Utilizing Bacterial Mechanisms of Epithelial Cell Entry: Invasin-induced Oral Uptake of Latex Nanoparticles

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Received August 6, 1997; accepted October 23, 1997

KEY WORDS: nanoparticles; oral uptake; invasin; Peyer's patches.

INTRODUCTION

It is now generally accepted that small amounts of inert, micro-particulate matter can breach the intestinal barrier and enter the systemic circulation. Our laboratory over the last decade has consistently demonstrated, both quantitatively and histologically, the oral translocation of nanoparticles through Peyer's patches and normal epithelial cells and that such transport processes can be modulated by engineering particle surface characteristics or by use of a bioadhesive surface ligand (1,2).

Recently we demonstrated significantly enhanced oral uptake of latex nanoparticles surface-coupled with tomato lectin into the systemic circulation (1). After 5 days continuous dosing, approximately 23% of particles were found in the serum, and the extent of absorption was 15 fold higher via villous tissue than by the way of Peyer's patches. Peyer's patches have been the intense focus of investigation in recent years. We thus surmised that the use of ligands that were bound, and could be internalised, by the normal epithelial cell compared to the heterogenously distributed and relatively scarce M-cells, could offer a very large, yet, untapped potential surface area for the absorption and subsequent systemic delivery of nanoparticles.

Facultative intracellular bacteria such as *Yersinia, Salmonella* and *Shigella* sp. (3) are able to penetrate epithelia and become internalised by normally non-phagocytic mammalian cells through either inducible or constitutive expression of enteroinvasive bacterial surface proteins. We have coupled to the surface of latex nanoparticles a truncated form of an outer membrane protein from *Yersinia pseudotuberculosis* (4) termed invasin that is capable of evoking non-professional phagocytic cells e.g. enterocytes to internalise the carrier displaying this protein. Invasin, a 986-amino acid outer membrane protein isolated both from *Yersinia pseudotuberculosis* and *Yersinia enterocolitica*, is a chromosomally encoded virulance factor (5) that allows these pathogens to enter the intracellular epithelial compartment that confers protection of bacteria from the host's immune system and chemotherapeutic agents. Although several invasion factors exist, the highest level of penetration into cultured mammalian cells is promoted by invasin, which when surface-expressed in non-invasive bacteria (4,5) or coated on metabolically inert latex microspheres (6), is alone sufficient to confer invasive ability into cultured cells. Subsequent genetic and molecular analysis of truncated derivatives of this protein has revealed that the carboxyl-terminal 192 amino acids are necessary and sufficient to promote attachment and entry (7) and latex beads coated with this terminal region have also been reported to be efficiently internalised by mammalian cells (3).

Upon binding to non-phagocytic cells, invasin-bearing carriers are internalised rapidly without requiring any other bacterial factors, as shown by the uptake of latex beads coated with invasin containing membranes (6) or purified truncated derivatives (3) by cultured mammalian cells This is in sharp contrast to tomato lectin where high uptake of coupled nanoparticles was only apparent after chronic dosing in animals (1), and little or no uptake was detectable after a single day's administration (unpublished observations). The use of invasin may prove to be a more fruitful approach in unlocking the endocytic potential of the epithelial cell. It was therefore of interest to assess the effect *in vivo* of the carboxyl-terminal 192 amino acid fragment of invasin in promoting the translocation of orally gavaged invasin coupled nanoparticles. The relative simplicity of invasin-mediated internalisation suggests that it might be exploited to introduce macromolecules or colloidal materials into a variety of mammalian cells. In this communication we demonstrate that up to 13% of maltose binding protein (MBP)-invasin-C192-coupled nanoparticles were present in the cardiovascular compartment within 24h of oral administration, whereas those blocked by pre-incubation with porcine mucin (8) or only displaying MBP were significantly impaired in their ability to translocate.

MATERIALS AND METHODS

Isolation and Purification of Carboxyl-terminal 192 Amino Acid Truncated Derivative of Invasin

Maltose-binding protein (MBP) and the fusion protein containing the carboxyl-terminal 192 amino acids of invasin fused to MBP were produced in an *E. coli* strain using the p-MAL-p2 plasmid fusion system (New England Biolabs, Massachusetts, USA). These recombinant bacterial strains expressing either the MBP or the fusion protein composed of MBP coupled to the C-terminal 192 amino acids fragment, that is alone sufficient to confer the invasive phenotype, were constructed by Dr John Tite and colleagues at the former Wellcome Research Laboratories (Dartford, United Kingdom). Their generous gift of the *E. coli* strain allowed the experiments described herein to be conducted (9). For brevity, the isolation of MBP-invasin-C192 and MBP itself was conducted as described in detail (7). The only alterations made in our isolation was the use of a Branson sonicator (50% amplitude, 3 × 15s pulses) on an ice-water bath for the lysis of bacterial cells and the use of a micro-ultrafiltration system (Model 8MC stirred cell, Amicon, UK) using a low protein-binding ultra-filter membrane (25mm, YM10, Amicon, UK) with 10kDa molecular weight cut-off to concentrate the purified proteins (1mL). Typically between

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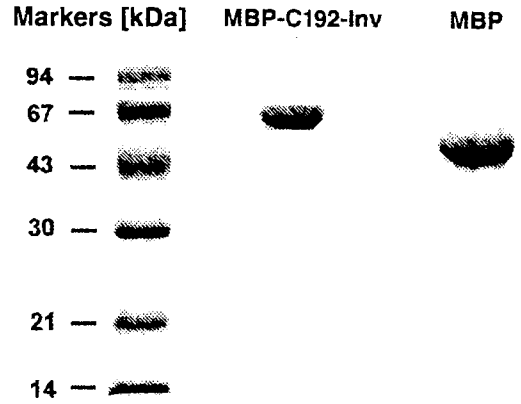

Fig. 1. SDS-Page (12.5%) analysis of purified MBP and MBP-Inv-192 fusion proteins. The predicted molecular mass for MBP and MBP-invasin fusion protein are 45kDa and 63kDa respectively (7).

2–5mg of each protein could be purified from 500mL of cultured cells as estimated by the Bradford assay. Expression of both proteins was confirmed using SDS-Page analysis (Figure 1). Invasin-C192 was not cleaved from MBP using factor Xa, as the fusion protein is more stable than the truncated invasin-C192 carboxyl fragment alone (7).

Coupling of MBP-Inv-C192 Fusion Protein to Nanoparticles

Purified proteins were coupled to 500 nm fluorescent carboxylated nanoparticles (Polysciences® Inc. Warrington, USA) using water-soluble carbodiimide as described in detail for tomato lectin (1). Nanoparticles were sized before and after the coupling procedure using photon correlation spectroscopy (Malvern Autosizer 2C, Malvern, United Kingdom); coupling increased the apparent particle size by an average of 30nm. Coupled nanoparticles were incubated with BSA to prevent non-specific adsorption. Peptide conjugation to nanoparticles can occur either through the smaller invasin-C192 moiety or within the carrier molecule (MBP) via their basic amino acid. Thus multiple conjugation points are possible including intramolecular cross-linking, that could result in a biologically inactive molecule. The fact that β1-integrin receptors from the human placenta could be purified with high yield using invasin-agarose affinity chromatography, in which truncated invasin derivatives (MBP-Inv-479) were covalently crosslinked to agarose beads, strongly indicates the retention of biological activity following immobilisation onto inert supports (10).

In addition to MBP coupled nanoparticles as a control, a further control consisting of fusion protein-coupled (ie. MBP-Inv-C192) nanoparticles pre-incubated with 2% porcine mucin was employed. Comparison of the ability of simple and complex carbohydrates to inhibit bacterial invasion of *E. coli* expressing invasin on its outer membranes indicated that porcine mucin was by far the most effective competitive inhibitor of invasin (8).

Administration, Extraction and Quantification of Nanoparticles

A single dose (0.1mL or 12.5mg/Kg or 6×10^9 particles) of the nanoparticle suspension was given orally to a group of 4 rats which were sacrificed 24h later. Quantification of tissue extracted latex from excised systemic organs and intestinal regions using gel permeation chromatography (GPC) was accomplished as described in detail in earlier reports (1,2). Extracted latex was quantified using a standard curve (signal vs amount injected in μg) generated from tetrahydrofuran-dissolved unmodified nanoparticles from the same batch as those used in the experiments (1,2).

RESULTS AND DISCUSSION

After a single dose 13% of MBP-Inv-C192 nanoparticle conjugates (thereafter referred to as invasin conjugates) were found in the systemic circulation significantly higher than the 2 controls [MBP-conjugates and MBP-Inv-C192 conjugates pre-incubated with mucin (=blocked)] which both attained ~2% (n=1) in the blood (Table 1). Our results are in line with previous reports in which *E. coli* expressing invasin on its outer surface (5) or *Staphylococcus aureus* coated with antibody-immobilized invasin derivatives (11), and inert particles coated with invasin containing membranes (6) or purified truncated derivatives (3) all entered cultured cell lines. However the preliminary *in vivo* results here indicate invasin's capability of enhancing the transcytosis of nanoparticles across the epithelial barrier. It also demonstrates and confirms previous findings that other bacterial components are not necessary for internalisation to succeed, nor does it require energy derived from the metabolic activity of the microbe (6). Furthermore the results also suggest that the MBP moiety does not appear to be involved in the epithelial entry process of nanoparticles despite its possible lectin-like activity, thus confirming previous reports that the C-192 terminal domain of invasin *per se* can induce particulate uptake.

The site of intestinal uptake of the nanoparticles was less clear. From previous reports 1μM latex nanoparticles coated with invasin-containing membranes are internalised in a matter of minutes upon binding to apical membrane of cultured epithelial cells via β-1 integrin receptors (6). This may explain the lack of detection of invasin conjugates from both the large and small intestine due to the rapid ingestion and clearance of invasin-conjugated nanoparticles over the 24h period prior to sacrificing of the rats. Despite the fact that the levels of invasin- and MBP-conjugated nanoparticles in the intestine were not readily detectable using GPC, histological evidence presented

Table 1. Organ Uptake of Invasin- and MBP-Conjugated Latex Nanoparticles (500 nm) Upon Oral Administration (% of Administered Dose)

	Invasin-C192-MBP	Invasin-C192-MBP and mucin	MBP
Peyer's patches	n/d	n/d	0.25 n = 1
Villous tissue	n/d	6.4 n = 1	n/d
Colon	n/d	n/d	0.3 n = 1
Liver	n/d	n/d	n/d
Blood[a]	1.47 ± 0.9	0.31 n = 1	0.24 n = 1
Systemic[b]	13.0 ± 5.3*	1.98* n = 1	2.0* n = 1

N = 4 n/d = not detectable by GPC *P ≤ 0.05.
[a] Approximately 2 mL blood taken directly via cardiac puncture.
[b] Extrapolation to whole blood [12.8 mL for 200g rat, or 64 mL/Kg (15)].

in Figure 2A shows clearly the great abundance of invasin-conjugated nanoparticles deposited in the serosal layer of distal ileum compared to the sparse appearance of MBP-conjugates and blocked nanoparticles in the same region (not shown). These histological observations correspond with the quantified systemic data indicating that invasin conjugates are able to enter the epithelial layer, and that nanoparticles solely coupled to the carrier itself (MBP) or blocked with porcine mucin were unable to bind or translocate the epithelia despite the presence of a carbohydrate-binding moiety. Further, the latter result suggests that in spite of the possible lectin-like activity of MBP, there appears to be a deficiency of maltose residues on the rat apical enterocyte membrane and translocation of invasin-conjugates is probably due to the truncated invasin derivative. The lack of maltose residues/sequences has also been previously reported by other workers probing the rat intestinal epithelium with glucose/mannose binding lectins (12). This is further supported by the low systemic appearance of invasin-conjugates pre-incubated with mucin, a highly sialylated polymeric glycoprotein that has been previously shown to be a strong competitive inhibitor of the entire invasin molecule (8). The anomalous high level of intestinal adsorption associated with blocked invasin conjugated nanoparticles in one rat (out of a group of 4) may either be due to the incomplete removal of intestinal contents during excision of this organ (since very few nanoparticles were seen in intestinal serosal layers from this mouse), or it may be the reason why the same animal gave rise to the low systemic uptake of blocked nanoparticles (1.98%, n = 1). From this result one would expect that invasin-coupled nanoparticles should also interact with the rat mucin, yet 13% of the administered dose is found in the blood. The reason for this anomaly is presently unclear but it perhaps reflects the differing properties of the two mucins or the fact that some of the administered dose is able to escape and reach the apical enterocyte surface (13).

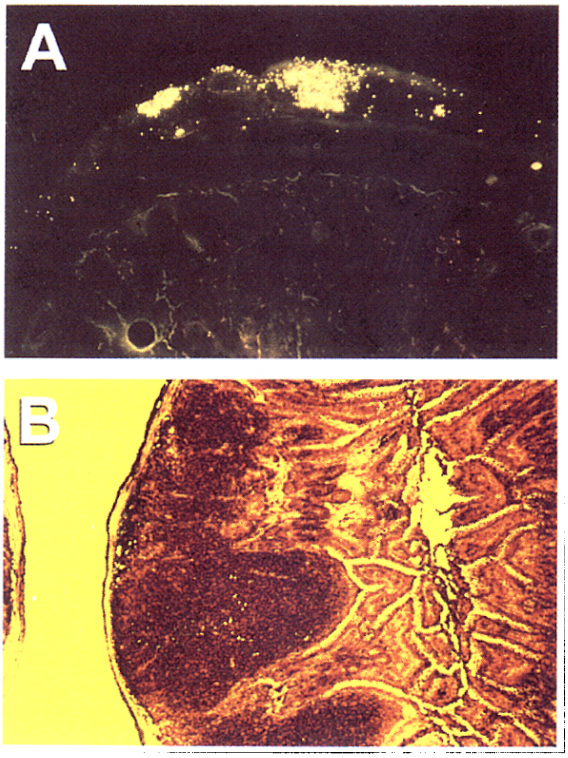

Fig. 2(A). Photomicrograph (taken under UV-light) of invasin-coupled nanoparticles abundantly deposited in the serosal layer of the distal ileum, the nanoparticles accumulating in the serosa outside the thick layer of muscular tissue (× 100); **(B)** Photomicrograph of haematoxylin/eosin stained (14) distal ileal tissue from rats dosed with invasin coupled nanoparticles. Notice the Peyer's patch characterised by the two dense follicular regions with overhanging and intermediate villi (× 40). No apparent damage is observable, such as loosening of villi and cryptic regions.

Although throughout our studies there has been a direct correlation between the levels of nanoparticles seen in tissue sections and the magnitude of uptake in such tissues as assessed by quantification of extracted latex by GPC (1,2), the uncoupling observed here with invasin-conjugated nanoparticles serves as a reminder of the limitations of using optical methods as the sole method of quantifying intestinal particulate absorption. Histological examination of cryostat tissue sections from different regions of the intestine (ie jejunum, proximal and distal ileum as well as Peyer's patches from such regions), revealed varying levels of distribution, binding and true translocation to the serosal layers of invasin-conjugated nanoparticles, with the distal ileum being the most heavily deposited region (Figure 2A) and the proximal ileum/duodenum the least. Examination of haematoxylin and eosin stained tissue sections (14), as well as unstained tissues without UV light, indicated no apparent damage that may have resulted in such selective congregation of invasin conjugates in the distal portion of the intestine (Figure 2B).

This study indicates that a unique bacterial protein involved in the epithelial translocation of *Yersinia* is able to retain its functionality upon conjugation to latex nanoparticles and to induce their epithelial absorption. As enterocytes are the most abundant cells in the intestine it is logical to target these for absorption of colloidal carriers. Despite our use of two controls to determine the specificity of uptake, further studies are required employing a greater number of animals and employing specific inhibitors such as the recently discovered disintegrin molecules.

Little is known about the intracellular biochemical pathways that leads to the mobilisation of the endocytic apparatus and cytoskeletal rearrangements, the complexity of which precludes detailed discussion here. Rather, research in the area of bacterial and viral cell entry should not only yield novel potential ligands to overcome biological barriers to particulate uptake but more information on the untapped latent endocytic potential of our own epithelial cells for the design of novel strategies to circumvent epithelial membrane barriers.

ACKNOWLEDGMENTS

N. H. was supported by the joint RPSGB/Syntex Research CRISP Award. We are grateful to Mr. David McCarthy for the cryostat histology and preparation of the photomicrographs. In addition we would like to extend our gratitude to Dr. John Tite of Glaxo-Wellcome, United Kingdom for the generous gift of the recombinant *E. Coli* vector and to the Biomedical Communication/Art department at St. Jude Children's Research Hospital for their additional photographic work.

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