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Experimental Salmonellosis

VIII. Postinfective Immunity and Its Significance for Conferring Cellular Immunity

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In the process of live-vaccine immunization of *Salmonella enteritidis* infection in mice, the relation between the number of bacteria in the organs of mice and their protecting effect was studied. Treatment with antibiotics was used to control the number of immunizing bacteria in the tissues. Mice, which were infected with 10^{-5} mg (1,000 mouse MLD) of virulent *S. enteritidis* and treated with kanamycin simultaneously, acquired high antilethal resistance against infection with the same organisms. However, the administration of large amounts of kanamycin, which caused a rapid decrease in bacterial numbers in the organs of infected mice, was incapable of conferring immunity. This indicated the necessity of persistence of live bacteria in the host for the production of immunity. A large number of microorganisms were maintained for 53 weeks in a diffusion chamber inserted into the mouse abdominal cavity. The mice implanted with diffusion chambers containing large numbers of virulent *S. enteritidis* did not acquire antilethal resistance against infection with the same organisms, although agglutinins against *S. enteritidis* were observed in these mice. Agglutinin was also found in the fluid contained in diffusion chambers inserted into mice immunized with a killed vaccine of *S. enteritidis*. This indicated that antibody penetrated the membrane filter of diffusion chambers from outside to inside and vice versa. From these results, it is suggested that contact of live microorganisms with the host cell is necessary for conferring postinfective immunity in salmonellosis.

In previous papers (17, 22), it was reported that mice immunized with a live vaccine of *Salmonella enteritidis* acquire high antilethal resistance against reinfection with the same organisms. By contrast, killed vaccines have been largely ineffective in protection from infection, in spite of the administration of 10^3 to 10^4 times as many bacterial cells and of the presence of high titers of agglutinins against a virulent strain of *S. enteritidis* (14, 17, 31). Some evidence has been presented to answer the question of how animals immunized by living vaccines acquire the antilethal resistance. The attenuated microorganisms inoculated into mice as a living vaccine become located mainly in the cells of spleen, liver, and lymph nodes and persist for a long time. On the basis of some of the characteristics of the bacteria in these organs, such as smaller size and altered sensitivity to antibiotics, it has been suggested that they differ from those cultured in vitro (21). It was also found that the mononuclear phagocytes of mice immunized with a live vaccine acquired cellular immunity and inhibited intracellular growth of bacteria in the absence of immune sera in the culture medium (23, 28).

In a previous paper (13), it was postulated that a plausible explanation of the effectiveness of living vaccine and the lack of effect with killed vaccines was that only bacteria in the body were able to produce an effective substance for immunization as a result of host-parasite interaction in the infected animals.

More precise knowledge is desirable concerning the influence of the duration, as well as location, of bacterial infection in the host on the acquisition of immunity produced by infection with living bacteria.

To elucidate the mechanism of the immunity conferred by a live vaccine, diffusion chambers were employed. This paper describes the results of certain immune effects of a virulent strain of *S. enteritidis*, and a lack of effect from living microorganisms without contact with host cells.

An outline of this study was presented at the 17th Kanto Branch Meeting of the Japan Bacteriological Association, Tokyo, November 1962. Some of the diffusion-chamber experiments were also presented independently by Akiyama et al. at the same meeting.

MATERIALS AND METHODS

Experimental animals. Seven-week-old mice of strain ddN (raised by the Central Animal Laboratory, Gunma University), weighing 20 to 22 g each, were used throughout.

Microorganisms. A virulent strain of *S. enteritidis*, 116-54, was used for immunization and challenge. An attenuated strain of *S. enteritidis*, SER, was used for immunization. The virulence and antigenic structure of these strains were described previously (19). The mouse LD_{50}, tested by intravenous infection, of the virulent strain was $10^{-8.2}$ mg and that of the attenuated strain was 10^{-2} mg. *S. enteritidis* SMR49, which was resistant to 1.0 mg of streptomycin per ml, was used in the diffusion-chamber experiments and for challenge. The virulence of the latter strain was the same as that of its parent strain, 116-54.

Viable counts were obtained by plating. Estimates of the total number of bacteria were made photometrically by reference to a standard curve relating optical density at 530 mμ to number of microorganisms.

Immunization. All killed vaccines, namely, Formalin vaccine, heat-killed vaccine, and chrome alum vaccine (9), were prepared and used for immunization of mice by methods previously reported (17).

Two different procedures were used for immunization with living bacteria. One group of mice was inoculated intravenously with living bacteria corresponding to 10^{-5} mg (dry weight) of the attenuated strain, SER, and, 21 days later, with 10^{-7} mg of the living virulent strain, 116-54. These amounts correspond to 3×10^4 and 3×10^2 viable cells, respectively. Immunized mice were used 21 days after the last injection. Mice of another group were given 5 mg of kanamycin subcutaneously 24 hr prior to intravenous infection with 3×10^4 viable virulent bacteria (strain 116-54). They were treated by subcutaneous injection with 5 mg of kanamycin 1, 2, 4, 7, 10, 14, and 18 days after infection, and were used as immunized mice 24 days after the last injection of kanamycin.

Serological methods. Serum was obtained by heart puncture. The sera from at least five mice were pooled and were inactivated by heating at 56 C for 30 min. The O agglutinin titer was assayed by use of heat-killed cells of strain 116-54 as antigen. Freshly grown cells of strain 116-54 were suspended in 0.85% NaCl. After heating at 100 C for 30 min and washing by centrifugation, the cells were resuspended in saline to a final concentration of 0.2 mg (dry weight) per ml. For the agglutination assays, each 0.5 ml of this suspension was mixed with 0.5 ml of serial twofold dilutions of sera, and the mixture was first incubated at 37 C for 2 hr and then allowed to stand at 4 C overnight. Agglutination was observed in the agglutinoscope. The H agglutinin titer was determined with Formalin-killed cells. To an overnight culture of strain 116-54 in nutrient broth, Formalin was added to a concentration of 0.6%. After incubation of Formalin-treated cells at 37 C for 2 hr, the cells were used for assay as antigen. Mixtures of antigen and diluted serum were incubated at 52 C for 2 hr. The recorded titer was the highest dilution which gave a positive agglutination reaction.

Antibody titer was also determined by the immune adherence hemagglutination (IAHA) method of R. A. Nelson, Jr., and H. C. Woodworth (Walter Reed Institute of Medical Research, Washington, D.C.), as modified by Nishioka (24). In this test, agglutination of human erythrocytes results from the formation of an antigen-antibody-complement complex. Freshly prepared Veronal buffer (*p*H 7.5) containing 0.038 M sucrose, 0.0005 M $MgCl_2$, 0.00015 M $CaCl_2$, and 0.09 M NaCl was used for the diluent of serum and bacterial suspension. Veronal buffer (*p*H 7.5) containing 0.01% gelatin, 0.01 M disodium-ethylenediaminetetraacetate (EDTA), and 0.15 M NaCl was used for the suspension of human erythrocytes.

Fresh human erythrocytes, type O, obtained from a healthy donor, were used as an indicator for IAHA (24; Nelson and Woodworth). Fresh human serum freed from antibacterial antibody by absorption with *S. enteritidis* was used as a complement source.

The mixtures containing 0.4 ml of a serial twofold dilution of serum, 0.4 ml of bacterial suspension (10^8 cells per milliliter), and 0.2 ml of 30 times-diluted human complement were incubated at 37 C for 30 min with shaking. Thereafter, 0.1 ml of human erythrocytes was added to the mixture and incubated for an additional 10 min with shaking. Finally, the mixture was kept stationary for 60 min at 37 C without shaking, and the shape of the erythrocyte deposit which settled on the bottom of test tubes was recorded. Degree of agglutination was expressed as 1 to 4 according to the pattern of the erythrocyte deposit. The titer was recorded as the dilution which gave a second-degree reaction. The titer of antiserum was recorded as the maximal dilution of antiserum giving a positive IAHA reaction.

Diffusion chamber. The two types of diffusion chamber were modifications of that described by Algire (4). They were modified according to Osawa et al. (27) and are shown in Fig. 1. Type I was used for immunization of mice, and type II, for determination of bacterial count and titration of antibody in the chamber. Both chambers were made of heat-resistant rubber rings affixed to membrane filters (type VF, Millipore Corp., Bedford, Mass.). The adhesive used was a mixture of Cemedine no. 700 (Cemedine Co., Tokyo, Japan) and Desmodur R (Farbenfabriken Bayer A. G., Leverkusen, Germany). Rubber rings and filter discs were sterilized by autoclaving at 121 C for 15 min and ultraviolet irradiation, respectively. A small rubber block was attached to the ring of the type II chamber with the same adhesive. The bacterial suspension was introduced into this chamber by use

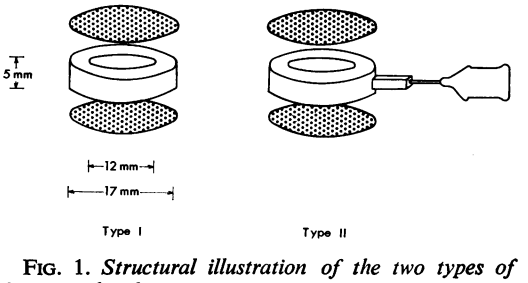

FIG. 1. *Structural illustration of the two types of diffusion chamber.*

of a needle inserted through the rubber block. After the needle was withdrawn, the tip of the rubber block was sterilized by gentle flaming.

A laparotomy was performed on mice anesthetized with sodium pentobarbital. The chamber was inserted into the abdominal cavity of mice, and, after insertion, the abdomen was closed with surgical silk thread, and 1 mg of streptomycin was given to each mouse.

At appropriate time intervals after insertion, a leak test of chambers was performed by checking for the presence of bacteria in the organs of mice, with the use of nutrient agar containing streptomycin (50 μg/ml) for the detection of escaped organisms. Only 2% leakage was detected in these tests.

The number of bacteria in the organs of mice were determined by counting colonies which developed on nutrient agar after the organs were blended in saline (21).

RESULTS

Control of infecting population by antibiotic treatment. To study the effect of bacterial numbers and duration of infection on the resistance produced by immunization with living bacteria, mice were infected with a virulent strain and the growth of microorganisms was controlled by treatment with various amounts of antibiotics. Quantitative experiments showed that the maximal number of bacteria in the organs was reached 1 week after infection and decreased gradually thereafter (Fig. 2).

During kanamycin treatment, 15.5% of the mice died from the injection of 10^{-5} mg of strain 116–54. In contrast, the same infection killed all of the mice within 1 week when kanamycin treatment was omitted. The number of bacteria in the livers of these mice reached about 10^8 per g at death. It was also noted that viable counts of 10^2 and 10^3 microorganisms per g of liver and spleen were found in kanamycin-treated mice even after 7 weeks of infection.

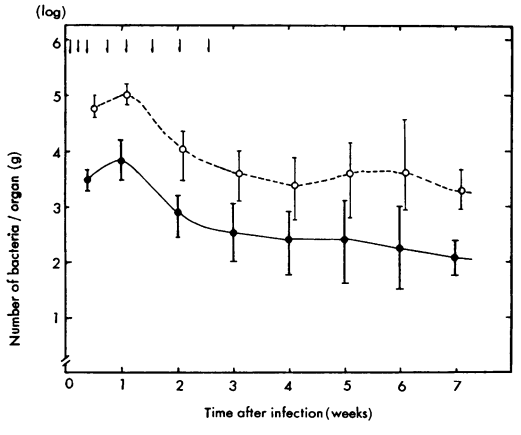

FIG. 2. *Bacterial counts in the organs of mice infected with a virulent strain, 116-54, of Salmonella enteritidis and treated with kanamycin thereafter. Mice were infected intravenously with 10^{-5} mg of 116-54 and were treated with 5 mg of kanamycin subcutaneously at the time indicated by arrows. Open circles, number of living bacteria in spleen; closed circles, number of living bacteria in liver. Vertical line indicates standard distribution of each value calculated from the data on five mice.*

The surviving mice of the kanamycin-treated group acquired high antilethal resistance against challenge infection with 10^{-5} mg (1,000 MLD) of a virulent strain, SMR49, of *S. enteritidis*, and 96 to 100% of the mice survived the challenge (Tables 1 and 2).

Vaccination was unable to confer antilethal resistance when larger amounts of kanamycin were administered to mice, resulting in a rapid decrease in the numbers of vaccinating bacteria in the organs of the infected mice. The number of microorganisms in the organs of infected mice decreased rapidly after frequent treatment with kanamycin over a period of 2 weeks. These mice did not seem to acquire resistance, however, because the number of bacteria in the organs increased markedly again 4 weeks after the cessation of kanamycin treatment (Table 3).

Antilethal resistance of these mice was tested 6 weeks after the first kanamycin treatment by a challenge infection with 10^{-5} mg (3×10^4 viable cells) of strain SMR 49. All 10 mice died 2 weeks after the challenge infection. These facts suggest that it is necessary to maintain a certain number of live bacteria in the organs of mice for the establishment of antilethal immunity.

Number of bacteria in a diffusion chamber.

TABLE 1. *Antilethal resistance of mice immunized with Salmonella enteritidis 116-54, and treated with kanamycin thereafter*[a]

Expt	Survival rate during immunization (%)	Survival rate after challenge (%)
1	95 (30/32)	100 (30/30)
2	85 (24/28)	100 (24/24)
3	87 (28/32)	96 (27/28)
4	81 (97/120)	99 (96/97)
Control		0 (0/10)

[a] Mice were infected with 10^{-5} mg of living *S. enteritidis* 116-54, and were treated with kanamycin at different time intervals, as shown in Fig. 2. Six weeks after the first injection of kanamycin, mice were challenged with the virulent strain, SMR-49 (10^{-5} mg, corresponding to 1,000 mouse MLD). The survival mice were scored 30 days after challenge. The numerator and denominator in parentheses indicate the survivals and total number of mice used, respectively.

TABLE 2. *Comparison of immunizing effect of live and killed vaccines against infection by Salmonella enteritidis*

Mice immunized with	No. of mice tested	Challenge dose	Survival rate after challenge
		mg	%
Live vaccine			
SER (10^{-5} mg) + 116-54 (10^{-7} mg)	200	10^{-5}	100
116-54 (10^{-5} mg) + kanamycin treatment	179	10^{-5}	99
Killed vaccine			
Heat-killed vaccine of 116-54, 10^{-2} mg	50	10^{-7}	0
Formalin vaccine of 116-54, 10^{-2} mg	50	10^{-7}	0
Chrome alum vaccine of 116-54, 2.8 mg	50	10^{-7}	10
	20	10^{-5}	0
Control			
Without immunization	100	10^{-7}	0

TABLE 3. *Number of microorganisms in the organs of infected mice treated with frequent administration of kanamycin*[a]

Weeks after bacterial infection introduced	No. of bacteria per g of	
	Liver	Spleen
1	3×10^2	1.65×10^3
2	10	5×10
6	4.0×10^4	7.1×10^6
7	2.1×10^7	4.6×10^7

[a] Mice were injected with 5 mg of kanamycin 24 hr before infection, infected with 10^{-5} mg of living *S. enteritidis* 116-54 by the intravenous route, and then treated with 5 mg of kanamycin twice a day for 3 days, and once a day for 11 days successively.

Histological studies have shown that infecting bacteria are found in reticuloendothelial cells in liver, spleen, and lymph nodes (18, 25). These observations raised the question of whether contact by living bacteria with host cells is necessary for the production of immunity. To resolve this problem, an experiment was designed in which bacteria in the host were separated from host cells by a filter. A diffusion chamber containing 0.5 ml of a bacterial suspension was inserted into the abdominal cavities of mice, and the bacteria in the chambers were counted at intervals. As shown in Fig. 3 and 4, the number of bacteria reached a maximum 24 hr after insertion and this level continued for 53 weeks without any decline. It was surprising that the bacterial concentration in the chamber was about 10 times greater than that of an overnight culture of the same bacteria in nutrient broth, and that the mice survived with so many bacteria in the chamber. The total number of bacteria determined photometrically, however, increased gradually and reached 10^{11} per ml 53 weeks after insertion. This indicates that the chamber contained both living and dead microorganisms in a ratio of about 1:10.

Permeability of antigen and antibody through the diffusion chamber. The anti-O titers in the sera of mice which received a diffusion chamber containing a bacterial suspension of *S. enteritidis* SMR49 were determined. Antibody was found in these mice (Table 4), which indicates that the chambers were permeable to the O antigens of *S. enteritidis*. Furthermore, anti-O antibody was detected in the

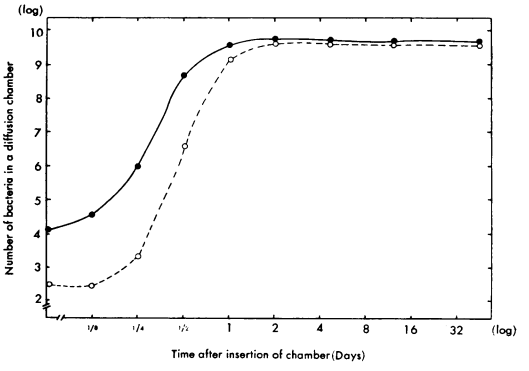

FIG. 3. *Number of bacteria in the diffusion chamber inserted into the abdominal cavity of a mouse. The number of bacteria (virulent strain, SMR49, of Salmonella enteritidis) at insertion was 4 × 10² (open circles) or 2 × 10⁴ (closed circles).*

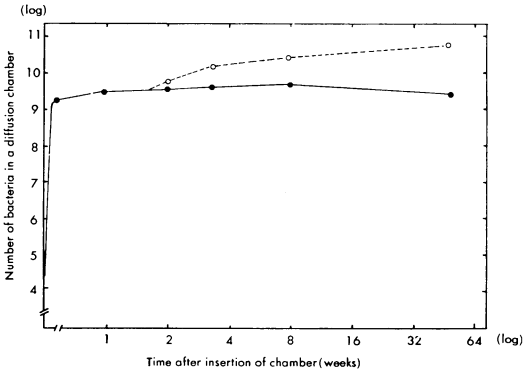

FIG. 4. *Number of bacteria in the diffusion chamber inserted into the abdominal cavity of a mouse. The number of bacteria at insertion was 2 × 10⁴. Open circles, number of bacterial cells estimated from turbidity; closed circles, number of living bacteria counted by plating.*

chamber fluid when chambers were inserted into mice immunized previously with killed vaccine. This indicates that antibody also penetrated into the diffusion chambers (Table 5).

Inability to confer immunity with diffusion chambers containing living S. enteritidis. Mice were implanted with diffusion chambers containing live microorganisms of strain 116-54 or SMR 49, and were then tested for antilethal resistance against infection with the same organisms. Two mice from each group in this experiment were used to check bacterial numbers in diffusion chambers. About 10^{10} bacteria in each chamber were again observed as in the experiment shown in Fig. 4.

All mice implanted with diffusion chambers containing about 10^{10} living microorganisms, as well as the mice on which sham operations were performed, died after infection (Table 6). However, the mice which were given the sham operation acquired antilethal resistance when immunized with live vaccine of *S. enteritidis*.

TABLE 4. *Antibody titer of mouse sera after insertion of the diffusion chamber containing bacteria*[a]

Time after insertion of diffusion chamber	Antibody titer of serum determined by	
	Bacterial agglutination (anti-O)	IAHA
weeks		
2	1:6.6	1:400
3	1:10	1:800
8	1:16	1:1,600
53	1:13.2	1:800
Control without diffusion chamber	<1:2	1:100

[a] Each diffusion chamber contained 0.5 ml (1.0×10^9) of overnight culture of *Salmonella enteritidis* SMR49 in nutrient broth.

TABLE 5. *Comparison of anti-O titer between serum and the fluid in the diffusion chamber of mice immunized with killed vaccine*[a]

Time after immunization	Anti-O titer of	
	Serum	Fluid in diffusion chamber
weeks		
6	1:160	1:80
	1:160	1:160
	1:40	1:80
8	1:160	1:160
	1:40	1:80
	1:40	1:40

[a] Mice were immunized with chrome alum vaccine of *Salmonella enteritidis* 116-54. A diffusion chamber containing 0.5 ml of 0.85% NaCl was inserted into each mouse. After 18 hr of insertion, mice were killed and the anti-O values were titrated. Three experiments are shown in the table, and each experimental value shows the titer of pooled sera of five mice.

TABLE 6. *Inability to confer immunity by use of the diffusion chamber containing live Salmonella enteritidis*[a]

Bacterial strain in diffusion chamber	Time interval between insertion of diffusion chamber and challenge	Mean survival time of dead mice	Per cent survival (survival/total)
	days	*days*	
SMR49	7	7	0 (0/7)
SMR49	14	11.5	0 (0/8)
SMR49	28	7.7	0 (0/10)
SMR49	35	8.4	0 (0/12)
116-54	56	9.3	0 (0/6)
Control[b]	28	8.5	0 (0/10)
Control[c]			100 (10/10)

[a] Each mouse was inserted with diffusion chamber containing 1.5×10^4 living microorganisms or 0.85% NaCl.

[b] Control mice inserted with a diffusion chamber containing 0.85% NaCl.

[c] Control mice which were inserted with a diffusion chamber containing 0.85% NaCl and which were superimmunized with live vaccine 30 days after insertion.

DISCUSSION

Earlier reports from this and other laboratories have described the effect of live and killed vaccines against mice infected with *S. enteritidis* (14, 17, 22, 31). In experimental infections with other species of group D salmonellae, e.g., *S. dublin* (16) and *S. typhimurium* (14, 30), killed vaccines have increased the survival time of mice after challenge, but have been largely ineffective in preventing death from a challenge infection. By contrast, suitable immunization with live vaccine was found to confer high antilethal protection against challenge with the virulent strain of the same organism. Experimental salmonellosis is a model of some infectious diseases, such as tuberculosis, brucellosis, and *Listeria monocytogenes* infection (10, 11, 15, 26).

Although a single immunization with live vaccine of an attenuated strain, SER, is much more

effective than multiple immunization with killed vaccines, only 55% of mice immunized with live vaccine resist a 10-MLD challenge infection. Higher grades of antilethal resistance have been observed in mice which were immunized with living attenuated bacteria, strain SER, and which survived challenge infection with the virulent strain, 116-54. About 100% of mice surviving this treatment are resistant to challenge with 1,000 MLD of virulent *S. enteritidis* (17, 20, 22).

In the present studies, it was found that convalescence, i.e., infection with 10^{-5} mg of strain 116-54 combined with kanamycin treatment, also conferred the same degree of protection as afforded by superimmunization with live, attenuated, and virulent bacteria. Moreover, the efficiency of immunization by the combined method was higher than that of superimmunization. Approximately 84.5% of mice subjected to the combined method became resistant, whereas only 55% of mice subjected to superimmunization became resistant. It was also noted in this experiment that large numbers of virulent bacteria were present in the convalescent mice and that convelescence did not protect mice when large amounts of kanamycin, which caused a rapid decrease in bacterial numbers, were administered.

To elucidate the mechanism of convalescent immunity, the diffusion chamber was used. The diffusion chamber has been widely used for cell transplantation immunity (4, 5, 6). The chamber has also been used to study the following experimental infections: streptococcal and staphylococcal infections of mice (12, 29), tuberculosis (1), and salmonellosis (2, 27).

In the present study, a diffusion chamber inserted into the abdominal cavity offered good conditions for bacterial growth, maintaining 10^{10} living cells and more than 10^{11} total bacterial cells for as long as 53 weeks after insertion. It was also found that mice with diffusion chambers containing 10^{10} (2.5 mg) living microorganisms of virulent strain 116-54 or SMR49 did not acquire antilethal resistance against the same organisms, although the number of living microorganisms in the mouse abdominal cavity was 10^7 times higher than necessary for effective immunization with a conventional living vaccine. Furthermore, O antigen from the organisms could diffuse out of the chamber and antibody against this antigen could enter it.

Histopathological studies of experimental salmonellosis in mice have shown that most bacteria of attenuated strains persist in organs rich in reticuloendothelial elements, i.e., spleen, liver, lymph node, and bone marrow, and that they are found primarily in mononuclear phagocytes in the form of nodules (18, 25). At the time of challenge, there were 10^3 and 10^4 bacteria in the liver and spleen, respectively (21).

From these facts, it is suggested that immunization with live vaccine or convalescent immunity can be achieved only by direct contact between host cells (probably mononuclear phagocytes) and living bacterial cells, perhaps necessitating actual intracellular parasitism.

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