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Modulation of Intestinal Permeability: A Novel and Innovative Approach for the Oral Delivery of Drugs, Macromolecules and Antigens

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Introduction

In the past few years, we have witnessed an explosion in research aimed at creating new oral drug delivery systems. This research has been fuelled by unprecedented challenges, such as the need to deliver newer and more complex drugs (such as proteins, hormones, etc.) that are becoming available through genetic engineering. Consequently, the need has arisen for further investigation into utilizing the intestine as a prime site for targeting the absorption on these new compounds. One potential and attractive mechanism would be to exploit avenues that increase intestinal permeability. Theoretically, three transepithelial pathways are available for the passage of molecules from the intestinal lumen into the bloodstream (*Figure 16.1*): (1) transcellular (ie through the cell) carrier-mediated active or facilitated transport; (2) transcellular passive transport; and (3) paracellular (ie between adjacent cells) transport. With the exception of those molecules that are transported by active or facilitated transcellular mechanisms, the absorption of large hydrophilic macromolecules is mainly limited to the paracellular pathway (Lee *et al.*, 1991). Under normal conditions, however, this pathway is restricted to molecules with molecular radii < 11 Angstroms and, therefore, is not accessible to large compounds.

To overcome the intestinal barrier, several strategies have been developed to target either the transcellular or the paracellular pathway for drug delivery. The most

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Abbreviations: ASC, antibody secreting cell; SigA, secretory IgA; Tj, tight junction; Zot, zonula occuldens toxin; CT, cholera toxin; PEG, polyethylene glycol; NALT, nasal associated lymphoid tissue.

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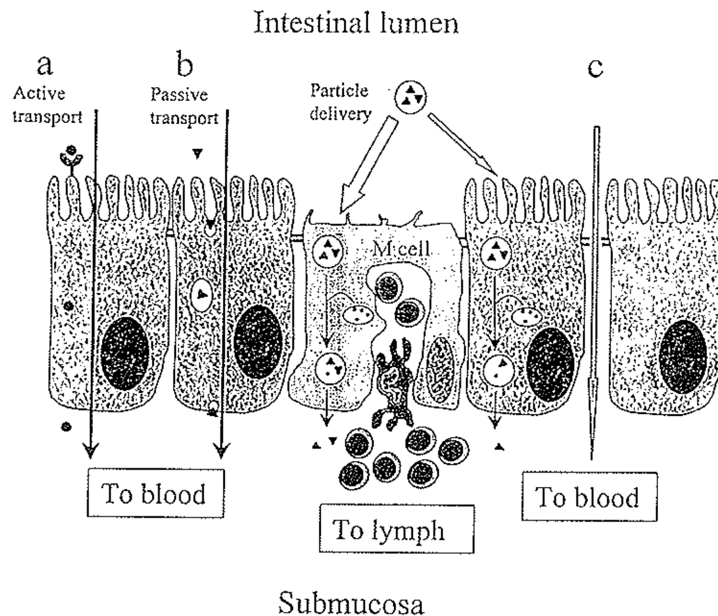

Figure 16.1. Schematic representation of the three transepithelial intestinal pathways: (a) transcellular active transport; (b) transcellular passive transport; (c) paracellular transport. The carrier-mediated, transcellular active transport is limited to small molecules, such as sugars and amino acids, but the other two pathways are theoretically available for oral delivery of drugs and vaccines because they do not require the presence of specific carriers for the transepithelial transport of molecules. The transcellular passive transport may be enhanced by entrapment of the active components in microspheres that are more efficiently taken up through the M cells; particles absorbed through intestinal epithelial cells (enterocytes) are subject to degradation by lysosomes and, therefore, less efficiently absorbed. The paracellular pathway may be used for drug and peptide delivery by modulating the permeability of tight junctions.

promising techniques currently available will be reviewed, highlighting the advantages and disadvantages of each system.

Transcellular pathway

The intestinal epithelium represents the largest interface (more than 200 m^2) between the external environment and the internal host milieu, and constitutes a major barrier through which molecules can either be absorbed or secreted. Conceptually, the phospholipid bilayer of the plasma membrane of the epithelial cells that normally line the intestine (the enterocytes) is considered to be the major factor restricting the free movement of substances from the lumen to the bloodstream through the transcellular pathway. The uptake of hydrophobic molecules usually occurs by passive diffusion because the cell membrane behaves like an inert barrier and the molecules enter the cell by endocytosis through the apical cell membrane. Facilitated and active transcellular transport occurs via specific carriers for smaller molecules, including sugars and amino acids, while the enterocyte membrane is almost impermeable to large and hydrophilic substances, such as proteins. Therefore, strategies have been developed that apply the principle of the 'Trojan horse': the macromolecules to be

delivered are hidden inside hydrophobic, biodegradable microspheres which intestinal cells take up by endocytosis (*Figure 16.1*). Even if, theoretically, this seems to be a solution to the problem, several factors may affect the extent of uptake of particles across the gut.

PARTICLE SIZE, SURFACE AND INTESTINAL TARGET

Particles currently used for drug delivery fall into two classes: (1) nanoparticles, ranging in size from 10 to 1,000 nm, and (2) microparticles, in the size range 1–1,000 μm. For oral delivery, nanoparticles seem to be more efficiently absorbed because the uptake of particles within the intestine increases with decreasing particle size and increasing hydrophobicity (Kreuter, 1996). Furthermore, the extent and pathway of nanoparticle uptake is different in different parts of the intestine (Michel *et al.*, 1991). The M cells of the Peyer's patches (*Figure 16.1*) represent a sort of lymphatic island within the intestinal mucosa, and possibly the major gateway through which particles can be absorbed.

The M cells of Peyer's patches make an attractive target site for the absorption of peptides. These cells are largely devoid of lysosomes, thereby greatly reducing intracellular proteolysis. Anatomically, M cells have a shorter distance between the apical and basolateral surfaces when compared with adjacent columnar epithelia, reducing transit time (Brayden and Baird, 1994). Lastly, the first pass effect is no longer a limiting factor because macromolecules taken up within M cells are presented to the thoracic duct of the lymphatic system bypassing the hepatic circulation. Taken together, there are several key structural features of M cells which make their continued study an important avenue of active research. Moreover, studies addressing the potential targeting of pharmaceutical agents to these cells, particularly antigens (vaccines) and immunoglobulins, may enhance uptake efficacy, which is a limiting factor in drug delivery research.

Although the uptake of micro- and nanoparticles is an accepted biological phenomenon, the intracellular mechanisms involved remain to be elucidated. Without an accepted model detailing the events of absorption, it is not possible to address one of the major limitations currently hampering drug delivery research: efficacy. At present, the extent to which these particles are absorbed varies, making their general use in pharmaceutical research difficult, if not impossible.

DOSE AND ADMINISTRATION VEHICLE

A critical parameter affecting intestinal delivery of pharmaceutical agents resides within selection of the administration vehicle. There are several key areas that can affect the absorption of drugs via the gastrointestinal system. Namely, rapid transit through the gastrointestinal system, degradation, and low transmucosal permeability (Harding *et al.*, 1999). Selection of a proper vehicle can potentially prolong transit time and enhance mucosal permeability. Liposomes and mucoadhesives are the main vehicles currently under investigation as a means for increasing efficacy of oral delivery. As mentioned previously, the M cells are prime target sites in drug delivery paradigms. Additional features of M cells include bioadhesive properties for peptide encasing polymers, namely latex microspheres and cyanoacrylate nanoparticles.

Mucoadhesives encompass a broad class of administration vehicles with interesting properties. One class of mucoadhesive polymers, poly(acrylic acid), has been shown to inhibit proteolytic enzymes, thereby adding an additional layer of protection for protein during transit through the gastrointestinal system (Lehr, 1994). Additionally, there is growing evidence that interactions between mucoadhesive polymers and epithelial cells may directly influence the permeability of the gastrointestinal epithelia. Non-specific interactions between mucoadhesives and the gut have been shown to temporarily 'loosen' intercellular tight junctions, allowing passage of some molecules via the paracellular pathway (Lehr, 1994).

Several studies have shown that the intestinal uptake of nanoparticles is dose dependent (Le Fevre and Joel, 1984; Ebel, 1990). Le Fevre and Joel (1984) have shown that nanoparticles were identified in Peyer's patches with difficulty after one day of feeding, but were readily identified following chronic feeding. Peroral drug delivery may be further enhanced by addition of mucoadhesive substances to the nanoparticles, thus providing longer interaction of the particles with the cell membrane (Kreuter, 1996). An alternative strategy to increase the interaction of nanoparticles with their target cells is to mix them with lipid delivery vehicles, such as lecithin (Thomas *et al.*, 1996). However, an important caveat to consider with lipid vehicles is that they have the ability to both enhance and depress the absorptive process. Comparative studies utilizing oleic acid and lecithins clearly demonstrates that lipid interactions vary widely, and interactions taking place at the plasma membrane of the enterocyte can potentially influence absorption processes (Thomas *et al.*, 1996). When compared to saline controls, lecithins promoted a two-fold increase in particle absorption, whereas oleic acid preparations failed with respect to controls (Thomas *et al.*, 1996).

ANIMAL SPECIES, AGE AND FOOD INGESTION

The extent of uptake of nanoparticles in rabbits seems to be at least an order of magnitude greater than in mice, probably because of the much greater abundance of M cells in rabbit Peyer's patches (Pappo and Ermak, 1989). The age of the animal also seems to affect particle uptake, with greater absorption observed in older animals (Le Fevre *et al.*, 1989). The presence of food seems to be another enhancing factor for particle uptake, possibly because it may increase the intestinal transit time (Harding *et al.*, 1999).

Clearly, there are several compounding variables that can dramatically alter the effects of particle uptake in *in vivo* animal models. Since the rate-limiting step for particle uptake is permeation across enterocytes, human intestinal epithelial cell lines grown on porous filters provide an attractive alternative for the screening of molecules for oral administration (Brayden, 1997). Using this approach, it is possible to gain insights into the permeation pathway of a particular compound, minimizing the confounding factors present *in vivo*. The many useful experimental questions that can be deciphered using cell models are not within the scope of this review. However, it is worth stressing the concept that experimental oral drug delivery systems can be exploited using *in vitro* intestinal cell models before moving to *in vivo* studies.

LIMITATIONS OF THE TRANSCELLULAR PATHWAY FOR DRUG DELIVERY

It should be pointed out that the term 'uptake' of particles for gut tissues may include both adsorbed particles (ie particles that remain on the surface of the intestinal cells) and absorbed particles (ie particles that are actually translocated to the bloodstream and are therapeutically relevant). This means that the high figure reported in some of the literature for the particle uptake (Jani *et al.*, 1990; Jani *et al.*, 1992) is perhaps an over-estimation of the levels of actual absorption through the gut. Furthermore, the macromolecules contained within the microspheres, once taken up by the intestinal cell, must escape degradation by cellular lysosomes and then cross the basolateral membrane in order to reach the bloodstream.

For successful exploitation of particle uptake, it is necessary that the process be both predictable and reproducible. Currently, there are contrasting data available describing the extent of particle uptake following repeated administration to the same animal. While some researchers have reported high levels of uptake (Jani *et al.*, 1990; Eyles *et al.*, 1995), low levels of uptake have been reported by others (Brayden and Baird, 1994; Harding *et al.*, 1999; Jenkins *et al.*, 1994; Ebel, 1990). The preferred methodology to quantify total particle uptake remains unknown, and many of the studies reviewed here were not designed with this objective in mind. For example, Jenkins *et al.* (1994) were concerned only with evaluating the relative extent of uptake of alternative microparticle formulations of different sizes. This study did not accurately determine the total extent of particle uptake, because particle counting was performed only from lymph and Peyer's patches samples.

The variable uptake of particles reported in the aforementioned studies make it unlikely that the process could be successfully applied to the delivery of a wide range of drugs. It may be possible, however, to use this technology for the oral delivery of drugs that have a wide 'therapeutic window', that is, drugs that are active at very low concentrations and show limited toxicity at much higher doses.

TRANSCELLULAR PATHWAY FOR VACCINE DELIVERY

A pathway responsible for the uptake of small numbers of particles is unlikely to be appropriate as a delivery mechanism for a therapeutic dose of a drug, but it might be adequate as a mechanism for stimulating a significant immune response to an orally delivered microencapsulated antigen. Traditionally, the induction of systemic immunity concomitant with parenteral administration of vaccines was the favoured method for vaccine administration. With the evidence of the protective role of IgA antibodies against agents attacking mucosal surfaces, there is heightened interest and research into the use of oral vaccine delivery systems.

The advantage of oral delivery for the induction of immune responses is two-fold. Mucosal immunization stimulates both the production of mucosal and systemic immunity via secretion and production of IgA and IgG antibodies, thereby affording the host with protection from invading infections if mucosal surfaces become breached. The concern as to whether mucosal immunity elicits a sufficient systemic immune response has been addressed in several recent studies. In a study conducted by Hertiage and co-workers (1998), mice orally immunized with human serum albumin showed proliferation and antibody secretion from Peyer's patches following

a single exposure to the antigen. In addition, progressive dissemination of antigen-specific lymphocytes from Peyer's patches to the spleen was also observed (Hertiage *et al.*, 1998). Additionally, Hertiage *et al.* (1998) observed a more robust increase in human serum albumin IgG antibodies following oral administration of the antigen compared to antigen initially delivered parenterally. Taken together, these observations suggest that the mucosal immunity stimulated via oral antigen administration and the concomitant production of systemic immunity may indeed be an attractive alternative avenue for vaccine delivery.

The oral route for vaccine delivery offers several advantages, including high potential patient acceptance and compliance, less pain and discomfort, and lower costs for production and administration because trained personnel would not be required to carry out immunizations. Consequently, a number of vaccines would be significantly improved if they could be administered orally. Oral immunization might also result in improvements in vaccine efficacy because oral immunization can stimulate mucosal immunity. This might prove to be particularly advantageous in the elderly. Unlike systemic immunity, mucosal immunity does not appear to be subjected to age-associated dysfunction. Additionally, oral immunization might also be attractive in the very young because mucosal immunity appears to develop earlier than systemic immunity.

The majority of the gut-associated lymphoid tissue is organized into aggregates of lymphoid follicles called Peyer's patches. The relative number and distribution of Peyer's patches is dependent upon the microbial and antigenic load presented to the species throughout evolution. In humans, the largest Peyer's patches are found in the terminal ileum and are covered with a specialized epithelium that is adapted to allow antigen sampling from the lumen. The major physiological role of the Peyer's patches is the induction of a secretory immune response to ingested antigens. On contact, antigens are then delivered into the underlying dome structures of the patches through specialized cells called M cells. There are two important aspects of the uptake and transport of antigens by M cells: (1) antigens will probably escape degradation; and (2) the antigen will be released into an environment rich in immunocompetent cells (*Figure 16.1*). Thus, uptake by M cells can enable the delivery of intact antigens into the immuno-inductive environment of the Peyer's patches, thus producing a substantial immune response. As mentioned above, the M cell represents the favoured route for nanoparticle uptake. Therefore, a great deal of research has been focused on the delivery of antigens trapped in particles. Oral immunization with fimbriae from *Bordetella pertussis* entrapped in nanoparticles protected mice from intranasal challenge with the pathogen (Jones *et al., 1996*), and whole viruses entrapped in nanoparticles also induced protective immunity (Moldovenwanu *et al.*, 1993; Ray *et al.*, 1993). Oral immunization in mice with nanoparticles induced significant serum IgG and secretory IgA antibody responses (Challacombe *et al.*, 1992); the secretory IgA response was disseminated throughout the common mucosal immune system (Challacombe *et al.*, 1992). Hence, oral immunization with microencapsulated vaccines potentially offers protection against pathogens that infect the gut, the oral cavity, and the respiratory and genital tracts.

Several alternative approaches to the oral delivery of vaccines using polymeric delivery systems other than microencapsulation have also been described, including the use of enteric coated polymers (Klipstein *et al.*, 1983), swellable hydrogels and

the encapsulation of antigens in water-soluble polymers (Offit *et al.*, 1994). Each of these approaches may have potential advantages over the use of microparticles, but these have yet to be demonstrated.

Clearly, there are many advantages for implementing oral vaccine delivery systems. However, the question arises as to how vaccine preparations can be packaged so as to enter the gastrointestinal system and retain biological activity. In an innovative study by Tacket *et al.* (1998), volunteers fed antigens delivered in transgenic potatoes were able to mount an immune response. Transgenic potatoes containing the gene from the B sub-unit of Entertoxigenic *Escherichia coli*, which binds to GM1 ganglosides on the apical surface of enterocytes, facilitates the entry of heat labile entertoxin which causes the diarrhoea associated with this form of *E. coli* infection (Tacket *et al.*, 1998). Healthy volunteers fed transgenic potatoes elicited anti-LT IgG ASC (antibody secreting cells) seven days after ingestion of the antigen (Tacket *et al.*, 1998). The appearance of gut associated ASC's within this timeframe represents immunological priming of the small intestine. Prior to ingestion of the antigen, gut associated ASC were not detectable (Tacket *et al.*, 1998). Additionally, a four-fold increase in serum IgG was observed in 91% of the volunteers, which remained elevated 59 days after the ingestion of the first dose. Seventy-six percent of the tested volunteers developed neutralizing titres (Tacket *et al.*, 1998).

A fascinating, alternative approach has recently been proposed by Kerneis *et al.* (1997), who described in an animal model how to stimulate the conversion of enterocytes to an M cell lineage, which more efficiently transports antigens across the intestinal barrier to the underlying immune system. While there are many exciting avenues currently under investigation for the oral delivery of vaccines (ie the use of live vectors, liposomes, and controlled release preparations), the ideal target site within the gastrointestinal system appears to be the M cell, which allows for targeted drug delivery, a necessary specificity not often achieved by using the gastrointestinal system as the prime delivery site.

Despite the promising results obtained in animal models, there are still major limitations to antigen delivery through the transcellular pathway. These limitations are mainly related to the small number of M cells present within the intestinal mucosa ($< 0.1\%$ of epithelial cells). In the search for alternative solutions, several investigators have used the B sub-unit of cholera toxin (produced by *Vibrio cholerae*) as an adjuvant to deliver antigens orally, via the enterocytes (Elson and Ealding, 1984). It has been demonstrated that, under certain conditions, enterocytes themselves can directly present antigens (Mayer and Schlien, 1987). These observations suggest that the delivery of oral vaccines might also be enhanced by harnessing the transcellular pathway of the major enterocyte population for antigen delivery, and perhaps even initial antigen processing.

In addition to the oral delivery of vaccines, oral delivery systems for antibodies are also in development. Since the immune system is capable of great amplification following presentation of only a small antigen load, the systemic response to orally delivered antibodies may provide a valid approach for the diagnosis and therapy of cancer, detoxification, and passive immunotherapy. Potential pitfalls include proteolytic digestion. However, orally administered antibodies that are proteolytically degraded into their $F(ab')_2$, Fab, and Fc fragments, retain some of their neutralizing activity within the local environment of the gastrointestinal system (Reily *et al.*, 1997).

M cells selectively bind and transport secretory IgA (sIgA) via an undefined mechanism that may play a role in mucosal immune responses. Additionally, oral administration of secretory IgA (sIgA) to adult BALB/c mice induced IgA+, IgM+, and IgG+ lymphoblasts in Peyer's patches, which subsequently fused with myeloma cells resulting in hybridomas producing antibodies to the secretory component. The secretory component is a derivative of the polymeric immunoglobulin receptor and is secreted with IgA (sIgA) in mucosal tissues. In addition to promoting clearance of pathogens, sIgA selectively adheres to M cells. These observations prompted the study by Corthesy and co-workers, who proposed that the secretory component of IgA could be used as a potential vector to target protective epitopes into mucosal lymphoid tissue and elicit an immune response.

In order to utilize sIgA complex as a potential vaccine delivery system, insertion of the antigen should not alter the molecular folding of the secretory component or the function of sIgA. The invasion B epitope of *Shigella flexneri* was inserted into recombinant sIgA and orally administered to mice. Systemic and mucosal immune responses were observed, suggesting that antigenized sIgA may be a potential mucosal vaccine delivery system.

Transcellular delivery of pharmaceutical agents remains the most explored pathway for drug delivery through the gastrointestinal system, although it does not represent the ideal solution. Within the gastrointestinal tract, there are four potential target sites for uptake: the tip of the villus, enterocytes, the Peyer's patch, and intestinal macrophages. We have already discussed in some detail the role of Peyer's patches and intestinal enterocytes as the major targets within the transcellular pathway. However, recent evidence suggests that dentritic cells, a specialized differentiated cell of the leukocyte lineage, may be another site for antigen presentation and delivery within the gastrointestinal system.

One of the major limitations for transcellular delivery of drugs remains the lack of targeting specificity within the system. The mechanisms whereby micro- and nanoparticles become ingested within cells remain to be clearly established. As a result, while the therapeutic agent, encased within the delivery vehicle, has a clear target and mechanism of action, a critical component is missing with regard to targeting uptake and transcytosis from the time of ingestion. While the therapeutic preparations targeting M cells allows for an additional level of targeting specificity, these cells are in the striking minority with respect to the epithelial cells comprising the gastrointestinal tract. Therefore, research emphasis should also focus on understanding the mechanisms of endocytosis and how to target endocytotic pathways as a means for increasing oral bioavailability of pharmaceutical agents.

Paracellular pathway

There is now a large body of evidence suggesting that tight junctions (tj) play a pivotal role in epithelial permeability. However, the utility of the paracellular route for oral drug delivery has remained unexplored due to our limited understanding of tj physiology and the lack of substances capable of increasing the tj permeability without irreversibly compromising intestinal integrity and function (Lee *et al.*, 1991; Muranishi, 1990; Hochman and Artursson, 1994; Citi, 1992). Indeed, attempts to find ways to increase paracellular transport by loosening intestinal tj have been hampered

by unacceptable side effects induced by the potential absorption enhancing agents tested thus far (Lee *et al.*, 1991; Muranishi, 1990; Hochman and Artursson, 1994; Citi, 1992). For the most part, these agents fall within two classes: (1) calcium chelators, and (2) surfactants (Hochman and Artursson, 1994). Both types have properties that limit their general utility as a means to promote absorption of various molecules. In the case of calcium chelators, Ca^{2+} depletion induces global changes in the cells, including disruption of actin filaments, disruption of adherent junctions, and diminished cell adhesion (Citi, 1992). In the case of surfactants, the potential lytic nature of these agents may cause exfoliation of the intestinal epithelium, irreversibly compromising its barrier functions (Hochman and Artursson, 1994).

Considering these limitations, it was reasonable to explore whether findings from basic research on tj regulation can be applied to developing new approaches to enhancing drug absorption through the paracellular route. Before addressing these issues, it is worth reviewing some of the structural and biochemical features of tj.

Molecular composition of the intestinal tight junctions

Tj play a major role in regulating intestinal paracellular flow of fluid and solute. Variations in transepithelial conductance can usually be attributed to changes in the permeability of the paracellular pathway, since the resistance of the enterocyte plasma membrane is relatively high (Cereijido, 1992). The tj represents the major barrier within this paracellular pathway and the electrical resistance of epithelial tissues seems to depend on the number of transmembrane protein strands and their complexity within the tj as observed by freeze-fracture electron microscopy (Magnuson *et al.*, 1978). There now exists evidence that the tj, once regarded as static structures, are in fact dynamic, and readily adapt to a variety of developmental (Schneeberger *et al.*, 1978; Madara and Dharmsathaphorn, 1985; Madara and Pappenheimer, 1987; Mazariegos *et al.*, 1984), physiological (Sardet *et al.*, 1979; Milks *et al.*, 1986; Nash *et al.*, 1988; Shasby *et al.*, 1988), and pathological (Anderson *et al.*, 1993; Furuse *et al.*, 1993; Gumbiner *et al.*, 1991) circumstances. These adaptive mechanisms are still not completely understood. In the presence of Ca^{2+}, tj assembly is the result of a complex cascade of biochemical events that ultimately lead to the formation of an organized network of tj elements, the composition of which has been only partially characterized (*Figure 16.2*). One candidate for the transmembrane protein strands, occludin, has been identified (Gumbiner *et al.*, 1991). Several proteins have been identified in a cytoplasmic submembraneous plaque underlying membrane contacts (*Figure 16.2*), but their function remains to be established. ZO-1 and ZO-2 each exist as a heterodimer (Stevenson *et al.*, 1988) in a detergent-stable complex with an uncharacterized 130 kD protein (ZO-3). Most immunoelectron microscopic studies have localized ZO-1 to precisely beneath membrane contacts (Citi *et al.*, 1988). Both ZO-1 and ZO-2 belong to the membrane associated guanylate kinase (MAGUK) family of proteins (Anderson, 1996). Several other peripheral membrane proteins have been localized to the tj, including cingulin (Zhong *et al.*, 1993), 7H6 (Zahraoui *et al.*, 1994), rab 13 (Ridley *et al.*, 1992), Ga_{i-2} (Denker *et al.*, 1996; Dodane and Kachar, 1996) and PKC (Dodane and Kachar, 1996). Recently, a novel protein (symplekin) has been described that not only associates with tj, but can also be localized to the nucleus (Keon *et al.*, 1996). Similar to ZO-1, symplekin is also

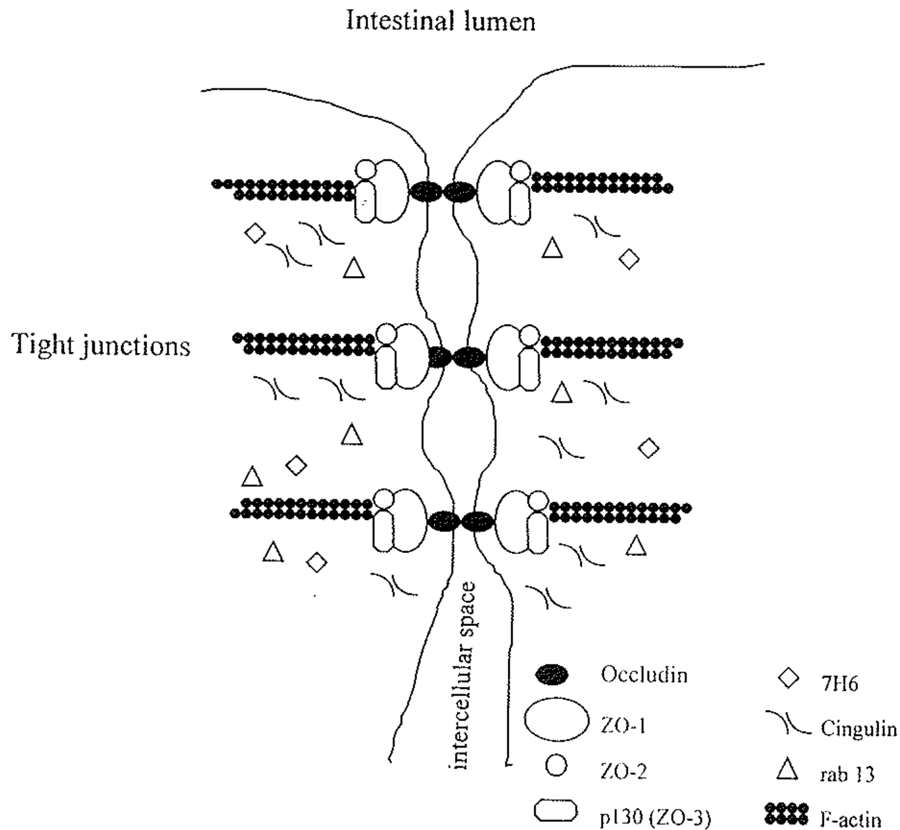

Figure 16.2. Model for components of the tight junction. Occludin, the transmembrane protein strand, is anatomically and functionally connected with the cell cytoskeleton via the junctional complex. This complex comprises a series of proteins, including ZO-1, ZO-2, and p130 (ZO-3). Other proteins, such as cingulin, 7H6, rab13, rho, and ras, are located further from the cell membrane. However, they also seem involved in the regulation of tight junction permeability.

expressed by cells that do not form tj, where it appears to be only in the nucleus. ZO-1 also can be localized to the nucleus, but unlike symplekin, only in growing but not in differentiated epithelial cells (Gottardi *et al.*, 1996). This dual localization for these tj components suggests that tj might also be involved in the regulation of gene expression, cell growth and differentiation (Balda and Matter, 1998). Beside rab 13, other small GTP-binding proteins are known to regulate the cortical cytoskeleton; Rho regulates actin polymerization and focal adhesion formation (Ridley and Hall, 1992). In polarized epithelial cells, Rho also regulates tj organization and permeability (Nusrat *et al.*, 1995). Other proteins, such as Rac and focal adhesion kinase (FAK), play a role in plasma membrane ruffling and focal adhesion formation (Hanks and Polte, 1997). Whether these molecules also participate in tj regulation is unknown (Denker and Nigam, 1998). On the basis of the bi-directional signalling that is transduced across focal adhesions (Guan and Shalloway, 1992), and the zonula adherens (Tsukita *et al.*, 1993), it is conceivable that tj-associated proteins are similarly involved in transducing signals in one or more directions across the cell

membrane and in regulating links to the cortical actin cytoskeleton. In eukaryotic cells, junctional complex proteins, actin filaments, microtubules, and intermediate filaments interact to form the cytoskeleton network involved in determination of cell architecture, intracellular transport, modulation of surface receptors, paracellular permeability, mitosis, cell motility, and differentiation (MacRae, 1992). There is now a large body of evidence that structural and functional linkage exists between the actin cytoskeleton and the tj complex of absorptive cells (Gumbiner, 1987; Madara *et al.*, 1986; Drenchahn and Dermietzel, 1988; Fasano *et al.*, 1995). The actin cytoskeleton is composed of a complicated meshwork of microfilaments whose precise geometry is regulated by a large cadre of actin-binding proteins. The architecture of the actin cytoskeleton appears to be critical for tj function. Most of the actin is positioned under the apical junctional complex where myosin II and several actin-binding proteins, including α-catenin, vinculin, and radixin have been identified (Denker and Nigam, 1998). Myosin movement along actin filaments is regulated by ATP and phosphorylation of the regulatory light chain by Ca^{2+}-calmodulin activated myosin light chain kinase (Hecht *et al.*, 1996). In several systems, increases in intracellular Ca^{2+} can affect phosphorylation of myosin regulatory light chain contraction of perijunctional actin and cause increased paracellular permeability (Tsuneo *et al.*, 1991). We have recently demonstrated PKCα-dependent actin polymerization associated with increments in paracellular permeability (Fasano *et al.*, 1995).

A variety of intracellular mediators have been shown to alter tj function and/or structure. Tj within the amphibian gallbladder (Duffey *et al.*, 1981), and goldfish (Bakker and Groot, 1984) and flounder (Krasney *et al.*, 1983) intestine display enhanced resistance to passive ion flow as intracellular cAMP is elevated. In addition, exposure of amphibian gallbladder to Ca^{2+} ionophore appears to enhance tj resistance and induce alterations in tj structure (Palant *et al.*, 1983). Lastly, activation of PKC either by the *Vibrio cholerae* produced zonula occuldens toxin (Zot) (Fasano *et al*, 1995) or by phorbol esters (Thelen *et al.*, 1991; Ellis *et al.*, 1992; Stenson, 1993), increases paracellular permeability. Alteration of epithelial tj is a well-described feature of infectious agents. *Clostridium difficile* toxin A (Hecht *et al.*, 1988) and B (Fiorentini and Thelestam, 1991), and influenza and vesicular stomatitis viruses (Marinaro *et al.*, 1999) have been shown to loosen tj in tissue culture monolayers. However, unlike what occurs after the Zot stimulus, these changes appear to be irreversible and are associated with destruction of the tj complex (Hecht *et al.*, 1988; Fiorentini and Thelestam, 1991).

Regulation of intestinal tight junctions

To meet the many diverse physiological and pathological challenges to which epithelia are subjected, tj must be capable of rapid and coordinated responses that require the presence of a complex regulatory system. The precise characterization of the mechanisms involved in the assembly and regulation of tj is an area of current active investigation. The discovery of Zot (Fasano *et al.*, 1991; Baudry *et al.*, 1992) shed some light on the intricate mechanisms involved in the regulation of tj permeability. As often occurs in science, the discovery of this protein was made by accident. A few years ago, researchers at the Center for Vaccine Development at the University of Maryland in Baltimore, U.S.A. engineered what was believed to be an ideal

attenuated vaccine for cholera. At that time, cholera toxin (CT) was the only described 'weapon' used by *Vibrio cholerae* to induce diarrhoea. Therefore, the deletion of the gene encoding the active sub-unit of CT appeared to be the best approach to eliminate the key pathogenic factor of the microorganism, while maintaining the expression of other *Vibrios* antigens necessary for a protective immune response. When fed to volunteers, these vaccine candidates still caused mild diarrhoea in more than one-half of the vaccinees (Levine *et al.*, 1988). In search of other factors responsible for this residual diarrhoea, our group identified Zot, a protein elaborated by *Vibrio cholerae* that increases the permeability of the small intestine by affecting the structure of tj (Baudry *et al.*, 1992).

We have subsequently demonstrated that Zot activates a complex intracellular cascade of events that regulate the intestinal permeability (*Figure 16.3*) (Fasano *et al.*, 1995). Zot induces a dose- and time-dependent PKCα-related polymerization of actin filaments strategically localized to regulate the paracellular pathway (Fasano *et al.*, 1995). These changes are a prerequisite to opening of tj and are evident at a toxin concentration as low as 1.1×10^{-13} M (Fasano *et al.*, 1997). The toxin exerts its effect by interacting with a specific surface receptor that is present on mature cells of small intestinal villi, but not in the colon (Fasano *et al.*, 1997). The regional distribution of

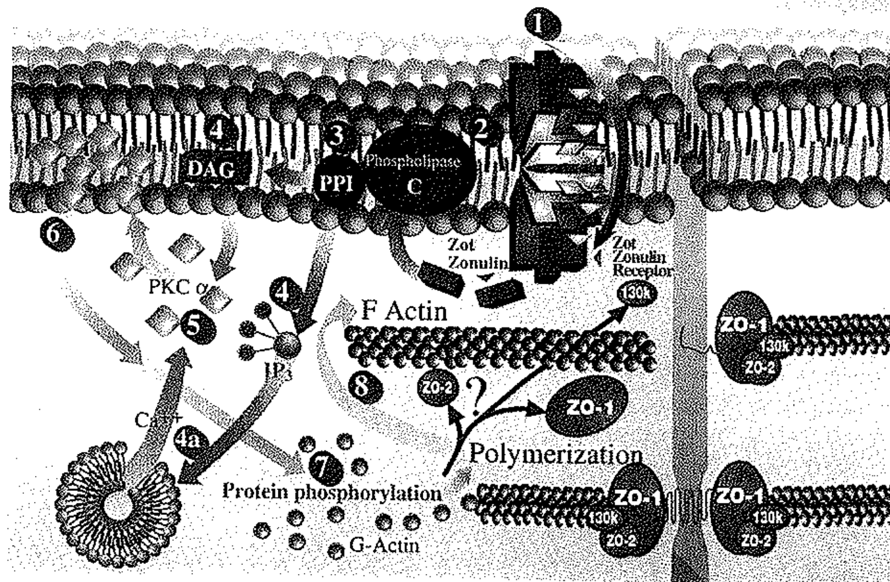

Figure 16.3. Proposed Zot intracellular signalling leading to the opening of intestinal tight junctions. Zot interacts with a specific surface receptor (1) whose distribution within the intestine varies. The protein is then internalized and activates phospholipase C (2) that hydrolyzes phosphatidyl inositol (3) to release inositol 1,4,5-tris phosphate (PPI-3) and diacylglycerol (DAG) (4). PKCα is then activated (5), either directly (via DAG) (4) or through the release of intracellular Ca^{++} (via PPI 3) (4a). PKCα catalyzes the phosphorylation of target protein(s), with subsequent polymerization of soluble G actin in F actin (7). This polymerization causes the rearrangement of the filaments of actin and the subsequent displacement of proteins (including ZO1) from the junctional complex (8). As a result, intestinal tight junctions become looser.

Zot receptor(s) coincides with the different permeabilizing effect of the toxin on the various tracts of intestine tested (Fasano *et al.*, 1997). Both *in vivo* (Fasano *et al.*, 1997; Fasano and Uzzau, 1997) and *in vitro* (Fasano *et al.*, 1991; Fasano *et al.*, 1997; Fasano and Uzzau, 1997) studies demonstrated that the effect of Zot on tissue permeability occurs within 20 minutes after the addition of the protein to the intestinal mucosa and is readily reversible once the toxin is removed.

Use of Zot as a tool for oral drug delivery

Zot displays multiple properties that make it the most promising tool currently available to enhance drug and peptide transport through the intestinal mucosa. Zot: (*a*) is not cytotoxic and does not affect the viability of the intestinal epithelium *ex vivo* (Fasano *et al.*, 1991; Fasano *et al.*, 1995); (*b*) fails to completely abolish the intestinal transepithelial resistance (Fasano *et al.*, 1991; Fasano *et al.*, 1995; Fasano and Uzzau, 1997); (*c*) interacts with a specific intestinal receptor whose regional distribution within the intestine varies (Fasano *et al.*, 1997); (*d*) is not effective in the large intestine where the presence of the colonic micro flora could be potentially harmful if the mucosal barrier was compromised (Fasano *et al.*, 1997; Fasano and Uzzau,

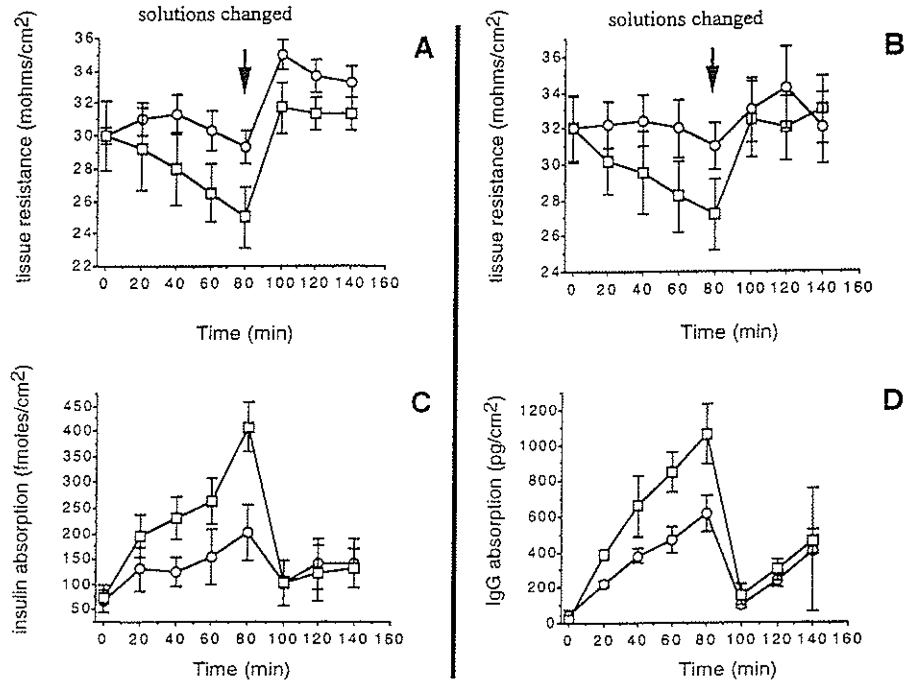

Figure 16.4. Reversible effect of purified Zot on tissue resistance (**A** and **B**) and transepithelial transport of insulin (**C**) and IgG (**D**) in rabbit ileum *in vitro*. Paired tissues, matched on the basis of their resistance, were exposed luminally to either ^{125}I-insulin 10^{-11} M (2µCi = 10^{-12} M) (left panel) or ^{125}I-IgG 156.25 ng (1 µCi = 83.3 ng) (right panel), alone (¡) or in the presence of 1.1×10^{-10} M Zot ("). After 80 min of incubation, the Ringer's solutions were replaced with solutions of identical composition but without Zot. Zot reversibly increased the transepithelial absorption of both insulin and IgG. These effects paralleled the Rt decrement induced by the toxin. Nr. of animals = 4.

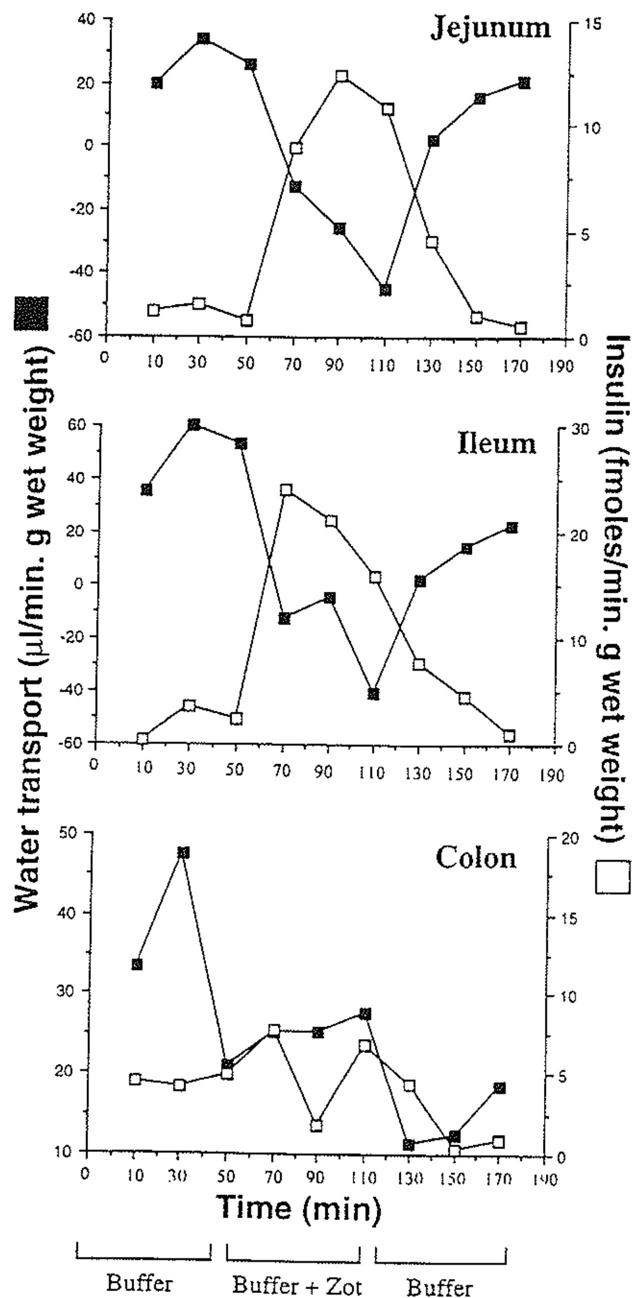

Figure 16.5. Effect of purified Zot on water (n) and insulin (¨) transport, as determined by the *in vivo* perfusion assay, in rabbit jejunum, distal ileum, and colon. Note the reversible increment of insulin absorption that Zot induced in the small, but not in the large intestine. This effect coincided with the decreased absorption of water evoked by the toxin.

1997); (*e*) does not induce acute systemic side effects (for at least 80–90 hrs) when orally administered (Fasano and Uzzau, 1997); and, lastly (*f*) induces a reversible increase of tissue permeability (Fasano *et al.*, 1991; Fasano *et al.*, 1995; Fasano and Uzzau, 1997).

To establish the efficacy of Zot as an intestinal absorption enhancer, we selected insulin and immunoglobulin G (IgG). This choice was based on the relative size and structure, biological activities, and the therapeutic relevance of these proteins. *In vitro* experiments in the rabbit ileum mounted in Ussing chambers demonstrated that Zot (at a molar concentration of 1.1×10^{-10} M) reversibly increases the intestinal absorption of both insulin (by 72%) and IgG (by 52%) in a time-dependent manner (Fasano and Uzzau, 1997). Zot permeabilizing effect peaked at 80 min and was completely reversible within 20 min after the withdrawal of the toxin from the Ussing chambers (*Figure 16.4*). This Zot-induced increase in absorption coincided with a reduction in tissue resistance (Rt) (*Figure 16.4*). When tested in the intact host by using the rabbit *in vivo* perfusion assay, Zot (1.1×10^{-10} M) increased the passage of insulin across both the jejunum and distal ileum 10-fold, whereas no substantial changes were observed in the colon (Fasano and Uzzau, 1997) (*Figure 16.5*). The increased absorption of insulin was reciprocal with a shift of water absorption toward secretion (*Figure 16.5*), a change that has been related to the permeabilizing effect of Zot on the paracellular pathway *in vivo* (Fasano *et al.*, 1997). This effect was detectable as soon as 20 min after Zot perfusion in the small intestine and was completely reversible within 60 min its withdrawal (*Figure 16.5*). Zot also reversibly increased the serum concentration of both insulin and the non-absorbable marker ^{14}C-polyethylene glycol (PEG)-4000 from the jejunum and ileum, but not from the colon (Fasano and Uzzau, 1997). Similar results were obtained with IgG, whereby Zot (1.1×10^{-10} M) induced 2-fold and 6-fold increases of IgG absorption in the jejunum and ileum, respectively. Again, no increases in absorption were detected in the colon (Fasano and Uzzau, 1997).

To evaluate the bioactivity of insulin after enteral co-administration with Zot, the hormone was orally administered to acute type I diabetic male BB/Wor rats with or without Zot and the blood glucose levels of the rats were serially measured. After oral administration of insulin alone, given at doses between 5 and 30 IU, blood glucose levels of treated animals were not appreciably lowered (Fasano and Uzzau, 1997). In contrast, when insulin at doses as low as 10 IU was orally co-administered with Zot at 1.1×10^{-10} moles (5 μg), a significant reduction in blood glucose concentration was observed (*Figure 16.6*). This decrement was comparable to that seen with a conventional dose of SQ insulin and returned to baseline by 6 h post-administration (*Figure 16.6*). None of the animals treated with insulin plus Zot experienced fever or other systemic symptoms, and no structural changes could be demonstrated in the small intestine on histological examination (Fasano and Uzzau, 1997). Furthermore, Zot administration did not induce diarrhoea, despite the secretory effect of the toxin. The lack of diarrhoea is probably related to the distribution of the Zot receptors within the intestine (Fasano *et al.*, 1997). Following the activation of the Zot receptors in the small intestine, tj are reversibly opened and fluid leaks into the intestinal lumen driven by the osmotic gradient. The excess of fluid that accumulates in the small intestine is completely reabsorbed in the colon (where tj regulation is not operative because of the lack of Zot receptors), preventing intestinal fluid loss and, therefore, diarrhoea.

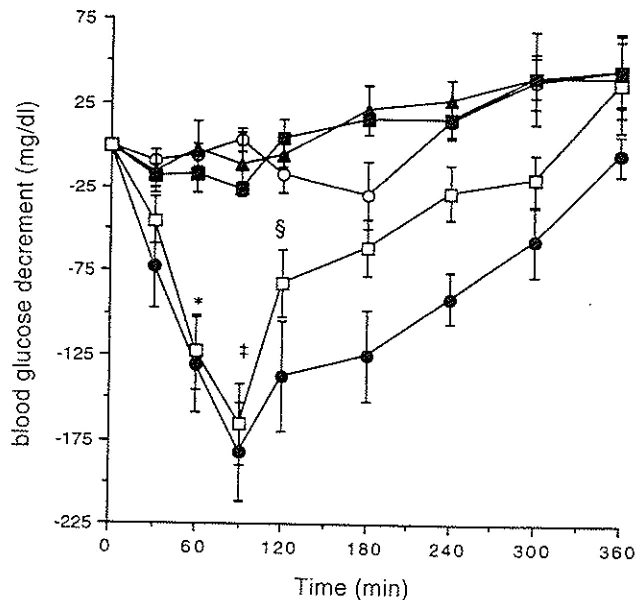

Figure 16.6. Effect of oral insulin 10 IU, alone (■) or in the presence of Zot 5μg (□) on serum glucose decrement in BB/Wor diabetic rats. The co-administration of Zot induced a reduction in blood glucose concentration comparable to that seen with a conventional dose of SQ insulin (●) and returned to baseline by 6 hr post-administration. Blood glucose decrement of untreated animals (O) and animals treated with oral Zot alone (▲) are shown for comparison. Nr. of observations = 3; *p = 0.005;‡p = 0.003; §p = 0.009, as compared to oral insulin alone.

Taken together, these results demonstrate that co-administration of Zot with biologically active ingredients enhances intestinal absorption of the active molecule, and that this enhancement is effective for both relatively small (5,733 Da: insulin) and large molecules (140–160 kDa: IgG). Furthermore, the experiments in BB/Wor diabetic rats demonstrate that orally delivered insulin can retain its biological activity without provoking severe hypoglycaemia within the range of the insulin administered, ie up to 15 times more than the effective parenteral insulin dose. These findings have important practical implications, since the insulin therapeutic index (ie the ratio between the median toxic dose and the median therapeutic dose) is relatively low.

PARACELLULAR PATHWAY FOR VACCINE DELIVERY

While the focus of this review centred on oral delivery systems, the future trends with respect to targeted drug delivery appears to be focused also on other mucosal systems. Much emphasis has been placed on the relative ease with which agents can be delivered in oral preparations. However, other avenues exist for the design of drug delivery systems to alternative mucosal sites, including the respiratory system. It has been shown that nasal delivery of antigens is able to stimulate secretion of antigen specific IgA antibodies at sites distant from the site of immunization, including the intestine. Interesting findings are emerging as researchers seek new avenues to deliver pharmaceutical agents. Zot has been shown to be a mucosal adjuvant for the intranasal delivery of antigens, supporting the concept that the paracellular pathway

is a viable method to introduce agents into the systemic circulation (Marinaro *et al.*, 1999). In addition, intranasal immunization with *B. pertussis* entrapped in microparticles has also been shown to induce protective immunity (Jones *et al.*, 1996).

In a recent report, De Magistris *et al.* co-administered Zot with ovalbumin in an intranasal preparation given to mice (Marinaro *et al.*, 1999). The results from this study were two-fold. Zot *via* its action on intercellular tj was able to allow the passage of ovalbumin into the systemic circulation, thereby inducing the production of anti-ovalbumin antibodies of the subclasses IgG and IgA (Marinaro *et al.*, 1999). In addition, antigen specific IgA was detected at distant mucosal sites from the initial immunization site, including the intestine and vaginal mucosa (Marinaro *et al.*, 1999). Comparative studies with LT, which is considered one of the most powerful mucosal adjuvants, demonstrated that the adjuvant effect of Zot on serum IgG was evident after two immunizations (Marinaro *et al.*, 1999). As mentioned previously, the effects of Zot on intercellular tj requires interaction with a specific receptor expressed on the surface of cells. The ability of Zot to permit passage of ovalbumin delivery to nasal epithelial cells, implies that a Zot receptor may be present in these tissues, thereby giving rationale to future studies utilizing Zot within mucosal vaccine preparations for pathogens of the respiratory, gastrointestinal, and genital systems (Marinaro *et al.*, 1999).

With respect to intranasal delivery of microparticles, systemic induction of immunity has been clearly demonstrated. The nasal associated lymphoid tissue (NALT), a well-defined entity in mouse, has been confirmed as the site of uptake for microparticles, a theme consistent with studies discussed for the gastrointestinal system. An important consideration, however, is that in humans the representative NALT tissue is housed within the tonsils and Waldeyer's rings. This may or may not be a limitation for microparticle intranasal administration in human models. While these two avenues for delivery of pharmaceutical agents provide promising new technologies, the global usefulness may be limited and does not circumvent the need to develop stronger oral delivery systems, but may perhaps provide a synergism between advances made in the pursuit of ideal oral preparations.

Conclusions

Of the many drug delivery paradigms presented within this review, one aspect remains unchanged: oral preparation of pharmaceutical agents is the most attractive mechanism for introducing compounds into the systemic circulation. The advantages are clear, and new agents are formulated on a daily basis. Therefore, the need to develop targeted delivery systems is paramount if we are going to continue the pursuit of oral administration of new drugs. Utilizing the paracellular pathway as a target for the oral delivery of drugs may provide a viable alternative to current oral drug delivery paradigms, particularly in light of our limited knowledge of how to target M cells for drug delivery. Research into the mechanisms that govern paracellular transport is currently an active area of investigation. Tight junctions are a hallmark of secretory epithelia and may also represent a novel target for mucosal drug delivery in general.

Current knowledge on the regulation of intestinal tight junctions by Zot was

applied to enhance the absorption of macromolecules normally not absorbed through the intestine. The promising results obtained in the animal model, both *in vitro* and *in vivo,* represent an encouraging basis for further studies to establish the possible clinical applications of this system for the treatment of human diseases that currently require frequent and long-life parenteral drug administration.

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