined (Table 1). The scavenging activity of EM-iron complex was less than that of SOD but equal to that of L-ascorbic acid.

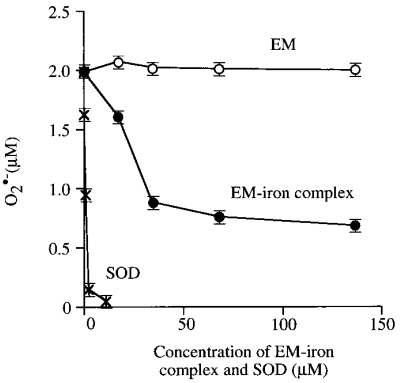

FIG. 3. Scavenging of $O_2^{\bullet-}$ by EM-iron complex and SOD. $O_2^{\bullet-}$ was generated by the XO/HPX reaction mixture. The $O_2^{\bullet-}$ scavenging activity was calculated from the relative intensity of the DMPO-OOH ESR spectrum [20].

The inhibition of XO activity by the EM-iron complex was only 6% at 170 μM and 225 μM.

Effect of EM and EM–Iron Complex against $O_2^{\bullet-}$ Release from PMN and AM

EM-iron complex (68.1 μM) appeared to inhibit the $O_2^{\bullet-}$ release from PMN or AM stimulated with PMA (Fig. 4). The time course and concentration-dependency of the inhibition were observed also (Fig. 5). The inhibition of $O_2^{\bullet-}$ release from PMN by EM-iron complex was increased in a concentration-dependent manner and the IC_{50} was 47.7 μM. However, EM (68.1 μM) inhibited only 14.1% of $O_2^{\bullet-}$ release from PMN. The effect of EM-iron complex was confirmed by determination of CL

TABLE 1

Scavenging Activity of EM–Iron (1:1) Complex and Other Scavengers (50% of Inhibitory Capacity IC_{50} (M) against $O^{\bullet-}_2$)

Radical scavengers	IC_{50} (M)
EM–iron (1:1) complex	3.00×10^{-5}
Ascorbic acid	3.32×10^{-5}
Epigallocatechin	1.16×10^{-4}
Cu, Zn-SOD	3.46×10^{-9}

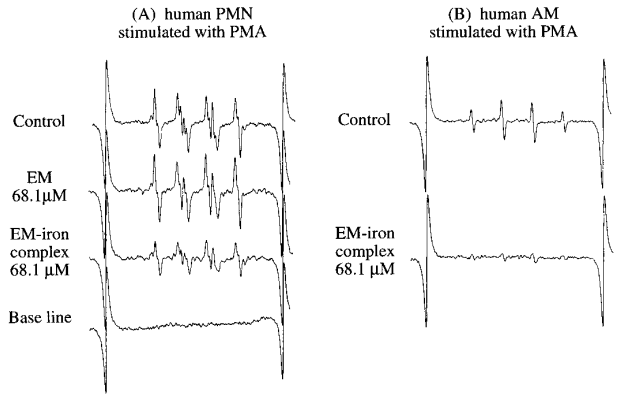

FIG. 4. Effects of EM and EM-iron complex on $O_2^{\cdot-}$ release from PMA-stimulated PMN or AM by ESR spin trapping. Cells were stimulated with 0.5 μg/ml of PMA in KRP containing 45 mM DMPO and 500 μM DTPA. ESR spectra were recorded under the same conditions as described for the XO/HPX system (Fig. 2).

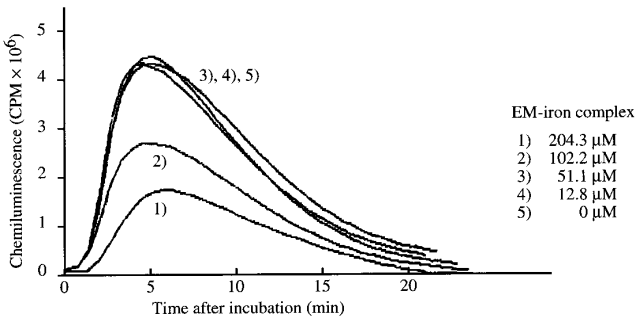

FIG. 6. Time course and dose dependency of the inhibition by EM-iron complex against $O_2^{\cdot-}$ release from PMA-stimulated PMN and AM observed by CL assay. The reaction was initiated by addition of PMA to PMN cells suspension containing luminol in the presence or absence of various concentrations of EM-iron complex.

assay and cytochrome *c* reduction assay. At concentrations of EM-iron complex greater than 51.1 μM, the CL response was inhibited in a concentration dependent manner (Fig. 6). The rate of ferricytochrome *c* reduction by $O_2^{\cdot-}$ from PMA-stimulated PMN indicated that EM-iron complex (17.0 μM) inhibited 52.8% of $O_2^{\cdot-}$ release. The same concentration of EM inhibited only 4.2% of $O_2^{\cdot-}$ release.

DISCUSSION

Results of the present study indicated that a stable EM-iron complex was readily produced by the 1:1 reaction between EM and iron chloride in water (Fig. 1) and the resulting complex was a radical scavenger. The $O_2^{\cdot-}$ scavenging activity of EM-iron complex in the XO/HPX system was equal to that of ascorbic acid

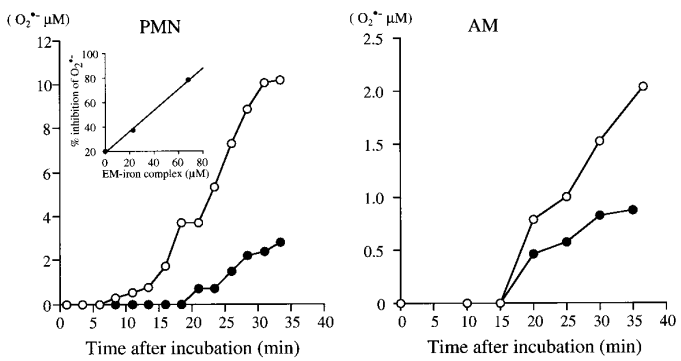

FIG. 5. Time course and dose dependency of the inhibition by EM-iron complex on $O_2^{\cdot-}$ release from PMA-stimulated PMN or AM observed by ESR spin trapping. Control or EM-iron-containing (68.1 μM) suspensions of PMN or AM were treated as in Fig. 4. Inhibition of $O_2^{\cdot-}$ release form PMN at 33 min after addition of PMA by EM-iron complex was observed in a concentration dependent manner as shown in the inset.

(Table 1). The chemical structure of EM is characterized by a large 14-membered lactone ring substituted with hydroxyl group in position 6 and carbonyl group in position 9, and it appears to make metal-complexes. A carbon centered radical possibly originated from EM was observed in the XO/HPX system by ESR (Fig. 2 C), indicating that not only iron in EM-iron complex but also its EM ligand might be involved in scavenging $O_2^{\cdot-}$.

We ruled out the possibility that EM-iron complex might inhibit the $O_2^{\cdot-}$ synthesis in cell-free system since EM-iron complex did not affect uric acid production in the XO/HPX system (data not shown). We interpret the $O_2^{\cdot-}$ scavenging activity as playing the principal role also in the whole-cell system. It is because the complex showed comparable IC_{50}, 30.00 μM and 47.65 μM, in both systems by ESR assay and because EM itself has been reported not to affect $O_2^{\cdot-}$ production of PMA-stimulated PMN up to 100 μM [4]. In other report, the IC_{50} was 500 μM and 200 μM in the whole-cell system and the NADPH oxidase cell-free system, respectively [3]. EM increases intracellular cyclic AMP [25, 26], but the effect of EM on $O_2^{\cdot-}$ production of PMN through the cyclic AMP-dependent protein kinase (PKA) is under discussion [4, 5]. The whole-cell system using PMA in the present study should minimize the effect of EM-iron complex on PKA. However, the contribution of PKA to the effect of EM-iron complex *in vivo* is worth investigating further.

The $O_2^{\cdot-}$ scavenging activity of EM-iron complex was observed at concentrations greater than 17.0 μM (12.5 μg/ml) in the XO/HPX system. And 52.8% inhibition of $O_2^{\cdot-}$ release from PMN with the complex (17.0 μM) was observed by the cytochrome *c* reduction assay. The effective concentration appeared to exceed the therapeutic serum concentrations of EM (0.5-2 μg/ml) but is achievable in PMN or pulmonary tissue cells which accumulate EM at 5 to 24 times extracellular levels [27, 28].

Free iron has been reported to be released from iron bound proteins at the inflammatory sites [29, 30, 31]. In *Pseudomonas aeruginosa*-infected lungs of patients with diffuse panbronchiolitis and other chronic lung diseases, the increased degradation of iron bound proteins was suggested to possibly contribute to their lung injury via the Haber-Weiss reaction [32]. However, the concentration of the free iron is still controversial; e.g. it was elevated to 37 μM in sera of patients with sepsis [33], but was decreased in bronchoalveolar lavage fluid of patients with adult respiratory distress syndrome [34].

In the present study, we demonstrated the $O_2^{\cdot -}$ scavenging activity of EM-iron complex by multiple assay systems *in vitro*. To confirm the anti-inflammatory action of EM-iron complex in chronic inflammatory lung diseases, further study *in vivo* is necessary.

ACKNOWLEDGMENT

We thank Dr. Sumio Tanase for critical advice on MALDI-MS.

REFERENCES

1. Ichikawa, Y., Ninomiya, H., Koga, H., Tanaka, M., Kinoshita, M., Tokunaga, N., Yano, T., and Oizumi, K. (1992) *Am. Rev. Respir. Dis.* **146,** 196–203.
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. Umeki, S. (1993) *Chest* **104,** 1191–1193.
4. Mitsuyama, T., Tanaka, T., Hidaka, K., Abe, M., and Hara, N. (1995) *Respiration* **62,** 269–273.
5. Hojo, M., Fujita, I., Hamasaki, Y., Miyazaki, M., and Miyazaki, S. (1994) *Chest* **105,** 520–523.
6. Goswami, S. K., Kivity, S., and Marom, Z. (1990) *Am. Rev. Respir. Dis.* **141,** 72–78.
7. 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.
8. Khair, O. A., Devalia, J. L., Abdelaziz, M. M., Sapsford, R. J., and Davies, R. J. (1995) *Eur. Respir. J.* **8,** 1451–1457.
9. Keicho, N., Kudoh, S., Yotsumoto, H., and Akagawa, K. (1994) *J. Antibiot.* **47,** 80–89.
10. Kooy, N. W., Royall, J. A., Ye, Y. Z., Kelly, D. R., and Beckman, J. S. (1995) *Am. J. Respir. Crit. Care. Med.* **151,** 1250–1254.
11. Akaike, T., Ando, M., Oda, T., Doi, T., Ijiri, S., Araki, S., and Maeda, H. (1990) *J. Clin. Invest.* **85,** 739–745.
12. Freeman, B. A., and Crapo, J. D. (1982) *Lab. Invest.* **47,** 412–426.
13. Anderson, R., and Lukey, P. T. (1987) *Ann. N. Y. Acad. Sci.* **498,** 229–247.
14. Yaping, T., Yunzhong, F., Cunpu, S., Wenmei, S., Qinhui, L., and Menchang, S. (1993) *Biochem. Biophys. Res. Commun.* **191,** 646–653.
15. Cuperus, R. A., Muijsers, A. O., and Wever, R. (1985) *Arthritis Rheum.* **28,** 1228–1233.
16. Bagchi, D., Prasad, R., and Das, D. K. (1989) *Biochem. Biophys. Res. Commun.* **158,** 52–57.
17. Kimura, E., Koike, T., Shimizu, Y., and Kodama, M. (1986) *Inorg. Chem.* **25,** 2242–2246.
18. Jay, D., Cuéllar, A., Zamorano, R., Muñoz, E., and Gleason, R. (1991) *Arch. Biochem. Biophys.* **290,** 463–467.
19. Itami, C., Matsunaga, H., Sawada, T., Sakurai, H., and Kimura, Y. (1993) *Biochem. Biophys. Res. Commun.* **197,** 536–541.
20. Strien, F. J. C. von, Fespersen, S., Greef, F. van der, Jenks, B. G., and Roubos, E. W. (1996) *FEBS Letters* **379,** 165–170.
21. Taketomi, T., Hara, A., Uemura, K., Kurahashi, H., and Sugiyama, E. (1996) *BBRC* **224,** 462–467.
22. Ando, Y., Sato, K., Sakashita, N., Tanaka, Y., Uchino, M., and Ando, M. (1995) *Pharm. Pharmacol. Lett.* **1,** 5–7.
23. Sato, K., Akaike, T., Kohno, M., Ando, M., and Maeda, H. (1992) *J. Biol. Chem.* **267,** 25371–25377.
24. Nakashima, H., Ando, M., Sugimoto, M., Suga, M., Soda, K., and Araki, S. (1987) *Am. Rev. Respir. Dis.* **136,** 310–315.
25. Aoshiba, K., Nagai, A., and Kohno, K. (1995) *Antimicrob. Agents Chemother.* **39,** 872–877.
26. Takeyama, K., Tamaoki, J., Chiyotani, A., Tagaya, E., and Kohno, K. (1993) *J. Pharma. Pharmacol.* **45,** 756–758.
27. Martin, J. R., Johnson, P., and Miller, M. F. (1985) *Antimicrob. Agents Chemother.* **27,** 314–319.
28. Brun, Y., Forey, F., Gamondes, J. P., Tebib, A., Brune, J., and Fleurette, J. (1981) *J. Antimicrob. Chemotherapy* **8,** 459–466.
29. Biemond, P., van Eijk, H. G., Swaak, A. J. G., and Koster, J. F. (1984) *J. Clin. Invest.* **73,** 1576–1579.
30. Brieland, J. K., and Fantone, J. C. (1991) *Arch. Biochem. Biophys.* **284,** 78–83.
31. Brieland, J. K., Clarke, S. J., Karmiol, S., Phan, S. H., and Fantone, J. C. (1992) *Arch. Biochem. Biophys.* **294,** 265–270.
32. Britigan, B. E., Hayek, B. N., Doebbeling, B. N., and Fick, R. J. (1993) *Infect. Immun.* **61,** 5049–5055.
33. Galley, H. F., and Weber, N. R. (1996) *Intensive Care Med.* **22,** 226–229.
34. Gutteridge, J. M. C., Mumby, S., Quinlan, G. J., Chung, K. F., and Evans, T. W. (1996) *BBRC* **220,** 1024–1027.