THE PROTECTIVE EFFECT OF MATERIALS EXTRACTED FROM BACTERIAL CELLS AGAINST INFECTION IN EXPERIMENTAL SALMONELLOSIS

1. PROTECTION WITH PHENOL EXTRACT

YOSHISATO HAGIHARA, MITSUKO SASAKI, AND SACHIKO HONMA

*Department of Microbiology, Kurume University School of Medicine,
Kurume, Japan*

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The protective effect of living as well as killed cells and phenol extracts obtained from an attenuated strain of *Salmonella enteritidis* against infection with a virulent strain in mice were studied. Results summarized as follows.

1. Killed cells of an attenuated strain had no protective effect against infection with a virulent strain, though living cells were remarkably effective.

2. Eight fractions were obtained from an attenuated strain by extraction with phenol. A large amount of RNA and a small amount of DNA with protein were contained in these fractions.

3. It was shown that fraction I and IV extracted with phenol pH 4 had some protective effects against infection, but the remaining fraction I-D, III and all of the fractions extracted with phenol pH 6 were ineffective.

It has been known that killed vaccine of *Salmonella enteritidis* is ineffective in preventing ultimate death from infection with a virulent strain. On the contrary, suitable immunization with living vaccine can protect against infection¹⁾. Kawakami et al.²⁾ reported on the immunizing effect of materials isolated from *Salmonella enteritidis* against infection with the same strain. On the other hand, Youmans et al.^{3) 4)} and Venneman et al.⁵⁾ reported that ribosomal fraction showed protective effect against infection. This paper concerns attempts to study on the protective effect of phenol extract obtained from bacterial cells against infection in experimental Salmonellosis.

MATERIALS AND METHODS

Bacteria. A virulent strain 116-54 (116 strain) and an attenuated strain SER-18M (18M strain) of *Salmonella enteritidis* acquired from Dr. Mitsunashi, Department of Microbiology, Gunma University School of Medicine, Maebashi, were used.

Experimental animals. Female mice of dd-N strain, weighing about 20 gm were used throughout this experiment.

Preparation of immunizing materials. Viable cell suspension of the 18M strain in physiological saline was prepared from overnight culture on agar slant, and adjusted to adequate concentration by using a densitometer. Heat

killed cells of the 18M strain were prepared by heating at 100°C for 30 min. Phenol extracts were prepared by the modified method of Shibatani et al.⁶⁾, Ingle et al.⁷⁾ and Georgiev et al.⁸⁾ Cells of the 18M strain cultured at 37°C overnight in five flasks containing 200 ml of heart infusion broth (HIB) were respectively pored into five flasks containing 1,500 ml of fresh HIB kept at 37°C. After cultivation at 37°C for 1 hour and addition of 7.5 gm of bentonite, bacterial cells were collected by centrifugation, and washed once with 10 mM tris buffer pH 7.4 containing 0.15M NaCl (tris-NaCl). To the bacterial suspension in 170 ml of the same buffer, 35 ml of 25 % sodium lauryl sulphate was added, and the mixture was stirred in ice for about 1 hour. Two hundred ml of 80 % phenol which contained tris-NaCl at the concentration of 20 % (listed as phenol pH 4) was further added, and stirred in ice for 15 min. The phenol adjusted to pH 6.0 with 1N NaOH was used for extraction, and this phenol was listed as phenol pH 6.

After centrifugation at 10,000 g for 15 min, aqueous phase was removed, and designated as Fraction I. The denatured protein between aqueous phase and phenol phase, and a centrifugated residue, were respectively resuspended in 30 ml and 70 ml of tris-NaCl. After heating at 70°C for 15 min and mixing with 80 % phenol for 15 min, these mixtures were centrifugated, and two removed aqueous phases were designated as Fraction III and IV, respectively.

These three fractions were further deproteinated with equal volume of 80 % phenol 3 to 5 times, and nucleic acids were precipitated by addition of 2 volumes of cold ethanol. After standing at -20°C overnight, the precipitates were collected by centrifugation and washed with a series of ethanol, and dried by suction. A flocculent precipi-

tate occurred in Fraction I was separated, washed in the same manner, and designated as Fraction I-D.

Chemical analysis. Ribonucleic acid (RNA) was estimated by the modified orcinol method⁹⁾, and deoxyribonucleic acid (DNA) and protein by the method of Dishe¹⁰⁾ and Lowry¹¹⁾, respectively.

Immunization. Each group containing 10 mice was used for immunization. The mice received immunizing materials by intramuscular injection on femur or on femur and upper arm.

A one tenth ml of suspension of viable cells and killed cells of the 18M strain was only once injected for immunization

The phenol extract was dissolved in tris-NaCl at the concentration of 10 mg/ml as RNA. To this solution, a half volume of 20 mg/ml of methylated bovine serum albumin was added and mixed, and one and half volumes of Freund's complete adjuvant was further added¹²⁾. A three tenth of this mixture was administered to mice. Five weeks later, the second immunization was carried out by the same procedure.

Challenging infection. A one tenth ml of suspension containing 1×10^4 to 1×10^5 cells of the 116 strain cultured on agar slant at 37°C overnight was intraperitoneally injected for challenging infection 3 weeks after the last immunization.

RESULTS

1. *Virulence of 116 strain and 18M strain.* The virulence of the 116 strain of *Salmonella enteritidis* was examined by intraperitoneal injection to mice. The results are shown in Fig. 1. No mice injected with 6.6×10^3 and more than cells of the 116 strain survived over 10 days. On the other hand, all of the mice injected with under 1.4×10^6 cells of the 18M strain survived over 30 days.

2. *Protective effects of living and killed cells of the 18M strain against infection with the 116 strain.* Mice immunized with living cells of the 18M strain were strongly resistant against

infection with 1×10^4 cells of the 116 strain, but all mice immunized with killed cells died within 10 to 15 days (Fig. 2).

Fig. 1 Pathogenicity of the 18 M strain and the 116 strain of *S. enteritidis* in mice.

Fig. 2 Protective effect of living and killed cells of the 18 M strain against infection with 1×10^4 cells of the 116 strain. Infection was carried out 3 weeks after immunization.

3. *Chemical analysis of phenol extracts.* The results of chemical analysis of fractions extracted with phenol pH 4 and pH 6 are shown in Table 1 and 2, respectively. Each fraction contained 82 to 96 % of RNA, except I-D fraction which contained a lot of DNA. Protein was contained at a concentration 1.4 to 6.6 μg per 100 μg of RNA. The ultraviolet adsorption spectra of these fractions are shown in Fig. 3. The ratio of $\text{OD}_{260}/\text{OD}_{230 \text{ nm}}$ and the ratio of $\text{OD}_{260}/\text{OD}_{280 \text{ nm}}$ are also shown in Table 1. The ratio of fraction I, I-D, III and IV extracted with phenol pH 4 were 2.37, 2.23, 2.12 and 2.19, respectively.

Protective effect of phenol extract. Protective effects of each fraction extracted with phenol pH 4 and pH 6 are examined, and the results are shown in Fig. 4. Each fraction extracted with phenol pH 6 had no preprotective effect against infection with a virulent strain. On the other hand, two fractions (I and IV) extracted with phenol pH 4

protected mice from the death by infection. However, in these cases, the survival rates were 50 % and 30 %.

Fig. 3 Ultraviolet adsorption spectra of each fraction extracted from the 18M strain with phenol pH 4.

TABLE 1
Chemical composition of each fraction extracted with phenol pH 4

Fraction	RNA	DNA	Protein/RNA	$\text{OD}_{260}/\text{OD}_{230}$	$\text{OD}_{260}/\text{OD}_{280}$
I	96.3%	1.6%	3.9%	1.78	2.37
I-D	65.6	48.5	4.1	1.91	2.23
III	92.7	6.0	1.4	2.19	2.12
IV	95.5	3.9	1.9	2.23	2.19

TABLE 2
Chemical composition of each fraction extracted with phenol pH 6

Fraction	RNA	DNA	Protein/RNA
I	82.4%	12.9%	3.5%
I-D	74.0	23.6	3.2
III	96.4	2.0	1.7
IV	86.2	8.1	6.6

Fig. 4 Protective effect of each fraction extracted from 18 M strain with phenol pH 4 and 6 against infection with 1×10^5 cells of the 116 strain. Infection was carried out 3 weeks after last immunization.

DISCUSSION

In the experimental Salmonellosis, it has been well known that killed cells had no protective effect against infection with a virulent strain, though living cells were effective. In the present experiment, it was reconfirmed that these effects were reproducible. However, the true effective factor in living vaccine is not yet known.

Since the reports concerning the immunogenicity of ribosomal fraction in experimental tuberculosis were described by Youmans et al.^{3) 4)}, studies on the protective effects of ribosomal fraction and its components against infection were carried out by several investigators^{5) 13)}. Smith et al.¹⁴⁾ reported that RNA and protein isolated from the cell lysate showed low protection compared with ribosomal fraction against infection with *Salmonella typhimurium*. In our experiment, studies were carried

out on the protective effect of RNA fraction extracted from destructured whole cells of attenuated strain of *S. enteritidis* against infection with a virulent strain. It was shown that two RNA fractions extracted with phenol pH 4 protected mice from the death by infection, though all of RNA fractions extracted with phenol pH 6 had no protective effect. As the fractions used in the present experiment contained small amounts of DNA and protein, besides a large volume of RNA, the problem on the relation of these components in protective effect is remained in further studies.

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REFERENCES

- 1) MITSUHASHI, S., KAWAKAMI, M. and YAMAGUCHI, M. : Studies on the experimental typhoid 1 A comparative study of living and killed vaccine against the infection of mice with *Salmonella enteritidis*. Jap. J. Exp. Med., **28**, 249-258, 1958.
- 2) KAWAKAMI, M., OSAWA, N. and MITSUHASHI, M. : Experimental Salmonellosis VII Comparison of the immunizing effect of live vaccine and materials extracted from *Salmonella enteritidis*. J. Bacteriol., **92**, 1585-1589, 1966.
- 3) YOUMANS, A. S. and YOUMANS, G. P. : Preparation of highly immunogenic ribosomal fraction of *Mycobacterium tuberculosis* by use of sodium dodecyl sulfate. J. Bacteriol., **91**, 2139-2145, 1966.
- 4) YOUMANS, A. S. and YOUMANS, G. P. : Effect of trypsin and ribonuclease on the immunogenic activity of ribosomes and ribonucleic acid from *Mycobacterium tuberculosis*. J. Bacteriol., **91**, 2146-2154, 1966.
- 5) VENNEMAN, M. R. and BIGLEY, N. J. : Isolation and partial characterization of an immunogenic moiety obtained from *Salmonella typhimurium*. J. Bacteriol., **100**, 140-148, 1969.
- 6) SIBATANI, A. and KIMURA, K. : Fractionation of RNA of animal cells with phenol. Protein Nucleic acid and Enzym., **8**, 266-277, 1963.
- 7) INGLE, J., LEY, J. L. and HOLM, R. E. : Demonstration and characterization of DNA-like RNA in excised plant tissue. J. Mol. Biol., **11**, 730-746, 1965.
- 8) GEORGIEV, G. P., MANTIVA, V. L. and ZBALSKY, I. B. : RNA fraction in cell nuclei isolated by phenol and by sucrose-glycophosphate. Biochem. Biophys. Acta., **37**, 373-374, 1960.
- 9) HAGIHARA, Y. and SHIKADA, K. : The base composition of deoxyribonucleic acid isolated from *Mycobacteria*. Kekkaku, **44**, 321-324, 1969.
- 10) DISHE, Z. : The Nucleic Acid, Vol. 1, p. 287, Academic Press N. Y.
- 11) LOWRY, O. H., ROSENBURROUGH, N. J., FARR, A. L. and RUNDALL, R. J. : Protein measurement with the folin phenol reagent. J. Biol. Chem., **193**, 265-275, 1951.
- 12) PLESCIA, O. J., BRAUN, W. and PALCZUK, N. C. : Production of antibodies to denaturated deoxyribonucleic acid (DNA). Proc. N. A. S., **52**, 279-285, 1964.
- 13) JENSEN, R., GREGORY, B., NAYLOR, J. and ACTOR, P. : Isolation of protective somatic antigen from *Vibrio cholerae* (Ogawa) ribosomal preparations. Infection and Immunity, **6**, 156-161, 1972.
- 14) SMITH, R. A. and BIGLEY, N. J. : Ribonucleic acid-protein fraction of virulent *Salmonella typhimurium* as protective immunogens. Infection and Immunity, **6**, 377-383, 1972.