Studies on Thermophile Products. VI.¹⁾ Activation of Mouse Peritoneal Macrophages by Bis(2-hydroxyethyl) Trisulfide

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The biological effects of a cytotoxic substance (BS-1), isolated from *Bacillus stearothermophilus* UK563 and identified as bis(2-hydroxyethyl) trisulfide, on elicited mouse peritoneal macrophages induced by a casein injection, were investigated *in vitro*. BALB/c mouse macrophages treated or pretreated with BS-1 (1–10 μ g/ml) showed cytotoxicity against syngeneic DBA/2 mouse P815 mastocytoma. BS-1 also showed weak cytotoxicity directly against P815 in the absence of macrophages. BS-1 significantly increased the glucose consumption of macrophages without producing cytotoxicity. This trisulfide compound increased nitric oxide formation, interleukin-1 production and prostaglandin E₂ release in macrophages. It did not, however, increase the production of active oxygen species in macrophages, but it reduced cytochrome c in the presence of phagocytes. These results indicate that BS-1 activates macrophages to the cytolytic stage.

Keywords bis(2-hydroxyethyl) trisulfide; cytotoxicity; macrophage activation; glucose consumption; nitric oxide; IL-1

Recently, great interest has been shown in the application of thermostable enzymes produced by thermophile in bioreactor, biosensor and clinical reagents, in addition to its uses in industrial technology.²⁾ Thermophile contains a variety of components such as lipids and polyamines which are chemically different from those of mesophile.³⁾ In connection with our studies on products and constituents of thermophile with potentially useful biological activity, we previously reported the isolation of a cytotoxic substance, named BS-1, from the autolysate and culture filtrate of *Bacillus stearothermophilus* UK563.⁴⁾ The structure of BS-1 was determined as bis(2-hydroxyethyl) trisulfide on the basis of spectral data.⁵⁾ Although this compound has been synthesized and used as a useful reagent in the manufacture of liquid polymers and in vulcanization technology,⁶⁾ our report on BS-1 was the first demonstration of the natural occurrence and the biological role of the compound.⁵⁾ The present paper deals with the biological effects of BS-1 on macrophages which contain the cytotoxic mechanism involved in natural immunity.

Materials and Methods

Materials BS-1 used in this study was the preparation previously synthesized.⁵⁾ Casein, cytochrome c (Cyt.c) and the glucose and lactic dehydrogenase (LDH) assay kits were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Hank's balanced salt solution (HBSS) and RPMI1640 medium were obtained from Nissui Pharmaceutical Co., Ltd. (Tokyo, Japan) and phenol red free Dulbecco's modified Eagle's medium (DMEM) was obtained from Cosmo Bio (Tokyo, Japan). Fetal calf serum (FCS) was obtained from Gibco (Gland Island, NY, U.S.A.). Heparin, xanthine, xanthine oxidase (XOD), superoxide dismutase (SOD), actinomycin D, prostaglandin (PG) E₂, phytohemagglutinin (PHA) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Co. (St. Louis, MO, U.S.A.). [¹²⁵I]Iododeoxy uridine (¹²⁵I-UdR, 2000 Ci/mmol) was obtained from Amersham (Braunschweig, Germany), [³H]arachidonic acid (³H-AA, 200 Ci/mmol) from ARC (St. Louis, MO, U.S.A.), lipopolysaccharide (LPS, *E. coli*, serotype 0.55:B5) from Difco (Detroit, MO, U.S.A.) and human recombinant tumor necrosis factor alpha (hTNF- α) from R & D Systems (Minneapolis, MN, U.S.A.). All other reagents were of analytical grade.

Macrophages Male BALB/c mice (8 weeks old, Nippon Slc., Shizuoka, Japan) were injected intraperitoneally with 2.5 ml 2% sterilized casein

dissolved in saline. Four days later peritoneal exudate cells (PECs) were removed by washing the HBSS containing 10 units/ml heparin. The PECs were washed 3 times with HBSS and suspended in RPMI1640 medium supplemented with 100 units/ml penicillin, 100 μ g/ml streptomycin and 10% FCS (complete RPMI1640 medium). The cell suspension was plated in tissue culture dishes (100 i.d. $\times$ 20 mm) and allowed to adhere for 2 h at 37°C in a humidified atmosphere of 5% CO₂ in air. The non-adherent cells were removed by shaking and washing the dishes with complete RPMI1640 medium. The adherent cells (macrophages) were collected with a soft, wide-tripped rubber "policeman" and suspended in the medium.⁷⁾

The number of viable cells was determined by a trypan blue dye exclusion method.⁸⁾ LDH in the supernatant of cultured macrophages was measured using an assay kit to ensure that the test sample induced no release of cytoplasmic marker.⁹⁾

Macrophage-Mediated Cytotoxicity DBA/2 mouse mastocytoma P815 (Japanese Cancer Research Resources Bank, JCRB) and syngeneic BALB/c mouse macrophages were used as target and effector cells, respectively, for the cytolytic assay. P815 cells (10⁶ cells/ml) were labelled with ¹²⁵I-UdR (2 μ Ci/ml) by incubation at 37°C for 1 h in complete RPMI1640 medium. The labelled cells were washed 3 times with medium to remove unbound ¹²⁵I-UdR. Then, the labelled P815 cells (2 $\times$ 10⁴ cells), in fresh complete RPMI1640 medium, were mixed with macrophages (4 $\times$ 10⁵ cells) at a ratio of 20:1 (macrophages to P815 cells). In separate experiments, macrophages were preincubated with the sample. After incubation at 37°C for 24 h in a humidified atmosphere of 5% CO₂ in air, the radioactivity released into the supernatant was determined using a Aloka γ -counter TD-103. The cytotoxicity was calculated from the following formula: cytotoxicity (%) = $(R - S) \times 100 / (T - S)$ with R = cpm released in the presence of sample, S = cpm spontaneously released and T = total cpm.⁷⁾

Glucose Consumption Macrophages (4 $\times$ 10⁵ cells) were incubated with the sample at 37°C for 24 h under 5% CO₂ in air for the times indicated in 24-well culture plates. The glucose concentration in the medium was determined by the glucose oxidase method in kit form and calculated using the following formula: glucose consumption (mg) = (glucose in fresh medium) – (glucose in the medium cultured with sample).

Nitric Oxide (NO) Formation NO formation was evaluated by measuring generated nitrite, a breakdown product of NO in macrophages, as described by Zembowicz and Vane.¹⁰⁾ Nitrite concentrations in the supernatants from macrophages (4 $\times$ 10⁵), cultured with or without sample in the medium, were measured by the Griess reaction.¹¹⁾ Briefly, supernatants (50 μ l) were mixed with 50 μ l Griess reagent (1% sulfanilamide, 3% H₃PO₄ and 0.1% *N*-1-naphthylethylenediamine dihydrochloride) and incubated for 10 min to form a chromophore. Absorbance was read at 550 nm, and nitrite dissolved in the culture medium was used as a standard.

Tumor Necrosis Factor (TNF) Production Macrophages (1 $\times$ 10⁶) were incubated with or without sample for 48 h in DMEM–10% FCS medium and the supernatant was assayed for TNF by the L929 cytotoxicity assay

described by Ruff and Gifford.¹²⁾ Briefly, L929 cells (6×10^4), dilutions of the TNF sample or standard hTNF- α and actinomycin D (100 ng) were incubated for 18 h in 100 μ l DMEM-5% FCS. Then the number of viable cells was measured by the MTT method.¹³⁾

Biological Determination of Interleukin (IL)-1 Activity The IL-1 activity was measured as the ability to increase the proliferation of thymocytes in the presence of mitogenic lectin PHA (thymocyte costimulation activity). The ability of complement PHA-induced mitogenesis is known as thymocyte costimulation, and is characteristic of IL-1.¹⁴⁾ Macrophages were incubated with the sample for 24 h to give the conditioned media. Thymocytes from C3H/HeJ mice (Nippon Slc, Shizuoka, Japan) were incubated for 72 h in RPMI 1640 medium supplemented with 5% FCS and 1 μ g/ml PHA, with conditioned media. [3 H]Thymidine (3 H-TdR, 10 μ Ci/ml) was added during the last 18 h of this incubation, and the radioactivity incorporated was determined by liquid scintillation counting.

AA and PGE₂ Release According to the method described by Hepburn *et al.*,¹⁵⁾ macrophages (1×10^6) were incubated for 16 h in 2 ml DMEM-10% FCS containing 2 μ Ci 3 H-AA. The cells were rinsed three times with DMEM-1% bovine serum albumin (BSA) to remove unincorporated label and then incubated in DMEM-10% FCS, with or without sample. After 24 h, aliquots of the medium were removed and counted in an Aloka LS-1000 liquid scintillation counter. For further analysis of PGE₂, 20 μ l 1 N HCl and 2 ml chloroform-ethanol (2:1, v/v) were added to a 0.5 ml aliquot of the supernatant. The mixture was then shaken vigorously and separated into two phases by centrifugation at 1000 rpm for 5 min. The lower phase (1 ml) was evaporated and used together with standard PGE₂ preparation for the separation of prostaglandins by thin-layer chromatography on Silica gel 60 (Merck) using a solvent system of ethyl acetate-chloroform-ethanol-acetic acid (20:20:4:1, v/v) as described by Humes *et al.*¹⁶⁾ PGE₂ (*Rf*: 0.30) was detected by I₂-vapor and spots were scraped off, and the radioactivity was counted.

Cyt. c Reduction The Cyt. c reduction assay was performed in microtiter plates, to determine superoxide anions (O₂⁻) produced from phagocytes, as previously described.¹⁷⁾ Macrophages (5×10^5 cells) were incubated with the sample and 80 μ M Cyt. c, with or without 50 μ g/ml SOD, at 37 °C in 200 μ l HBSS. Cyt. c reduction was monitored spectrophotometrically by determining the difference in absorbance (550–490 nm) of the supernatant utilizing a Immuno Reader NJ-2001 (Inter Med, Osaka, Japan). In separate experiments, superoxide anions were generated by the reaction of xanthine with XOD.¹⁸⁾

Results

Macrophage-Mediated Cytotoxicity 125 I-UdR-labeled P815 mouse mastocytoma cells were plated *in vitro* with macrophages obtained from casein-treated BALB/c mice. When both cells were incubated in the presence of BS-1, P815 cytotoxicity increased in a dose-dependent manner over the range 1–10 μ g/ml BS-1 (Fig. 1a). In addition, weak cytotoxicity was produced by 5 to 10 μ g/ml BS-1 even in the absence of macrophages. Macrophage-mediated P815

Fig. 1. Effect of BS-1 on Cytotoxicity of Macrophages

a) 125 I-UdR-labelled P815 (2×10^4) were incubated with BS-1 for 24 h at 37 °C (●) in the presence of macrophages (4×10^5) or (○) in their absence. b) Macrophages, preincubated with BS-1 for 24 h at 37 °C and washed, were incubated with 125 I-UdR-labelled P815 for 24 h at 37 °C. Data represent the mean $\pm$ S.E. ($n=3$).

cytotoxicity was calculated from the following equation: [macrophage-mediated cytotoxicity (%)] = (cytotoxicity in the presence of macrophages) – (direct cytotoxicity). Macrophage-mediated cytotoxicity was 4, 17, 28 and 32% at 0.5, 1, 2.5 and 10 μ g/ml BS-1, respectively. BS-1 increased macrophage-mediated cytotoxicity in a dose-dependent manner over the range 1–10 μ g/ml. Furthermore, when macrophages were preincubated with BS-1, P815 cytotoxicity increased in a dose-dependent manner (Fig. 1b), as well as the calculated macrophage-mediated cytotoxicity.

No significant differences in the supernatant LDH levels and the number of viable cells were found when comparing controls and cells treated with 10 and 25 μ g/ml BS-1 at 24 and 48 h (data not shown), indicating that BS-1 was not cytolytic to macrophages.

Glucose Consumption BS-1 significantly enhanced glucose consumption in macrophages at doses of 1–10 μ g/ml (Table I).

NO Formation Cultures of casein-elicited macrophages from BALB/c mice were treated with BS-1 (0.1–10 μ g/ml) for 24 h and the final culture supernatant NO₂⁻ concentrations were compared with those of control cultures not treated with BS-1. Figure 2 shows that BS-1 (0.1–10 μ g/ml) significantly increases nitrite level in a dose-dependent manner. LPS (0.1 μ g/ml) used as a positive control increased nitrite accumulation.

TNF Production BALB/c mouse peritoneal macrophages incubated for 24 h with BS-1 (10 μ g/ml) increased TNF production slightly, compared with the effect of LPS (0.1 μ g/ml), used as a positive control (Fig. 3).

TABLE I. Effect of BS-1 on Glucose Consumption in Macrophages

	Concentration (μ g/ml)	Glucose consumption (mg/10 ⁶ cells)	
		24 h	48 h
Control	—	0.069 $\pm$ 0.006	0.110 $\pm$ 0.010
BS-1	0.1	0.087 $\pm$ 0.005	0.209 $\pm$ 0.020 ^{a)}
	1	0.139 $\pm$ 0.008 ^{b)}	0.372 $\pm$ 0.013 ^{c)}
	10	0.243 $\pm$ 0.027 ^{a)}	0.430 $\pm$ 0.032 ^{c)}

Macrophages were incubated for the indicated times at 37 °C with BS-1. After incubation, the glucose concentration in the supernatant was determined. All values represent the mean $\pm$ S.E. ($n=3$). Significantly different from control; a) $p < 0.05$, b) $p < 0.01$, c) $p < 0.001$.

Fig. 2. Effect of BS-1 on Nitrite Formation in Macrophages

Macrophages (10^6) were incubated with BS-1 for 24 h and the concentration of nitrite in the supernatant was measured. Each column represents the mean $\pm$ S.E. ($n=3$). Significantly different from control; a) $p < 0.05$, b) $p < 0.01$, c) $p < 0.001$.

Fig. 3. Effect of BS-1 on TNF Production in Macrophages

Macrophages (10^6) were cultured with BS-1 for 48 h and the supernatant was assayed for TNF activity using the L929 cytotoxicity assay. Each column represents the mean $\pm$ S.E. ($n=6$). Significantly different from control; a) $p < 0.01$, b) $p < 0.001$.

Fig. 4. Effect of BS-1 on IL-1 Production in Macrophages

Macrophages (10^6) were incubated with the sample for 24 h. The conditioned medium was assayed for thymocyte costimulation activity. Each column represents the mean $\pm$ S.E. ($n=4$). Significantly different from control; a) $p < 0.05$, b) $p < 0.01$, c) $p < 0.001$.

Fig. 5. Effect of BS-1 on AA Release from Macrophages

Macrophages (10^6) labelled with ^3H -AA were incubated with BS-1 for 24 h and the radioactivity in the supernatant measured. Each column represents the mean $\pm$ S.E. ($n=3$). Significantly different from control; a) $p < 0.01$, b) $p < 0.001$.

IL-1 Production The generation of IL-1 activity in macrophages was assayed by thymocyte costimulation with PHA, as shown in Fig. 4. The medium collected from BS-1 (0.1 and 1 $\mu\text{g/ml}$)-stimulated macrophages exhibited biological activity typical of IL-1, being comparable with the activity of LPS (0.1 $\mu\text{g/ml}$)-stimulated cells. At 10 $\mu\text{g/ml}$,

Fig. 6. Effect of BS-1 on PGE_2 Release from Macrophages

Macrophages (10^6) labelled with ^3H -AA were incubated with BS-1 for 24 h and the supernatant was examined for PGE_2 release. Each column represents the mean $\pm$ S.E. ($n=3$). Significantly different from control; a) $p < 0.05$.

Fig. 7. Reduction of Cyt.c by BS-1 in the Presence of Macrophages

Macrophages (5×10^5) were incubated with BS-1 and 80 μM Cyt.c, and the reduction of Cyt.c was monitored spectrophotometrically. Each point represents the mean of triplicate determinations. (○) Control, (●) BS-1 1 $\mu\text{g/ml}$, (▲) 2.5 $\mu\text{g/ml}$, (■) 5 $\mu\text{g/ml}$, (▼) 10 $\mu\text{g/ml}$, (□) BS-1 10 $\mu\text{g/ml}$ + SOD 50 $\mu\text{g/ml}$.

BS-1 showed on very weak thymocyte costimulation activity.

AA and PGE_2 Release When incubated with ^3H -AA-labelled macrophages at 37 $^\circ\text{C}$ for 24 h, BS-1 (0.1–10 $\mu\text{g/ml}$) increased dose-dependently the release of significant amounts of radioactivity from macrophages (Fig. 5). BS-1 also promoted PGE_2 release from macrophages in a dose-dependent manner (Fig. 6).

Cyt.c Reduction BS-1, at concentrations of 1–10 $\mu\text{g/ml}$, reduced Cyt.c in the presence of mouse peritoneal macrophages (Fig. 7). The reduction of Cyt.c took place in a dose-dependent manner, but did not occur in the absence of the cells. SOD (50 $\mu\text{g/ml}$) had no significant effect on the Cyt.c reduction induced by 10 $\mu\text{g/ml}$ BS-1. BS-1 (25 $\mu\text{g/ml}$) did not affect the scavenging effect (68% inhibition of Cyt.c reduction) of SOD (1 $\mu\text{g/ml}$) on O_2^- production in the xanthine-XOD system, indicating that BS-1 did not inactivate the SOD protein (data not shown).

Discussion

Activated macrophages *in vivo* and *in vitro* exhibit a

number of morphological and functional differences compared with the control macrophages taken from uninfected animals; there are also biochemical differences.¹⁹⁾ The elicited (inflammatory) macrophages that are released into the peritoneum in response to an injection of sterile casein, increased glucose consumption and cytotoxicity against P815 mastocytoma when treated with BS-1. It is known that inhibition of glycolysis markedly inhibits tumor cell killing by mediator-activated macrophages, indicating that glucose consumption is related to cytotoxicity.²⁰⁾ Furthermore, the cytotoxicity of BS-1 pretreated macrophages was similar to the macrophage-mediated cytotoxicity seen when BS-1 and cells were present together. Therefore, BS-1 undoubtedly activates inflammatory macrophages to the cytolytic stage.

Important roles of NO and TNF have been evaluated as effector mechanisms of macrophage-mediated cytotoxicity.²¹⁾ NO is generated by mammalian cells from one of the N^G-guanidino nitrogens of L-arginine and this reaction is catalyzed by NO synthase.²²⁾ A calcium-independent inducible NO synthase is formed in macrophages after activation by bacterial LPS or cytokines.²³⁾ NO, made by inducible NO synthase, is responsible for killing microbial pathogens and tumor cells by activated macrophages.²⁴⁾ Cytokines produced by macrophages play important roles in immunoregulation and cytotoxicity. Macrophages activated *in vitro* with bacterial LPS produce TNF, which is toxic to tumor cells.²⁵⁾ It has been reported that IL-1 also induces cytotoxicity mediated *via* NO or TNF formation.²⁶⁾ In the present study, BS-1 apparently increased the production of NO and IL-1, and to a much lesser extent that of TNF, compared LPS. P815, used as the target cell in the present study, is resistant to TNF. It is highly probable that the cytotoxicity of BS-1 to P815 *via* macrophages relates, at least in part to the increased production of NO and IL-1.

Macrophages are a major source of certain arachidonate metabolites, which, endogenously produced, also modulate the synthesis or release of certain mediators by macrophages. One of these metabolites, PGE₂, which is abundantly produced in LPS-stimulated macrophages,²⁷⁾ enhances NO production in the presence of TNF²⁸⁾ and inhibits IL-1 release,²⁹⁾ and it may represent an important endogenous autoregulator of NO and IL-1 release. The present study demonstrates the increasing effect of BS-1 on PGE₂ release, indicating that macrophage activation, resulting in cytotoxicity and cytokine production by BS-1, must be regulated by PGE₂. The observation that BS-1, at 10 µg/ml, showed significant but weak stimulating effect on IL-1 production may possibly be explained by inhibition of PGE₂.

Oxygen-derived reaction products are also known to play a significant role in effector mechanisms of macrophage and PMN cytotoxicity.^{20,30)} The elicited phagocytes spontaneously continue to produce a small amount of O₂⁻ (Fig. 7).³¹⁾ In fact, we discovered BS-1 during our search for compounds stimulating active oxygen generation in products and constituents of thermophile using the Cyt. c reduction method. Although BS-1 reduced Cyt. c and nitroblue tetrazolium in the presence of phagocytes, it did not produce any active oxygen species for the following reasons: i) the Cyt. c reduction of BS-1 is not neutralized

by SOD which catalyzes the disproportionation of O₂⁻ and ii) BS-1 does not induce hydrogen peroxide production and luminol-enhanced chemiluminescence (data not shown). Thus, the activation of macrophages by BS-1 does not result in enhanced production of active oxygen species. This report is the first description of the activation of macrophages by a trisulfide compound. As for the biological action of BS-1, further studies are needed to examine this.

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References

- 1) Part V: Y. Kohama, K. Tanaka, N. Murayama, M. Itoh, T. Suda, S. Takae, T. Mimura, *Chem. Pharm. Bull.*, **40**, 3017 (1992).
- 2) H. Kondo, K. Nagata, *Kagaku To Kogyo* (Tokyo), **60**, 428 (1986); K. Tomita, *Bio Industry*, **3**, 707 (1986).
- 3) S. M. Friedman, "Biochemistry of Thermophily," Academic Press, New York, 1978, pp. 1—400.
- 4) Y. Kohama, T. Teramoto, K. Iida, N. Murayama, T. Semba, N. Tatebatake, Y. Kayamori, K. Tsujikawa, T. Mimura, *Chem. Pharm. Bull.*, **39**, 2468 (1991).
- 5) Y. Kohama, K. Iida, T. Semba, T. Mimura, A. Inada, K. Tanaka, T. Nakanishi, *Chem. Pharm. Bull.*, **40**, 2210 (1991).
- 6) D. N. Harpp, K. Stelion, T. H. Chan, *J. Am. Chem. Soc.*, **100**, 1222 (1978); Z. Wu, T. G. Back, R. Ahmad, R. Yamadagni, D. A. Armstrong, *J. Phys. Chem.*, **86**, 4417 (1982); A. Duda, S. Penczek, *Makromol. Chem. Commun.*, **9**, 151 (1983).
- 7) K. C. Norbury, I. J. Fidler, *J. Immunol. Methods*, **7**, 109 (1975).
- 8) H. M. Shapiro, "Practical Flow Cytometry," 2nd ed., Wiley-Liss, New York, 1988, p. 129.
- 9) H. J. Showell, R. J. Freer, S. H. Zigmond, E. Schiffmann, S. Aswanikumar, B. Corcoran, E. L. Becker, *J. Exp. Med.*, **143**, 1154 (1976).
- 10) A. Zembowicz, J. R. Vane, *Proc. Natl. Acad. Sci. U.S.A.*, **89**, 2051 (1992).
- 11) P. Griess, *Chem. Ber.*, **12**, 426 (1879); L. C. Green, D. A. Wagner, J. Glogowski, P. L. Skipper, J. S. Wishnok, S. R. Tannenbaum, *Anal. Biochem.*, **126**, 131 (1982).
- 12) M. R. Ruff, G. E. Gifford, *J. Immunol.*, **125**, 1671 (1980).
- 13) M. C. Alley, D. A. Scudiero, A. Monks, M. L. Hursey, M. J. Boyd, *Cancer Res.*, **48**, 4827 (1988).
- 14) L. J. Rosenwasser, C. A. Dinarello, *Cell. Immunol.*, **63**, 134 (1981).
- 15) A. Hepburn, J.-M. Boeynaems, W. Fiers, J. E. Dumont, *Biochem. Biophys. Res. Commun.*, **149**, 815 (1987); R. W. Godfrey, W. J. Johnson, S. T. Hoffstein, *ibid.*, **142**, 235 (1987).
- 16) J. L. Humes, S. Burger, M. Galavage, F. A. Kuehl, Jr., P. D. Wightman, M. E. Dahlgren, P. Davies, R. J. Bonney, *J. Immunol.*, **124**, 2110 (1980).
- 17) E. Pick, D. Mizel, *J. Immunol. Methods*, **46**, 211 (1981); Y. Kohama, Y. Kayamori, Y. Katayama, N. Murayama, M. Itoh, K. Tanaka, H. Tsuboi, N. Sakaguchi, T. Mimura, *Chem. Pharm. Bull.*, **40**, 3013 (1992).
- 18) J. M. McCord, I. Fridovich, *J. Biol. Chem.*, **244**, 6049 (1969).
- 19) R. J. Soberman, M. L. Karnovsky, "Lymphokines," Vol. 3, ed. by E. Pick, Academic Press, New York, 1981, p. 11.
- 20) W. F. Piessens, W. E. Churchill, S. D. Sharma, "Lymphokines," Vol. 3, ed. by E. Pick, Academic Press, New York, 1981, p. 293.
- 21) K. Klostergaard, M. E. Leroux, M.-C. Hung, *J. Immunol.*, **147**, 2802 (1991).
- 22) M. A. Marletta, P. S. Yoon, R. Iyengar, C. D. Leaf, J. S. Wishnok, *Biochemistry*, **27**, 8716 (1988).
- 23) D. J. Stuehr, M. A. Marletta, *Proc. Natl. Acad. Sci. U.S.A.*, **82**, 7738 (1985).
- 24) J. B. Hibbs, Jr., R. R. Taintor, Z. Vavrin, E. M. Rachlin, *Biochem. Biophys. Res. Commun.*, **157**, 87 (1988).
- 25) S. Sone, K. Tachibana, K. Ishii, M. Ogawara, E. Tsubura, *Cancer Res.*, **44**, 646 (1984).
- 26) R. Philip, L. B. Epstein, *Nature* (London), **323**, 86 (1986); L. Bergmann, K.-D. Knöck, C. Suschek, H. Kolb, V. K. Bachofen, *FEBS Lett.*, **299**, 103 (1992); D. A. Rappollee, Z. Werb, "Macrophage Biology and Activation," ed. by S. W. Russell, S. Gordon, Springer-Verlag, Tokyo, 1992, pp. 87—140.

- 27) T. Decker, M.-L. Lhmann-Matthes, U. Karck, T. Peters, K. Decker, *J. Leuko. Biol.*, **45**, 139 (1989).
- 28) T. Gaillard, A. Mulsch, R. Busse, H. Kein, K. Decker, *Pathobiology*, **59**, 280 (1991).
- 29) S. L. Kunkel, S. W. Chensue, S. H. Phan, *J. Immunol.*, **136**, 186 (1986).
- 30) C. F. Nathan, S. C. Silverstein, L. H. Brukner, Z. A. Cohn, *J. Exp. Med.*, **149**, 100 (1979); K. Morikawa, S. Kamegaya, M. Yamazaki, D. Mizuno, *Cancer Res.*, **45**, 3482 (1985).
- 31) J. A. Badwey, J. M. Robinson, J. K. Lazdino, R. T. Briggs, M. J. Karnousky, M. L. Karnovsky, *J. Cell. Physiol.*, **115**, 208 (1983).