Discovery of vesicular exocytosis in procaryotes and its role in Salmonella invasion*

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Intracellular uptake of bacterial pathogens may require a microbe-directed process of macropinocytosis\(^1\) of the organisms involving complex invasive activity following surface adhesion to the host cell\(^2,3\). Notorious for inflicting in calculable losses to egg, poultry and meat industry, Salmonella infections are a global concern as they cause serious food poisoning problems in man and animals. Only if we understood how these organisms accomplished an essential step of entry into the host cells, a lot many newer interventions could be envisaged to ameliorate the prevailing difficult situation. Active involvement via specialized bacterial surface protrusions acquired during close host–pathogen interactions\(^4\), is thought to play an important role in this process. As epithelial cell surfaces are known to induce salmonella proteins required for bacterial adherence and invasion\(^5\), such bacterial surface protrusions\(^4\) are considered to contain the necessary invasion factors. Discovery\(^6\) of a novel exocytic mechanism of bulk transport of bacterial secretions in the form of membrane vesicles, for subsequent ‘fusion’ with host cells, has practically removed the energetic and conformational constraints\(^7\) on protein secretion through the bacterial outer membrane\(^8,9\). The observed ‘fusion’ of membrane vesicles released from the pathogen with the host cell plasma membrane\(^6\), is therefore proposed here to deliver the necessary biochemical signals so as to induce the required morphological and cytoskeletal changes in the host cells\(^10,11\), culminating in the entry of the pathogen through the membrane ruffle so produced, into the host cell. These studies also present a structural mechanism for entry of Salmonella organisms into the host epithelial cells in vivo. The traditional belief that the vesicular secretion is a prerogative of eucaryotic cells alone, too is debated.

During their passage through chicken ileum, Salmonella 3,10:1:- organisms almost invariably present undulating amorphous surface structure when they are found located near-centrally in the lumen, i.e. more than 2000 nm away from the epithelial cell microvilli (Figures 1 and 2, arrowhead). On the other hand, over 70% (± 5) of the organisms located in close proximity (within 500 nm) of microvilli of the ileal epithelial cells, present a markedly different surface structure (Figures 2 and 3), consisting of numerous periplasmic protrusions bounded by bacterial outer membrane, obviously detached from the underlying cell wall (Figure 4). These organisms are also associated with several membrane vesicles, pinched off from the periplasmic protrusions (Figure 5). These vesicles are also seen ‘fused’ with the microvillus plasma membrane of the ileal epithelial cells (Figure 5). Such a ‘fusion’ would obviously discharge the vesicular contents into the interacting host epithelial cells.

Interestingly, similar membrane vesicles released by Pseudomonas aeruginosa have been recently shown to contain several virulence factors\(^8\), and have been demonstrated to ‘fuse’ with other gram-positive and gram-negative organisms in cultures, resulting in their bacteriolyis\(^12\). Comparably, disruption of the epithelial cell microvilli is seen (Figure 6) as a result of ‘fusion’ of membrane vesicles with epithelial cell microvillus membrane. In addition, a reorganization of the cytoskeleton of the co-interacting epithelial cells into ruffle-like structures (Figure 6) is also observed in vivo in the chicken ileum. In vitro observations of similar ruffles in cultured kidney (MDCK) epithelial cells (used as a model for intestinal epithelial cells) while interacting with Salmonella typhimurium organisms\(^10\) corroborate the present in vivo observations under report. Likewise, Salmonella organisms also lose their periplasmic protrusions when they are located at the ruffle (Figure 6), quite comparably with the observations from the in vitro experiments\(^10\). It has now become practically clear that Salmonella organisms enter the host epithelial cells through such ruffles\(^1,11,13,14\).

Formation of surface protrusions by Salmonella organisms, during close interactions with microvilli of host epithelial cells is a highly significant process as has been observed both under in vivo (present report) and in vitro conditions. When the microvilli-bearing cultured Madin-Darby canine kidney (MDCK) epithelial cells are used as a model for intestinal epithelial cells for studying in vitro interactions with Salmonella typhimurium organisms, about 70% of bacteria developed

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Figure 1. A representative organism of the 'non-interactive' salmonellae located, in the ileal lumen, more than 2000 nm away from the epithelial cell lining. Amorphous surface structure of the organisms is notable (scale bar 500 nm).

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Figure 2. A section of the chicken ileum showing numerous transversely cut Salmonella 3,10,t- organisms closely (within 500 nm distance) interacting with epithelial cell microvilli (MV). A large majority of organisms show several periplasmic protrusions (arrows) on their surface. Periplasmic space (arrows) contains some secretory material, observed better in Figure 4 (p). The periplasmic protrusions are not observable in some non-interacting organisms (arrowhead) located a little farther from the microvilli as shown more specifically in Figure 1.

surface appendages (protrusions) in 15 min which increased to 90% in 30 min (ref. 10). With passage of time, these bacterial protrusions were progressively lost with concomitant increase in the number of ruffle-bearing host cells and subsequent bacterial uptake\(^6\). These studies\(^6\) thus corroborate the active physiological role, assigned in this report, to the pathogen surface protrusions for creating membrane ruffles in the host cells. With a technically lower resolution capability of scanning electron microscopy, Ginocchio et al.\(^10\) could not have resolved the precise ultrastructure of these protruding appendages or their felling as vesicles, as revealed by transmission electron microscopy from this laboratory\(^4,6\). Such membrane-bound surface protrusions\(^7\) which were subsequently shown to pinch off as bacterial outer membrane-bound vesicles\(^6,8\) thus substantiated the discovery\(^8\) of exocytosis from procaryotes, as these vesicles appeared to 'fuse' with the host cell membrane. This novel finding of the release of membrane-bound vesicles from Salmonella has now been extended to another gram-negative organism, Pseudomonas aeruginosa\(^9,12\), which has been shown to release membrane vesicles containing some virulence enzymes such as phospholipase C, protease, haemolysin and alkaline phosphatase, besides the endotoxin lipopolysaccharide (LPS) as an integral part of the vesicle-membrane\(^8\).

The observed intimate contact between the host microvillus membrane and the bacterial outer membrane-bound vesicles (Figure 5) confirms the earlier report\(^6\). This process of vesicular exocytosis is proposed here, as a mechanism for carrying the necessary biochemical signals from the invading pathogen (Salmonella) to the host epithelial cells. This new process of vesicular exocytosis also answers the long-pending question on the existence of a viable mechanism for mass transport (export) of virulence proteins and toxins across the outer membrane.

Figure 3. Structure of an organism (B) representing over 70% of salmonellae (also see Figure 2) found in close proximity (less than 500 nm) of microvilli (empty arrow) of epithelial cell lining of the chicken ileum. Numerous membrane-bound protrusions (bold arrows) are observable on the surface of these organisms. Some of these protrusions (p) are quite large (scale bar, 500 nm). Bacterial outer-membrane structure of the lining of these periplasmic protrusions is clear in Figure 4.

Figure 4. Electron micrograph of the edge of a Salmonella 3,10,t- organism (empty arrow) located in close proximity of the microvilli (curved bold arrow). A large periplasmic protrusion (p) is seen bounded by the outer membrane (short arrow) obviously 'detached' from the cell wall (long arrow) of the organism. The double-layer structure of the bounding outer membrane is clear in the space enclosed by the empty arrow (scale bar, 100 nm).
typhimurium, coincident with the appearance of ruffles in host MDCK cells and also the reported bacteriolysis of certain gram-negative bacteria upon fusion with similar membrane vesicles released by P. aeruginosa.

The concept of acquisition of a 'virulence state' by gram-negative organisms, during host-pathogen interactions, draws credence from (i) expression of invasion factors by salmonellae while they are in intestinal lumen, (ii) change in chromosomal DNA-supercolding for internalization of salmonellae, (iii) homology of salmonella secretory proteins with type III secretion system of other bacteria, (iv) dependence of this intracellular parasite (Salmonella) on its protein synthesis for invasion, (v) exocytic release of outer-membrane bound vesicles from Salmonella and their fusion with host cell membrane prior to invasion, (vi) secretion of virulence enzymes contained in membrane-bound vesicles by other gram-negative organisms and their predatory bacteriolytic role.

What triggers the process of acquisition of virulence characteristics by salmonellae is still unknown. However, the role of membrane permeabilizing antimicrobial peptides and proteins such as defensins, cationic antimicrobial proteins and polymyxins, released by host cells cannot be ruled out at present, which could directly or indirectly activate one or more of Salmonella genes for internalization of the pathogen. Ruffle formation (Figure 6) in the host epithelial cell is another important aspect of interest in studying the modus operandi of pathogen internalization which is achieved essentially through specific cytoskeletal rearrangement in the host cell.

In this report, ultrastructural observations on the release of membrane vesicles from 'interactive' Salmonella organisms and their 'fusion' with ileal epithelial cell microvillus membrane (Figure 5) is proposed to function as a novel mechanism of bulk transport for carrying the necessary biochemical signals. This initiates focal disruption of microvilli and formation of membrane ruffles in the epithelial cells, leading to the intracellular entry (Figure 7) of the Salmonella organisms (Figures 1, 3, 5, 6). It is also concluded that this new process of secretion of membrane-bound vesicles (exocytosis), which was earlier thought to be confined to eucaryotic cells, has its definite role in the gram-negative organisms for the release of outer-membrane bound vesicles containing virulence factors. These 'virulence bombs' may in turn undergo 'fusion' with host cells for invasion, as observed in this laboratory, or for bacteriolysis of other competing gram-negative and gram-positive organisms in the milieu. In addition, microbial DNA being transported through these membrane vesicles, into another cell may also perhaps control the recipient cell activities for a facilitated host cell invasion or bacteriolysis, as the case may be. The mechanism proposed here (see diagrams in Figure 7) also explains how lipopolysaccharide (endotoxin) may be released by gram-negative organisms as a...
Figure 7. A schematic representation of sequential steps (I, II & III) for entry of Salmonella organisms into host epithelial cells in chicken ileum in vivo. Diagrammatic interpretation (a, b, c & d) of electron microscopic observations (A, B, C & D, respectively in Figures 1, 3, 5, 6) is self-explanatory. Close interaction of salmonellae (b, c) with host epithelial cell (HEC) microvilli produces numerous periplasmic protrusions (p) filled with virulence factors (I), which pinch off as membrane vesicles (MV) from the organism (c) "fusing" with plasma membrane of microvilli thus discharging its contents into the host cell (II). This results in the formation of a ruffle (R) by rearrangement of host cell cytoskeleton (III), facilitating the entry (short empty arrow) of the organisms into the cell, accompanied by focal disruption of microvilli (curved empty arrows).

constituent of the bacterial outer membrane bounding the exocytotic vesicles, so released.

Culture of Salmonella 3,10:--, organisms isolated from cases of human food poisoning were received from the National Salmonella Centre, IVRI, Izatnagar. In vivo interactions were studied 18 h after injection (100 μl per ileal loop) of the Salmonella organisms (10⁶ CFU) into the chicken ileum in 24 h fasted birds by following standard ligated ileal loop methodology. Ileal tissue pieces were fixed in 2.5% glutaraldehyde in phosphate buffer (pH 7.0), block-stained in 1% aqueous osmium tetroxide, embedded in araldite and sectioned with LKB ultratome III. Mounted on 3 mm diameter grids, the sections were stained with uranyl acetate and lead citrate and finally examined under JEM-1200EX (JEOL) transmission electron microscope, following standard procedures.

Induced spawning and establishment of a captive population for an endangered fish, *Ompok bimaculatus* in India

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*Ompok bimaculatus* is an endangered fish species of high commercial value. Over the last few decades its wild population is declining rapidly (> 50%). This fish was induced to spawn by a single intramuscular injection of ovaprim (dosage 0.5 ml/kg body weight). Spawning was observed 5–6 h after injection. An average of 4012 ± 100 eggs were spawned by each female. Hatching occurred 24 h after spawning. Hatchlings were reared up to fingerling size after which they were released into an earthen pond and cultured for 6 months. Induced breeding of this fish enabled us for the first time to produce a captive population of 548 individuals.

*Ompok bimaculatus* popularly known as the *butter fish* is a freshwater teleost native to South-east Asia. In India its current distribution is the plains and submontane regions. It is a piscivorous and carnivorous fish inhabiting the lakes, ponds and rivers from an elevation of 100 to 2500 m. Over the last 10 years, its wild population has undergone a steady decline (> 50%) mainly due to over exploitation, loss of habitat, disease, pollution, siltation, poisoning, dynamite and other destructive fishing, due to which it is listed among the 91 endangered species of India according to IUCN status 2. With a view to re-establishing the population in wild and to develop breeders for stock management programme, induced breeding of this fish was attempted.

Spawners (Figure 1) were collected (2 ♦ ; 3 ♀) from Tamiraparani riverian associated wetlands around Tirunelveli (8.15°N; 77.45°E), Tamil Nadu, during April 1997 and were stocked in an earthen pond (7.5 × 5 × 1.5 m) at the Centre for Aquaculture Research and Extension (CARE) for six months after which they were induced to spawn by a single intramuscular injection of ovaprim (Syndel laboratory, Canada) at a dosage of 0.5 ml/kg body weight to both males and females. After injections at 17.30 h, the breeding set consisting of two males and a single female was released into a cement tank (capacity 1500 l). Aquatic macrophytes like *Hydrilla verticillata* and *Eichhornia crassipes* were introduced into the tank for hiding purposes. The results of induced breeding experiments are summarized in Table 1.

Figure 1. Breeders of *Ompok bimaculatus*. a, Male; b, Female.

Figure 2. 10-h-old embryo of *O. bimaculatus*.

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