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Effect of *Streptoverticillium cinnamoneum* Cell Wall on *Staphylococcus aureus* Infection in Mice

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A cell wall fraction of *Streptoverticillium cinnamoneum*, a non-pathogenic strain of Streptomycetaceae, was obtained by centrifugation of sonicated mycelia. After treatment with proteases, deoxyribonuclease, ribonuclease, and organic solvent mixture, the cell wall specimen (P-CW) was found to consist of homogeneous fibrous material when examined under an electron microscope. The survival of mice in the infection–protection assay against the *Staphylococcus aureus* βH 248 strain, 1.5×10^9 cells/mouse, was increased by treatment with 50 mg/kg of P-CW as compared with the control group. Remarkable elevation of serum lysozyme and carbon clearance activities was observed in mice treated with the same dose of P-CW.

Keywords——*Streptoverticillium*; cell wall; *Staphylococcus aureus*; infection; protection; serum lysozyme; phagocytic activity

It is well-known that higher animals have many defense mechanisms against bacterial infection, and that the role of macrophages is quite important in these host-defense mechanisms. Lysozyme, a secretory product of the macrophage-monocyte series,[1] was found to be a strong bacteriolytic factor, also modulating antitumor and phagocytic activities of the same host animal.[2] Serum lysozyme has been considered as an index of the state of macrophage.[3] It is also well-known that some microorganisms[4] and cell wall components[4b,5] are able to enhance nonspecific resistance to bacterial infections, with accompanying elevation of lysozyme activity.

Recently, we reported the antitumor effect of a cell wall fraction of a non-pathogenic species of Streptomycetaceae, *Stv. cinnamoneum*, against mice implanted with Ehrlich and Meth–A ascites tumors.[6] In order to examine whether this cell wall fraction is also capable of protecting against bacterial infection, a series of infection-defense assays has been carried out in mice challenged with a virulent strain of *Staphylococcus aureus*. The present paper reports the anti-Staphylococcal activity of the cell wall of *Stv. cinnamoneum* with special reference to the modulation of serum lysozyme and carbon clearance activities.

Experimental

Materials——*Streptoverticillium* (*Stv.*) *cinnamoneum* IFO 12852 was used throughout. Lyophilized *Mycobacterium bovis* was obtained from the Japan BCG Laboratory Ltd., Tokyo, Japan. Male ddY mice, 22 ± 2 g, were used.

Preparation of Cell Wall——The *Stv. cinnamoneum* IFO 12852 strain was cultured as described previously.[7] The cell wall was prepared according to the method of Nakamura *et al.*[8] and was further purified by the following procedures. A suspension of the cell wall, 320 mg in 100 ml of 0.1 M Tris–HCl buffer, pH 7.5, containing 20 mg each of trypsin and chymotrypsin, was gently stirred at 37°C for 15 h and washed once with the same buffer. The trypsin-chymotrypsin treatment was repeated twice, and the residue was resuspended in the same buffer, 100 ml, containing pronase, 20 mg. After incubation at 37°C for 15 h, the solid mass was centrifuged and washed with the buffer. Then it was suspended in 100 ml of the buffer containing 10 mg each of deoxyribonuclease and ribonuclease, and the mixture was kept at 37°C for 15 h. Then, the solid mass was extracted with a mixture of chloroform–methanol–water (1: 1: 1, v/v) by mechanical stirring at room temperature for 15 min. After centrifugation, insoluble material suspended between water and solvent layers was collected and further extracted 3 times by the same procedure. Finally, the purified cell walls (P-CW) of the middle layer were freeze-dried. The cell wall preparations were routinely checked the shadowing method with a Hitachi type HU11DS electron microscope.

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Analytical Methods——Total sugar was determined by the modified method of Molisch.[9] Total hexosamine was determined by the modified method of Elson and Morgan.[10] Total phosphorus was quantitated by the method of Ames and Dubin.[11] Amino acid analysis was conducted using a Hitachi model 835 amino acid analyzer after hydrolysis of the sample for 15 h at 105°C in 6 N HCl. Glucosamine and galactosamine were quantitated using the same amino acid analyzer after hydrolysis for 4 h at 105°C in 4 N HCl. Neutral sugars were analyzed qualitatively by descending chromatography on Toyo filter paper No. 51A.[12]

Treatment of Mice——Mice received intraperitoneal injections of various doses of P–CW or BCG on days 6, 4 and 2 before challenge with *S. aureus*, or assay of serum lysozyme and carbon clearance activities.

Infectious Challenge——*Staphylococcus aureus* βH 248 strain was grown in Staphylococcus No. 110 medium (Nissui Seiyaku Co., Tokyo, Japan) for 43 h at 37°C and the harvested cells were suspended in saline. The cells (1.5×10^9) were injected intravenously in the tail vein of ddY mice, then the mice were observed for 60 d.

Enzyme Activity of Serum——Blood was drawn from the carotid, and serum was separated by centrifugation. Lysozyme activity was assayed according to Parry *et al.*[13] by using lyophilized cells of *Micrococcus lysodeikticus* (Miles Laboratories Inc., Kankakee, U.S.A.) as a substrate. The change in transmission percentage at 540 nm per min was recorded and compared to a standard curve prepared with known concentration of egg white lysozyme (Merck, Darmstadt, Germany).

Assay of Phagocytic Activity——The phagocytic activity of the reticuloendothelial system was examined by measuring the clearing rate of colloidal carbon in the mouse blood according to the method of Biozzi *et al.*[14] The mice were injected intraperitoneally with P-CW and BCG (50 mg/kg) on days 0, 2 and 4. Phagocytic activity was measured on day 6. The phagocytic index, K, was calculated according to the description in the previous paper.[7]

Results and Discussion

A significant decrease of the cell envelope content was observed as the purification procedure of P–CW was repeated. Finally, P–CW no longer contained electron-dense material, as shown in Fig. 1, and was therefore confirmed to consist of the so-called cell wall skeletons. From the results of chemical analyses (Table I), the components of P–CW were amino acid (39.2%), hexosamine (14.4%), neutral sugar (4.9%), and phosphorus (1.6%), on a weight basis with respect to lyophilized P–CW. Galactose and mannose were identified as the neutral sugar components of P–CW. The amino acid and amino sugar compositions (μmol/mg) of P–CW were glutamic acid 0.58, muramic acid 0.41, glycine 0.64, alanine 1.04, diaminopimelic acid 0.70, glucosamine 0.59 and galactosamine 0.04 (Table II). The total amino acid and phosphorus contents of P–CW were much higher than those of the cell wall skeletons of BCG and *Corynebacterium*,[15] whereas the neutral sugar content was less than those in the other

TABLE I. Chemical Composition of P–CW from *Stv. cinnamoneum*

Total sugar (as glucose) (%)	Total hexosamine (%)	Total amino acid (%)	Total phosphorus (%)
4.9	14.4	39.2	1.6

These values are expressed on a weight basis of dried P–CW of *Stv. cinnamoneum*.

TABLE II. Amino Acids and Amino Sugars in P–CW from *Stv. cinnamoneum*

Amino acids and amino sugars	μmol/mg	Molar ratio
Glutamic acid	0.58	1.00
Muramic acid	0.41	0.71
Glycine	0.64	1.10
Alanine	1.04	1.70
Diaminopimelic acid	0.70	1.21
Glucosamine	0.59	1.02
Galactosamine	0.04	0.07

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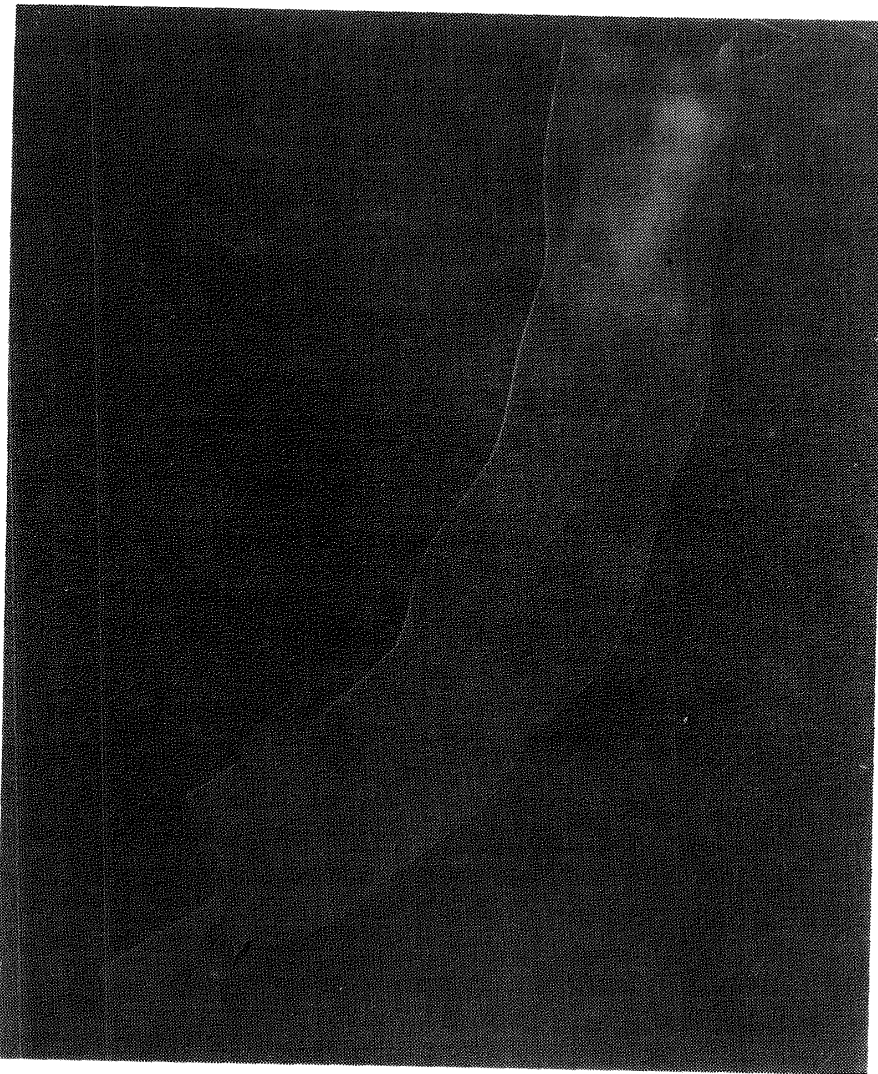

Fig. 1. Observation of P–CW from *Stv. cinnamoneum* by Electron Microscopy ($\times 37000$)

TABLE III. Effect of P–CW Treatment on the Survival of ddY Mice with *Staphylococcus aureus* Infection

Samples	Dose (mg/kg × 3)	Mortality (death/total)	Survival (%)
Control		13/16	19
P–CW	1	5/6	17
P–CW	50	1/16	94
BCG	50	5/16	69

P–CW or BCG was administered intraperitoneally on days 6, 4 and 2 prior to intravenous challenge with the bacteria (1.5×10^9 cells per mouse). This table shows the results 60 d after the infections challenge.

organisms. The protecting effect of P–CW in mice against *S. aureus* is shown in Table III. P–CW at doses of 1 and 50 mg/kg was given to mice intraperitonally at intervals of 6, 4 and 2 d prior to an intravenous challenge with *S. aureus*. The survival percent did not increase with 1 mg/kg of P–CW, but increased with 50 mg/kg of P–CW. Only 19% of mice in the control group survived as opposed to 94% survival in the P–CW-treated group ($p<0.01$). Figure 2 shows the lysozyme activity of serum of ddY mice treated 3 times with 50 mg/kg of P–CW, indicating that P–CW was able to stimulate the serum lysozyme activity. The

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curve reached a peak at 2—3 d after the third injection of P–CW, then the activity gradually decreased to the control level. The effect of doses of P–CW on the lysozyme activity of serum is shown in Fig. 3. Remarkable stimulation of serum lysozyme activity was seen in the mice pretreated with 50 mg/kg of P–CW, while no stimulation of this activity was seen in the mice pretreated with 1 mg/kg of P–CW, as compared with the control group. P–CW and BCG were administered intraperitoneally and intravenously on days 0, 2 and 4, and serum lysozyme activity was measured on day 6 (Fig. 4). Serum lysozyme activity of mice injected with P–CW using both routes increased about 2 times as compared with the control group. However, the lysozyme activity of mice treated with BCG by intravenous injection was not elevated, though the activity slightly increased after intraperitoneal injection relative to the control group. A significant increase of carbon clearance activity was observed in mice treated with P–CW and BCG as compared with the control group (Fig. 5).

Although the protection mechanism against microbial infection is not fully understood, participation of released lysosomal enzymes and active oxygens is considered to be very important. In a previous presentation, we reported that the lysosomal enzyme activity of peritoneal macrophages of mice increased after treatment with P–CW,[6] suggesting that lysozyme has an important role in the initial stages of microbial invasion.[16] It is thought that lysozyme acts as an antibacterial agent directly through its bacteriolytic action. Biggar and Sturgess have reported the significant role of lysozyme in mediating the microbicidal action of rat alveolar macrophages,[16a] demonstrating that phagocytosis of intact bacteria did not occur, whereas lysozyme-degraded bacteria were rapidly phagocytized. In the present paper, it is shown that serum lysozyme activity in mice increased on treatment with P–CW,

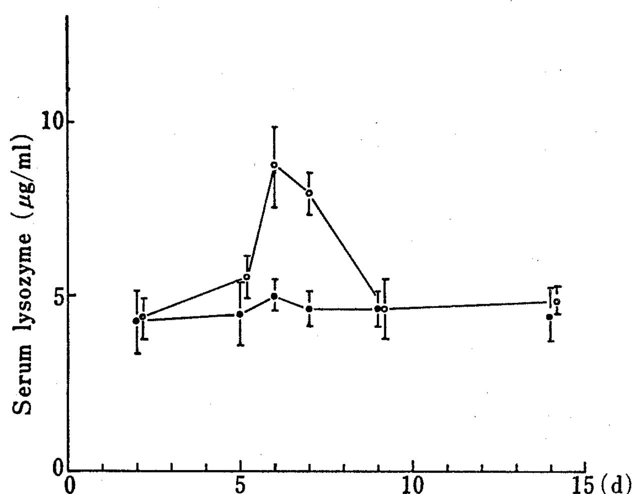

Fig. 2. Serum Lysozyme in Mice administered with P–CW

The mice were injected intraperitoneally with P–CW (50 mg/kg) on days 0, 2 and 4. The mice were examined at 2, 5, 6, 7, 9 and 14 d after the beginning of P–CW administration.
●; control, ○; P–CW.

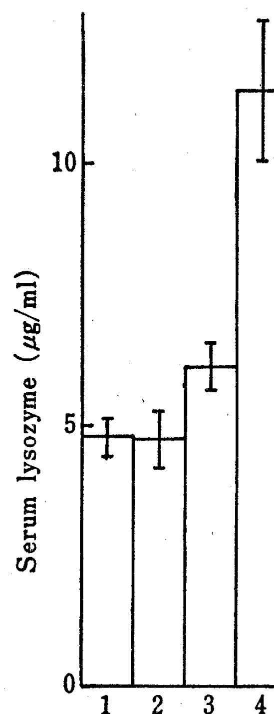

Fig. 3. Dose Response Relation for Serum Lysozyme in Mice administered with P–CW

The mice were injected intraperitoneally with P–CW on days 0, 2 and 4. Serum lysozyme concentration was measured on day 6.
1; control.
2; P–CW 1 mg/kg.
3; P–CW 5 mg/kg.
4; P–CW 50 mg/kg.

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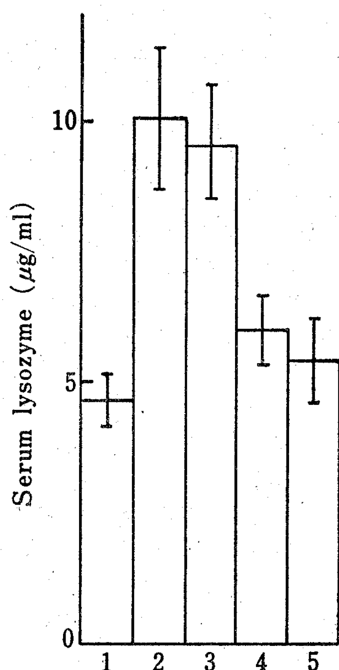

Fig. 4. Effect of Intraperitoneal and Intravenous Administrations of P–CW and BCG on Serum Lysozyme

These samples were administered on days 0, 2 and 4. Serum lysozyme concentration was measured on day 6.
1; control.
2; P–CW 50 mg/kg *i.p.*
3; P–CW 50 mg/kg *i.v.*
4; BCG 50 mg/kg *i.p.*
5; BCG 50 mg/kg *i.v.*

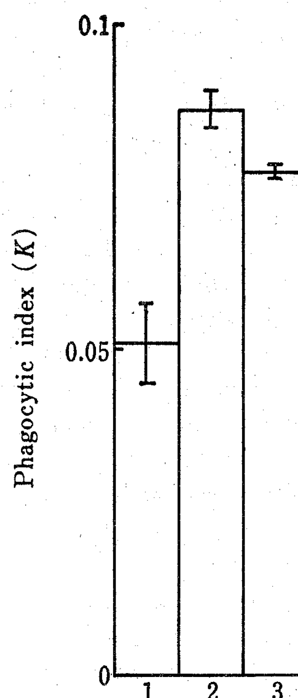

Fig. 5. Elevation of Carbon Clearance Activity of Mice administered with P–CW and BCG

The mice were injected intraperitoneally with P–CW and BCG on days 0, 2 and 4. Phagocytic activity was measured on day 6.
1; control.
2; P–CW 50 mg/kg.
3; BCG 50 mg/kg.

and when the activity finally peaked (Fig. 2), the mice were challenged intravenously with *S. aureus*. As shown in Table III, treatment of mice with P–CW significantly increased their survival potential. Moreover, the increase of resistance to infection was correlated well with the increase in serum lysozyme activity induced with P–CW. Cappuccino *et al.*[17] suggested that the augmentation of lysozyme activity may be the result of stimulation of the reticuloendothelial system of the host. Therefore, the carbon clearance activity of mice treated with P–CW was examined. The activity was remarkably increased as compared with the control group (Fig. 5). Numbers of peritoneal exudate cells at this time were increased about 6—10 times over the control group. From this result, P–CW was found to increase the number and phagocytic function of macrophages.

In view of these findings, it is considered that P–CW contributes to the removal of foreign entities from the host animal *via* direct or indirect stimulation of the phagocytic mechanisms.

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References

1) S. Gordon, J. Todd, and Z.A. Cohn, *J. Exp. Med.*, **139**, 1228 (1974).
2) *a*) E.F. Osserman, M. Klockars, J. Halper, and R.E. Fischel, *Nature* (London), **243**, 331 (1973); *b*) M. Klockars and P. Roberts, *Acta Haemat.*, **55**, 289 (1976).
3) *a*) G.A. Currie and S.A. Eccles, *Br. J. Cancer*, **33**, 51 (1976); *b*) N.R. DiLuzio, *J. Reticuloendothel. Soc.*, **26**, 67 (1979).
4) *a*) C. Adlam, E.S. Broughton, and M.T. Scott, *Nature New Biol.*, **235**, 219 (1972); *b*) N.A. Sher, S.D. Chaparas, L.E. Greenberg, and S. Bernard, *Infect. Immun.*, **12**, 1325 (1975).

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5) N.R. DiLuzio, and D.L. Williams, *Infect. Immun.*, **20**, 804 (1978).
6) Y. Okawa, K. Hashimoto, and M. Suzuki, The 101st Annual Meeting of the Pharmaceutical Society of Japan, Kumamoto, April 1981, Abstract Paper p. 285.
7) Y. Okawa, M. Kadowaki, M. Suzuki, and S. Suzuki, *Ann. Rep. Tohoku Coll. Pharm.*, **24**, 113 (1977).
8) T. Nakamura, G. Tamura, and K. Arima, *J. Ferment. Technol.*, **45**, 869 (1967).
9) A.W. Devor, *Anal. Chem.*, **24**, 1626 (1948).
10) S. Gardell, *Acta Chem. Scand.*, **7**, 207 (1953).
11) B.N. Ames and D.T. Dubin, *J. Biol. Chem.*, **235**, 769 (1960).
12) V. Riveros-Moreno, R. Bomford, and M.T. Scott, *J. Natl. Cancer Inst.*, **60**, 653 (1978).
13) R.M. Parry, R.C. Chandan, and K.M. Shahani, *Proc. Soc. Exp. Biol. Med.*, **119**, 384 (1965).
14) G. Biozzi, B. Benacerraf, and B.N. Halpern, *Brit. J. Exp. Pathol.*, **34**, 441 (1953).
15) *a*) I. Azuma, E.E. Ribi, T.J. Meyer, and B. Zbar, *J. Natl. Cancer Inst.*, **52**, 95 (1974); *b*) I. Azuma, K. Sugimura, T. Taniyama, A.A. Aladin, and Y. Yamamura, *Jpn. J. Microbiol.*, **19**, 265 (1975).
16) *a*) W.D. Biggar and J.M. Sturgess, *Infect. Immun.*, **16**, 974 (1977); *b*) M.E. Selsted, and R.J. Martinez, *ibid.*, **20**, 782 (1978).
17) J.G. Cappuccino, S. Winston, and G.C. Perri, *Proc. Soc. Exp. Biol. Med.*, **116**, 869 (1964).

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