Biochemical and Biophysical Research Communications **271,** 229–233 (2000)
doi:10.1006/bbrc.2000.2602, available online at http://www.idealibrary.com on IDEAL®

Chitinous Materials Inhibit Nitric Oxide Production by Activated RAW 264.7 Macrophages

Shiaw-Min Hwang,[1] Chiung-Yun Chen, Shan-Shan Chen, and Jian-Chyi Chen
Food Industry Research and Development Institute, P.O. Box 246, Hsinchu 30099, Taiwan, Republic of China

Received March 28, 2000

Chitinous materials have been studied in wound healing and artificial skin substitutes for many years. Nitric oxide (NO) has been shown to contribute to cytotoxicity in cell proliferation during inflammation of wound healing. In this study, we examined the effect of chitin and its derivatives on NO production by activated RAW 264.7 macrophages. Chitin and chitosan showed a significantly inhibitory effect on NO production by the activated macrophages. Hexa-*N*-acetylchitohexaose and penta-*N*-acetylchitopentaose also inhibited NO production but with less potency. However, *N*-acetylchitotetraose, -triose, -biose, and monomer of chitin, *N*-acetylglucosamine and glucosamine had little effect on NO production by the activated cells. These results suggest that the promotive effect of chitinous material on wound healing be related, at least partly, to inhibit NO production by the activated macrophages.

Chitinous materials, the second most abundant biopolymer on earth after cellulose, have been recognized as a multifunctional, biocompatible, and biodegradable biomass resource, especially chitin and chitosan (1, 2). Chitin is the major structural constituent of the exoskeleton of crustaceans and insects and as a component of cell wall of fungi (3, 4). Chitin is composed of 2-acetamido-2-deoxy-β-D-glucose (*N*-acetylglucosamine) by β-(1,4)-linkage. Chitosan is the high degree of deacetylation of chitin and is the amino polysaccharide, poly(β-(1,4)-glucosamine). Both chitin and chitosan have been applied in food, agriculture, textile, polymers, wastewater treatment and pharmaceutics due to their specific physiochemical and biological properties (2, 5–7). In pharmaceutical uses and biomedical materials, much interest has been focused on the wound healing effect and applications in artificial skin substitutes and sutures of chitin and chitosan (8–11). Not only the antimicrobial activity (12, 13) but also stimulation of immune response (14, 15), chitinous materials were reported to accelerate the wound healing (9, 16). But the promotive healing mechanisms are still unclear.

Nitric oxide (NO) is a highly reactive free radical and plays as an important second messenger molecule in many cell types (17). NO is synthesized from conversion of L-arginine to citrulline *in vivo* by nitric oxide synthase (18). In host defense system, activated macrophages by inflammatory agents such as interferon-γ (IFN-γ) and bacterial lipopolysaccharide (LPS) are known to produce a large quantity of NO as major cytotoxic mediator and inhibit the growth of invading microorganisms and tumor cells (19, 20). However, this strong inflammatory response to foreign cells could also induce further damage for the neighboring cells and tissues around the wound area and thus, inhibit the healing process (21–23). There are reports that decreasing NO production by adding inhibitors of nitric oxide synthase and L-arginine analogues could protect some forms of injury (24, 25). In order to elucidate the promotive capability of chitinous materials on wound healing, we examined the effect of chitin and its derivatives on NO production by the IFN-γ and LPS activated RAW 264.7 macrophages. Our initial results indicated that chitin and chitosan significantly inhibited NO production by the activated cells. Thus, the promotive healing effect of chitinous materials should be related to inhibit NO production by the activated macrophages.

MATERIALS AND METHODS

Cell culture. The mouse monocyte-macrophage, RAW 264.7 cell line was obtained from Culture Collection and Research Center (CCRC 60001), Taiwan. Cells were cultured in Dulbecco's modified essential medium (DMEM, GIBCO BRL, Rockville, MD) containing 1.5 g/L $NaHCO_3$ supplemented with 10% fetal bovine serum (Hyclone, Logan, UT) and incubated in 5% CO_2, 37°C incubator. Cell cultures were examined without mycoplasma contamination with

Abbreviations used: NO, nitric oxide; IFN-γ, interferon-γ; LPS, lipopolysaccharide; NAGA, *N*-acetylglucosamine; $(NAGA)_2$, di-*N*-acetylchitobiose; $(NAGA)_3$, tri-*N*-acetylchitotriose; $(NAGA)_4$, tetra-*N*-acetylchitotetraose; $(NAGA)_5$, penta-*N*-acetylchitopentaose; $(NAGA)_6$, hexa-*N*-acetylchitohexaose.

[1] To whom correspondence should be addressed. Fax: 886-3-521-4016. E-mail: hsm@firdi.org.tw.

0006-291X/00 $35.00

Hoechst 33258 fluorescent staining method and direct culture method on a regular basis (26).

Collagen-/chitin-coated well. Collagen type I (rat tail, Sigma, St. Louis, MO), 1 mg/ml, was dissolved in 0.1 N acetic acid. Chitin was suspended in 0.1 N acetic acid at 1 mg/ml and sonicated for 3 min. (Labsonic U, B. Braun Biotech International, Germany). After autoclaved (121°C, 15 min), chitin was finely wiry and slurry in solution. 96-well tissue culture plates were rinsed with Dulbecco's phosphate-buffer saline without Ca^{2+} and Mg^{2+} (D-PBS, GIBCO BRL) and then, added 5 μl collagen or different amount of chitin solution. The plates were air dried on the laminar flow hood and could be stored at 4°C for a week.

Effect of chitin and chitosan on cell growth. For chitin experiments, cells were subcultured into the 96-well tissue culture plates precoated with different amount of chitin solution at 2000 cells/well and incubated for 48 h. For chitosan experiments, cells were subcultured into 96-well tissue culture plates and incubated overnight. Next day culture media were discarded and refed with fresh media containing different amount of chitosan for a further 48 h. Effect of chitin and chitosan on cell growth was performed with MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) (Merck Co. Germany) method and measured the absorbance at 590 nm by a microplate reader (MRX, Dynex Technologies, Inc., VA) (27).

Measurement of NO (as NO_2^-) production. Nitric oxide (NO) production by cells was quantified spectrophotometrically by measuring the accumulation of nitrite (NO_2^-) in the culture media using the Griess reagents with sodium nitrite as standard (28, 29). For nitrite assays, RAW 264.7 cells were subcultured into 96-well tissue culture plates at 1×10^5 cells/well and incubated for 24 h. The culture media were changed to phenol red-free and serum-free DMEM containing 100 U/ml interferon-γ (IFN-γ, murine, recombinant, GIBCO BRL) and 100 ng/ml lipopolysaccharide (LPS, *Escherichia coli* serotype O128:B12, Sigma) and test samples (30). To measure the effect of chitin on NO production, cells were subcultured directly into the chitin-coated 96-well tissue culture plates. After overnight incubation, cells were attached on the bottom of chitin-coated wells. The culture media were changed and refed with media supplemented with IFN-γ and LPS as above. After 48 h incubation, aliquots (50 μl) of culture supernatants were mixed sequentially with equal volumes of 60 mM sulfanilamide (Sigma) in 3 N HCl and 4 mM *N*-1-naphthylethylenediamine (Sigma) and shaken for 5 min at room temperature (30). Nitrite concentration was measured by the absorbance at 540 nm using a microplate reader (MRX, Dynex Technologies, Inc., VA).

Other chemicals. Chitin (MW $\approx$ 450,000) and chitosan (MW $\approx$ 150,000, deacetylation degree: >95%) were obtained from Sun-Chiu Chemical Co., Taiwan. Chitosan colud be completely dissolved at 1 mg/ml in 0.01 N acetic acid. Both chitin and chitosan have been tested as endotoxin free. Glucosamine and *N*-acetylglucosamine (NAGA) were purchased from Sigma Chemical Co. Di-*N*-acetylchitobiose (2-acetamido-2-deoxy-4-*O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-D-glucopyranose, $(NAGA)_2$), tri-*N*-acetylchitotriose ($(NAGA)_3$), tetra-*N*-acetylchitotetraose ($(NAGA)_4$), penta-*N*-acetylchitopentaose ($(NAGA)_5$) and hexa-*N*-acetylchitohexaose ($(NAGA)_6$) were purchased from Calbiochem Co. (La Jolla, CA).

RESULTS

Effect of Chitin and Chitosan on Cell Growth

The growth effect of chitin and chitosan was performed on the mouse macrophage RAW 264.7 cells. Different amount of chitin slurry solution had been added and air-dried on the 96-well tissue culture plates before cells were seeded. After 48 h incubation, cell growth was assayed by MTT method. As shown in Fig. 1A, chitin could significantly enhance the growth of RAW 264.7 cells at low dosage ($\leq$2 μg/well). Above 5 μg/well, chitin showed an inhibitory effect on cell growth. However, chitosan, up to 200 μg/ml (equivalent 30 μg/well), showed no significantly cytotoxic effect on the growth of RAW 264.7 cells. Moreover, the low concentration of chitosan, 0.32 μg/ml, had a promotive effect on the growth of RAW264.7 cells, as shown in Fig. 1B.

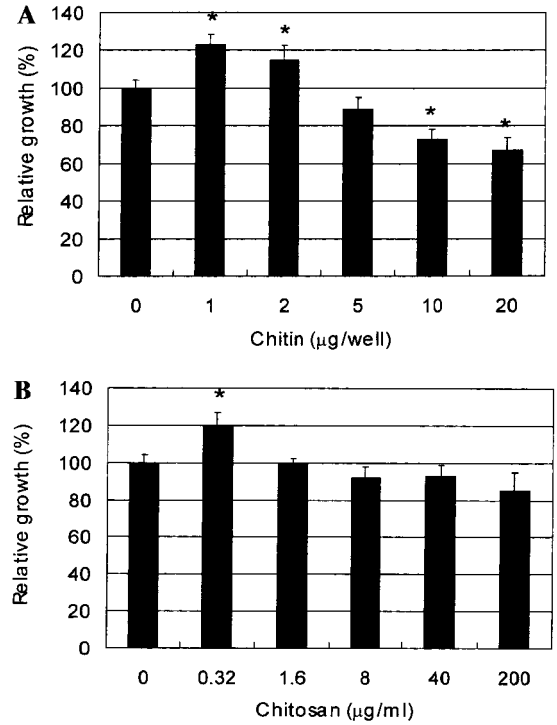

FIG. 1. Effect of (A) chitin and (B) chitosan on the growth of RAW 264.7 cells. Chitin was sonicated and dissolved in 0.1 N acetic acid (1 mg/ml) and sterilized by autoclave. Each well was added different amount of chitin solution and air-dried on the laminar flow hood before seeding the cells. Cell growth was measured by MTT methods after 48 h incubation. The asterisks indicate a statistically significant difference ($P < 0.05$).

Inhibition of NO Production by Chitin and Chitosan

Macrophage RAW 264.7 cells produced a large amount of nitric oxide (as measured nitrite) when they were incubated with the inflammatory mediators, such as interferon-γ (IFN-γ) and lipopolysaccharide (LPS) for a period of time (30). As shown in Fig. 2, chitosan suppressed the nitrite production by the IFN-γ and LPS activated RAW 264.7 cells in a dose-dependent manner. When RAW 264.7 cells were plated on the chitin-coated tissue culture plates (5 μg/well) and activated by IFN-γ and LPS, nitrite production was profoundly inhibited to 52%, as shown in Table 1. Collagen-coated wells had no effect on NO production

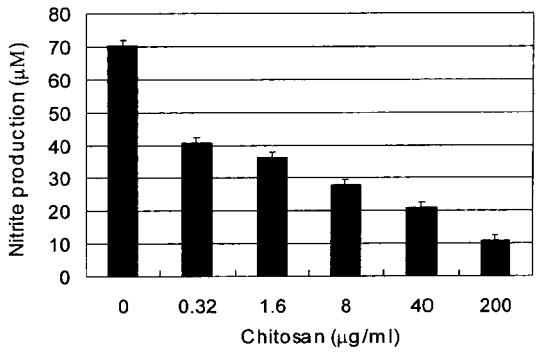

FIG. 2. Effect of chitosan on nitrite production by the activated RAW 264.7 cells. Cells, plated on 96-well dishes (1×10^5/well), were treated with IFN-γ (100 U/ml), LPS (100 ng/ml) and different amount of chitosan. Nitrite accumulation in the culture medium was quantified after incubation for 48 h.

by the activated cells. Furthermore, addition of chitosan to the activated RAW 264.7 cells grown on the chitin-coated wells had a further inhibitory effect on NO production.

Effect of Oligosaccharides of Chitin on NO Production

Chitin and chitosan remarkably inhibit NO production by IFN-γ and LPS activated RAW 264.7 cells. Since chitin and chitosan are built up by the units of N-acetylglucosamine (NAGA) and glucosamine, the effect of these molecules and oligosaccharides of chitin on NO production were also investigated in the activated RAW 264.7 cells. As shown in Table 2, glucosamine, NAGA, $(NAGA)_2$, $(NAGA)_3$ and $(NAGA)_4$ had little effect on the nitrite production by the activated RAW 264.7 cells. However, $(NAGA)_5$ and $(NAGA)_6$ had a significant inhibitory effect on the nitrite production by the activated RAW 264.7 cells. $(NAGA)_6$ is more potent than $(NAGA)_5$ as the inhibitor of NO production at the same dosage level. Glucosamine, NAGA and oligosaccharides of chitin were no cytotoxicity in our test dosages for RAW 264.7 cells. (Data no shown).

TABLE 1

Effect of Chitin and Chitosan on Nitrite Production by the Activated RAW 264.7 Cells

Treatment	Nitrite (μM)	%
Control	74.0 ± 1.2	100
Collagen-coated	73.4 ± 0.9	99
Chitin-coated	38.5 ± 0.8	52
Chitin-coated + chitosan 2 μg/ml	24.0 ± 2.2	32
Chitin-coated + chitosan 10 μg/ml	21.4 ± 1.4	29

Note. Chitin was sonicated and dissolved in 0.1 N acetic acid (1 mg/ml) and sterilized by autoclave. To each well 5 μg of chitin or collagen solution was added and air-dried on the laminar flow hood before seeding the cells (see Materials and Methods). RAW 264.7 cells were activated with 100 U/ml IFN-γ and 100 ng/ml LPS and incubated for 48 h with or without chitosan. The nitrite concentrations were the mean ± standard error from triplicates.

TABLE 2

Effect of Oligosaccharides of Chitin, NAGA, and Glucosamine on Nitrite Production by the Activated RAW 264.7 Cells

Treatment	(μg/ml)	Nitrite (μM)	%
Control		65.6 ± 0.7	100
$+(NAGA)_6$	10	35.8 ± 2.9	54.5*
	2	41.3 ± 0.8	63.0*
$+(NAGA)_5$	10	42.7 ± 1.2	65.1*
	2	47.0 ± 0.5	70.6*
$+(NAGA)_4$	10	61.3 ± 0.6	93.4
	2	63.4 ± 0.5	96.6
$+(NAGA)_3$	10	61.1 ± 0.2	93.1
	2	61.5 ± 0.9	93.8
$+(NAGA)_2$	10	64.1 ± 0.9	97.7
	2	64.9 ± 0.8	98.9
+NAGA	10	66.7 ± 0.9	101.7
+glucosamine	10	64.3 ± 0.8	98.0

Note. RAW 264.7 cells were activated with 100 U/ml IFN-γ, 100 ng/ml LPS, and different amount of compounds. Nitrite accumulation in the culture medium was quantified after 48 h incubation. The nitrite concentrations were the mean ± standard error from triplicates. NAGA, *N*-acetylglucosamine. The asterisks indicate a statistically significant difference ($P < 0.05$).

DISCUSSION

Wound healing is a complicated process including cell proliferation, migration and tissue reconstruction etc. (31). During the inflammation and infection, the attracted and activated macrophages produce a large amount of NO around the wound tissues (24, 32). NO has been proposed to be a major destructive factor in the wound healing process (33, 34), although some reports showed that the presence of low amount of NO could help the wound repair in the early phase of healing (35, 36). As wound healing progress, excess NO is detrimental to fibroblast regeneration and collagen accumulation (37). In order to elucidate the effect of chitinous materials on NO production by macrophages, we first characterized the effect of chitin and chitosan on the growth of mouse macrophage RAW 264.7 cells. It has been shown that chitin and its derivatives had a significantly promotive effect on cell proliferation (38, 39). However, some reports indicated that chitin and its derivatives also showed an inhibitory effect on proliferation of cells (39, 40). The different results of chitin and its derivatives on cell proliferation might be due to different targeted cells and different dosages, sources, and preparation methods of chitin and its derivatives. Consistent with the assumption of dosage effect, the growth effect of chitin on RAW 264.7 cells was correlated to the dosage (Fig. 1A). Chitin, at low dosage,

could provide a matrix for cell anchorage and migration and enhanced the proliferation of cells (39). However, chitin at high dosage could cause a marked inhibition of cell proliferation. It has been shown that the strong interaction of the positive charge of chitin with cell surface would lead cell membrane damage and retard the cell grow (39, 40). On the other experiments, chitosan up to 200 μg/ml showed no significant effect on cell growth except that 0.32 μg/ml chitosan could enhance growth of RAW 264.7 cells (Fig. 1B).

Both chitin and chitosan under the dosage of no inhibitory effect on cell growth were capable of decreasing NO production by the IFN-γ and LPS activated RAW 264.7 cells (Table 1 and Fig. 2). Chitosan could further suppress the degree of NO production when combined with chitin treatment. Given the known cytotoxicity of NO, it is likely that the promotive effect of chitinous materials for the wound healing is partly due to effectively reducing NO production by the activated cells. Moreover, the oligosaccharides of chitin were also investigated their ability to suppress NO production by the activated macrophages. The results showed that penta-*N*-acetylchitopentaose and hexa-*N*-acetylchitohexaose exhibited a significant effect on the inhibition of NO production by the activated RAW 264.7 cells, whereas glucosamine, NAGA, di-*N*-acetylchitobiose, tri-*N*-acetylchitotriose and tetra-*N*-acetylchitotetraose had little effect on this issue (Table 2). It has been shown that the biological activities of oligosaccharides of chitin were proportional to their molecular weights, such as activation of phagocytes (42), antitumor activity (43) and microbicidal activity (44). We found that the minimal length of oligosaccharides of chitin to significantly suppress NO production by the activated cells should be beyond tetramer. Hexamer was more potent than pentamer of NAGA on inhibition of NO production. The mechanism was still not known.

Peluso *et al.* (45) have shown that chitosan, NAGA and glucosamine could activate rat peritoneal exudate macrophages to produce NO *in vitro.* We tested whether our chitinous materials and oligosaccharides of chitin alone could stimulate RAW 264.7 cells to produce NO. The result showed that chitin, chitosan, oligosaccharides of chitin, NAGA and glucosamine were not able to induce RAW 264.7 cells to produce NO (data not shown). LPS alone is a poor stimulator for NO production. But LPS and IFN-γ can synergistically stimulate cells to produce a large amount of NO (46). In order to test if chitinous materials and IFN-γ could synergistically stimulate cells to produce NO, we added chitinous materials and IFN-γ into media of RAW 264.7 cells. The results showed that chitin, chitosan, penta-N-acetylchitopentaose and hexa-N-acetylchitohexaose also inhibited NO production by the IFN-γ activated RAW 264.7 cells (data not shown). The character of chitinous materials to inhibit NO production was also found in many other types of cells, including keratinocytes and fibroblasts (in preparation). *In vivo* experiments and the mechanism of chitinous materials to inhibit NO production will be investigated further.

ACKNOWLEDGMENT

This work was supported by Grant 89-EC-2-A-17-0263 from the Ministry of Economic Affairs (MOEA), Taiwan.

REFERENCES

1. Hirano, S. (1996) *Biotechnol. Annu. Rev.* **2,** 237–258.
2. Li, Q., Dunn, E. T., Grandmaison, E. W., and Goosen, M. F. A. (1992) *in* Applications of Chitin and Chitosan (Goosen, M. F. A., Ed.), pp. 3–29. Technomic, Lancaster, PA.
3. Jeuniaux, C., Voss-Foucart, M. F., Poulicek, M., and Bussers, J. C. (1989) *in* Chitin and Chitosan (Skjak-Braek, G., Anthonsen, T., and Sandford, P., Eds.), pp. 3–11. Elsevier Applied Science, New York.
4. Bartnicki-Garcia, S. (1968) *Annu. Rev. Microbiol.* **22,** 87–108.
5. Shahidi, F., Arachchi, J. K. V., and Jeon, Y. (1999) *Trends Food Sci. Technol.* **10,** 37–51.
6. No, H. K., and Meyer, S. P. (1995) *J. Aquatic Food Prod. Technol.* **4,** 27–52.
7. Shigemesa, Y., and Minami, S. (1996) *Biotechnol. Genet. Eng. Rev.* **13,** 384–420.
8. Su, C. H., Sun, C. S., Juan, S. W., Hu, C. H., Ke, W. T., and Sheu, M. T. (1997) *Biomaterials* **18,** 1169–1174.
9. Balassa, L. L. (1975) U.S. Patent 3,903,268.
10. Austin, P. R., Brine, C. J., Castle, J. E., and Zikakis, J. P. (1981) *Science* **212,** 749–753.
11. Tachibana, M., Yaita, A., Taniura, H., Fukasawa, K., Nagasue, N., and Nakamura, T. (1988) *Jpn. J. Surg.* **18,** 533–539.
12. Tsai, G. J., and Su, W. H. (1999) *J. Food Prot.* **62,** 239–243.
13. Yalpani, M., Johnson, F., and Robinson, L. E. (1992) *in* Advances in Chitin and Chitosan (Brine, C. J., Sandford, P. A., and Zikakis, J. P. Eds.), pp. 543–548. Elsevier Applied Science, New York.
14. Nishimura, K., Ishihara, C., Ukei, S., Tokura, S., and Azuma, I. (1986) *Vaccine* **4,** 115–156.
15. Nishimura, K., Nishimura, S., Nishi, N., Tokura, S., and Azuma, I. (1990) *Mol. Biother.* **2,** 115–120.
16. Biagini, G., Muzzarelli, R. A. A., Giardino, R., and Castaldini, C. (1992) *in* Advances in Chitin and Chitosan (Brine, C. J., Sandford, P. A., and Zikakis, J. P., Eds.), pp. 16–24. Elsevier Applied Science, New York.
17. Lowenstein, C. J., and Snyder, S. H. (1992) *Cell* **70,** 705–707.
18. Palmer, R. M., Ashton, D. S., and Moncada, S. (1988) *Nature* **333,** 664–666.
19. Moncada, S., and Higgs, A. (1993) *N. Engl. J. Med.* **329,** 2002–2012.
20. Stuehr, D. J., and Nathan, C. F. (1989) *J. Exp. Med.* **169,** 1543–1555.
21. Beckman, J. S., Beckman, T. W., Chen, J., Marshall, P. A., and Freeman, B. A. (1990) *Proc. Natl. Acad. Sci. USA* **87,** 1620–1624.
22. Laskin, D. L., and Pendino, K. J. (1995) *Annu. Rev. Pharmacol. Toxicol.* **35,** 655–677.
23. Bauer, J. A., Rao, W., and Smith, D. J. (1998) *Wound Repair Regeneration* **6,** 569–577.
24. Heck, D. E., Laskin, D. L., Gardner, C. R., and Laskin, J. D. (1992) *J. Biol. Chem.* **267,** 21277–21280.

25. Mulligan, M. S., Moncada, S., and Ward, P. A. (1992) *Br. J. Pharmacol.* **107,** 1159–1162.
26. Hay, R. J., Caputo, J., and Macy, M. L. (1992) ATCC Quality Control Methods for Cell Lines, pp. 23–33. American Type Culture Collection, Manassas, VA.
27. Mosmann, T. (1983) *J. Immunol. Methods* **65,** 55–63.
28. Green, L. C., Wagner, D. A, Glogowski, J., Skipper, P. L., Wishnok, J. S., and Tannenbaum, S. R. (1982) *Anal. Biochem.* **126,** 131–138.
29. Ignarro, L. J., Fukuto, J. M., Griscavage, J. M., Rogers, N. E., and Byrns, R. E. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 8103–8107.
30. Rollo, E. E., Laskin, D. L., and Denhardt, D. T. (1996) *J. Leukocyte Biol.* **60,** 397–404.
31. Wilson, K. (1997) *Nursing Crit. Care* **2,** 291–296.
32. DeGeorge, G. L., Heck, D. E., and Laskin, J. D. (1997) *Biochem. Pharmacol.* **54,** 103–112.
33. Rubbo, H., Tarpey, M., and Freeman, B. A. (1995) *Biochem. Soc. Symp.* **61,** 33–45.
34. Mulligan, M. S., Hevel, J. M., Marletta, M. A., and Ward, P. A. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 6338–6342.
35. Schaffer, M. R., Tantry, U., Thornton, F. J., and Barbul, A. (1999) *Eur. J. Surg.* **165,** 262–267.
36. Murrell, G. A., Szabo, C., Hannafin, J. A., Jang, D., Dolan, M. M., Deng, X. H., Murrell, D. F., and Warren, R. F. (1997) *Inflamm. Res.* **46,** 19–27.
37. Shearer, J. D., Richards, J. R., Mills, C. D., and Caldwell, M. D. (1997) *Am. J. Physiol.* **272,** E181–E190.
38. Su, C. H., Sun, C. S., Juan, S. W., Ho, H. O., Hu, C. H., and Sheu, M. T. (1999) *Biomaterials* **20,** 61–68.
39. Chung, L. Y., Schmidt, R. J., Hamlyn, P. F., Sagar, B. F., Andrews, A. M., and Tuner, T. D. (1994) *J. Biomed. Mater. Res.* **28,** 463–469.
40. Mori, T., Okumura, M., Matsuura, M., Ueno, K., Tokura, S., Okamoto, Y., Minami, S., and Fujinaga, T. (1997) *Biomaterials* **18,** 947–951.
41. Levine, D. W., Wang, D. I. C., and Thilly, W. G. (1979) *Biotechnol. Bioeng.* **21,** 821–845.
42. Suzuki, K., Tokoro, A., Okawa, Y., Suzuki, S., and Suzuki, M. (1986) *Microbiol. Immunol.* **30,** 777–789.
43. Suzuki, K., Mikami, T., Okawa, Y., Tokoro, A., Suzuki, S., and Suzuki, M. (1986) *Carbohydr. Res.* **151,** 403–408.
44. Suzuki, K., Tokoro, A., Okawa, Y., Suzuki, S., and Suzuki, M. (1985) *Chem. Pharm. Bull.* **33,** 886–888.
45. Peluso, G., Petillo, O., Ranieri, M., Santin, M., Ambrosio, L., Calabro, D., Avallone, B., and Balsamo, G. (1994) *Biomaterials* **15,** 1215–1220.
46. Nathan, C. (1992) *FASEB J.* **6,** 3051–3064.