Susceptibility to Active Oxygen Species of Protective and Nonprotective Strains on the Challenge of *Salmonella enteritidis* by Immunization with Culture Filtrate

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1. Summary

Protective ability against the challenge of different strains by immunization with culture filtrate (CF) obtained from *Salmonella enteritidis* was investigated. It was shown that the different strains of *S. enteritidis* can be separated into two distinct groups of protective (2547, 116M, 116—54, SR-98G, and 3775) and nonprotective strains (2822, 3975, and IID-604). Using a cell-free microbicidal system, the susceptibilities of these strains to active oxygen species was evaluated. *S. enteritidis* was found to be susceptible to these active oxygen species, however no differences between the protective and nonprotective strains were observed. Both catalase (H_2O_2 scavenger) and histidine ($^1\text{O}_2$ scavenger) inhibited the bactericidal activity of the xanthine-xanthine oxidase system. Therefore, among the various oxygen intermediates, H_2O_2 and $^1\text{O}_2$ appears to be necessary for killing of *S. enteritidis*. In tests for the ability to trigger an oxidative burst in murine peritoneal macrophages, strain 2547 triggered O_2^- generation at levels as high as those observed with strain 2822. These studies indicate that the difference between the protective and nonprotective strains is not attributed to susceptibility against active oxygen species nor to the ability to trigger an oxidative burst. From these observations, it is suggested that the difference is not due to differences in resistance to the killing of different strains within macrophages.

2. Introduction

Salmonella enteritidis is a facultative intracellular pathogen that causes a systemic infection in mice. Infections caused by this organism appear to correlate with the cellular immune system of the host¹⁾. Living cell vaccines have been shown to induce a cellular immune response, and are thought, by some investigators, to be more effective than non-viable vaccines for the induction of immunity to mouse typhoid infection^{2,3)}. However, other investigators have reported excellent levels of protection with non-viable vaccines^{4,5,6)}. Thus there is no consensus among investigators concerning the vaccine efficacy of protection against *Salmonella* infection.

Previous studies in this laboratory have shown that protection against infection with *S. enteritidis* by immunization with culture filtrate (CF) varies in accordance with the different challenge strains⁷⁾. This result suggests that the differing reports on vaccine efficacy against *Salmonella* infection may be attributable to differences in the challenge strain used.

In this study, nine challenge strains were investigated to determine differences in protection afforded

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against infection with *S. enteritidis* by CF. The challenge strains of *S. enteritidis* could be separated into two distinct groups of protective and nonprotective strains. To investigate differences between these groups, the susceptibilities of these strains to killing by reagent H_2O_2 and by active oxygen species generated by the xanthine-xanthine oxidase system was evaluated. Further comparisons were made concerning the abilities of these strains to trigger the generation of superoxide anions in vitro by macrophages.

3. Materials and Methods

3.1. Bacterial strains

The microbial strains used in this study and their sources were as follows: *Salmonella enteritidis* strains 2547, 2822, 3975 and 3775 (Niigata Prefectural Institute of Public Health, Japan), 116M (Mie Prefectural Institute of Public Health, Japan), SR-98G (Gifu Prefectural Institute of Public Health, Japan), 116-54 (Dr. S. Mitsuhashi, Gunma University, Maebashi, Japan). IID 604 (Dr. T. Watanabe, Institute of Medical Science, University of Tokyo, Tokyo, Japan), ATCC 13076 (Dr. E. Yabuuchi, Gifu University, Gifu, Japan). *Escherichia coli* strain ATCC 25922 (Dr. K. Tomono, Nagasaki University, Nagasaki, Japan) was used in control experiments. Bacteria were cultured in liver and beef papain digestion agar medium at 37°C for 18 hr, and adjusted to working concentrations with a spectrophotometer (590 nm).

3.2. Mice

Female mice of ddy strain were purchased from the Shizuoka Laboratory Animal Center, Hamamatsu, Japan. Mice were used at the age of 7-8 weeks.

3.3. Reagents

Xanthine (grade III), xanthine oxidase (grade III), superoxide dismutase (SOD) (type 1), catalase (from bovine liver), ferricytochrome *c* (type VI, from horse heart) and gentamicin were purchased from Sigma Chemical Co., St. Louis, Mo. Histidine was from Wako pure Chemical Industries Ltd., Japan. RPMI 1640 medium and fetal calf serum (FCS) were from GIBCO Laboratories, Grand Island, N.Y. and Flow Laboratories, Inc. Rockville, N.Y., respectively. Other chemicals used were of analytical grade from commercial sources.

3.4. Preparation of CF

Culture filtrate (CF) was prepared as described previously⁷⁾. Bacterial strains were inoculated on a synthetic TSA medium without protein, cultured at 37°C for 4 days, and the culture medium was filter-sterilized using a Seiz EK filter.

3.5. Determination of protection

Groups of five mice were immunized intraperitoneally (i.p.) with 0.5 ml of CF obtained from each strain, and challenged i.p. with various doses of each strain in 0.5 ml of 5% (W/V) mucin 14 days following immunization. The protective ability was demonstrated by the mathematical difference observed between the LD_{50} of control mice and the LD_{50} of immunized mice.

3.6. Microbicidal assays

In the microbicidal assay of the xanthine-xanthine oxidase system, 1×10^6 bacteria were suspended in 1 ml of Phosphate buffered saline (PBS) containing 0.5 mM xanthine, 0.1 mM $FeSO_4$, 0.1 mM EDTA, and various concentrations of xanthine oxidase. After incubation for 1 hr at 37°C in a shaking water bath, samples were serially diluted 10-fold with physiological saline, and spread on nutrient agar plates in duplicate. Plates were incubated for 24 hr at 37°C, and colonies were counted. Bactericidal activity of reagent H_2O_2 was assessed similarly. Bacteria suspended in 0.1 M sodium phosphate buffer (pH 7.0) containing various concentration of H_2O_2 were incubated for 1 hr at 37°C. The number of colonies was determined as described above.

3.7. Preparation of peritoneal macrophage monolayer

Peritoneal cells were collected by washing the peritoneal cavity of non-treated mice with RPMI 1640 medium containing 5 µg/ml of gentamicin (RPMI 1640-GM). The cells were washed three times and suspended in RPMI 1640-GM medium supplemented with 10% heat-inactivated FCS. The number of viable cells was determined by the trypan blue dye exclusion method using a hemacytometer. Viability was always >95%. Differential cell counts were made with Giemsa-stained smears under light microscopy. The suspension was found to contain at least 40 to 45% macrophages on the basis of morphology. A total of 1×10^6 macrophages were plated in each well of a 24-well tissue culture plate (Falcon no. 3047; Becton Dickinson Labware, Oxnard, Calif.) and incubated for 24 hr at 37°C in 5% CO₂-95% air (CO₂ incubator). Following incubation, macrophage monolayers were washed three times with warmed RPMI 1640 medium to remove nonadherent cells.

3.8. Assay for superoxide anion (O₂⁻) generation

O₂⁻ released from macrophages was measured using the ferricytochrome *c* reduction method⁸⁾. Briefly, 2×10^7 bacteria were suspended in 0.9 ml of Krebs-Ringer phosphate buffer (137 mM NaCl, 4.9 mM KCl, 1.2 mM MgSO₄, 0.5 mM CaCl₂, 5.7 mM sodium phosphate buffer, 5.5 mM glucose, pH 7.35) containing 10% fresh mouse serum with or without SOD (30 µg/ml). After opsonization for 10 min at 37°C, 0.1 ml of 1 mM ferricytochrome *c* was added, and the bacterial suspensions were placed onto the macrophage monolayers. The plates were incubated for 1 hr at 37°C in a CO₂ incubator. The assay was stopped by centrifugation at $1,500 \times g$ for 10 min at 4°C, and the absorbance of each supernatant was determined at 550 nm in a Hitachi model 220A spectrophotometer (molar absorption coefficient, $21.0 \times 10^3 \text{ cm}^{-1}$). Superoxide anion generation (nanomoles per 1×10^6 cells) was measured by the following formula: O₂⁻ generation = $\Delta\text{OD}/21.0 \times 10^3$, where OD is optical density.

4. Results

4.1. Protection

Mice were immunized with CF obtained from each strain of *S. enteritidis* and examined for the protective ability against infection afforded by different strains. As shown in Table 1, protective effects by CF were observed in the groups of mice challenged with strains 2547, 116M, 116-54, SR-98G, and 3775

Table 1 Protective ability against the challenge of different strains by immunization with culture filtrate (CF)

Challenge strains	Protective ability ^{a)}
2547	>2.8
116M	>2.2
116-54	1.5
SR-98G	1.3
3775	1.0
2822	0.4
3975	0.4
IID-604	0.3
ATCC-13076	N.D. ^{b)}

a) Mice were immunized i.p. with CF obtained from each strain, and challenged i.p. with various doses of different strains 14 days after immunization. Protective ability was demonstrated by differences between the LD₅₀ of control mice and the LD₅₀ of immunized mice. (LD₅₀ of immunized mice...10¹¹ mg, LD₅₀ of control mice...10¹²mg, Protective ability = n1-n2)

b) Not determined

Fig. 1 Susceptibility of *S. enteritidis* and *E. coli* to xanthine-xanthine oxidase system. Bacteria (1×10^6) were incubated at 37°C for 60 min in 50 mM sodium phosphate buffer, pH 7.5 with 0.1 mM Xanthine, 0.1 mM FeSO_4 , 0.1 mM EDTA, and xanthine oxidase (XO) at the indicated concentrations in a total volume of 1 ml. * $p < 0.01$; significantly different from value for *S. enteritidis* strain 2822. Each point represents the mean $\pm$ S.E. of five experiments.

Fig. 2 Susceptibility of different *S. enteritidis* strains to the xanthine-xanthine oxidase system. The reaction mixture was as described in Fig. 1, except that xanthine oxidase was added at a concentration of 0.1 units/ml. Each column represents the mean $\pm$ S.E. of four experiments.

(Protective strains). On the other hand, when strains 2822, 3975, and IID-604 were used as the challenge strain, no protective effects were observed (Nonprotective strains). Similar results were obtained using formalin-killed cells obtained from *S. enteritidis* as a vaccine instead of CF (data not shown).

4.2. Susceptibility of different strains to the xanthine-xanthine oxidase system.

The susceptibilities of *S. enteritidis* strains 2547, 2822, and *E. coli* to the xanthine-xanthine oxidase system were investigated (Fig. 1). The killing of *S. enteritidis* reached a maximum at the concentration of 0.1 units/ml of xanthine oxidase. Strain 2822 was found to be more susceptible than strain 2547, while *E. coli* proved most resistant to this system. In the absence of xanthine or xanthine oxidase, no bacteria killing occurred (data not shown). Figure 2 compares the susceptibility of different strains to the xanthine-xanthine oxidase (0.1 units/ml) system. No difference between protective and nonprotective strains in susceptibility to this system could be detected.

4.3. Effects of oxygen intermediate scavengers on microbicidal activity of the xanthine-xanthine oxidase system.

Table 2 shows the effects of various oxygen intermediate scavengers on the killing of *S. enteritidis* strains 2547, 2822, ATCC-13076, and *E. coli* using the xanthine-xanthine oxidase system. Bactericidal activity of the xanthine-xanthine oxidase system against these bacteria was inhibited by the addition of catalase (H_2O_2 scavenger), whereas SOD (O_2^- scavenger) was not effective in inhibiting the killing. Histidine ($^1\text{O}_2$ scavenger) and Mannitol ($\cdot\text{OH}$ scavenger) resulted in a lower yet significant inhibition for the killing of strains 2822, ATCC-13076, and *E. coli*, respectively. These results indicate that H_2O_2 and $^1\text{O}_2$ play important roles in the expression of microbicidal activity against the *S. enteritidis* tested.

4.4. Susceptibility of different strains to H_2O_2

The susceptibilities of *S. enteritidis* strains 2547, 2822, and *E. coli* to varying concentrations of H_2O_2 were investigated (Fig. 3). The three organisms were killed in a concentration-dependent manner, with

Table 2 Effects of oxygen intermediate scavengers on the bactericidal activity of the xanthine-xanthine oxidase system

Scavengers	Viability (%)			
	2547	2822	ATCC-13076	<i>E. coli</i>
None	60.4±4.7	50.5±5.2	2.3±0.8	57.0±0.6
SOD (100 µg/ml)	52.0±7.1	18.8±0.6	0.5±0.3	43.9±3.0
Catalase (100 µg/ml)	89.8±5.3*	109.7±4.3***	103.6±3.5***	91.1±3.1***
Mannitol (10 mM)	63.6±5.3	61.1±3.3	4.3±1.8	69.5±2.4**
Histidine (10 mM)	75.6±9.9	88.2±4.1**	18.6±3.6*	55.0±8.2

The reaction mixture was as described in Fig. 2, except that the various oxygen intermediate scavengers were added at the indicated concentrations. The activities of SOD and catalase were 300 and 14,100 units/mg protein, respectively. Values represent the mean ± S.E. of four experiments. Asterisks indicate significant differences from the system without scavengers (None) at $p < 0.05$ (*), $p < 0.01$ (**) or $p < 0.001$ (***).

Fig. 3 Susceptibility of *S. enteritidis* and *E. coli* to H₂O₂. Bacteria (1×10^6) were incubated at 37°C for 60 min in 0.1 M sodium phosphate buffer, pH 7.0 with H₂O₂ at the indicated concentrations in a total volume of 10 ml. * $p < 0.05$; significantly different from *S. enteritidis* strain 2822. Each point represents the mean ± S.E. of five experiments.

Fig. 4 Susceptibility of different *S. enteritidis* strains to H₂O₂. The reaction mixture was as described in Fig. 3, except that H₂O₂ was added at a concentration of 1 mM. Each column represents the mean ± S.E. of four experiments.

Table 3 Superoxide production of macrophages by phagocytosis of *S. enteritidis* or *E. coli*

Bacteria	O ₂ ⁻ Production (nmoles/10 ⁶ cells)
2547	4.59±0.35
116M	2.22±0.24
2822	4.28±0.21
<i>E. coli</i>	4.38±0.44

Macrophages (1×10^6 cells) were incubated with bacteria (2×10^7 CFU) in the presence of 10% normal mouse serum at 37°C for 1 hr. The results are expressed as the mean ± S.D. of four experiments.

strain 2822 showing more susceptibility to H_2O_2 than strain 2547. *E. coli* was most sensitive to H_2O_2 and was almost destroyed at concentrations of 1 mM. Figure 4 compares the susceptibility of the different strains to H_2O_2 at 1 mM concentration. As is the case with the xanthine-xanthine oxidase system, no difference between the protective and nonprotective strains in susceptibility to H_2O_2 was observed.

4.5. Superoxide production of macrophages by phagocytosis of different strains

Table 3 shows the abilities of *S. enteritidis* strains 2547, 116M, 2822, and *E. coli* to trigger the generation of O_2^- in vitro by macrophages. Although the O_2^- generated by the triggering of strain 116M to macrophages was small, the degree of O_2^- generation by strains 2547 and 2822 was similar to that seen in *E. coli*. From these results, no differences in the ability to trigger an oxidative burst in macrophages were observed between the protective and nonprotective strains.

5. Discussion

Although the importance of cell-mediated immunity induced by live vaccines in controlling *Salmonella* infections is well documented, there has been the viewpoint that killed vaccines induce humoral immunity and can provide a high level of protection against *Salmonella* infection^{9,10}. Eisenstein *et al*¹¹ reported that these discrepancies in vaccine efficacy may be attributable to differences in the mouse strains used in the experiments. In a previous paper, this laboratory demonstrated that protection against infection with *S. enteritidis* differs in accordance with the different challenge strains⁷. This result indicates that the selection of the challenge strain is significant in the study of protection against *Salmonella* infection. In this study, the protective ability against challenge from different strains by immunization with CF obtained from *S. enteritidis* was investigated. The different challenge strains of *S. enteritidis* could be separated into distinct groups of protective (2547, 116M, 116—54, SR-98G, and 3775) and nonprotective strains (2822, 3975, and IID-604). By sodiumdodecyl sulfate-polyacrylamide gel electrophoresis of lipopolysaccharides (LPS), it was shown that all LPS obtained from these strains are typically smooth-type LPS (data not shown).

The importance of macrophages in Salmonellosis has been suggested by a number of investigators^{12,13,14}. *S. enteritidis*, ingested by murine macrophages, can usually survive and resist the protection of infected hosts^{15,16}. Therefore, the resistance to killing of *S. enteritidis* within macrophages is considered an important factor in the protective immunity against infection caused by this organism. In general, mechanisms for survival within macrophages of facultative intracellular parasites are thought to include resistance or inactivation of active oxygen species, lysosomal enzymes, and cationic peptides^{17,18,19}; inhibition of the respiratory burst; inhibition of phagosome-lysosome fusion and escape from the phagosome into the cytoplasm²⁰. Active oxygen species such as the superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), hydroxy radical ($\cdot OH$), and singlet oxygen (1O_2) generated by macrophages and polymorphonuclear leucocytes exhibit a potent microbicidal activity²¹. The xanthine-xanthine oxidase system can produce active oxygen species in vitro and has been employed as a model for the oxygen-dependent microbicidal system of these phagocytic cells. This system exhibited a potent microbicidal effect on the various strains tested in the present study. Bactericidal activity of this system against *S. enteritidis* was inhibited by the addition of catalase (H_2O_2 scavenger) and histidine (1O_2 scavenger), indicating that among the oxygen intermediates, H_2O_2 and 1O_2 play an important role in the expression of microbicidal activity of *S. enteritidis*. Additionally, susceptibility differences of the protective and nonprotective strains to H_2O_2 were investigated. As was the case with the xanthine-xanthine oxidase system, the rate of susceptibility of these strains to H_2O_2 varied. These results show that the difference between the two distinct groups can not be attributed to susceptibility against active oxygen species.

There are reports that *Mycobacterium leprae* and *Toxoplasma gondii* fail to trigger the respiratory burst

of macrophages, and, consequently, readily multiply within the cells^{22,23}. The ability of *S. enteritidis* strain 2547 to trigger the generation of O₂ by macrophages was very similar to that observed in strain 2822. From these results, it was concluded that the difference between the two distinct groups is not correlated with the ability to trigger an oxidative burst.

Some facultative intracellular parasites are known to inhibit phagolysosomal fusion of macrophages after phagocytosis^{24,25}. Preliminary experiments revealed that although *S. enteritidis* was more effective inhibiting phagolysosomal fusion than *E. coli*, the rate of inhibition of phagolysosomal fusion of the protective and nonprotective strains varied (unpublished data). The conclusion obtained from this study is that *S. enteritidis* is susceptible to active oxygen species generated in vitro or to reagent H₂O₂. However, differences in protection observed between protective and nonprotective strains can not be attributed to the susceptibility against these active oxygen species nor to the ability to trigger an oxidative burst. These results suggest that the difference in protection is not due to differences in resistance to the killing of different strains within macrophages.

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実験的サルモネラ症の培養濾液での感染防御成立における

有効株，無効株の各種活性酸素に対する感受性

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Salmonella enteritidis から得られた培養濾液での免疫による異なる菌株の攻撃に対する防御能力を調べた結果、攻撃菌株は有効株である 2547 株、116M 株、116-54 株、SR-98G 株、3775 株と無効株である 2822 株、3975 株、IID-604 株に分けられた。In vitro において、これらの菌株は各種活性酸素に対して感受性を示したが、有効株と無効株との間に違いは見られなかった。活性酸素の阻害剤である catalase と histidine は、このような殺菌系に対して阻害効果を示した事より、 H_2O_2 と 1O_2 が *S. enteritidis* の殺菌に対して重要であると考えら

れた。マウス腹腔マクロファージが有効株である 2547 株を貪食した時に起こる活性酸素放出は、無効株である 2822 株を貪食した時に起こるそれと同程度であった。これらの結果より、有効株と無効株の違いは各種活性酸素に対する感受性ばかりでなく、マクロファージからの活性酸素放出に影響を及ぼす能力にも起因しないという事が判った。このような知見から、この感染防御に見られる違いはマクロファージ内でこれらの菌株の殺菌に対する抵抗性による違いに起因するものではないという事が示唆された。