Hijacking of macrophages by *Salmonella* (3, 10 : r : -) through 'type III' secretion-like exocytotic signaling: a mechanism for infection in chicken ileum

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**ABSTRACT**

Ultrastructural studies of experimentally-infected chicken ileum reveal that virulent *Salmonella* (3, 10 : r : -) signal their own phagocytosis by tissue macrophages, and are thus able to dodge host defence mechanisms. The observed fusion of membrane vesicles (MVs) liberated from virulent organisms with closely interacting macrophages is proposed here to constitute a mechanism for type III secretion-like system of gram-negative organisms in general, in order to translocate the necessary biochemical signals into the cytosol of macrophages (eucaryotic host cells). This leads to (i) membrane ruffling culminating in augmented phagocytosis of salmonellae, (ii) inhibition of phagosome-lysosome (P-L) fusion for survival and replication of the pathogens within the host cytoplasm and (iii) initiation of apoptosis of the infected macrophages and ultimate release of salmonellae from the migrating host cells.

Contains 5 figures

Key words: Exocytosis from *Salmonella*, membrane vesicular signaling for phagocytosis, macrophage invasion, mechanism of systemic infection.

Responsible for diarrhoea, gastroenteritis, septicemia, reticulo-endothelial hyperplasia, equine abortions, and human typhoid fever, *Salmonella* pathogens are a perpetual threat throughout the world. *Salmonella* has been linked to millions of illnesses every year, inflicting losses worth billions of dollars in productivity and increased medical expenses. Therefore, it has become essential to understand the mechanism of invasion and propagation of these pathogens affecting birds, animals and humans. Recently, a cellular mechanism of *Salmonella* invasion of the intestinal epithelium has been reported, wherein membrane vesicles (MVs) released by the activated *Salmonellae* were shown to signal cellular events culminating in the host-intracytoplasmic penetration of the pathogens (YashRoy, 1998). The present study investigates the second stage of *Salmonella* infection, wherein, after leaving the intestinal mucosa these pathogens are phagocytosed by macrophages.

Inside macrophages, these organisms persist within phagosomes, and this-way, they safely get transported to the organs of reticulo-endothelial system such as liver and spleen (Carrol *et al.*, 1979; Fields *et al.*, 1986). The present study on *Salmonella* 3, 10: r : - organisms, which are held responsible for serious infections in man, animals and poultry (Gupta *et al.*, 1980 and Kumar *et al.*, 1981) substantiates the first structural evidence (YashRoy, 1999) in favour of involvement of the exocytotic MVs released by the *Salmonella* pathogens in signaling stimulated phagocytosis in the closely-interacting macrophages, *in vivo*. The phagocytosed organisms, not only survive within the cytoplasmic phagosomes, but also are able to replicate within them. The new generation salmonellae finally disrupt and escape from the infected macrophages, thus causing a systemic spread of the infection.

**MATERIALS AND METHODS**

Five 3-month-old broiler chickens received from the Central Avian Research Institute, Izatnagar were used in this study. Cultures of *Salmonella* 3,10: r : - organisms isolated from human food poisoning cases were obtained from National *Salmonella* Centre of the Indian Veterinary Research Institute,
Izatnagar. Standard ligated ileal loop methodology as modified by Giannela et al. (1973) was employed for inoculation of the organisms in 24-h fasted chickens as already described (YashRoy, 1998, 1999). Ileal loops inoculated with live organisms showing fluid exsorption were drained off their fluid and cut into small pieces and immersed in the fixative solution (2.5% glutaraldehyde, buffered at pH 7 with phosphate buffer). The tissue pieces were further cut to about 1mm³ size and kept immersed in the same fixative at 10°C for about 6 h. The control loops injected with bacteria-free culture medium, did not show any fluid exsorption and were also similarly fixed. All tissues were block-stained by immersing in 1% aqueous osmium tetroxide solution in vials wrapped with aluminium foil for 2 h at room temperature and subsequently processed for dehydration in acetone series and embedding in araldite medium as per standard procedures. Tissue blocks were trimmed and cut into ultrathin sections with LKB ultratome III ultramicrotome and obtained on 3-mm diameter copper grids. The grids were air-dried overnight in a dust-free chamber and stained with uranyl acetate and lead citrate by following set procedures (Hunter, 1984 and YashRoy, 1993). The stained sections were examined under JEOL JEM 1200EX electron microscope working in transmission mode operated at 100 KV.

RESULTS AND DISCUSSION

Figure 1 represents the typical surface structure of Salmonella 3, 10 : r : - organisms closely-interacting (within 500 nm) with macrophages (M). The organisms reveal presence of several membrane-bound protrusions (p), which appear to pinch off (line arrow) as membrane vesicles (MV). Some of these MVs are also observed to dock (bold arrows) on cell membrane of the interacting macrophages. An intimate fusion-like contact observed between the MVs released by Salmonella 3, 10 : r : - and cell membrane of macrophages (curved bold arrow in fig. 1) would obviously discharge the vesicular contents into the macrophage cytoplasm.

Fig. 2 shows the phagocytic cup-like structures (p) formed by ruffling of the cell membrane of macrophages, closely interacting with the organisms (bold arrow). Direct
interactions between the bacterial periplasmic protrusions and macrophage cytoplasmic extensions also appear to take place (bold arrow in Fig. 2).

Fig. 3 shows that once phagocytic cup is formed, more than one organism (A,B) may enter which are observed as enclosed in cytoplasmic phagosomes (P) inside the macrophage. Even a single macrophage can this way, phagocytose (engulf) a large number of salmonellae, which appear as conspicuous inclusions in the cytoplasm of these macrophages (Fig. 4). Numerous lysosomes are also seen in the macrophages but, however, they are not found fused with phagosomes. The contents of these darkly-staining inclusions are difficult to resolve as they are electron-opaque tight phagosomes. Nonetheless, salmonellae 3, 10: r: - organisms seem to survive within them. These organisms obviously multiply within spacious cytoplasmic phagosomes (Fig. 5). Macrophages, with replicated Salmonella within the spacious phagosomes, also reveal structures similar to apoptotic vacuoles in the cytoplasm. Release of the replicated new-generation salmonellae also appears to occur from macrophages showing advanced apoptotic changes (Fig. 5).

Unlike many host-adapted salmonellae – S. Typhi (humans), S. pullorum (poultry), S. dublin (cattle) and S. arizonae (reptiles) (Hueck, 1998) – Salmonella 3, 10: r: - organisms, under report here, are known to cause food poisoning in humans, pigs, cattle and poultry, etc., and are therefore, of public health importance as well (Gupta et al., 1980 and Kumar et al., 1981). An essential virulence determinant for Salmonella pathogens is their ability to survive and replicate in professional phagocytes, which may lead to systemic typhoid-like infections (Fields et al., 1986, Alpuocha-Aranda et al., 1994 and Libby et al., 1994). Invasive Salmonella typhimurium strains can invade macrophages in 2 h in vitro, that is, at 7 to 10-fold higher levels than non-invasive strains (Monack et al., 1996). The present communication provides a structural evidence for involvement of a novel exocytotic signaling mechanism for infection of the host macrophages by invasive Salmonella organisms in chicken ileum (Fig. 1 to 5); the first initial report having also been published from this
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Laboratory (YashRoy, 1999). Fusion of MVs with the macrophage cell membrane, observed here (Fig. 1), can obviously translocate, directly into the host cytosol, even fully-conformed (globular) proteins. The bacterial secretions in the form of MVs may, therefore, signal actin polymerization, necessary for inducing phagocytic activity in macrophages (Fig. 1). This mechanism can also explain the observed efficient induction of phagocytic activity in macrophages (Fig. 1), which can obviously translocate, directly into the host cytosol, even fully-conformed (globular) proteins. The bacterial secretions in the form of MVs may, therefore, signal actin polymerization, necessary for inducing phagocytic activity in macrophages (Fig. 1). This mechanism can also explain the observed efficient induction of phagocytic activity in macrophages (Fig. 1).

Irrespective of host cell-type (macrophages for intestinal epithelial cells), a similar mechanism may operate for mediating host-pathogen interactions. It appears that the invaded intestinal epithelial cells may not provide a safe intracellular ‘corridor’ to the invasive Salmonella for moving from lumen to deeper layers of intestine, where the pathogens reach their second, but more important hosts namely, macrophages (Small et al., 1987). Earlier studies from this laboratory (YashRoy, 1994) revealed that Salmonella 3, 10: r: - pathogens travelled through the lymphatic vessels of ileum, where they were phagocytosed by macrophages. Then, however, it was not understood whether and how these organisms could induce phagocytic activity in macrophages.

Membrane vesicles, therefore, offer a novel modus operandi for bypassing the energetic and conformational constraints on export of periplasmic proteins across the barrier of the bacterial outer membrane for bulk secretion of virulence proteins of gram-negative organisms. Furthermore, fusion of MVs with plasma/cell membrane of host cells also explains the observed translocation of bacterial exo-proteins directly into the cytosol of the recipient/target cells, and thus, it is proposed to constitute a viable mechanism explaining the type III secretion system of gram-negative organisms, in general. This proposal is in agreement with the finding that type III secretion by S. typhimurium does not require its contact with eucaryotic host (DaeHae, 1999). Due to energetic and conformational constraints (Pugsley, 1993 and Hueck, 1998), the 3-dimensionally conformed proteins, are unlikely to pass through the outer-membrane bounding the periplasm for their secretion, nor could they pass through (eucaryotic) cell membrane so as to enter inside the recipient cells. (YashRoy, 1998). The present investigation thus provides a clear structural evidence for involvement of this novel mechanism in inducing membrane ruffling for stimulated phagocytosis by macrophages. Liberation of MVs from the invasive pathogens and their attachment to macrophage surface also explains how endotoxic lipopolysaccharide (LPS) – an integral component the MVs– may activate the macrophages during the progression of infection in vivo. Translocation of the biochemical contents of these MVs into the macrophage cytosol may induce cytoskeletal changes in macrophages leading to the augmented of phagocytic cups (Fig. 2) and engulfment of organisms (Fig. 3).

Recently, phospholipases present in the MVs of many gram-negative organisms, have also been linked with activation of phagocytic activity in leukocytes (Lannar tz, 1999).

Upon their engulfment, Salmonella 3, 10: r: - organisms are enclosed into darkly-staining phagosome-like inclusions in the cytoplasm of macrophages in vivo (Fig. 4). Presence of numerous such inclusions is conspicuously seen within the infected macrophages. The present in vivo observations of chicken ileum under report here, corroborate the results of in vitro interactions between macrophages and Salmonella typhimurium, wherein it was shown that this pathogen multiplied in those phagosomes which were not fused with lysosomes (Carrol et al., 1979). How virulent Salmonellae are able to prevent phagosome-lyosome (P-L) fusion within macrophages is not clearly understood (Buchmeier and Heffron, 1991). However, it has been observed that P-L fusion could possibly be inhibited by (i) decrease in fluidity of lysosomal membrane or by (ii) altering the cytoskeletal state within the cytoplasm of macrophages (Mozhenok et al., 1998). It is observed that synthesis of about 30 proteins is augmented in salmonellae present within the phagosomes (Buchmeier and Heffron, 1990; Abshire and Neidhart, 1993). Inhibition of bacterial protein synthesis with oxytetracycline depresses the...
capability of preventing P-L fusion within polymorphonuclear cells by another organism, \textit{E. (Cytoectes) phagocytophiliae} (Gokce et al., 1999). Increasing fluidity of lysosomal membrane with bilirubin and inducing cytoskeletal changes with farmorubicin and chelerythrine results in enhanced P-L fusion in peritoneal macrophages of mice (Buchmeier and Heffron, 1991).

\textit{Salmonella} organisms while within tight phagosomes (Fig. 4), may be synthetically very active but, however, replicated organisms are observed only within spacious phagosomes (Fig. 5). New-generation salmonellae (about four of which are normally seen in each spacious phagosome), appear to leave the infected macrophages, also simultaneously undergoing degenerative changes like vacuolisation (Fig. 5). These \textit{in vivo} observations corroborate the earlier \textit{in vitro} studies, wherein bone marrow macrophages infected with \textit{Salmonella typhimurium}, showed similar apoptotic changes. Cytotoxic signal for inducing apoptosis in macrophages was, however, linked with the ruffling process associated with phagocytosis of the salmonellae. On the contrary, though complement coated salmonellae did enter macrophages efficiently without the involvement of the ruffling process; but it only increased the intracellular bacterial load and did not induce apoptosis in macrophages (Monack et al., 1996). Therefore, it appears that MVs of \textit{Salmonella} 3, 10 : r : - which are proposed to translocate molecular signals for stimulating phagocytic activity in macrophages, may also bring in, the apoptosis-initiating biochemcals. It suggests that this programmed cell destruction of infected macrophages progresses side-by-side, with the survival of salmonellae in tight phagosomes first, and then their replication in spacious phagosomes, leading finally to their release from the disintegrating macrophages. During these parallel events of \textit{Salmonella} multiplication and progression of apoptosis in infected macrophages, a systemic spread of infection may be expected to take place due to the simultaneous movement of macrophages to other organs and tissues.

\textbf{Proposed mechanism of macrophage infection by \textit{Salmonella}:}

1. Close proximity (within 500 nm) between \textit{Salmonellae} and macrophages results in the acquisition of 'virulent state' of the organisms, characterized by the presence of numerous outermembrane-bound 'periplasmic organallae' on the surface of the pathogens. 2. MVs pinched off, from these organellae are proposed to contain the necessary biochemical signals, released by the pathogens and targeted for delivery into the macrophages. 3. The observed 'fusion' of MVs with the plasma membrane of macrophages is proposed to mediate the necessary translocation of biochemical signals directly into the cytosol of the host macrophages, besides also carrying LPS (endotoxin) as an integral component of the external leaflet of the their bounding outer-membrane. 4. Upon this translocation, the macrophages respond by (a) cytoskeletal reorganisation into ruffling and augmented phagocytosis of salmonellae and (b) inhibition of phagosome-lysome fusion for survival of salmonellae engulfed into numerous cytoplasmic phagosomes. 5. Multiplication of organisms within spacious phagosomes and continual release of new-generation salmonellae from the migrating macrophages, appear to be timed with the progression of the apoptotic disintegration of the macrophages. The apoptotic signals appear to be brought into macrophages through bacterial MVs. 6. Translocation of biochemical signal molecules (which may also include bacterial exoproteins) from \textit{Salmonella} organisms to directly inside the eucaryotic host cell (macrophage) cytosol, observed here and earlier (YashRoy; 1998) through this membrane vesicular exocytosis process, is proposed to provide a viable mechanism for Type-III secretion in gram-negative organisms.

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