Electronmicroscopy studies on the bactericidal action of inflammatory leukocytes in murine salmonellosis

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Summary. Inbred female C3H mice were given 2×10^7 cfu virulent *Salmonella typhimurium* by intraperitoneal injection. Peritoneal washings were harvested between 3 h and 72 h after infection and examined by electronmicroscopy. There was evidence of intracellular killing by polymorphs and macrophages. The degeneration of intracellular salmonellae was seen initially as enlarging central electron-lucent areas in the cytoplasm and peripheral condensation of cytoplasmic granules, followed by disruption of the bacterial envelope and disintegration of cellular structure. Alternatively, the initial injury appeared as an irregular and discontinuous bacterial envelope with compression of the bacterium and diffuse condensation of cytoplasmic granules. It was also evident that virulent salmonellae multiplied extracellularly in the peritoneal cavity of the infected mice.

Introduction

During the past decade, there has been controversy about whether pathogenic salmonellae survive and multiply within host phagocytes. Descriptions of these organisms as facultative intracellular parasites (Suter and Ramseier, 1964; Collins, 1974) imply that they are able to survive or multiply, particularly within host macrophages. They were based upon the results of cell-culture experiments (Mitsuhashi *et al.*, 1961; Sato *et al.*, 1962; Mackaness *et al.*, 1966) which were later considered unreliable and were based on the misconception that an increased bacterial population within the liver and spleen of infected mice inevitably reflected intracellular bacterial multiplication (Collins, 1974). We have consistently been able to show by quantitative recovery of viable intracellular bacteria in cell culture that virulent *Salmonella typhimurium* and *S. enteritidis* are inactivated by macrophages from peritoneal exudates of guinea-pigs and mice (Hsu and Mayo, 1973; Rhodes and Hsu, 1974; Marecki *et al.*, 1975; Mayo *et al.*, 1977). This observation is supported by more recent reports by other investigators which show the intracellular killing of salmonellae within macrophages (van Zwet *et al.*, 1975; Briles *et al.*, 1981) as well as within

polymorphs (Beckerdite *et al.*, 1974; Rest *et al.*, 1978; Modrzakowski and Spitznagel, 1979). Carrol *et al.* (1979) have also examined cultured mouse peritoneal macrophages by electronmicroscopy 1 h after infection with *S. typhimurium* and described evidence of damage to the ingested bacteria.

The assays of intracellular destruction of salmonellae in most of these studies were based on a decrease in viable bacteria in a population of phagocytes and the experiments were usually done *in vitro*. Such methodology is subject to criticism and many workers remain sceptical about the conclusion that salmonellae are killed intracellularly. Advances in the elucidation of the mechanism of host immunity, and hence the development of vaccines for salmonellosis, are hampered by the lack of knowledge about whether virulent salmonellae are killed or multiply within host cells and where the pathogen proliferates in host tissues. The present investigation concerns the interaction between the pathogen and individual phagocytes at the site of infection. We used electronmicroscopy to provide a sequential description of the degenerative changes of salmonellae in the inflammatory cells of mice from a very early stage after an infectious challenge.

Materials and methods

Bacteria and growth conditions

A virulent strain of *Salmonella typhimurium* SR-11 was used. The bacteria were propagated in Tryptic Soy Broth

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(TSB; Difco Laboratories, Detroit, MI) for 6 h and were then washed in saline as previously described (Hsu and Radcliffe, 1968). The optically standardized bacterial suspension contained approximately 2.5×10^9 viable organisms/ml.

Mice

Three-month-old female mice of the inbred strain C3H/HeNMTV (Litton Bionetics, Charleston, SC) were used. They were highly susceptible to challenge with *S. typhimurium*; the LD50 was $< 10^2$ bacteria by intraperitoneal (ip) inoculation.

Infection of mice and harvesting of peritoneal exudates

Groups of 15–18 mice were each given 2×10^7 salmonellae suspended in 0.2 ml of saline by ip injection. At intervals of 3, 7, 24, 30, 48, 54 and 72 h after inoculation, two or three mice were killed with chloroform and immediately placed on a flat tray and their external skin was removed. About 6–8 ml of chilled Hanks's solution containing heparin 6 U/ml were injected into the peritoneal cavity of each mouse with a syringe and 18-gauge needle and 3–4 min later a small incision was made on the abdominal wall through which a glass tube with multiple small openings was inserted. The peritoneal washings were gently stirred, removed by suction and transferred into a 15-ml centrifuge tube containing 7 ml of chilled glutaraldehyde 4% in 0.1 M sodium cacodylate buffer and placed in an ice bath. After 1 h, the tubes were centrifuged at 1300g for 20 min to collect the peritoneal exudate cells (PEC) and extracellular bacteria. The pellet was resuspended in 1.5 ml of cacodylate buffer and the suspension was then allowed to stand for 5 min for the large clumps of tissue debris to sediment. The supernate containing PEC and extracellular bacteria was transferred to another tube.

Preparation of peritoneal exudate cells for electron-microscopy

The suspension of fixed PEC and extracellular bacteria was washed three times with 1.5 ml of chilled cacodylate buffer by centrifugation and postfixed with 1% osmium tetroxide for 1 h at room temperature. After dehydration with increasing concentrations of ethanol, the PEC pellets were embedded in Polybed epoxy resin (Polysciences, Inc., Warrington, PA). Ultrathin sections were stained with uranyl acetate and Reynold's lead citrate. They were examined in a Hitachi HS-8 electronmicroscope.

Results

Effect of bacterial challenge

Intraperitoneal challenge with 2×10^7 salmonellae initiated a rapid dissemination of the pathogen throughout the reticuloendothelial system and resulted in progressive infection. Microscopic lesions were seen in the liver and spleen within 72 h. Mice began to die 54 h after the infection.

Sequence of intracellular destruction of salmonellae

Soon after their ingestion by polymorphs and macrophages, there appeared to be two alternative sequences of destructive events of salmonellae by host phagocytes. The first was progressive cytoplasmic vacuolation followed by rupture of the bacterial envelope, and the second was an early distortion of the bacterial envelope and condensation of cytoplasmic granules. These changes are depicted in figs. 1 and 2. Fig. 1a shows the normal cytology of salmonellae in the extracellular environment of the peritoneal washings. Fig. 1b shows a longitudinal and a transverse section of organisms shortly after

Fig. 1. a. Intact extracellular salmonellae in peritoneal washings. b–d. A sequence of bacterial degeneration within peritoneal phagocytes in which the injury begins in the bacterial cytoplasm. $\times 12000$; bar = 0.5 μm .

fig. 2. c. A sequence of bacterial degeneration within peritoneal phagocytes in which the injury begins in the bacterial envelope. $\times 12000$; bar = $0.5 \mu\text{m}$.

gestion by a phagocyte but while still retaining their cytological integrity. The third organism in the middle of this phagocyte is undergoing an early stage of cytological degeneration, characterized by mild central vacuolation (electron-lucent areas) and early peripheral condensation of cytoplasmic granules. Then, as seen in fig. 1c, the degenerative process continues with a progressive enlargement

and confluence of central vacuoles along with increasing peripheral margination of condensed cytoplasmic granules around the inside of an apparently intact bacterial envelope. Finally, the bacterial envelope becomes irregular and discontinuous, leading to rupture of the bacterium as shown in fig. 1d. Alternatively, the initial injury to the intracellular bacterium begins with the disruption of the

Fig. 3. A peritoneal macrophage 3 h after infection. $\times 12700$; bar = $1.0 \mu\text{m}$.

Figs. 4 and 5. Macrophages 7 h after infection, one of which contains a polymorph. $\times 12\,700$; bar = $1.0\ \mu\text{m}$.

Fig. 6. Cytoplasm of a macrophage 24 h after infection. L = lipid droplet; $\times 12\,700$; bar = $1.0\ \mu\text{m}$.

Fig. 7. Extracellular bacteria in peritoneal washings 24 h after infection. Arrows show bacteria undergoing division. $\times 17\,000$; bar = $1.0\ \mu\text{m}$.

Fig. 8. A macrophage 30 h after infection. L = lipid droplet; $\times 12\,700$; bar = $1.0\ \mu\text{m}$.

Fig. 9. A macrophage after ingesting a polymorph 54 h after infection. $\times 12\,700$; bar = $1.0\ \mu\text{m}$.

Fig. 10. A polymorphonuclear leukocyte 54 h after infection. $\times 12\,700$; bar = $1.0\ \mu\text{m}$.

Figs. 11 and 12. Macrophages 72 h after infection. L = lipid droplet; $\times 12\,400$ and $\times 12\,700$, respectively; bar = $1.0\ \mu\text{m}$.

bacterial envelope and a diffuse condensation of the cytoplasmic granules as shown in fig. 2a. The bacterial morphology now appears compressed or shrunk and these bacteria disintegrate directly without complete disappearance of the cytoplasmic granules and vacuolation, as exemplified by the bacteria in the lower portion of figs. 2b and 2c.

Cytological evidence for the destruction of salmonellae within phagocytes

Figs. 3–12 are electronmicrographs taken from peritoneal washings of infected mice 3–72 h after infection. As early as 3 h, some bacteria were ingested by phagocytes as seen in fig. 3, which shows a macrophage soon after ingesting three bacteria whose cytological and morphological appearance remains intact. By 7 h, there was evidence of active ingestion and digestion of the pathogen. For example, in fig. 4, the macrophage has apparently just phagocytosed one or two bacteria as well as a degenerating polymorph, which in turn contains several intracellular bacteria in various stages of digestion. The macrophage after 7 h (fig. 5) harbours one bacterium at an early stage of cytoplas-

mic damage and several phagolysosomes containing what appears to be bacterial debris. The prominent ribosomes in the cytoplasm seen in fig. 6 are indicative of a macrophage which, at 24 h, has ingested and digested foreign particles. Its phagolysosomes show the various stages of bacterial digestion, beginning with a pair of dividing bacteria at an early stage of fragmentation and ending with the formation of lipid droplets. The macrophage after 30 h (fig. 8) also contains several bacteria at various stages of digestion. Its lipid droplets suggest that this phagocyte has been active at the site for some time. Fig. 9 shows a macrophage after phagocytosis of a polymorph and fig. 10 shows a polymorph after 54 h. Both phagocytes appear to be intact while the intracellular bacteria were in various stages of destruction by the host cells. The macrophage in this case has ingested bacteria and a polymorph which appears just to have engulfed three bacteria, one of which remains undamaged. The phagolysosome of the macrophage at 72 h (fig. 11) contains a large number of bacteria whose morphological integrity is distorted apparently after a relatively brief sojourn in the host cell. However, other evidence of bacterial debris and lipid droplets in the

cytoplasm of this macrophage suggest that it may have been ingesting bacteria continuously for some time without suffering serious injury. The large phagolysosome in the macrophage after 72 h (fig. 12) contains an example of what may be the final recognisable stage of bacterial disintegration within host cells.

Extracellular multiplication of salmonellae

There was no evidence of bacterial multiplication within host phagocytes. Even when seemingly dividing bacteria were observed intracellularly, they appeared to be in various stages of degeneration (figs. 1c and 6). These pairs of organisms were probably phagocytosed while undergoing division. On the other hand, extracellular salmonellae could frequently be seen in the process of division (fig. 7).

Discussion

To resolve the issue of whether virulent salmonellae can survive and multiply within host phagocytes, experimental procedures must be designed in such a way that the fate of the intracellular bacteria can be determined within a population of phagocytes. Cell-culture methods have been used by many investigators for this purpose. Unfortunately, several pitfalls inherent in cell-culture procedures render the observations inconclusive at best. The major criticisms revolve around the quantitative methods by which the changing intracellular bacterial population is assayed, and, if antibiotics are incorporated into the culture medium to suppress the extracellular bacterial multiplication, their possible interference with the viability of intracellular bacteria. These technical concerns have frequently been discussed (Hsu and Radcliffe, 1968; Hsu & Mayo, 1973; Rhodes and Hsu, 1974; Marecki *et al.* 1975).

In the present investigation, a relatively large dose (2×10^7 cfu) of virulent *S. typhimurium* was injected ip to initiate a rapidly progressive infection, such that inflammatory phagocytes, both polymorphs and macrophages, would swiftly accumulate at the site of challenge. This procedure was necessary to facilitate the early phagocytosis of bacteria by a relatively large proportion of the exudative cells so that intracellular bacteria could readily be examined by electronmicroscopy. This would allow the study on the interactions between individual inflammatory cells and salmonellae *in situ* at a very early stage of the infection and before the onset of acquired immunity. It should be emphasised that this study was not concerned with

the process and pathology of the disease in the intact host, nor with the antibacterial action of resident peritoneal macrophages in the host. The fate of the pathogen in the intracellular versus the extracellular location of host tissues was determined by direct electronmicroscopic observation of the morphology of the bacteria. This procedure had the advantage of avoiding various criticisms, including the counting of intracellular bacteria, the possible effect of antibiotics on intracellular bacteria and the artificial nature of cell-culture procedures.

Several general observations can be made. Peritoneal exudative phagocytes derived from innately susceptible mice, both polymorphs and macrophages, were capable of ingesting virulent salmonellae as early as 3 h after infection in the absence of specific antibodies. However, in spite of the initial inoculation of a relatively large number (2×10^7 cfu) of the pathogen ip, each phagocytic cell appeared to have fewer than five bacteria in the sections examined. This is consistent with the previous observation that the pathogenicity of salmonellae is directly related to their antiphagocytic properties (Wells and Hsu, 1970; Hsu and Piper, 1972). It was also clear that the destruction of the ingested bacteria occurred rapidly, resulting in their complete disintegration. The sequence of degenerative changes of intracellular bacteria appeared to proceed either through cytoplasmic deterioration (figs. 1b-d) or through damage to the bacterial surface and distortion of its morphological structure (figs. 2a-c). Although most of the photomicrographs presented here show bacteria within macrophages, there was early inflammatory exudation of polymorphs into the peritoneal cavity, followed by a later influx of monocytes. Figs. 4 and 9 show that polymorphs were actively ingesting bacteria before their subsequent engulfment by macrophages.

The phagocytes at the site of infection displayed an ability to ingest and digest the pathogen continuously without incurring serious injury to themselves, as suggested by the observation that their cytoplasm frequently contained bacteria at various stages of disintegration (figs. 5, 8, 10 and 11). There was no indication of active intracellular bacterial multiplication, although it would be possible to see dividing bacteria in these fixed tissue preparations. Several dividing bacteria were seen undergoing intracellular disintegration (figs. 1c and 6) but these were presumably phagocytosed at the time of division. Furthermore, if virulent salmonellae did multiply within phagocytic leukocytes, it should be possible to see large numbers of intact intracellular bacteria in many host cells. This was not the case. In contrast, whenever a relatively large number of

bacteria was seen within host cells, they had usually lost their structural integrity (fig. 11). Hence, in the absence of any evidence of intracellular bacterial multiplication, the rapid proliferation of salmonellae within the infected host must be attributed to their active extracellular replication as seen in fig. 7.

The representative electronmicrographs in this study provide a clear illustration of both the intracellular destruction and extracellular multiplication of virulent *S. typhimurium* at the site of infection in mice. The magnification in electronmicroscopy shown here allows the morphological integrity of several bacteria to be seen within one frame, from which their viability or degeneration can be assessed. No attempt was made to describe the mechanism or process of bacterial disintegration by examining the ultrastructural detail of cytological components of individual bacteria. The fixation method used was considered adequate for the proper fixation of a suspension of single cells. It is reasonable to discount the possibility that fixation artifacts were misinterpreted as degenerative changes of intracellular bacteria because intact bacteria were seen adjacent to degenerating ones within the same phagocytes (figs. 1b and 9). Since each tissue section represents a plane across the host cell amounting to less than 1% of the total spherical volumes of the whole cell, it would be impractical to determine the percentage of bacteria surviving or being destroyed within a phagocyte at a given time. In light of the electronmicroscopic evidence of intracellular killing of salmonellae in this study, the possibility that a few salmonellae might survive and multiply within host cells is rendered insignificant, because their contribution to the total increase in the bacterial population in host tissues would be negligible compared with the massive extracellular

bacterial proliferation in the peritoneal cavity of infected mice.

The evidence presented here, therefore, lends further support to the previous work from this laboratory (Hsu and Mayo, 1973; Marecki *et al.*, 1975; Mayo *et al.*, 1977; Nakoneczna and Hsu, 1980 and 1983) which showed that salmonellae have erroneously been described as facultative intracellular bacteria (Suter and Ramseier, 1964; Collins, 1974). A thorough search of the literature reveals that there is no satisfactory experimental evidence to show unequivocally the survival or multiplication of virulent salmonellae within host macrophages. Histopathological evidence derived from lesions in the liver and spleen of infected mice (Nakoneczna and Hsu, 1980 and 1983) further demonstrates that the early stage of a primary lesion and of lesions in infected mice protected by killed salmonella vaccine is characterised by the rapid accumulation of polymorphs, not macrophages. Collectively, therefore, the classification of salmonellae as facultative intracellular bacteria is untenable. The pathogenic mechanism of this organism is directly related to its antiphagocytic property and hence its ability to proliferate in the extracellular location of host tissues.

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