Phagocytosis of Bacteria and Their Intracellular Multiplication in Experimental Typhoid*

By Daizo Ushiba

Department of Bacteriology, School of Medicine Keio University, Tokyo

(Received for publication, September 18, 1961)

Experimental typhoid is an infection of small laboratory animals in which the inoculated organism invades organs through a certain route from the infecting site causing a systemic infection and produce the specific pathological lesions of granuloma (typhom) particularly in the liver, spleen and lymph nodes. This infection was provoked only by particular relationships between host and parasite, for instance, by inoculating the mouse or the guinea pig with Salmonella typhi murium or S. enteritidis, or the rabbit with S. cholerae suis; but it can never be caused by S. typhosa in the animals stated above (Kobayashi and Ushiba1), Ushiba2). In the study on this particular relationship between host and parasite conducted in our laboratory during the past years, we have accumulated data suggesting the importance of cellular factors in the mechanism of infection and immunity of typhoid disease; the intracellular multiplication of infectious organisms represent an important phase of infection on the one hand, and on the other the mechanism of immunity is not totally elucidated by the presence of conventional antibodies but considered as of cellular nature.

Two series of investigations which have contributed to the consideration mentioned above will be presented here; one is the light-microscopic study of the relation between tissue-cultured cells and infectious organisms and the other the electron-microscopic study on the mechanism of phagocytosis and disposition of phagocytized organisms.

MATERIALS AND METHODS

Bacterial strains

S. enteritidis No. 11: A high-virulent S-type strain which usually kills all

* This paper was read at the First Conference of Japan Society of Reticulo-endothelial System as a part of the symposium on phagocytosis, June 26, 1961, in Sendai.
mice in about one week after an intraperitoneal inoculation with $10^{-4}$ mg (ca. 1,000 cells).

*S. enteritidis “Jena”: An almost non-virulent S-type strain which cannot kill mice even with $10^{-5}$ mg (ip).

Strain 11-Rx: An R-type mutant obtained from strain No. 11. This strain lacks the majority of O antigens and shows an intermediate virulence for mice.

Also used are a few other mutants of strain No. 11 which show virulence of various degrees for mice, and a V-type strain (Ihara) of *S. typhosa*.

**Animals**

Guinea pigs weighing around 400 g and ddN mice of 6-7 weeks old were used. In some experiments DK 1 mice, an inbred strain reared for a special purpose in our laboratory were used.

**Method of the light-microscopic study**

The details were described in a paper of Saito et al. Briefly, glycogen-induced peritoneal cells (usually more than 80% of them are mononuclear macrophages) from mice or guinea pigs were infected in vitro with various strains of *S. enteritidis*, and, after having fixed on small cover glasses with the aid of “formvar”, cultured by the roller-tube method in a medium consisting of 30% serum-Hanks’ solution added with proper amounts of streptomycin and penicillin to prevent extracellular growth of bacteria.

**Method of the electron-microscopic study**

Phagocytosis by peritoneal mononuclear cells: The detailed description has been given by Yamamoto and Nakano. Cell suspensions were obtained by induction with glycogen from the peritoneal cavity of guinea pigs, and fixed by 1% osmic acid added with an equal volume of Hanks’ solution at various intervals after being infected with bacteria. Dehydration by ethylalcohol and embedding in methacrylate resin were followed by ultrathinsectioning with a Porter-Blum’s microtome.

Colloidal particles used as the control of bacteria were “inferon” (dextran-Fe) with diameters of 50-70 Å and “fatgen” (fat particles) with an average diameter of 7 Å. Both were phagocytized being mixed with bacteria.

For the sensitizing with strain No. 11, both 5% of rabbit immune serum agglutinin 1:10240, O-agglutinin 1:320) and 20% of normal guinea pig serum were added to the strain.

Phagocytosis by Kupffer cells: In this experiment only DK 1 mice were used. They were inoculated intraperitoneally with $10^{-5}$ mg of strain No. 11 or intravenously with 2 mg of strains Jena and 11-Rx, and sacrificed by bleeding 3 hours later. Small pieces of their livers were fixed by Paladé’s solution and followed by the above-mentioned procedures for electron-microscoping.

Quantitative cultivation of inoculated bacteria in organs of mice and various
stainings of liver sections for light-microscopic observation were also carried out.

**EXPERIMENTAL RESULTS**

1. The Light-Microscopic Study by Tissue Culture Method.

   It was found in the study of tissue-cultured peritoneal mononuclear cells from guinea pigs or mice that the degree of intracellular multiplication of infecting bacteria showed a close correlation with the virulence for animals of the particular strain used. Thus, strain No. 11 multiplied vividly in guinea pig cells after 3 days of incubation, gradually increasing in number at the 5th and 7th days and finally destroyed the cells. This phenomenon was observed more markedly and rapidly in mouse cells, the majority of which were destroyed by multiplying bacteria after 3 days of incubation. Compared with this finding, a mutant of No. 11 which killed mice after a more prolonged life time multiplied more slowly in cells, and strains Jena and 11-M mutant which are almost non-virulent for animals also failed to multiply intracellularly. It is worth mentioning here that a V-type strain Ihara of *S. typhosa* did not multiply at all in guinea pig or mouse cells, which indicated an intimate relation between the pathogenicity concerning typhoid and intracellular growth in host cells (Saito et al.3).

   Having recognized the above results as a basis of further possible studies on experimental typhoid by means of tissue culture, we proceeded to the investigation on the mechanism of immunity.

   It has been concluded from the results of many experiments carried out in our laboratory during the past years, that the most important facet of immunity for typhoid should be searched for mainly in the inhibition of bacterial multiplication in organs which is in reality the inhibition of intracellular growth of bacteria and is caused by means of some cellular factors, but not brought about by the antibodies that are effective in inhibiting the multiplication of bacteria in body fluids (outside of cells). The difference of both factors was clearly shown by the treatments of hosts with an R-type live vaccine and an S-type killed vaccine. Thus, after the treatment with the former many animals survived a challenge with an S-type virulent strain in spite of the absence of specific antibodies or no capability to kill infecting bacteria in body fluids and organs at the very beginning of challenge infection. On the contrary, after the treatment with the latter the animals only showed a prolonged life span compared to the control after the challenge, and almost none survived, in spite of a marked production of specific antibodies in the serum or apparent killing of infecting bacteria particularly in body fluids at the early stage of infection.

   The results mentioned above led to a further investigation on whether the intracellular multiplication of bacteria could be really inhibited in cells from animals previously immunized with the live vaccine. This was pursued by an
experiment with tissue culture and proved to be true. As shown in Figs. 1 and 2, the intracellular multiplication of strain No. 11 was clearly inhibited in cells from guinea pigs immunized with strain 11-Rx, but not in cells from animals immunized with a heat-killed vaccine of strain No. 11. Further, the results were the same regardless of the presence or absence of specific immune serum in culture medium (Ushiba et al.).

In all experiments mentioned above, the difference of phagocytic ratio could not be precisely observed because the experimental conditions were not set up to do so. However, in no case was the phagocytic ratio for low- or non-virulent strains not higher than that for high-virulent strains. The significance of the reticuloendothelial system should be considered in its influence upon the fate of bacteria after phagocytosis rather than in its phagocytic activity which used to be thought as the most important factor in non-specific immunities.

II. The Electron-Microscopic Study.

1. Experiment with peritoneal mononuclear cells:

At the beginning of the engulfing of bacteria by macrophages there was often found pseudopodium like processes at the surface of cells which surrounded

![Graph](image)

**Fig. 1.** Multiplication of *S. enteritidis* strain No. II within cultivated peritoneal macrophages from guinea pigs immunized with live vaccine or killed vaccine. Differently shaded bars represent the percentage of phagocytized macrophages classified according to the number of intracellular bacteria (See Fig. 2).
NORMAL CELLS

bacteria just as for colloidal particles (Fig. 3). At the next stage of phagocytosis when bacteria were found in cytoplasm, a continuum of cell membrane and limiting membrane enclosing bacteria was sometimes shown (Fig. 4), which suggested the participation of smooth-surfaced endoplasmic reticulum with phagocytosis.

At the final stage of phagocytosis when bacteria were usually found in vacuoles with a clear limiting membrane, strain No. 11 cells were seen as electron-dense bodies surrounded by thick cell walls, usually together with several bacterial cells in a vacuole (Fig. 5). As time passed vacuoles containing bacteria usually enlarged with wide space between the bacteria and limiting membrane, and sometimes figures indicating binary fission of the engulfed bacteria were seen. Strain Jena cells showed, however, quite different appearances at this stage. As seen in Fig. 6, some bacterial cells demonstrated rather degenerated or swollen bodies, while in others the electron density seemed to prevail in degenerated bacterial cells which were often surrounded by wide and dense substances. This material may have derived from cytoplasm or from the interaction of cytoplasm and disintegrated bacterial products.

One problem which remained to be solved in the above experiments was the comparison between the phagocytosis of bacteria and the colloidal particles in
relation to the participation of endoplasmic reticulum. For this purpose, the mixture of bacteria (No. 11) and “fatgen” or “inferon” was used with peritoneal mononuclear cells, but no concomitant phagocytosis of both materials in a cell was observed in electron-microscopic sections.

Another problem to be solved was the difference between high-virulent and low-virulent strains in relation to their intracellular disposition. In this connection, strain No. 11 cells were inactivated by heat or sensitized with immune serum prior to phagocytosis. The inactivation of No. 11 cells by heat (60°C, 30 minutes) resulted in markedly degenerated appearances of bacterial cells in vacuoles, but they were not swollen nor surrounded by dense substances like strain Jena cells. When sensitized with immune serum, phagocytized cells of

Fig. 3. A guinea pig peritoneal macrophage infected in vitro with Salmonella enteritidis strain No 11; a bacterium engulfed by two small processes of the cytoplasm is seen.

Fig. 4. Two macrophages adjacent each other; in the lower cell, membranous structure surrounding a bacterium continues to the cell membrane.
strain No. 11 showed a variety of appearances; some were degenerated rather markedly and others remained almost normal.

2. Findings of liver sections:

After intraperitoneal inoculation of strain No. 11, the number of infecting bacteria in the liver of mice (DK 1 strain) rapidly increased up to around $8 \times 10^7$ at the 3rd day, but by electron-microscopy few bacteria were seen only in Kupffer cells, in spite of apparent degeneration of the parenchym cells. On the 4th day, however, a number of bacteria were observed in Kupffer cells, some of which showed binary fission (Fig. 7). It was noticeable that still no bacteria were seen at this stage in parenchym cells which were in highly degenerated states.

When a large number (2 mg) of bacteria were injected intravenously into
mice, many bacterial cells seen in Kupffer cells 3 hours after injection. In this condition strain Jena cells showed various appearances: normal, degenerated, and intermediate forms (Fig. 8). As in the case of peritoneal cells, electron-dense substances were seen around bacterial bodies in contrast to the case of No. 11 cells which, under the same condition, showed only normal dividing bodies in Kupffer cells without surrounding substances.

DISCUSSION

As stated beforehand our past experiments have led to the conclusion that the intracellular growth of bacteria is an important phase in the course of experimental typhoid infection and the mechanism of immunity in this disease...
Phagocytosis of Bacteria and Their Intracellular Multiplication in Typhoid

should be explained by cellular or non-antibody factors (Ushiba). The light-microscopic studies by a tissue culture method in the present paper contributed an essential basis to this conclusion and were the first attempt to carry out such an approach in the study on infection and immunity in experimental typhoid (Salmonella infection in animals). It is needless to say that the similar studies with tissue culture technics, particularly in tuberculosis and brucellosis, were previously reported by many investigators. For example, Suter, Mackaness et al., Mackaness, Fong et al., Freerksen and Shellenberg, and Berthrong and Hamilton reported experiments with tubercle bacilli, and thereafter, Pomales-Lebrón and Stonebring, Braun et al., and Freeman and Van-conducted similar experiments with brucella strains. In experimental typhoid also, Sato et al. recently reported experiments with S. enteritidis similar to ours. All of these papers reported experiments concerning intracellular growth of bacteria in tissue cultured cells or destruction or cells infected with bacteria in vitro, although there were some differences in technics or in the standard used in judging the results.

As for the electron-microscopic study on phagocytosis, several works have been published since Goodman and Moore who had observed phagocytosis of staphylococci by human leukocytes. Electronmicroscopic observations of leprosy bacilli in cells were reported by Bi and Chapman et al. The latter authors were particularly interested in the relationships between cells and microorganism and reported the existence of “capsule enclosing membrane” derived from the host cytoplasm around phagocytized bacteria (Mycobacterium leprae murium) and the “capsule space” between this membrane and the bacterial body. Detailed observations of phagocytosis of pleuropneumonia-like organisms by FL cells were recently carried out by Edwards and Fogh, who presumed that the microorganisms penetrated into vesicles or vacuoles formed by the host cell membrane in a manner similar to the formation of vesicles of endoplasmic reticulum.

The participation of endoplasmic reticulum in bacterial phagocytosis was also suggested by our present study as mentioned before, but a final conclusion was not obtained. While Uchino insisted that colloidal particles were transported into cytoplasm through preexisting smooth-surfaced endoplasmic reticulum of phagocytes, Kondo in opposition to this opinion indicated no relations between the limiting membrane around phagocytized particles and the preexisting endoplasmic reticulum. A close similarity between pinocytosis and phagocytosis of colloidal particles was suggested by many workers (Felix and Dalton, Parks and Chiquoine, Harford et al. and Karre), but the participation of endoplasmic reticulum was not necessarily clarified. On the other hand, the difference between phagocytosis of colloidal particles and bacteria remains unsolved, and should be pursued by improved technics.
As far as the disposition of phagocytized bacteria is concerned, it is noticeable that some correlation between the virulence of bacteria and electron-microscopic appearances of intracellular bacterial bodies was obtained in the present study. The most remarkable findings were the degenerated, swollen bodies with low density of low-virulent microorganisms and the existence of electron-dense substance around them in peritoneal mononuclear cells as well as in Kupffer cells. Its origin and nature are of course unknown, and its relation to "shell" which was found by Goodman et al.31) around phagocytized non-virulent staphylococci is also not clear.

The electron-microscopic study concerning the relationships between host cells and microorganisms has only recently started. This approach seems more worth while when it is advanced on the basis of exact knowledge of host-parasite relationships and by improved technics.

SUMMARY

1. In the light-microscopic study on the relationships between tissue-cultured cells and bacteria, it was found that the degree of intracellular growth of infecting microorganisms was in parallel with virulence of the particular strain for animals in which it could cause typhoid infection. It was also revealed that the intracellular growth of a virulent strain of S. enteritidis was inhibited in the mononuclear cells from animals previously immunized with an R-type live vaccine, but not in the cells from animals immunized with an S-type killed vaccine.

2. Phagocytosis of S. enteritidis by peritoneal mononuclear cells from guinea pigs or Kupffer cells of mice was also observed electron-microscopically, and the mechanism of the penetration of bacteria into cytoplasm has been discussed being compared with that of colloidal particles.

3. Some differences were observed between high-virulent and low-virulent strains of S. enteritidis in electron-microscopic findings when they were phagocytized into the above-mentioned cells; many of low-virulent organisms showed degenerated and swollen appearances with low electron-density surrounded by electron-dense substance, while high-virulent organisms were usually electron-dense and lacked surrounding substance.

This study was conducted by Drs. K. Saito, M. Nakano, K. Hashimoto and T. Akiyama of the Department of Bacteriology, and Dr. I. Yamamoto of the Department of Pathology.

A part of this study was aided by a grant from the Ministry of Education, Japan (Cooperative Research Fund, 1933–1935).

Thanks are due to Dr. T. Kobayashi, Professor of Pathology, and Dr. Y. Watanabe, Assistant Professor of Pathology, for their valuable advice on the electron-microscopic study.
Phagocytosis of Bacteria and Their Intracellular Multiplication in Typhoid

References