Cellular Immunity with Special Reference to Monocytes of Mice Immunized with Live Vaccine of *Salmonella Enteritidis**

Susumu Mitsuhashi, Nobutaka Osawa, Kazuko Saito
and Satonori Kurashige

*Department of Microbiology (Prof. S. Mitsuhashi),
School of Medicine, Gunma University, Maebashi*

The mice which had been immunized with highly virulent live *S. enteritidis* acquired a strong immunity against the bacilli and could perfectly survive a challenge with intravenous injection of a highly virulent strain *S. enteritidis* 116-54 in a dose of $1,000 \times \text{MLD}$. The sera of the mice contained O and H antibodies but no demonstrable factor to prevent death due to infection. However, large mononuclear cells from liver, subcutaneous tissue and peritoneal cavity of the immunized mice exhibited an immune activity. The cells could suppress intracellular proliferation of phagocytized highly virulent bacilli and finally digested them without addition of exogenous immune sera. The activity was regarded as an expression of cellular immunity. It could be established only after contact with live bacilli.

The supernatant fluid of the culture of the immunized cells or the cellular fraction which was sedimented at 100,000 g contained a transfer agent which could transfer cellular immunity. The transfer agent was inactivated by RNase but resisted DNase or trypsin treatment. An antibody reacting to live *S. enteritidis* 116-54 was demonstrated in the immunized cells, even after absorption with formalin treated bacilli which contained O and H antigens. This antibody, defined as the cellular antibody, could suppress the growth of *S. enteritidis* 116-54 *in vitro* in collaboration with complement and lysozyme.

In experimental salmonellosis of mice with *Salmonella enteritidis*, an administration of killed vaccines increased the survival time of mice after challenge, but it was almost ineffective in preventing death from the infection, even after the administration of an amount of bacterial cells 10^3 to 10^4 times larger than in the case of live vaccine (Kobayashi and Ushiba,¹ 1952; Mitsuhashi *et al.*,² 1958; Mitsuhashi,³ 1959).

In contrast to that, the mice hyperimmunized with live vaccine of *S. enteritidis* survived intravenous infection with 10^{-5} mg (corresponding to 1,000 mouse MLD) of a virulent strain of the same microorganism (Mitsuhashi,³ 1959).

Received for publication, February 9, 1966.

* Delivered at the 5th General Meeting of the Japan Society of the Reticuloendothelial System, April 12, 1965, Fukuoka.

In the previous papers from this laboratory, it was demonstrated that the monocytes, obtained from the abdominal cavity, liver, or subcutaneous tissue of the mouse superimmunized with live vaccine of *S. enteritidis*, inhibited intracellular multiplication of a virulent strain of the same organism, in the absence of immune serum in the cell culture medium, whereas the cell of the mouse immunized with dead vaccine did not. This resistance of immune monocytes was referred to as cellular immunity (Mitsuhashi *et al.*,⁴ 1961; Sato *et al.*,⁵ 1962). The cellular immunity was found in immunized mice for the whole life after the last injection of live vaccine (Tanaka *et al.*,⁶ 1962).

For the purpose of investigation of the immune mechanism with live vaccine, the following experiments were conducted. In the present article, the outline of their results is presented.

METHODS AND RESULTS

1. Postinfective immunity

Mice were inoculated intravenously with 10^{-5} mg of virulent *S. enteritidis* 116-54 and administered subcutaneously 0.1 ml of Kanamycin (50 mg/ml) 1/2, 1, 2, 4, 7, 10, 14 and 18 days after the infection. At appropriate time intervals after infection, the number of bacteria in the liver and spleen was determined. Quantitative experiments showed that the number of microorganisms grown in the liver and spleen reached its maximum 1 week after the infection and decreased gradually thereafter. These results are shown in Fig. 1. Without Kanamycin treatment, all mice died within 1 week after the infection with 10^{-5} mg of virulent *S. enteritidis* 116-54, and the number of the bacteria in the liver reached about 10^8 per g of the organ. In contrast to that, the mice survived, at a rate of 85 to 94%, the infection with the same amount of *S. enteritidis* 116-54 when treated with Kanamycin. The mice which had once survived the infection of *S. enteritidis* by treatment with Kanamycin, acquired a high resistance against further infection, and 96 to 100% of mice tolerated a challenge with 10^{-5} mg of virulent *S. enteritidis* SMR 49. However, it was unsuccessful to confer resistance when a larger amount of Kanamycin was administered because it caused a rapid decrease in the bacterial cell number in the organs of infected mice.

2. Diffusion chamber experiment

A diffusion chamber⁷ containing 0.5 ml of bacterial suspension (3.0×10^4 organisms/ml) was inserted into the mouse abdominal cavity, and the number of microorganisms in the chamber was counted. The number of bacteria reached the maximum 1 day after the insertion of the diffusion chamber giving an about 10 times as large count as that in nutrient broth culture. Even after 53 weeks the number of bacteria was almost the same as that 1 day after the insertion. The mice, inserted with diffusion chambers containing a large number of virulent

Fig. 1. Number of microorganisms in the mice infected with *S. enteritidis* and treated with Kanamycin. Five mg/day of Kanamycin were administered subcutaneously to each mouse, as indicated by the arrows in the figure. —•—, number of microorganisms in the liver; o---o, number of microorganisms in the spleen. Each value is the mean of 5 determinations.

TABLE 1. Incapability of conferring immunity by diffusion chamber containing live *S. enteritidis*

Microorganism contained in diffusion chamber	Time interval between insertion of diffusion chamber and challenge (day)	Mean survival time of mice (day)	Survival rate after challenge (%)
SMR 49	7	7	0 (0/ 7)
SMR 49	14	11.5	0 (0/ 8)
SMR 49	28	7.7	0 (0/10)
SMR 49	35	8.4	0 (0/12)
116-54	56	9.3	0 (0/ 6)
Control ^{a)}	28	8.5	0 (0/10)
Control ^{b)}			100 (10/10)

Each mouse was inserted with a diffusion chamber containing live microorganisms (1.5×10^4) or physiological saline.

a) Control mice inserted with diffusion chambers containing physiological saline.

b) Control mice inserted with diffusion chambers containing physiological saline and superimmunized with live vaccine 30 days after insertion.

S. enteritidis could not acquire resistance against the infection with the same organism (Table 1), while their serum had agglutinin against *S. enteritidis*. The fluid in the diffusion chamber which was inserted into the mice immunized with killed vaccine of *S. enteritidis* contained agglutinin against the same organism.

From these facts, it is strongly suggested that immunization with live vaccine or convalescent immunity will be achieved by unobstructed contact of host cells, probably mononuclear phagocytes, with living bacterial cells; namely by establishing a host-parasite relationship, in which bacterial cells were phagocytized by mononuclear cells.

3. *In vitro* transfer of cellular immunity

Supernatant fluid of cell culture was prepared by cultivation of monocytes obtained from normal or immunized mice. As shown in Fig. 2, the supernatant fluid of cell culture obtained from the mice immunized with live vaccine of *S. enteritidis* was able to transfer cellular immunity to non-immune monocytes maintained *in vitro* and the treated monocytes inhibited intracellular multiplication of the virulent strain of *S. enteritidis* 116-54 and were free from degeneration which would otherwise be caused by phagocytized bacteria. In contrast, normal

Fig. 2. Transfer of cellular immunity by TA and cellular resistance against infection.
 —●—, SFC (supernatant fluid of cell culture) obtained from normal mouse monocytes;
 —▲—, SFC obtained from the monocytes of mice immunized with killed vaccine;
 —○—, SFC obtained from the monocytes of mice immunized with live vaccine.
 Each value indicates the mean in three experiments.

monocytes or monocytes obtained from the mice immunized with killed vaccine were unable to produce transfer agent of cellular immunity.

Hereafter, the agent which is able to confer cellular immunity on non-immune monocytes is referred to as transfer agent (TA) of cellular immunity. Enzymatic treatment was employed to determine the properties of TA. RNase or DNase was added to TA and incubated at 37°C. After overnight incubation, the transfer agents treated with the enzymes were added to the culture medium of non-immune monocytes and their transfer potency of cellular immunity was investigated. When the monocytes were incubated in RNase treated TA, the number of surviving monocytes decreased rapidly after infection, whereas both untreated and DNase treated TAs were able to confer cellular immunity on non-immune monocytes and the cells resisted the injury after phagocytosis. The ribosomal fraction was found to be obtainable from the monocytes of mice immunized with live vaccine, and could transfer cellular immunity from immune to non-immune monocytes. Ribosomal fraction was obtained by the method of Takanami,⁸ and the normal monocytes were incubated for 72 hours with immune ribosomal fraction. It was found that the monocytes acquired cellular immunity and inhibited intracellular multiplication of *S. enteritidis*. In contrast, the monocytes incubated with ribosomal fraction of normal monocytes or the monocytes of mice immunized with dead vaccine, were unable to inhibit intracellular multiplication of *S. enteritidis*. After treatment with RNase, the transfer activity of cellular immunity in immune ribosomal fraction was lost, as well as the TA in supernatant fluid of cell cultures.

4. Cell-bound antibody

Employing the method of immune transfer and immune adherence hemagglutination^{9,10} the cell-bound antibody was detected in the monocytes of mice immunized with live vaccine of *S. enteritidis*. As is shown in Table 2, the maximum reactivity with the monocytes of mice superimmunized with live vaccine was attained by employing 1.5×10^7 bacteria. This was followed by the reactivity of monocytes of mice immunized with formalin vaccine.

The antibodies present in monocytes were absorbed with formalinized bacteria. Thereafter, the monocytes were used for immune transfer and immune adherence hemagglutination tests.

After the absorption of monocytes with formalinized bacteria, the monocytes of mice superimmunized with live vaccine still showed a maximum reactivity, when 3×10^7 live organisms of *S. enteritidis* were employed, whereas the reactivity of the cells from mice immunized with formalin vaccine turned negative against live organisms of *S. enteritidis*. The results are shown in Table 2.

When a series of dilution of monocytes (Ab) was used, the maximum reactivity with the monocytes of mice superimmunized with live vaccine and

TABLE 2. *Study on*

Monocytes obtained from the mice	Absorption with formalinized <i>S. enteritidis</i>	Antigen for hemagglutination
Hyperimmunized with live vaccine Immunized with formalin vaccine	Before absorption	Formalinized <i>S. enteritidis</i> 116-54
Hyperimmunized with live vaccine Immunized with formalin vaccine	After absorption	Live <i>S. enteritidis</i> 116-54

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Monocytes obtained from the mice	Absorption with formalinized <i>S. enteritidis</i>	Antigen for hemagglutination
Hyperimmunized with live vaccine Immunized with formalin vaccine	Before absorption	Formalinized <i>S. enteritidis</i> 116-54
Hyperimmunized with live vaccine Immunized with formalin vaccine	After absorption	Live <i>S. enteritidis</i> 116-54

immunized with formalin vaccine was attained by employing 1×10^7 and 2×10^7 monocytes, respectively. After the absorption of monocytes with formalinized bacteria, the monocytes of mice superimmunized with live vaccine still showed a positive reaction against live organisms of *S. enteritidis*, whereas the cells of mice immunized with formalin vaccine did not (Table 3).

These results indicate that the monocytes of mice superimmunized with live vaccine of *S. enteritidis* contained antibody other than those reacting with formalinized bacteria, *i.e.*, O and H antigens.

cell-bound antibody

Number of <i>S. enteritidis</i> antigen particles per reaction mixture ($\times 10^7$)							
24	12	6	3	1.5	0.75	0.38	None
+	+	+	+	+	—	—	—
+	+	+	—	—	—	—	—
+	+	+	+	—	—	—	—
		—					

cell-bound antibody

Number of monocytes per reaction mixture ($\times 10^7$)							
8	4	2	1	0.5	0.25	0.125	None
+	+	+	+	—	—	—	—
+	+	+	—	—	—	—	—
+	+	+	—	—	—	—	—

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