

Review Article

Fantastic voyage: the journey of intestinal microbiota-derived microvesicles through the body

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As part of their life cycle, Gram-negative bacteria produce and release microvesicles (outer membrane vesicles, OMVs) consisting of spherical protrusions of the outer membrane that encapsulate periplasmic contents. OMVs produced by commensal bacteria in the gastrointestinal (GI) tract of animals are dispersed within the gut lumen with their cargo and enzymes being distributed across and throughout the GI tract. Their ultimate destination and fate is unclear although they can interact with and cross the intestinal epithelium using different entry pathways and access underlying immune cells in the lamina propria. OMVs have also been found in the bloodstream from which they can access various tissues and possibly the brain. The nanosize and non-replicative status of OMVs together with their resistance to enzyme degradation and low pH, alongside their ability to interact with the host, make them ideal candidates for delivering biologics to mucosal sites, such as the GI and the respiratory tract. In this mini-review, we discuss the fate of OMVs produced in the GI tract of animals with a focus on vesicles released by *Bacteroides* species and the use of OMVs as vaccine delivery vehicles and other potential applications.

Introduction

The production and release of membrane vesicles from microbial surfaces is a process that is conserved across the three domains of life. This includes Gram-negative and Gram-positive bacteria, archaea, fungi and protozoa [1]. It is only recently that compelling evidence for the production of EVs by microorganisms with thick cell walls, such as Gram-positive bacteria, mycobacteria and fungi, has been obtained [2]. Here, we focus on the more advanced findings of extracellular vesicles produced by Gram-negative bacteria and, in particular, by representatives of the genus *Bacteroides* that are prominent members of the vertebrate intestinal microbiota.

Gram-negative bacteria produce outer membrane vesicles (OMVs) which are spherical buds of the outer membrane that encapsulate periplasmic components. The molecular mechanism of their biogenesis is still unclear, although several models of OMV formation have been proposed and have been recently reviewed [3,4]. OMVs range in size from 20 to 400 nm and can transport a variety of biomolecules such as enzymes, toxins, antigenic determinants, nucleic acids [5] and metabolites [6,7]. OMV contents are protected from enzymatic degradation by a lipid bilayer envelope that protects against the harsh extracellular environments of the GI tract [8,9]. Here, we review our current understanding of the fate of OMVs produced in the GI tract of animals with a particular focus on those released by commensal *Bacteroides* species. We also provide a brief update on the use of OMVs as mucosal delivery vehicles for biologics.

Received: 13 May 2018
Revised: 23 July 2018
Accepted: 24 July 2018

Version of Record published:
28 August 2018

The fate of OMVs produced by commensal bacteria in the GI tract of animals

OMVs and digestion

OMVs from Gram-negative bacterial species are produced and released into the intestinal lumen [10,11] enabling them to affect their environment remotely from their parent cells. Members of the *Bacteroides* genus distribute hydrolases including proteases and glycosidases [12] within the GI tract lumen using OMVs as delivery vehicles (Figure 1) that contribute to the communal breakdown of complex polysaccharides, the products of which serve as a source of nutrients for other members of the intestinal microbiota and the host [13]. Other examples of enzymes distributed via *Bacteroides* OMVs include multiple inositol polyphosphatases [8] which degrade dietary phytate to release phosphate, inositol phosphates and inositol (Figure 1), mucin sulfatases [10] that make mucin glycans more susceptible to degradation by bacterial glycosidases (Figure 1) and β -lactamases involved in antibiotic resistance which contributes to antibiotic resistance of other members of the microbiome [14].

Interaction with the intestinal mucosa

OMV–epithelial cell interactions

Gram-negative OMVs can use several routes to cross the intestinal epithelial barrier, which differ according to bacterial species [15]. Both phagocytic and non-phagocytic pathways have been implicated in host–cell interactions with the GI tract, although it should be noted that most studies have, to date, focused on OMVs of pathogenic rather than commensal bacteria. The observation that microbiota-derived OMVs are phagocytosed by

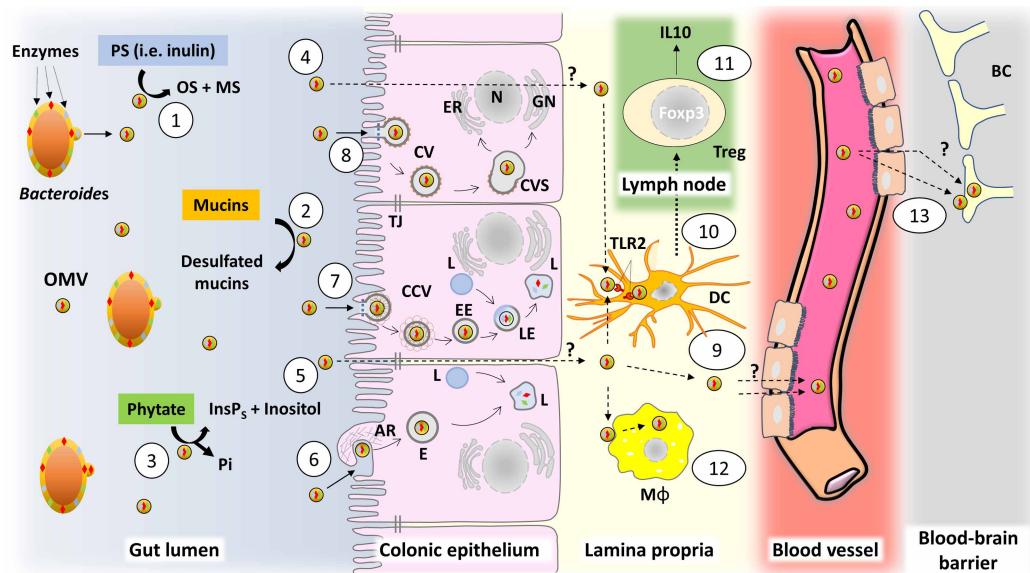


Figure 1. The predicted journey of a bacterial membrane vesicle from the gut to the brain.

The schematic depicts the (numbered) pathways and means by which OMVs produced in the lower GI tract by members of the intestinal microbiota can access and cross the epithelial barrier to gain access to underlying immune cells and the systemic circulation to access other organ systems and possibly the brain. 1. Degradation of polysaccharides by OMVs. 2. Mucin sulfatase activity carried by OMVs. 3. Degradation of inositol polyphosphates (i.e. phytate) by OMVs. 4. Transcellular transmigration. 5. Paracellular transmigration. 6. Macropinocytosis. 7. Clathrin-mediated endocytosis. 8. Caveolin-mediated endocytosis. 9. TLR2-dependent OMV internalisation by DCs. 10. DC migration. 11. Induction of Treg by DC modulated by OMVs. 12. Internalisation of OMVs by macrophages. 13. Hypotheses of OMV translocation across the blood–brain barrier. Abbreviations: OMV, outer membrane vesicle; PS, polysaccharide; OS, oligosaccharide; MS, monosaccharide; InsPs, inositol polyphosphates; TJ, tight junction; AR, actin remodelling; ER, endoplasmic reticulum; N, nucleus; GN, Golgi network; E, endosome; L, lysosome; CCV, clathrin-coated vesicle; EE, early endosome; LE, late endosome; CV, caveolar vesicle; CS, caveosome; DC, dendritic cell; Treg, regulatory T cell; M ϕ , macrophage; BC, brain cells.

immune cells in the lamina propria [16] and can be detected in the blood and urine [17,18] suggests that OMVs can cross the intestinal epithelium and vascular endothelium to reach sites beyond the GI tract. To do this, OMVs can utilise two distinct pathways to cross the intestinal epithelium or vascular endothelium: the paracellular (between cells) or transcellular (through cells) pathways.

Pathogenic OMVs can alter the intestinal barrier by modulating the permeability of cellular junctional complexes. *Campylobacter jejuni* OMVs cleave the adherens junction proteins E-cadherin and the tight junction (TJ) protein occludin [19]. *Helicobacter pylori* OMVs, containing the cytotoxin CagA, localise in the vicinity of cellular junctions and cause the redistribution of the TJ protein ZO-1 to the cytoplasm [20]. Among the few studies to date on commensal OMV–host cell interactions, the probiotic *Escherichia coli* Nissle 1917 strain has been shown to up-regulate expression of barrier-enhancing ZO-1 and ZO-2 proteins [21]. Together, these studies suggest that OMVs can directly or indirectly alter the composition of junctional complexes to enable them to cross epithelial barriers with this mechanism of trafficking being associated with virulence. It is possible that OMVs produced by commensal bacteria may also reduce TJ permeability, potentially limiting paracellular transport [22]. A challenge to identifying the specific routes of uptake and trafficking through host cells is visualising the localisation of nanosize OMVs and OMV-cargo alongside host proteins and organelles.

Using chemical inhibitors of endocytic pathways, OMVs have been shown to use all four endocytosis pathways: actin-dependant macropinocytosis, clathrin-mediated endocytosis, caveolin-mediated endocytosis or clathrin- and caveolin-independent mechanisms such as membrane fusion or lipid raft formation (reviewed in ref. [5]). Identifying the specific pathways of OMV and OMV-associated cargo entry into host cells has, however, proved challenging as chemical inhibitors are not always specific [23]. Also, as OMV preparations are heterogenous and consist of a range of vesicle sizes, they are likely to be internalised in a size-dependant manner, with potentially several endocytic pathways in simultaneous use [24–27].

The majority of endocytic routes of OMV uptake culminate in sequestration and degradation by lysosomes. Depending on the mechanism of endocytosis, internalised OMVs generally join either the host endolysosomal pathway or autophagy pathway (Figure 1). Caveolin-dependant endocytosis is an exception as it involves invagination of the host plasma membrane and delivery into specialised endosomes termed caveosomes which transport their cargo via the cytoskeleton to the host endoplasmic reticulum/Golgi complex. As this route of uptake does not merge with lysosomes, it may be utilised by OMVs to escape lysosomal degradation (Figure 1).

OMV–immune cell interactions

There are several lines of evidence that OMVs produced by *Bacteroides* interact with the mucosal immune system. Shen et al. [16] have demonstrated that OMVs from *Bacteroides fragilis* harbouring the capsular polysaccharide A (PSA) are sensed and internalised by dendritic cells (DCs) in an actin-dependent manner involving the Toll-like receptor (TLR) 2. This results in enhanced regulatory T cells (Treg) and production of the anti-inflammatory cytokine IL-10 as well as the Foxp3 transcription factor (Figure 1), a marker of Treg cells associated with protection from inflammatory and autoimmune diseases [28,29]. This finding has been interpreted as OMVs inducing host immunological tolerance to PSA or OMV-associated PSA. Within the context of OMVs promoting a protective effect in experimental models of colitis, Chu et al. [30] have shown that OMVs require the Crohn's disease (CD) susceptibility alleles ATG16L1 and NOD2, to activate a non-canonical autophagy pathway. For example, ATG16L1-deficient DCs lose the ability to induce Treg which in turn compromises the suppression of mucosal inflammation. Moreover, immune cells from human subjects with a major CD risk variant in the ATG16L1 allele are defective in Treg responses to OMVs [30]. Hickey et al. [10] have shown that access of *Bacteroides thetaiotaomicron* OMVs to the mucosal immune system is dependent on the activity of one or more sulfatases. Considering that mucin glycans are protected from enzymatic breakdown by sulfate residues [31,32], it is tempting to speculate that localised mucin degradation by OMVs facilitates their access to the intestinal epithelium and underlying cells of the mucosal immune system.

OMVs cross the intestinal mucosa and enter the bloodstream

Detection of OMVs in the blood

Results from various studies suggest that serum from healthy individuals contains genomic DNA of bacterial origin [33–35] in a phenomenon termed DNAemia. In these studies, DNA was extracted from blood samples using protocols primarily designed for bacterial cell disruption and cell lysis during denaturation steps in PCRs. While the presence of these blood-associated bacterial DNA sequences was inexplicable when first described

[33], it has recently been shown in a mouse model that blood-linked bacterial DNA is associated with bacterial membrane microvesicles that appear to be natural blood compounds, the diversity of which reflects the bacterial diversity of the intestinal microbiota [17]. This finding implies that bacterial microvesicles produced by bacteria in the GI tract may normally cross the intestinal epithelial barrier to reach the bloodstream. In their study, Park et al. claimed that based upon metagenomic sequencing, blood-derived microvesicles represent an alternative to faecal sampling for profiling the human intestinal microbiota and could constitute a new tool for evaluating variations of the intestinal microbiota (microbial dysbiosis) in the context of neurodegenerative diseases.

Nucleic acids of bacterial origin in the brain

Access to the brain via the blood stream is controlled primarily by the blood–brain barrier (BBB) composed of brain microvascular endothelial cells which line brain capillaries and effectively regulate molecular and cellular trafficking between the bloodstream and neural tissue. Maintaining the integrity of the BBB is essential for limiting the entry of potentially neurotoxic plasma components, blood cells and pathogens into the brain [36,37]. Additionally, TJs and adjacent perivascular cells, including astrocytes and pericytes, reinforce the BBB function [38]. A handful of studies have provided evidence for the presence of rRNA and rDNA from commensal bacteria in the brain and that it may have a unique microbiota dominated by fastidious obligate intracellular α -proteobacteria [39–41]. Although one study [39] has provided evidence of culturable bacteria in the brain, the majority of studies have relied on culture-independent, sequence-based approaches to identify brain-associated microbes. In view of the presence of bacterial microvesicles in the bloodstream and their ability to cross boundary epithelial cells, it is interesting to speculate that a fraction of these microbial nucleic acids might originate from, and gain access to, the brain via bacterial microvesicles. Alternatively, microvesicles may also be produced by brain-resident bacteria.

OMV-based vaccines

The nanosize and non-replicative status of OMVs together with their resistance to enzymes and low pH, alongside their ability to interact with mucosal and systemic host cells, makes them ideal candidates for drug delivery. Moreover, they possess innate adjuvant properties and the ability to activate immune cells via interactions between microbe-associated molecular patterns (MAMPs) or capsular polysaccharides they carry and TLRs on the surface of host antigen-presenting cells and, in particular, DCs [16].

Meningococcal OMV vaccines

Commercial formulations of *Neisseria meningitidis*-generated OMV vaccines have been used for epidemic control of group B *N. meningitidis* infections in New Zealand and Cuba [42,43]. In these outbreaks, group B *N. meningitidis* OMVs were effective in controlling infection and conferring protection in both adults and children [42,43]. More recently, a meningitis serogroup B vaccine, Bexsero, which contains *N. meningitidis* OMVs, was approved by the European Medicines Agency (EMA) and the Food and Drug Administration (FDA) [44].

The *N. meningitidis* OMV vaccines used, to date, in commercial preparations are administered parenterally [45] although several reports have shown the effectiveness of meningococcus OMV vaccines at eliciting mucosal responses [46–48]. OMVs from other Gram-negative bacteria have been formulated for use as experimental vaccines, with some showing induction of protective immune response upon mucosal administration via the intranasal or oral route [49–51].

Development of new mucosal OMV vaccines

The high stability of OMVs together with their ability to interact with the host immune system makes them attractive candidates for the development of new mucosal vaccines. As mucosal sites are the main location of entry of most pathogens, with induction of the mucosal immune system and neutralising (IgA) antibody production being required for immune protection, the development of such vaccines is of extreme importance, especially in light of the increase in antibiotic resistance among major bacterial pathogens.

Studies carried out to date using pathogen-derived and modified OMV vaccine formulations show that they are effective at inducing protective systemic and mucosal antigen-specific antibody and T-cell responses. Of interest, Schager et al. [52] have recently shown that administration of OMVs produced by hyperblebbing strains of *Salmonella* Typhimurium can generate protective antibodies in mice. OMVs produced by different bacteria are equipped with specific sets of proteins that can bind to receptors on host intestinal antigen sampling M cells and DCs. M cells are present in the follicle-associated epithelium of the small intestine as well as

the colon and rectum and are responsible for the sampling and uptake of luminal particulate antigens. Luminal antigens can also be acquired by DCs either directly or indirectly via epithelial cells that then initiate immune responses via activation of antigen-specific B cells and T cells.

M cells express an array of receptors that enable them to recognise various food/microbe/host cell-derived ligands that have been exploited to enhance M cell uptake by mucosal vaccines. For example, nanoparticles coated with UEA-1, a specific lectin and ligand for α -L-fucose present on the apical membrane of M cells, are able to effectively induce both mucosal and systemic immune responses in mice [53]. Bacterial OMVs, which have been used as vaccines (described above), are decorated with MAMPs which allow them to interact with the immune system, in particular, they can be recognised by TLR-4 and α 5 β 1 integrin receptors present in M cells [54,55]. M cell-restricted receptors have been identified including PrPC and the C5a molecules, which interact with the Hsp60 of *Brucella abortus* and with OmpH of *Yersinia enterocolitica*, respectively [56,57]. Another example is glycoprotein 2 (GP2) expressed on the luminal surface of M cells which interacts with the type-I pili of bacterial outer membranes [58]. Type-I pili are composed of FimA monomers and are present in Gram-negative pathogens including *E. coli* and *Salmonella* Typhimurium [59]. The genes encoding FimA superfamily proteins are often represented in the intestinal microbiome and in particular, in *Bacteroides* genomes where they can be found as putative pilus biogenesis operons [60]. Our proteomic analysis of OMVs produced by *B. thetaiotaomicron* has revealed at least one FimA-like protein (unpublished data). Thus, OMVs from bacteria containing FimA-like proteins, Hsp60 or OmpH might be ideal candidates for mucosal antigen delivery vaccines.

Concluding remarks

Extracellular microbial structures, such as OMVs and more generally microvesicles, are viewed as a new type of secretion system that enables the dissemination of membrane-encapsulated cellular materials including proteins, nucleic acids and metabolites into the extracellular milieu [4]. Microvesicles released by commensal microorganisms into the intestinal lumen have the potential to contribute to host physiology including digestion by distributing hydrolase activities across the lumen to help maximise digestion of macromolecules. They are internalised by boundary epithelial cells using different routes. They can also cross epithelial barriers enabling them to interact with mucosal immune cells to induce host immunological tolerance and to disseminate more widely around the body and perhaps the brain, via the bloodstream. It is becoming clear that microvesicles produced by commensal bacteria can mediate interkingdom cross-talk between the intestinal microbiota and the host and that these interactions confer a health benefit to the host. In addition, the potential of OMV as vehicles for the delivery of biologics and vaccine antigens is beginning to emerge. Different approaches to expressing heterologous proteins in these microvesicles [61,62] open new possibilities for the use of OMVs as vaccine delivery vehicles with significant advantages over current vaccine strategies and those that aim to protect mucosal surfaces from pathogen invasion.

Abbreviations

BBB, blood–brain barrier; CD, Crohn's disease; DC, dendritic cell; GI, gastrointestinal; MAMPs, microbe-associated molecular patterns; OMV, outer membrane vesicle; PSA, polysaccharide A; TJ, tight junction; TLR, Toll-like receptor; Treg, regulatory T cell.

Funding

This work was supported by an institutional grant from the Biotechnology and Biological Sciences Research Council [BBSRC; BB/J004529/1, S.R.C.].

Competing Interests

The Authors declare that there are no competing interests associated with the manuscript.

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