Protective Effect of Baker's Yeast Mannan against *Listeria monocytogenes* and *Pseudomonas aeruginosa* Infection in Mice

Makiko KOBAYASHI,[a] Yoshio OKAWA,[b] Shigeo SUZUKI,[b] and Masuko SUZUKI*,[a]

Department of Microbiology[a] and Second Department of Hygienic Chemistry,[b] Tohoku College of Pharmacy, Komatsushima 4-4-1, Aoba-ku, Sendai, Miyagi 981, Japan. *Received September 5, 1989*

The neutral mannan (WNM) and the acidic mannan (WAM025) fractions from baker's yeast (*Saccharomyces cerevisiae*) were found to manifest significant protective effects against intraperitoneal and intravenous infections of *Listeria monocytogenes* and *Pseudomonas aeruginosa* in mice. A remarkable decrease in the number of microbial cells in spleen and liver was observed in mice inoculated with these microorganisms after administration of either mannan fraction. In order to clarify the mechanism of the protective effects, we investigated *in vitro* the bactericidal activity and lysosomal enzyme activities such as myeloperoxidase, acid phosphatase, and neutral protease, in Kupffer cells (KCs) from mice pretreated with either mannan fraction. KCs from mice administered with these mannan fractions showed an enhanced killing effect on these bacteria *in vitro*, and neutral protease activity was considered to be one of the important factors in the kiling effect on both *L. monocytogenes* and *P. aeruginosa*.

Keywords mannan; protective effect; infection; Kupffer cell; *Listeria monocytogenes*; *Pseudomonas aeruginosa*

Several papers have dealt with the immunosuppressive response to mannan elaborated by *Candida albicans*.[1-4] Namely, Fischer *et al.*[1] described the inhibition of lymphocyte proliferation by polysaccharide antigens present in the serum of patients with chronic mucocutaneous candidiasis. Thereafter, Domer *et al.*[2] separated components enhancing and suppressing the immune response from *C. albicans* bulk mannan by chromatography on a diethylaminoethyl (DEAE)-Sephadex A-50 column, and observed that the fraction eluated with 0.2 M NaCl enhanced the antibody response to sheep red blood cells. Moreover, it has been reported by these workers that mannan obtained from the cells of *Saccharomyces cerevisiae* (baker's yeast) also displayed an immunosuppressive effect. Wright *et al.* also found that a baker's yeast mannan inhibits the release of myeloperoxidase and the respiratory burst of neutrophils.[3] They suggested that these properties are responsible for the immunosuppressive effect of this mannan by blocking the mannose receptors existing on the surface of the cell membranes.[4]

In the present paper, we describe the protective effect of baker's yeast mannan in mice infected separately with *L. monocytogenes* and *P. aeruginosa*, which have been shown to be facultative intracellular and extracellular bacteria, respectively, possessing low sensitivities to conventional antimicrobial agents. Changes in the bactericidal effects of the Kupffer cells (KCs) of the liver, one of the target organs of these bacteria, were examined in order to clarify the bactericidal mechanisms.

Materials and Methods

Animals Male 5- to 8-week-old BALB/c mice were obtained from Shizuoka Laboratory Animal Center, Hamamatsu, Japan.

Bacteria *Listeria monocytogenes* serotype 4b and *Pseudomonas aeruginosa* PA-103 strains were cultured in trypticase soy broth (BBL Microbiology System) and nutrient broth under shaking at 37 °C for 20 h, respectively.

Preparation of Mannan Fractions The mannan subfractions were prepared as previously reported.[5] Briefly, the bulk mannan fraction extracted from the parent baker's yeast, a wild-type strain of *Saccharomyces cerevisiae* cells, was fractionated by DEAE-Sephadex A-50 column chromatography, and the neutral and the strongly acidic fractions which were eluted with water and 0.25 M NaCl (designated as WNM and WAM025, respectively) were used in this study.

Treatment of Mice Mice were injected intraperitoneally (i.p.) with mannan fraction (WNM or WAM025) at a dose of 150 mg/kg/d for 5 d.

Determination of the Survival Ratio of Mice after Inoculation with Bacteria One day after the last injection of WNM or WAM025, the mice were injected intraperitoneally or intravenously with $0.25-5.0\times10^7$ viable *L. monocytogenes* or $1.0-1.5\times10^7$ viable *P. aeruginosa* cells. The survival of the mice was followed for 30 d.

Determination of Bacterial Growth Two days after infection, spleens and livers were removed and homogenized, and bacteria were enumerated by the pour-plate method, as previously reported by Okawa *et al.*[6]

Preparation of KCs KCs were prepared by a method based on those of Crofton *et al.*[7] and Page and Garvey.[8] Briefly, the liver was perfused with 0.2% Pronase E (Kaken Pharmaceutical), then digested for 60 min in the same solution at 37 °C. After 20 or 40 min of incubation, 0.5 mg of deoxyribonuclease (DNase) type I (Merck) was added to digest the cellular debris. KCs were then separated by Percoll (Pharmacia) gradient centrifugation (density 1.080).

Determination of Bactericidal Activity In 96-well microplates, *L. monocytogenes* or *P. aeruginosa* (1×10^6 cells/10 μl of Hanks' balanced salt solution (HBSS) was mixed with 5×10^5 KCs in 0.1 ml of the same medium and 10 μl of normal mouse serum. After incubation for 3 h, the culture supernatant was transferred to other wells, and intracellular bacteria were released by lysing phagocytes with water. The total number of extracellular and intracellular bacteria was determined by colony counting. Calculation of the growth inhibition was done by using the following formula: Growth inhibition $(\%)=[(A-B)/A]\times100$, where A is the average number of colonies that developed from the incubation mixture without KCs and B is the average number of colonies that developed from the incubation mixture containing KCs.

Assay of Chemiluminescence Response The chemiluminescence (CL) response was measured for 10 min and expressed as counts per minute (cpm) using a Biolumat LB 9500 (Berthold), in accordance with the previous description by Sakai *et al.*[9]

Assay of Myeloperoxidase, Acid Phosphatase and Neutral Protease Activity Myeloperoxidase (MPO) activity was measured by the *o*-tolidine method as described in our previous paper.[6] The activity of acid phosphatase was determined by measuring the amount of *p*-nitrophenol liberated from *p*-nitrophenyl phosphate according to the method of Komatsu *et al.*[10] The neutral protease activity of the supernatant was assayed by measuring the release of trichloroacetic acid (TCA)-soluble peptides from casein, according to a previously reported method.[11] It should be noted that only neutral protease activity can be determined by this method, as described previously.[12]

Intracellular Neutral Protease KCs (5×10^5 cells/well) were mixed with 5×10^5 heat-killed *L. monocytogenes* or *P. aeruginosa* cells, and incubated at 37 °C for 3 h in HBSS in microplates. After incubation for 3 h, the cells were destroyed by freeze/thawing 3 times. Assay of the neutral protease activity was carried out by the same method as mentioned above.

Results and Discussion

The effects of pretreatment of mice with either mannan fraction administered daily for 5 d prior to challenge with *L. monocytogenes* or *P. aeruginosa* are shown in Fig. 1. It is clear that both WNM and WAM025 can display potent protective effects against i.p. infection of *L. monocytogenes* and *P. aeruginosa* in comparison with normal mice. On the other hand, in the case of i.v. infection of *L. monocytogenes*, stronger protective effects were found using WAM025 than WNM ($p<0.001$; χ^2-test). Additionally, WNM was not effective against *L. monocytogenes* when administered intravenously. In the case of either i.p. or i.v. infection of *P. aeruginosa*, however, both WNM was WAM025 exhibited stronger protective effects than that observed in non-treated mice. In the case of i.p. infection of *L. monocytogenes*, the number of bacteria in liver and spleen of mice administered

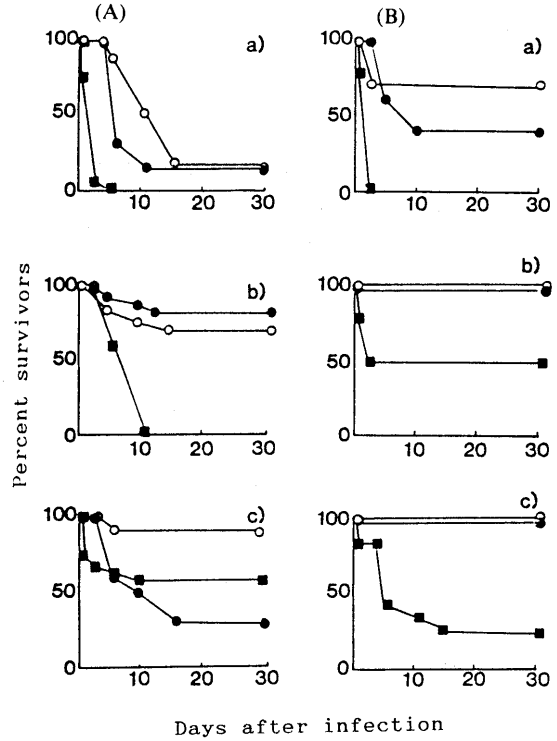

Fig. 1. Protective Effect of WNM and WAM025 on the Survival of Mice Infected with *L. monocytogenes* (A) or *P. aeruginosa* (B)

WNM or WAM025 (150 mg/kg) was administered i.p. to mice on days 0—4. One day after the final administration of mannan, the mice were infected with 2.6×10^6—2.5×10^7 cells of *L. monocytogenes*; 5.0×10^7 (a) or 2.5×10^7 (b) cells of *L. monocytogenes via* the i.p. route, or 2.6×10^6 (c) cells of *L. monocytogenes via* the i.v. route. On the other hand, the mice were infected with 1.0—1.5×10^7 cells of *P. aeruginosa*; 1.5×10^7 (a) or 1.0×10^7 (b) cells of *P. aeruginosa via* the i.p. route, or 1×10^7 (c) cells of *P. aeruginosa via* the i.v. route. Twenty to 35 mice were used for each group. ■, control; ●, WNM (150 mg/kg); ○, WAM025 (150 mg/kg).

Table I. Effect of WNM or WAM025 on the Growth of Bacteria in Mouse Spleen or Liver

Mice[a]	Number of viable bacteria in organs ($\times10^3$) (mean±S.E.)	
	Spleen	Liver
Exp. 1[b]		
L. monocytogenes (i.p.)		
Control	900± 23	13000± 680
WNM-treated	7± 1[c]	14± 5[c]
WAM025-treated	3± 2[c]	4± 2[c]
P. aeruginosa (i.p.)		
Control	490± 56	4600± 200
WNM-treated	47± 2[d]	330± 13[d]
WAM025-treated	31± 1[d]	180± 2[d]
Exp. 2[b]		
L. monocytogenes (i.p.)		
Control	1600±100	20000±1300
WNM-treated	13± 6[c]	10± 2[c]
WAM025-treated	6± 2[c]	8± 1[c]
P. aeruginosa (i.p.)		
Control	1000± 41	16000±1500
WNM-treated	97± 7[d]	400± 16[d]
WAM025-treated	45± 10[d]	160± 7[d]

a) WNM, WAM025 (150 mg /kg/d) were administered i.p. to mice on days 0—4. The mice were infected with 2.5×10^6 *L. monocytogenes* cells or 1.5×10^7 *P. aeruginosa* cells *via* the i.p. route 1 d after the final administration of mannan. *b*) Mice were dissected 1 d after challenge with bacteria, and the number of viable bacteria in the organs was determined. Results are expressed as the mean value±S.E. of three or five mice. *c*) $p<0.001$ *versus* control by Student's *t* test. *d*) $p<0.01$ *versus* control by Student's *t* test.

Table II. Bactericidal Activity of KCs in Mice Administered with WNM or WAM025

Source of KCs	Number of viable *L. monocytogenes* CFU[a]	Growth inhibition (%)	Number of viable *P. aeruginosa* CFU[a]	Growth inhibition (%)
Exp. 1				
None (after incubation)	320± 80	0	162± 3	0
Untreated KC	233±110	27	198±21	−22
WNM-treated KC	92± 42	71	133± 2	18
WAM025-treated KC	66± 18[b]	79	100± 2[c]	38
Exp. 2				
None (after incubation)	469± 11	0	155± 5	0
Untreated KC	501± 34	−7	152±16	2
WNM-treated KC	432± 23	8	100± 8[c]	35
WAM025-treated KC	221± 12[c]	53	70± 5[c]	55
Exp. 3				
None (after incubation)	266± 80	0	196± 2	0
Untreated KC	298±110	−12	290± 3	−48
WNM-treated KC	199± 42	25	269± 7	−37
WAM025-treated KC	150± 18[c]	44	129± 2[b]	34

WNM or WAM025 (150 mg/kg) was administered i.p. on days 0—4. KCs were separated on day 5. Bactericidal activity was determined as described in Materials and Methods. Results are expressed as the mean value±S.E. of three experiments. *a*) CFU was expressed as number of colonies per KCs (5×10^5). *b*) $p<0.001$ *versus* untreated group by Student's *t* test. *c*) $p<0.05$ *versus* untreated group by Student's *t* test.

TABLE III. Effect of Pretreatment of Mice with WNM or WAM025 on Chemiluminescence Response, and Activities of Myeloperoxidase, Acid Phosphatase, and Neutral Protease in KCs

Source of KCs/ 1×10^6	Chemiluminescence[a] (cpm $\times 10^3$)	Myeloperoxidase[b] (U)	Acid phosphatase[c] (mU)	Neutral protease[d] (U)
Untreated	28 ± 5	31 ± 4	60 ± 1	0.3 ± 0.1
WNM-treated	31 ± 14	25 ± 8	122 ± 1[e]	1.4 ± 0.2[f]
WAM026-treated	68 ± 20	45 ± 6	212 ± 1[e]	1.7 ± 0.2[e]

WNM of WAM025 (150 mg/kg/d) was administered i.p. on days 0—4. KCs were separated on day 5. Results are expressed as the mean value ± S.E. of three experiments. *a*) CL response was expressed as counts per minute (cpm). *b*) One unit of MPO activity was defined as an increase in absorbency at 460 nm of 0.001 per minute. *c*) One milli unit (mU) of this activity was defined as the amount of enzyme releasing one nmol of *p*-nitrophenol in 1 min. *d*) One unit of the enzyme was defined as the activity causing an increase in absorbency at 275 nm of 0.01 per min. *e*) $p < 0.001$ *versus* untreated KCs group by Student's *t* test. *f*) $p < 0.01$ *versus* untreated KCs group by Student's *t* test.

TABLE IV. Neutral Protease Activity of KCs from Mice Administered with WNM or WAM025

Source of KCs	Stimulation with *L. monocytogenes*	Neutral protease activity (U)	Stimulation with *P. aeruginosa*	Neutral protease activity (U)
None	+	0.10 ± 0.06	+	0.10 ± 0.02
Untreated	−	0.60 ± 0.06	−	0.60 ± 0.04
	+	1.20 ± 0.03	+	3.30 ± 0.06
WNM-	−	0.80 ± 0.02[b]	−	0.80 ± 0.06[b]
treated	+	5.50 ± 0.06[a]	+	9.20 ± 0.06[a]
WAM025-	−	1.00 ± 0.06[c]	−	0.90 ± 0.02[b]
treated	+	9.40 ± 0.02[a]	+	10.10 ± 0.06[a]

KCs (5×10^5) were mixed with 5×10^5 heat-killed bacteria, and incubated at 37 °C for 3 h. Neutral protease activity of bacteria-stimulated KCs was assayed by measuring the release of trichloroacetic acid-soluble peptide from casein. Results are expressed as the mean value ± S.E. of three experiments. *a*) $p < 0.001$ *versus* neutral protease activity from untreated KCs (stimulation; −) by Student's *t* test. *b*) $p < 0.05$ *versus* neutral protease activity from untreated KCs (stimulation; −) by Student's *t* test. *c*) $p < 0.01$ *versus* neutral protease activity from untreated KCs (stimulation; −) by Student's *t* test.

with WNM or WAM025 was significantly smaller ($p < 0.001$; Student's *t* test) than that observed in normal mice (Table I). In the case of i.p. infection of *P. aeruginosa*, the number of bacteria in the spleen and liver of mice administered with WNM or WAM025 was also significantly decreased, but the elimination of *P. aeruginosa* from infected mice by treatment with mannan fractions was less effective than in the case of *L. monocytogenes*-infected mice pretreatment with these mannan fraction. As shown in Table II, the bactericidal activity against *P. aeruginosa* cells of KCs treated with WAM025 was significantly greater than that displayed by normal KCs or WNM-treated cells ($p < 0.01$). Treatment of mice with both mannan subfractions did not enhance the CL response or the MPO activity significantly, as compared with the untreated group. However, increases in acid phosphatase and neutral protease activities in KCs were evident when either WNM or WAM025 was administered (Table III). As shown in Table IV, the intracellular neutral protease activity of KCs was significantly enhanced by treatment with both WNM and WAM025.

We conclude that both mannans, WNM and WAM025, are affective as biological response modifiers against infection of both *L. monocytogenes* and *P. aeruginosa* in mice, and that certain neutral protease induced in activated KCs by mannan fractions can serve as important factors in protecting the host animal against infection by these facultative intracellular and extracellular bacteria.

References

1) A. Fischer, J. J. Ballet, and C. Griscelli, *J. Clin. Invest.*, **62**, 1005 (1978).
2) J. E. Domer, P. W. Stashak, K. Elkins, B. Prescott, G. Caldes, and P. J. Baker, *Cell. Immunol.*, **101**, 403 (1986).
3) C. D. Wright, J. U. Bowie, G. R. Gray, and R. D. Nelson, *Infect. Immun.*, **42**, 76 (1983).
4) C. D. Wright, J. U. Bowie, and R. D. Nelson, *Infect. Immun.*, **43**, 467 (1984).
5) S. Suzuki, M. Suzuki, H. Hatsukaiwa, H. Sunayama, T. Suzuki, M. Uchiyama, F. Fukuoka, M. Nakanishi, and S. Akiya, *Gann*, **60**, 273 (1969).
6) Y. Okawa, K. Suzuki, M. Kobayashi, M. Asagi, K. Sakai, S. Suzuki, and M. Suzuki, *Microbiol. Immunol.*, **30**, 957 (1986).
7) R. W. Crofton, M. M. C. D. Dulk, and R. Van Furth, *J. Exp. Med.*, **148**, 1 (1969).
8) D. T. Page and J. S. Garvey, *J. Immunol. Meth.*, **27**, 159 (1979).
9) K. Sakai, S. Suzuki, and M. Suzuki, *J. Pharmacobio-Dyn.*, **7**, 943 (1984).
10) N. Komatsu, K. Egawa, and N. Nagumo, *Jpn. J. Antibiot.*, **26**, 61 (1973).
11) Y. Suzuki and T. Murachi, *J. Biochem.* (Tokyo), **82**, 215 (1977).
12) D. O. Adams, *J. Immunol.*, **124**, 286 (1980).