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Review

Recent Advances in Intestinal Macromolecular Drug Delivery via Receptor-Mediated Transport Pathways

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Receptor-mediated transport mechanisms provide a pathway for the trafficking of extracellular macromolecules into (endocytosis) and across (transcytosis) the cell. This comprises the binding of a ligand to a specific cell-surface receptor, clustering of the ligand-receptor complexes in endocytotic vesicles and vesicular sorting. This review focuses on recent advances in cellular and molecular biology pertaining to receptor-mediated endocytosis. A concise overview is presented of current and potential future applications of targeting to RME mechanisms to improve oral macromolecular drug delivery.

KEY WORDS: drug delivery; intestine; potocytosis; receptor-mediated endocytosis; transcytosis.

INTRODUCTION

The rapid expansion of applied biotechnology research in current pharmaceutical drug discovery has resulted in the development of increasing numbers of novel macromolecular therapeutics. Oral bioavailability of these compounds is usually poor due to a combination of incompatible physicochemical properties, resulting in low cellular penetration, and high susceptibility to metabolic enzymes present within the gastrointestinal tract. For chronic drug therapy, however, the oral pathway is generally considered to be the most convenient route of administration. As a consequence, systemic delivery of macromolecules via the oral pathway remains one of the most challenging aspects in the field of intestinal drug delivery and has experienced an increased interest over the last decade.

Thus far, limited success has been observed in oral macromolecular delivery by either concomitant administration of penetration enhancers or the use of prodrug strategies involving carrier-mediated transport systems. An alternative approach may be targeting to receptor-mediated endocytosis (RME) systems. Although not completely novel, the area of receptor-mediated endo- and transcytosis has recently gained a renewed interest due to the rapid progress in the fields of cellular and molecular biology. It is the aim of this review to present a concise overview of the latest developments in RME research and RME systems that are either currently targeted for the oral delivery of macromolecules or may appear to become potential targets for future oral drug delivery strategies.

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ABBREVIATIONS: ARC, apical recycling compartment; BFA, Brefeldin A; BLRC, basolateral recycling compartment; CRC, common recycling compartment; CURL, compartment of uncoupling receptor and ligand; Cbl, cobalamin; DT, diphtheria toxin; Fn, ferritin; FcRn, neonatal Fc receptor; GALT, gut-associated lymphoid tissue; GPI, glycosylphosphatidylinositol; Ig, immunoglobulin; IF, intrinsic factor; LHRH, luteinizing hormone releasing factor; pIgR, polymeric immunoglobulin receptor; RME, receptor-mediated endocytosis; Tf, transferrin; TfR, transferrin receptor.

RECEPTOR-MEDIATED TRANSPORT

In order to define terminology used in this review and avoid confusion, a distinct difference between carrier- and receptor-mediated systems should be pointed out. Carrier-mediated systems involve transport proteins that are anchored to the membrane by multiple membrane-spanning fragments or protein loops, whereas receptor-mediated systems utilize receptor proteins that span the membrane only once. Carriers operate by shuttling their substrates across the membrane via an energy-dependent (ATP or cotransport) flip-flop mechanism and receptors are internalized in vesicles after binding to their substrate, as described in more detail below.

Receptor-Mediated Endocytosis

Mammalian cells have developed an assortment of mechanisms to facilitate the internalization of specific substrates and target these to defined locations inside the cytoplasm. Collectively, these processes of membrane deformations are termed 'endocytosis' and comprises phagocytosis, pinocytosis, receptor-mediated endocytosis (clathrin-mediated), and potocytosis (non-clathrin-mediated RME). The emphasis of this review is receptor-mediated endocytosis in the intestinal tract, but the interested reader is gladly referred to alternative reviews covering the complete spectrum of endocytotic processes (1,2).

RME is a highly specific cellular biologic process by which, as its name implies, various ligands bind to cell surface receptors and are subsequently internalized and trafficked within the cell. In many cells the process of endocytosis is so

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active that the entire membrane surface is internalized and replaced in less than a half hour (3).

RME can be dissected into several distinct events. Initially, exogenous ligands bind to specific externally oriented membrane receptors. Binding occurs within 2 minutes and is followed by membrane invagination until an internal vesicle forms within the cell (the early endosome, "receptosome", or CURL (compartment of uncoupling receptor and ligand) (4). Localized membrane proteins, lipids and extracellular solutes are also internalized during this process. When the ligand binds to its specific receptor, the ligand-receptor complex accumulates in coated pits. Coated pits are areas of the membrane with high concentration of endocellular clathrin subunits. The assembly of clathrin molecules on the coated pit is believed to aid the invagination process. Specialized coat proteins, which are actually a multisubunit complex, called adaptins, trap specific membrane receptors—which move laterally through the membrane—in the coated pit area by binding to a signal sequence (Tyr-X-Arg-Phe, where X = any amino acid) at the endocellular carboxy terminus of the receptor. This process ensures that the correct receptors are concentrated in the coated pit areas and minimizes the amount of extracellular fluid that is taken up in the cell. RME appears to require the GTP-binding protein dynamin, but the process by which dynamin is recruited to clathrin-coated pits remains unclear (5).

Following the internalization process, the clathrin coat is lost through the help of chaperone proteins, and proton pumps lower the endosomal pH to approximately 5.5, which causes dissociation of the receptor-ligand complex (6). CURL serves as a compartment to segregate the recycling receptor (e.g. transferrin) from receptor involved in transcytosis (e.g. transcobalamin) (7). Endosomes may then move randomly or by saltatory motion along the microtubules (8) until they reach the trans-Golgi reticulum where they are believed to fuse with Golgi components or other membranous compartments and convert into tubulovesicular complexes and late endosomes or multivesicular bodies. The fate of the receptor and ligand are determined in these sorting vesicles. Some ligands and receptors are returned to the cell surface where the ligand is released into the extracellular milieu and the receptor is recycled. Alternatively, the ligand is directed to lysosomes for destruction while the receptor is recycled to the cell membrane. Figure 2 presents an overview of the existing possibilities in the fate of ligands and receptors.

The endocytotic recycling pathways of polarized epithelial cells (Figure 1) such as enterocytes, are generally more complex than in non-polarized cells. In these enterocytes a common recycling compartment (CRC) exists that receives molecules from both apical and basolateral membranes and is able to correctly return them to the appropriate membrane or membrane recycling compartment (ARC or BLRC) (9). The signals required for this sorting step have not been defined as of yet, but are presumably similar to the peptide sequences required for proper sorting in the trans-Golgi network.

Structure of Cell Surface Receptors

Our general understanding of RME receptor structure and related structure-function relationships has been significantly enhanced by ongoing efforts to clone mRNA sequences coding for endocytotic receptors. It appears that most RME receptors

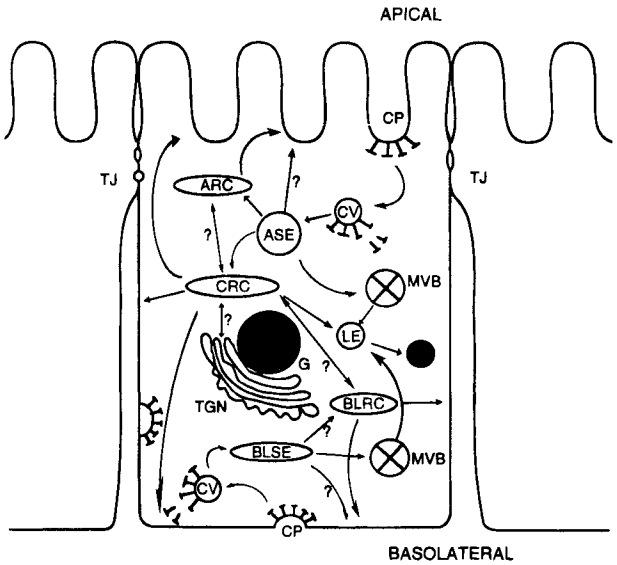

Fig. 1. Schematic representation of endocytotic pathways in polarized cells. Question marks along an arrow indicate that only circumstantial evidence exists for those pathways at present time. Double-headed arrows indicate that similar magnitudes of transfer occur in either direction. Ligand and receptor complex is initially take up from coated pits (CP) into coated vesicles (CV). The CV loses its clathrin coat as described in the text and transforms into an apical sorting endosome (ASE). The fate of receptor and ligand can be: a degradative pathway where the material is passaged to multivesicular bodies (MVB) and eventually late endosomes (LE), which fuse with lysosomes (LY); a recycling pathway to the central recycling compartment (CRC) and/or the apical recycling compartment (ARC) which eventually exocytose the contents of the vesicle; or direct exocytosis of the ASE to apical membrane. In polarized cells, similar pathways occur at the basolateral membrane. The CRC, in combination with the trans-Golgi network (TGN) interconnects the apical and basolateral sorting pathways and enables the potential for transcytosis. Explanation of additional abbreviations: BLRC, basolateral recycling compartment; BLSE, basolateral sorting endosome; G, Golgi apparatus; N, cell nucleus; TJ, tight junction.

share several structural features, such as an extracellular ligand binding site, a single hydrophobic transmembrane domain (unless the receptor is expressed as a dimer), and a cytoplasmic tail encoding endocytosis and other functional signals (10). Two classes of receptors are proposed based on their orientation in the cell membrane: the amino terminus of Type I receptors is located on the extracellular side of the membrane, whereas Type II receptors have this same protein tail in the intracellular milieu. Although protein orientation may appear trivial, it strongly influences the eventual endocytotic mechanism (10).

Transcytosis

One of the least understood aspects in vesicular trafficking and sorting, and possibly one of the most important aspects for successful oral drug delivery via RME, is the transport of endocytotic vesicles to the opposite membrane surface, more commonly referred to as transcytosis. Recent studies in the area of cellular biology have reported specific proteins, named TAPs (transcytosis-associated protein), that are particularly found on transcytotic vesicles and are believed to be required for fusion with the target membrane (11,12). Other methods to stimulate

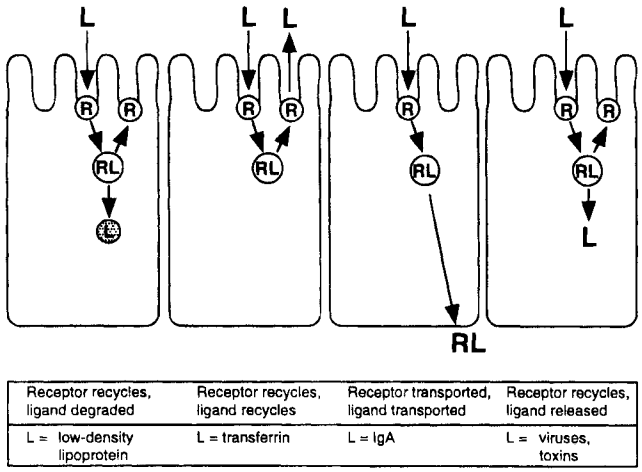

Fig. 2. Intracellular sorting pathways of RME. The initial binding and uptake steps (including receptor clustering in coated or non-coated pits, internalization of the receptor-ligand complex into coated vesicles (non-coated in case of potocytosis), and fusion of vesicles to form endosomes) are common to all pathways. After entry into acidic endosomes, ligand and receptors are sorted and trafficked independently which may result in degradation, recycling or transcytosis of either molecule (see text). L= ligand; R= receptor; lysosomes are depicted as shaded circles. After (10).

transcytosis have been recently explored. Transcytosis of transferrin (Tf) was found to be enhanced in presence of Brefeldin A (BFA), a fungal metabolite which has profound effects on the structure and function of the Golgi apparatus. Shah and coworkers (13) showed in Caco-2 cell monolayers that BFA causes a marked decrease in the number of basolateral Tf receptors (TfR) along with a slight increase in the number of apical TfR. BFA enhanced the TfR-mediated transcytosis of both [^{125}I]-Tf and the horseradish peroxidase-Tf conjugate across Caco-2 cells in both apical-to-basolateral and basolateral-to-apical directions. Prydz and colleagues (14) found that BFA treatment rapidly increased apical endocytosis of both ricin and HRP in MDCK cells, whereas basolateral endocytosis was unaffected.

Potocytosis

It was not until recently that potocytosis has been accepted as a distinct RME pathway (15–17). Potocytosis, or non-clathrin coated endocytosis, takes place through caveolae, which are uniform omega- or flask-shaped membrane invaginations (50–80 nm diameter) (1), and was first described as the internalization mechanism of the vitamin folic acid in a mouse keratinocyte cell line (18). Years before the name 'potocytosis' was coined, various ligands had been reported to localize in non-clathrin coated membrane regions, including cholera and tetanus toxins (19).

Morphological studies have implicated caveolae in (i) the transcytosis of macromolecules across endothelial cells; (ii) the uptake of small molecules via potocytosis involving GPI-linked receptor molecules and an unknown anion transport protein; (iii) interactions with the actin-based cytoskeleton; and (iv) the compartmentalization of certain signaling molecules involved in signal transduction, including G-protein coupled receptors. Caveolae are characterized by the presence of an integral 22-kDa membrane protein termed VIP21-caveolin, which coats the cytoplasmic surface of the membrane (20,21).

From a drug delivery standpoint, the advantage of potocytosis pathways over clathrin-coated RME pathways lies in the absence of the pH lowering step, thereby circumventing the classical endosomal/lysosomal pathway (16). This may be of invaluable importance to the effective delivery of pH-sensitive macromolecules.

RECEPTOR-MEDIATED ORAL ABSORPTION SYSTEMS

RME in Enterocytes vs. M-cells

According to Walker and Sanderson (22) the preferred route of intestinal uptake of low concentrations of antigens is

Table 1. Types of Ligands Transported by RME Mechanisms

Hormones and growth factors	Toxins and lectins	Viruses and bacteria	Serum transport proteins and antibodies	Vitamins and metal ions
Calcitonin	Cholera toxin	Adenovirus	IgE	Iron/transferrin
Catecholamines	Concanavalin A	Rous sacroma virus	IgG, via Fc receptors	Folate
Epidermal growth factor	Diphtheria toxin	Semliki forest virus	Low density lipoprotein	Riboflavin
	Pseudomonas toxin	Vesicular stomatitits virus	Maternal IgG	Vitamin B_{12}
Glucagon	*E. coli* heat labile toxin	Rotavirus	Polymeric IgA	
Growth hormone	Staphylococcal enterotoxin A & B	Varicella Zoster	Transcobalamin	
Insulin	Toxic shock syndome toxin 1	Adenovirus	Transferrin	
Interferon	Ribosome-inactivating proteins:	Reovirus	Yolk proteins	
Luteinizing hormone	ricin	Potato leafroll virus		
Nerve growth factor	saporin	*L. plantarum*		
Platelet derived growth factor	viscumin	*V. cholerae*		
	modeccin	*E. coli*		
Prolactin	nigrin b	*Y. pseudotuberculosis*		
Thyroid stimulating factor	α-sarcin	*Klebsiella* strains		
		Enterobacter strains		
Thyroid hormone		*Serratia* strains		

Note: This table represents RME uptake in all mammalian cell types.

through the M-cells (microfold or membranous), located in Peyer's patches, but at higher concentrations the regular enterocytes are also involved. M-cells are specialized epithelial cells of the gut-associated lymphoid tissue (GALT) that transport antigens from the lumen to cells of the immune system, thereby initiating an immune response or tolerance. Soluble macromolecules, small particles (23), and also entire microorganisms are transported by M-cells. The importance of M-cells in the uptake of particles is still a point of discussion. Recently, Hussain and colleagues (24) deduced from their own work and the work of others that "the importance of Peyer's patches as the principal site of particulate absorption may have been over-emphasized, and that normal epithelial cells can also be induced, with appropriate ligands such as plant lectins and bacterial adhesins, to absorb particulate matter." In this light, it should be stressed that the surface area of M-cells is only 10% compared to the surface area of normal epithelial cells.

Immunoglobulin Transport

Maternal and Neonatal IgG Transport

Receptor-mediated transcytosis of immunoglobulin G (IgG) across the neonatal small intestine serves to convey passive immunity to many newborn mammals (25). In rats, IgG in milk selectively binds to neonatal Fc receptors (FcRn) expressed on the surface of the proximal small intestinal enterocytes during the first three weeks after birth. FcRn binds IgG in a pH-dependent manner, with binding occurring at the luminal pH (6–6.5) of the jejunum and release at the pH of plasma (7.4). The Fc receptor resembles the major histocompatibility complex (MHC) class I antigens in that it consists of two subunits: a transmembrane glycoprotein (gp50) in association with β2-microglobulin (25). In mature absorptive cells both subunits are co-localized in each of the membrane compartments that mediate transcytosis of IgG. IgG administered *in situ* apparently causes both subunits to concentrate within endocytic pits of the apical plasma membrane, suggesting that ligand causes redistribution of receptors at this site. These results support a model for transport in which IgG is transferred across the cell as a complex with both subunits. Interestingly, Benlounes and co-workers (26) recently showed that IgG is effectively transcytosed at lower concentrations (<300 μg/ml), whereas a degradative pathway dominates at higher mucosal IgG concentrations.

Site-directed mutagenesis of a recombinant Fc hinge fragment has been used to localize the site of the mouse IgG1 molecule that is involved in the intestinal transfer of recombinant Fc in neonatal mice. These studies definitively indicate that FcRn is involved in transcytosis across both yolk sac and neonatal intestine, in addition to the regulation of IgG catabolism (27,28). Continued binding to vesicle membranes appears to be required for successful transfer since unbound proteins are removed from the transport pathway before exocytosis (29).

Drug carriers such as liposomes are not readily transported intact across epithelial barriers. Patel and Wild (30) showed that coating liposomes with appropriate IgG enhances their transport across rabbit yolk sac endoderm and enterocytes of suckling rat gut proximal small intestine. They measured the effect of liposomal transcytosis both by radiolabel assay of entrapped [^{125}I]-PVP and [^{3}H]-inulin, and by the hypoglycemic effect of entrapped insulin. Their results suggest that transported liposomes follow a pathway of transcytosis in clathrin-coated vesicles, thus escaping lysosomal degradation.

Polymeric IgA and IgM Transport

Polymeric IgA is produced by plasma cells and found in all external excretions, including bile and saliva (31,32). In the small intestine, polymeric IgA and IgM bind to the polymeric immunoglobulin receptor (pIgR) which is located on the basolateral surface of the cell. Expression of pIgR can be upregulated by cytokines (33). The pIgR-IgA complex is internalized into endosomes where it is sorted into vesicles that transcytose it to the apical surface. At the apical surface the pIgR is proteolytically cleaved, and the large extracellular fragment (known as secretory component) is released together with the ligand. pIgR includes a cytoplasmic domain of 103 amino acids that contains several sorting signals. Targeting from the trans-Golgi network to the basolateral surface is determined by the membrane-proximal 17 residues of this domain. For endocytosis there are two signals, both of which contain essential tyrosines. Transcytosis of pIgR is signaled by serine phosphorylation and may be regulated by the heterotrimeric Gs protein, protein kinase C and calmodulin. IgG is transcytosed from the apical to basolateral surface in several epithelial tissues such as the placenta and the small intestine of newborn rats. The receptor for intestinal transport of IgG is structurally similar to class I MHC molecules (34–36).

Bacterial Adhesins and Invasins

For many bacterial species, adherence to host cells is the initial key step towards colonization and establishing an infectious disease. Two components are necessary for the adherence process: a bacterial 'adhesin' (adherence or colonization factor) and a 'receptor' on the host (eukaryotic) cell surface. Bacteria usually express various cell adherence mechanisms depending on the environmental conditions and nature of the adhesins as well as receptors.

Bacteria causing gastrointestinal infections need to penetrate the mucus layer before attaching themselves to the epithelial surface. This attachment is usually mediated by bacterial fimbriae or pilus structures, although other cell surface components may also take part in the process. Adherent bacteria colonize intestinal epithelium by multiplication and initiation of a series of biochemical reactions inside the target cell through signal transduction mechanisms (with or without the help of toxins) (38). In a study on the colonization mechanism of *Klebsiella, Enterobacter,* and *Serratia* strains, Livrelli and co-workers (37) found no relationship between the adhesive pattern and the production of specific fimbriae, suggesting that several unrecognized adhesive factors are involved that remain to be identified.

Several adhesin and invasin molecules have been identified, such as a mannose-specific adhesin in *Lactobacillus plantarum* (39) and *V. cholerae* (40). Metcalfe and co-workers (41) found that adherence of *Escherichia coli* K-12(K88ab) to immobilized porcine small intestine mucus was caused by a 40- to 42-kDa glycoprotein K88-specific receptor. Using monoclonal antibodies against fimbrial adhesins of porcine enterotoxigenic

E. coli, K99 and K88 adhesin were detected, but not F41 and 987P adhesins (42).

The colonization mechanism of the enteropathogenic bacterium *Yersinia pseudotuberculosis* has been studied in most detail. In contrast to other infective agents, such as *Salmonella* strains or enteroinvasive *E. coli* (EIEC), invasion and transcytosis of *Y. pseudotuberculosis* is mediated by a single 986 amino acid protein, invasin, on the bacterial surface that binds to $\alpha 5\beta 1$ integrin (43). This single factor is sufficient to promote entry of inert particles by binding multiple integrin receptors during cellular uptake (44). This phenomenon has also recently been found by Mengaud and co-workers, who identified E-cadherin as the ligand for internalin, a *Listeria monocytogenes* protein essential for entry into epithelial cells. The internalization process of many microorganism is impressively fast: it was recently shown that within 45 min after introduction of *Y. pseudotuberculosis* into the lumen of BALB/C mice, wild-type bacteria can be found in the Peyer's patch (45). Mutants expressing defective invasin derivatives were unable to promote efficient translocation into the Peyer's patch and instead colonized on the luminal surface of the intestinal epithelium.

The study of bacterial adhesins and invasins for the application in drug delivery strategies has recently become the focus of much attention. Paul and colleagues used an invasin fusion protein system for gene delivery strategies (46) and Easson and co-workers (47,48) used a similar approach for intestinal delivery of nanoparticles. The latter group found that latex microspheres up to 1 μm coupled to maltose-binding protein which was fused with invasin can be internalized by MDCK cell monolayers (47,48).

Bacterial and Plant Toxins

After reaching early endosomes by RME, diphtheria toxin (DT) molecules have two possible fates. A large pool enters the degradative pathway whereas a few molecules become cytotoxic by translocating their catalytic fragment A (DTA) into the cytosol (49)

The B subunit of the *E. coli* heat labile toxin binds to the brush border of intestinal epithelial cells in a highly specific, lectin-like manner. Uptake of this toxin and transcytosis to the basolateral side of the enterocytes was observed *in vivo* (50) and *in vitro* (51).

Fisher and co-workers expressed the transmembrane domain of diphtheria toxin in *E. coli* as a maltose-binding fusion protein and coupled it chemically to high-M_w poly-L-lysine. The resulting complex was successfully used to mediate the internalization of a reporter gene *in vitro* (52).

Staphylococcus aureus produces a set of proteins (e.g., staphylococcal enterotoxin A (SEA), SEB, toxic shock syndrome toxin 1 (TSST-1)) which act both as superantigens and toxins. Hamad and co-workers (53) found dose-dependent, facilitated transcytosis of SEB and TSST-1 in Caco-2 cells, but not SEA. They extended their studies in mice *in vivo* by showing that ingested SEB appears in the blood more efficiently than SEA.

Various plant toxins, mostly ribosome-inactivating proteins (RIPs), have been identified that bind to any mammalian cell surface expressing galactose units and are subsequently internalized by RME (54). Toxins such as nigrin b (55), α-sarcin (56), ricin and saporin (57), viscumin (58), and modeccin (59) are highly toxic upon oral administration (i.e., are rapidly internalized). The possibility exists, therefore, that modified and, most importantly, less toxic subunits of these compound can be used to facilitate the uptake of macromolecular compounds or microparticulates.

Viral Haemagglutinins

The initial step in many viral infections is the binding of surface proteins (haemagglutinins) to mucosal cells. These binding proteins have been identified for most viruses, including rotaviruses (60), varicella zoster virus (61), semliki forest virus (62), adenoviruses (63), potato leafroll virus (64), and reovirus (65).

Recently, Etchart and colleagues (66) compared the immune response to a vaccinia virus recombinant, expressing the measles virus haemagglutinin (VV-HA), after parenteral or mucosal immunizations in mice. Oral immunizations with 10^8 p.f.u. of VV-HA generated low numbers of HA-specific IgA-producing cells in the lamina propria of the gut, whereas oral co-immunization with VV-HA and cholera toxin greatly enhanced the level of HA-specific spot-forming cells (IgA>IgG). Interestingly, intrajejunal immunizations with 10^8 p.f.u. VV-HA alone induced high levels of anti-HA IgG-producing cells in the spleen and anti-HA IgA-secreting cells in the lamina propria of the gut. This study shows that VV-A can induce measles-specific immunity in the intestine provided that it is protected from degradation in the gastrointestinal tract, or that cholera toxin is used as an adjuvant.

Lectins (Phytohaemagglutinins)

Lectins are plant proteins that bind to specific sugars which are found on the surface of glycoproteins and glycolipids of eukaryotic cells. Such binding may result in specific haemagglutinating activity. Since lectins are relatively heat stable, they are abundant in the human diet (e.g., cereals, beans and other seeds). Concentrated solutions of lectins have a 'mucotractive' effect due to irritation of the gut wall, which explains why so-called 'high fiber foods' (rich in lectins) are thought to be responsible for stimulating bowel motility (67,68).

In another study demonstrating the rapid RME-uptake of lectins, Weaver and colleagues (69) directly infused concanavalin A, conjugated with 10 nm colloidal gold particles, into the lumen of the jejunum in neonatal guinea pigs. Within 60 min, both villous and crypt epithelial cells contained gold particles, demonstrating the rapid accessibility of crypt cells to the lectin.

Hussain and co-workers showed that the uptake mechanism for lectins can be utilized for intestinal drug targeting *in vivo* (24). They covalently coupled polystyrene nanoparticles (500 nm) to tomato lectin and observed 23% systemic uptake after oral administration to rats. Control animals exerted a systemic uptake of <0.5%, indicating a 50-fold increase in oral absorption. Interestingly, they showed the intestinal uptake of tomato lectin-conjugated nanoparticles via the villous tissue to be 15 times higher than uptake by GALT (24).

Although lectins are generally believed to be transported via an RME mechanism, there is substantial evidence that these compounds have significant affinity to intestinal M-cells. (70,71). Binding studies have revealed that M-cells exhibit pronounced regional and species variation in glycoconjugate

expression. Sharma and colleagues (72) studied the nature of cell-associated carbohydrates in the human intestine that may mediate transepithelial transport of bacterial and dietary lectins and their processing by the lymphoid cells of Peyer's patches. Upon comparison of human and mouse glycoconjugates of follicle-associated epithelium and GALT, they found a distinct difference in glycosylation between mouse and human Peyer's patches and their associated lymphoid cells. Thus, choosing the appropriate lectin is apparently important when considering cell surface glycoconjugates as target molecules for intestinal drug delivery strategies. In the future, knowledge of the site and species related variations in M-cell surface glycoconjugate expression may allow lectins to be utilized to selectively target antigenic material and oral vaccines to the mucosal immune system at specific locations (71). Again, it should be pointed out that the overall contribution of M-cells to the absorptive surface area of the gastrointestinal tract is minimal, which could jeopardize the widespread application of drug targeting to these specialized cells.

RME OF VITAMINS AND METAL IONS

Folate

The cellular uptake of free folic acid is mediated by the folate receptor and/or the reduced folate carrier. The folate receptor is a glycosylphosphatidylinositol (GPI)-anchored 38 kDa glycoprotein clustered in caveolae mediating cell transport by potocytosis (16). While the expression of the reduced folate carrier is ubiquitously distributed in eukaryotic cells, the folate receptor is principally overexpressed in human tumors. Two homologous isoforms (α and β) of the receptor have been identified in humans. The α-isoform is found to be frequently overexpressed in epithelial tumors, whereas the β-form is often found in non-epithelial lineage tumors (73). Consequently, this receptor system has been used in drug-targeting approaches to cancer cells (74), but also in protein delivery (75), gene delivery (76), and targeting of antisense oligonucleotides (77) to a variety of cell types. Although considerable success has been met in other areas of drug targeting, there are currently—to our knowledge—no reports in the literature describing the utilization, or attempted utilization, of this system for intestinal drug delivery purposes. This may, in part, be attributable to the low expression level of the receptor in (healthy) enterocytes. However, the fact that the α-isoform of the folate receptor is overexpressed in epithelial cell lines, local targeting to intestinal cancer cells (e.g. colon carcinoma) appears to be a fertile approach.

Riboflavin

Although not as extensively investigated as the transferrin and folate pathways, it was recently shown by Low and co-workers (78) that serum albumin coupled to riboflavin showed RME-mediated uptake in distal lung epithelium. In this paper the authors indicate that a similar uptake process exists in the small intestine, although these (yet unpublished) results need to be further investigated.

Vitamin B_{12}

Vitamin B_{12}, the colloquial name for cobalamin (Cbl), is a large polar molecule that must be bound to specialized transport proteins to gain entry into cells. After oral administration it is bound to intrinsic factor (IF), a protein released from the parietal cells in the stomach and proximal cells in the duodenum. The Cbl-IF complex binds to an IF-receptor located on the surface of the ileum, which triggers a yet undefined endocytotic process. After internalization, the fate of the IF-Cbl complex has yet to be clarified. It was reported that IF-Cbl complex dissociates at acidic pH, and Cbl is transferred to transcobalamin II by Ramasamy and co-workers (79), whereas Dan and Cutler (80) found evidence of free Cbl in endosomes and the basolateral side of the membrane after administration to the apical surface of Caco-2 cell monolayers. It is clear, however, that Cbl is transported into all other cells only when bound to transcobalamin II.

The vitamin B_{12} RME system is probably the most extensively studied system for the oral delivery of peptides and proteins. In humans, the uptake of cobalamin is approximately 1 nmol per intestinal passage, with a potential for multiple dosing (2–3 times per hour) (81). Russell-Jones and co-workers have shown that this particular system can be employed for the intestinal uptake of luteinizing hormone releasing factor (LHRH)-analogs (82), granulocyte colony stimulating factor (G-CSF, 18.8 kDa), erythropoietin (29.5 kDa), α-interferon (83,84), and the LHRH-antagonist ANTIDE (81). More recently, they showed (*in vitro* as well as *in vivo*) the intestinal uptake of of non-biodegradable polymeric nanoparticles coupled to cobalamin to be 2–3 fold higher compared to control (non-specific uptake of nanoparticles) (85,86). Thus far, the universal application of this transport system for the oral delivery of peptides and proteins seems only to be hampered by its limited uptake capacity: 1 nmol per dose. Even though this amount of uptake may be adequate for molecules such as LHRH or erythropoietin, it is clearly not sufficient for the delivery of insulin or G-CSF. However, the recent successes with cobalamin-conjugated nanoparticles *in vivo* are promising and eliminate the requirement of covalently coupling cobalamin and the substrate to be delivered. This, in turn, would permit effective delivery of any macromolecule via the vitamin B_{12} uptake mechanism.

Transferrin

Transferrin, an 80 kDa iron-transporting glycoprotein, is efficiently taken up into cells by RME. Transferrin receptors are found on the surface of most proliferating cells, in elevated numbers on erythroblasts and on many kinds of tumors. According to current knowledge of intestinal iron absorption, transferrin is excreted into the intestinal lumen in the form of apotransferrin and is highly stable to attacks from intestinal peptidases. In most cells, diferric transferrin binds to transferrin receptor (TfR), a dimeric transmembrane glycoprotein of 180 kDa (87), and the ligand-receptor complex is endocytosed within clathrin-coated vesicles. After acidification of these vesicles, iron dissociates from the transferrin/TfR complex and enters the cytoplasm, where it is bound by ferritin (Fn). The role that transferrin, TfR and Fn have in regulating dietary iron uptake and maintaining total body iron stores is unknown. Both the TfR and Fn genes can be detected on small intestinal mRNA (87), and both proteins have been isolated from intestinal enterocytes (88,89). The uptake of iron in the intestinal tract amounts up to 20 mg/

day and is primarily mediated by transferrin. Recently, Shah and Shen (90) showed that insulin covalently coupled to transferrin, was transported across Caco-2 cell monolayers by RME. More recently, they showed that oral administration of this complex to streptozotocin-induced diabetic mice significantly reduced plasma glucose levels (≈28%), which was further potentiated by BFA pretreatment (≈41%) (91).

SUMMARY AND FUTURE DIRECTIONS

Effective and efficient drug targeting to intestinal receptor-mediated endocytosis and transcytosis pathways is still in its infancy. One factor that may attribute to the lack of RME-targeting studies found in the literature is our limited understanding of the complex mechanisms of endocytotic membrane transport at the present time. However, recent advances in this area have resulted in a sufficiently well documented morphology of many of the major pathways. This knowledge, together with the multitude of potential intestinal molecular targets, should initiate the design of novel macromolecular drug delivery strategies based on RME pathways.

As with any drug delivery strategy, targeting to RME systems will have some disadvantages as well. For example, the use of plant toxins may be seriously jeopardized by their high immunogenicity which could trigger an antibody response locally in the intestine or systemically. Furthermore, there may be high interindividual variation in intestinal tolerance as well as allergy to these substances. Another problem may lie in the 'black hole' scenario: the drug delivery device is successfully taken up by RME, but the endocytotic vesicle fuses immediately with lysosomes, thus degrading the active compound. The limited uptake capacity of these systems may also appear problematic. However, the intrinsically high systemic activity of many macromolecular compounds should be sufficient to warrant a systemic response resulting from the intestinal absorption of compounds in the ng range. In this light, especially the uptake of nanoparticles (44–46) into and across the enterocyte open exciting new possibilities for the oral delivery of polymeric devices containing macromolecular therapeutics. Locally, these systems may be applied for gene therapy to fight genetically inherited diseases of the gut, such as irritable bowel syndrome or Crohn's disease. Furthermore, the current knowledge of the etiology of many viral and bacterial infections indicate an important role for intestinal internalization in the onset of disease. With the structural knowledge of viral and bacterial attachment proteins it would be possible to design antibodies against these internalization factors. Thus, we could work toward anti-adhesion therapy for microbial diseases.

In summary, the area of RME appears a promising target for the development of oral drug delivery devices for macromolecules. Basic research in cellular biology has presented us with new tools to rationally design drug delivery devices aimed at novel intestinal targets. It shall be exciting to monitor the progress in this area of drug delivery as current knowledge is applied and new and updated information on endocytotic mechanisms appear.

REFERENCES

1. S. Mukherjee, R. N. Ghosh, and F. R. Maxfield. Endocytosis. *Physiol. Rev.* **77**:759–803 (1997).
2. C. M. Lehr. The transcytosis approach. In A. G. de Boer (ed.), *Drug Absorption Enhancement: Concepts, Possibilities, Limitations and Trends*, Harwood, Switzerland, 1994, pp. 325–365.
3. M. Marsh and A. Helenius. Adsorptive endocytosis of Semliki Forest virus. *J. Mol. Biol.* **142**:439–454 (1980).
4. A. Pol, D. Ortega, and C. Enrich. Identification and distribution of proteins in isolated endosomal fractions of rat liver: involvement in endocytosis, recycling and transcytosis. *Biochem. J.* **323**:435–43 (1997).
5. P. Wigge, Y. Vallis, and H. T. McMahon. Inhibition of receptor-mediated endocytosis by the amphiphysin SH3 domain. *Curr. Biol.* **7**:554–60 (1997).
6. D. J. Yamashiro and F. R. Maxfield. Acidification of endocytic compartments and the intracellular pathways of ligands and receptors. *J. Cell. Biochem.* **26**:231–46 (1984).
7. H. J. Geuze, J. W. Slot, G. J. Strous, J. Peppard, K. von Figura, A. Hasilik, and A. L. Schwartz. Intracellular receptor sorting during endocytosis: comparative immunoelectron microscopy of multiple receptors in rat liver. *Cell* **37**:195–204 (1984).
8. B. Herman and D. F. Albertini. Ligand-induced rapid redistribution of lysosomes is temporally distinct from endosome translocation. *Nature* **304**:738–40 (1983).
9. E. J. Hughson and C. R. Hopkins. Endocytotic pathways in polarized Caco-2 cells: identification of an endosomal compartment accessible from both apical and basolateral surfaces. *J. Cell Biol.* **110**:337–348 (1990).
10. A. L. Schwartz. Receptor Cell Biology: Receptor-Mediated Endocytosis. *Pediatr. Res.* **38**:835–843 (1995).
11. M. Barroso, D. S. Nelson, and E. Sztul. Transcytosis-associated protein (TAP)/p115 is a general fusion factor required for binding of vesicles to acceptor membranes. *Proceed. Nat'l. Acad. Sci. USA* **92**:527–31 (1995).
12. P. P. Lemons, D. Chen, A. M. Bernstein, M. K. Bennett, and S. W. Whiteheart. Regulated secretion in platelets: identification of elements of the platelet exocytosis machinery. *Blood* **90**:1490–1500 (1997).
13. D. Shah and W. C. Shen. The establishment of polarity and enhanced transcytosis of transferrin receptors in enterocyte-like Caco-2 cells. *J. Drug Target* **2**:93–9 (1994).
14. K. Prydz, S. H. Hansen, K. Sandvig, and B. van Deurs. Effects of brefeldin A on endocytosis, transcytosis and transport to the Golgi complex in polarized MDCK cells. *J. Cell Biol.* **119**:259–72 (1992).
15. N. J. Severs. Caveolae: static inpocketings of the plasma membrane, dynamic vesicles or plain artifact? *J. Cell Sci.* **90**:341–8 (1988).
16. R. G. Anderson, B. A. Kamen, K. G. Rothberg, and S. W. Lacey. Potocytosis: sequestration and transport of small molecules by caveolae. *Science* **255**:410–1 (1992).
17. R. G. Anderson. Caveolae: where incoming and outgoing messengers meet. *Proceed. Nat'l. Acad. Sci. USA* **90**:10909–13 (1993).
18. H. Matsue, K. G. Rothberg, A. Takashima, B. A. Kamen, R. G. Anderson, and S. W. Lacey. Folate receptor allows cells to grow in low concentrations of 5-methyltetrahydrofolate. *Proceed. Nat'l. Acad. Sci. USA* **89**:6006–9 (1992).
19. T. V. Kurzchalia, P. Dupree, and S. Monier. VIP21-Caveolin, a protein of the trans-Golgi network and caveolae. *FEBS Let.* **346**:88–91 (1994).
20. K. G. Rothberg, J. E. Heuser, W. C. Donzell, Y. S. Ying, J. R. Glenney, and R. G. Anderson. Caveolin, a protein component of caveolae membrane coats. *Cell* **68**:673–82 (1992).
21. R. Montesano, J. Roth, A. Robert, and L. Orci. Non-coated membrane invaginations are involved in binding and internalization of cholera and tetanus toxins. *Nature* **296**:651–653 (1982).
22. W. A. Walker and I. R. Sanderson. Epithelial barrier function to antigens. An overview. *Ann. NY Acad. Sci.* **664**:10–17 (1992).
23. M. P. Desai, V. Labhasetwar, G. L. Amidon, and R. J. Levy. Gastrointestinal uptake of biodegradable microparticles: effect of particle size. *Pharm. Res.* **13**:1838–45 (1996).
24. N. Hussain, P. U. Jani, and A. T. Florence. Enhanced oral uptake of tomato lectin-conjugated nanoparticles in the rat. *Pharm. Res.* **14**:613–8 (1997).
25. M. Berryman and R. Rodewald. Beta 2-microglobulin co-distributes with the heavy chain of the intestinal IgG-Fc receptor

throughout the transepithelial transport pathway of the neonatal rat. *J. Cell Sci.* **108**:2347–60 (1995).
26. N. Benlounes, R. Chedid, F. Thuillier, J. F. Desjeux, F. Rousselet, and M. Heyman. Intestinal transport and processing of immunoglobulin G in the neonatal and adult rat. *Biol. Neonate* **67**:254–63 (1995).
27. C. Medesan, C. Radu, J. K. Kim, V. Ghetie, and E. S. Ward. Localization of the site of the IgG molecule that regulates maternofetal transmission in mice. *Eur. J. Immunol.* **26**:2533–6 (1996).
28. C. Medesan, D. Matesoi, C. Radu, V. Ghetie, and E. S. Ward. Delineation of the amino acid residues involved in transcytosis and catabolism of mouse IgG1. *J. Immunol.* **158**:2211–7 (1997).
29. D. R. Abrahamson and R. Rodewald. Evidence for the sorting of endocytic vesicle contents during the receptor-mediated transport of IgG across the newborn rat intestine. *J. Cell Biol.* **91**:270–80 (1981).
30. H. M. Patel and A. E. Wild. Fc receptor-mediated transcytosis of IgG-coated liposomes across epithelial barriers. *FEBS Let.* **234**:321–5 (1988).
31. J. Mestecky. The common mucosal immune system and current strategies for induction of immune responses in external secretions. *J. Clin. Immunol.* **7**:265–276 (1987).
32. R. P. Hirt, G. J. Hughes, S. Frutiger, P. Michetti, C. Perregaux, O. Poulain-Godefroy, N. Jeanguenat, M. R. Neutra, and J. P. Kraehenbuhl. Transcytosis of the polymeric Ig receptor requires phosphorylation of serine 664 in the absence but not the presence of dimeric IgA. *Cell* **74**:245–55 (1993).
33. K. R. Youngman, C. Fiocchi, and C. S. Kaetzel. Inhibition of IFN-gamma activity in supernatants from stimulated human intestinal mononuclear cells prevents up-regulation of the polymeric Ig receptor in an intestinal epithelial cell line. *J. Immunol.* **153**:675–81 (1994).
34. K. E. Mostov. Transepithelial transport of immunoglobulins. *Ann. Rev. Immunol.* **12**:63–84 (1994).
35. K. Mostov. The polymeric immunoglobulin receptor. *Sem. Cell Biol.* **2**:411–8 (1991).
36. K. Mostov. Protein traffic in polarized epithelial cells: the polymeric immunoglobulin receptor as a model system. [Review] [47 refs]. *J. Cell Sci.—Suppl.* **17**:21–6 (1993).
37. V. Livrelli, C. De Champs, P. Di Martino, A. Darfeuille-Michaud, C. Forestier, and B. Joly. Adhesive properties and antibiotic resistance of Klebsiella, Enterobacter, and Serratia clinical isolates involved in nosocomial infections. *J. Clin. Microbiol.* **34**:1963–9 (1996).
38. A. C. Ghose. Adherence & colonization properties of Vibrio cholerae & diarrhoeagenic Escherichia coli. *Ind. J. Med. Res.* **104**:38–51 (1996).
39. I. Adlerberth, S. Ahrne, M. L. Johansson, G. Molin, L. A. Hanson, and A. E. Wold. A mannose-specific adherence mechanism in Lactobacillus plantarum conferring binding to the human colonic cell line HT-29. *Appl. & Envir. Microbiol.* **62**:2244–51 (1996).
40. S. Crennell, E. Garman, G. Laver, E. Vimr, and G. Taylor. Crystal structure of Vibrio cholerae neuraminidase reveals dual lectin-like domains in addition to the catalytic domain. *Structure* **2**:535–44 (1994).
41. J. W. Metcalfe, K. A. Krogfelt, H. C. Krivan, P. S. Cohen, and D. C. Laux. Characterization and identification of a porcine small intestine mucus receptor for the K88ab fimbrial adhesin. *Infection & Immunity* **59**:91–6 (1991).
42. C. J. Thorns, G. A. Wells, J. A. Morris, A. Bridges, and R. Higgins. Evaluation of monoclonal antibodies to K88, K99, F41 and 987P fimbrial adhesins for the detection of porcine enterotoxigenic Escherichia coli in paraffin-wax tissue sections. *Vet. Microbiol.* **20**:377–81 (1989).
43. R. R. Isberg and J. M. Leong. Multiple beta 1 chain integrins are receptors for invasin, a protein that promotes bacterial penetration into mammalian cells. *Cell* **60**:861–71 (1990).
44. R. R. Isberg. Pathways for the penetration of enteroinvasive Yersinia into mammalian cells. [Review] [50 refs]. *Mol. Biol. & Med.* **7**:73–82 (1990).
45. A. Marra and R. R. Isberg. Invasin-dependent and invasin-independent pathways for translocation of Yersinia pseudotuberculosis across the Peyer's patch intestinal epithelium. *Infection & Immunity* **65**:3412–21 (1997).
46. R. W. Paul, K. E. Weisser, A. Loomis, D. L. Sloane, D. LaFoe, E. M. Atkinson, and R. W. Overell. Gene transfer using a novel fusion protein, GAL4/invasin. *Human Gene Ther.* **8**:1253–62 (1997).
47. J. H. Easson, D. Jahn, and C.-M. Lehr. Utilizing bacterial mechanisms of cell entry for the design of bioinvasive drug carriers. *Proceed. Int'l. Symp. Control. Rel. Bioact. Mater.* **24**:101–102 (1997).
48. J. H. Easson, D. Jahn, and C.-M. Lehr. Invasin-modified latex beads display bioinvasive properties to cultured epithelial cells. *Pharm. Res.* **14**:S21 (1997).
49. E. Lemichez, M. Bomsel, G. Devilliers, J. vanderSpek, J. R. Murphy, E. V. Lukianov, S. Olsnes, and P. Boquet. Membrane translocation of diphtheria toxin fragment A exploits early to late endosome trafficking machinery. *Mol. Microbiol.* **23**:445–57 (1997).
50. J. Lindner, A. F. Geczy, and G. J. Russell-Jones. Identification of the site of uptake of the E. coli heat-labile enterotoxin, LTB. *Scand. J. Immunol.* **40**:564–72 (1994).
51. L. Lazorova, A. Sjolander, G. J. Russell-Jones, J. Linder, and P. Artursson. Intestinal tissue distribution and epithelial transport of the oral immunogen LTB, the B subunit of E. coli heat-labile enterotoxin. *J. Drug Target.* **1**:331–40 (1993).
52. K. J. Fisher and J. M. Wilson. The transmembrane domain of diphtheria toxin improves molecular conjugate gene transfer. *Biochem. J.* **321**:49–58 (1997).
53. A. R. Hamad, P. Marrack, and J. W. Kappler. Transcytosis of staphylococcal superantigen toxins. *J. Exp. Med.* **185**:1447–54 (1997).
54. F. Stirpe and L. Barbieri. Ribosome-inactivating proteins up to date. [Review] [70 refs]. *FEBS Let.* **195**:1–8 (1986).
55. M. G. Battelli, L. Citores, L. Buonamici, J. M. Ferreras, F. M. de Benito, F. Stirpe, and T. Girbes. Toxicity and cytotoxicity of nigrin b, a two-chain ribosome-inactivating protein from Sambucus nigra: comparison with ricin. *Arch. Toxicol.* **71**:360–4 (1997).
56. I. D. Sylvester, L. M. Roberts, and J. M. Lord. Characterization of prokaryotic recombinant Aspergillus ribotoxin alpha-sarcin. *Biochim. Biophys. Acta* **1358**:53–60 (1997).
57. M. G. Battelli, L. Buonamici, L. Polito, A. Bolognesi, and F. Stirpe. Hepatoxicity of ricin, saporin or a saporin immunotoxin: xanthine oxidase activity in rat liver and blood serum. *Virchows Archiv.* **427**:529–35 (1996).
58. F. Stirpe, K. Sandvig, S. Olsnes, and A. Pihl. Action of viscumin, a toxic lectin from mistletoe, on cells in culture. *J. Biol. Chem.* **257**:13271–7 (1982).
59. S. Sperti, L. Montanaro, M. Derenzini, A. Gasperi-Campani, and F. Stirpe. Effect of modeccin on rat liver ribosomes in vivo. *Biochim. Biophys. Acta* **562**:495–503 (1979).
60. J. E. Ludert, F. Michelangeli, F. Gil, F. Liprandi, and J. Esparza. Penetration and uncoating of rotaviruses in cultured cells. *Intervirology* **27**:95–101 (1987).
61. J. K. Olson and C. Grose. Endocytosis and recycling of varicella-zoster virus Fc receptor glycoprotein gE: internalization mediated by a YXXL motif in the cytoplasmic tail. *J. Virol.* **71**:4042–54 (1997).
62. A. Irurzun, J. L. Nieva, and L. Carrasco. Entry of Semliki forest virus into cells: effects of concanamycin A and nigericin on viral membrane fusion and infection. *Virology* **227**:488–92 (1997).
63. B. S. Worthington and J. Syrotuck. Intestinal permeability to large particles in normal and protein-deficient adult rats. *J. Nutr.* **106**:20–32 (1976).
64. A. Garret, C. Kerlan, and D. Thomas. The intestine is a site of passage for potato leafroll virus from the gut lumen into the haemocoel in the aphid vector, Myzus persicae Sulz. *Arch. Virol.* **131**:377–92 (1993).
65. J. L. Wolf, R. S. Kauffman, R. Finberg, R. Dambrauskas, B. N. Fields, and J. S. Trier. Determinants of reovirus interaction with the intestinal M cells and absorptive cells of murine intestine. *Gastroenterology* **85**:291–300 (1983).
66. N. Etchart, F. Wild, and D. Kaiserlian. Mucosal and systemic immune responses to measles virus haemagglutinin in mice immunized with a recombinant vaccinia virus. *J. Gen. Virol.* **77**: 2471–8 (1996).
67. S. Bardocz, S. W. Ewen, G. Grant, and A. Pusztai. Lectins as

growth factors for the small intestine and the gut. In S. Bardocz, A. Pusztai (eds.) *Lectins—Biomedical Perspectives*, Taylor and Francis, London, 1995 pp. 103–117.
68. C.-M. Lehr and A. Pusztai. The potential of bioadhesive lectins for the delivery of peptide and protein drugs to the gastrointestinal tract. In S. Bardocz, A. Pusztai (eds.) *Lectins—Biomedical Perspectives*, Taylor and Francis, London, 1995 pp. 117–141.
69. L. T. Weaver and D. S. Bailey. Effect of the lectin concanavalin A on the neonatal guinea pig gastrointestinal mucosa in vivo. *J. Pediatr. Gastroenterol. & Nutr.* **6**:445–53 (1987).
70. A. Gebert, H. J. Rothkotter, and R. Pabst. M cells in Peyer's patches of the intestine. [Review] [310 refs]. *Int. Rev. Cytol.* **167**:91–159 (1996).
71. M. A. Jepson, M. A. Clark, N. Foster, C. M. Mason, M. K. Bennett, N. L. Simmons, and B. H. Hirst. Targeting to intestinal M cells. *J. Anat.* **189**:507–516 (1996).
72. R. Sharma, E. J. van Damme, W. J. Peumans, P. Sarsfield, and U. Schumacher. Lectin binding reveals divergent carbohydrate expression in human and mouse Peyer's patches. *Histochem. & Cell Biol.* **105**:459–465 (1996).
73. J. F. Ross, P. K. Chaudhuri, and M. Ratnam. Differential regulation of folate receptor isoforms in normal and malignant tissues in vivo and in established cell lines. Physiologic and clinical implications. *Cancer* **73**:2432–2443 (1994).
74. J. Fan, N. Kureshy, K. S. Vitols, and F. M. Huennekens. Novel substrate analogs delineate an endocytotic mechanism for uptake of folate via the high-affinity, glycosylphosphatidylinositol-linked transport protein in L1210 mouse leukemia cells. *Oncol. Res.* **7**:511–516 (1995).
75. C. P. Leamon and P. S. Low. Membrane folate-binding proteins are responsible for folate-protein conjugate endocytosis into cultured cells. *Biochem. J.* **291**:855–860 (1993).
76. K. A. Mislick, J. D. Baldeschwieler, J. F. Kayyem, and T. J. Meade. Transfection of folate-polylysine DNA complexes: evidence for lysosomal delivery. *Bioconjug. Chem.* **6**:512–5 (1995).
77. S. Wang, R. J. Lee, G. Cauchon, D. G. Gorenstein, and P. S. Low. Delivery of antisense oligodeoxyribonucleotides against the human epidermal growth factor receptor into cultured KB cells with liposomes conjugated to folate via polyethylene glycol. *Proceed. Nat'l. Acad. Sci. USA* **92**:3318–3322 (1995).
78. O. D. Wangensteen, M. M. Bartlett, J. K. James, Z. F. Yang, and P. S. Low. Riboflavin-enhanced transport of serum albumin across the distal pulmonary epithelium. *Pharm. Res.* **13**:1861–1864 (1996).
79. M. Ramasamy, D. H. Alpers, C. Tiruppathi, and B. Seetharam. Cobalamin release from intrinsic factor and transfer to transcobalamin II within the rat enterocyte. *American J.Physiol.* **257**:G791–G797 (1989).
80. N. Dan and D. F. Cutler. Transcytosis and processing of intrinsic factor-cobalamin in Caco-2 cells. *J. Biol. Chem.* **269**:18849–18855 (1994).
81. G. J. Russell-Jones. Oral delivery of therapeutic proteins and peptides by the vitamin B12 uptake system. In M. D. Taylor and G. L. Amidon (eds.) *Peptide-based drug design: controling transport and metabolism*, ACS, Washington, DC, 1995, pp. 181–198.
82. G. J. Russell-Jones, S. W. Westwood, P. G. Farnworth, J. K. Findlay, and H. G. Burger. Synthesis of LHRH antagonists suitable for oral administration via the vitamin B12 uptake system. *Bioconjug. Chem.* **6**:34–42 (1995).
83. A. D. Habberfield, K. Jensen-Pippo, L. Ralph, S. W. Westwood, and G. J. Russell-Jones. Vitamin B12-mediated uptake of recombinant therapeutic proteins from the gut. *Int. J. Pharm.* **145**:1–8 (1996).
84. G. J. Russell-Jones, S. W. Westwood, and A. D. Habberfield. Vitamin B12 mediated oral delivery systems for granulocyte-colony stimulating factor and erythropoietin. *Bioconjug. Chem.* **6**:459–465 (1995).
85. G. J. Russell-Jones, L. Arthur, S. Killinger, and S. W. Westwood. Vitamin B12-mediated transport of nanoparticles. *Proceed. Int'l. Symp. Control. Rel. Bioact. Mater.* **24**:11–12 (1997).
86. G. J. Russell-Jones, S. W. Westwood, and A. D. Habberfield. The use of the vitamin B12 transport system as a carrier for the oral delivery of peptides, proteins and nanoparticles. *Proceed. Int'l. Symp. Control. Rel. Bioact. Mater.* **23**:49–50 (1996).
87. A. Pietrangelo, E. Rocchi, G. Casalgrandi, G. Rigo, A. Ferrari, M. Perini, E. Ventura, and G. Cairo. Regulation of transferrin, transferrin receptor, and ferritin genes in human duodenum. *Gastroenterology* **102**:802–809 (1992).
88. G. J. Anderson, L. W. Powell, and J. W. Halliday. Transferrin receptor distribution and regulation in the rat small intestine. Effect of iron stores and erythropoiesis. *Gastroenterology* **98**:576–85 (1990).
89. G. P. Jeffrey, K. A. Basclain, and T. L. Allen. Molecular regulation of transferrin receptor and ferritin expression in the rat gastrointestinal tract. *Gastroenterology* **110**:790–800 (1996).
90. D. Shah and W. C. Shen. Transcellular delivery of an insulin-transferrin conjugate in enterocyte-like Caco-2 cells. *J. Pharm. Sci.* **85**:1306–1311 (1996).
91. J. Wang, D. Shen, and W.-C. Shen. Oral delivery of an insulin-transferrin conjugate in Streptozotocin-treated cf/1 mice. *Pharm. Res.* **14**:S469 (1997).