Ligand-Mediated Oral Uptake of Nanospheres in
the rat

A thesis presented by
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Centre For Drug Delivery Research
The School of Pharmacy
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London
Flight by machines heavier than air is unpractical and insignificant, if not utterly impossible

Simon Newcomb
(a contemporary of the Wright Brothers), 1835-1909
To my parents and family whose support and encouragement in difficult times proved decisive in completing this thesis
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ABSTRACT

The thesis is concerned with the exploitation of the phenomena of intestinal particulate absorption for delivery of biodegradable colloidal carriers incorporating gastrointestinal labile molecules thereby affording protection of the latter against the hostile and degrading milieu of the intestine.

Peyer’s patches, are the main portals of entry of both living and inert particulate matter mediated by its unique enterocyte, the M-cell, which displays considerable phagocytic activity enabling it to sample luminal contents for xenobiotics to underlying macrophages. Numerous agents have been shown to bind specifically to the M-cell and attempts to target using these ligands have met little success due partly to differential binding specificity of the cell according to the animal model employed. As the M-cell represents less than 1% of the total enterocyte population more success can be achieved if the intestine as a whole is used as the bioadhesive target. Bioadhesion of sub-micron particulates to the intestine significantly enhances their uptake as exemplified by certain invasive enterobacteriaceae. Thus three differing ligands covalently conjugated to 500nm polystyrene nanospheres were employed to enhance particulate intestinal absorption. Tissue extracted polymer was quantified using gel permeation chromatography.

The non-toxic Tomato lectin was investigated due to its epithelial bioadhesive properties. Oral administration of tomato lectin conjugated nanospheres for 5 days resulted in 25% systemic absorption compared to <1% absorption of blocked nanospheres with N-acetylchitotetraose. The reticuloendothelial system was unable to sequestrate the nanospheres indicating for the first time that orally absorbed nanospheres coated with glycoproteins behave in a similar manner to intravenously injected particles (eg pegylated-liposomes) designed to avoid the liver and spleen.

The final part of the thesis is concerned with exploiting bacterial mechanisms of epithelial internalization. Invasin is a 986-amino acid outer membrane protein that confers Yersinia to invade non-phagocytic mammalian cells such as enterocytes. The carboxyl-terminal 192 amino acids which is sufficient to promote bacterial uptake was isolated as maltose binding fusion protein (MBP) from genetically engineered E. coli and coupled to nanospheres. After a single dose approximately 13% was found in the systemic circulation compared to two controls, MBP-coupled nanospheres and invasin-coupled nanospheres blocked with mucin which both exhibited < 2% in the blood. All nanospheres avoided the reticuloendothelial system.
LIST OF ABBREVIATIONS

TL  Tomato lectin (*Lycopersicon esculentum*)
PP  Peyer's patch(es)
GALT Gut-associated lymphoid tissue
GIT Gastrointestinal Tract
GI Gastrointestinal
FAE Follicle-associated epithelium
RES Reticuloendothelial system
MBP Maltose Binding Protein
MBP-Inv-192 Terminal 192 amino-acids of Invasin of *Yersinia enterocolitica* coupled to maltose binding protein
PS Polystyrene
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Chapter one - Introduction
1.1 Problems associated with oral peptide delivery

Recent years have witnessed the infusion of recombinant DNA techniques, computational chemistry and molecular modelling in pharmaceutical chemistry leading to the production and molecular characterisation of potent peptides and isolation of virulence factors and bacterial epitopes for the design of novel vaccines. Simultaneous advances in the field of biotechnology and fermentation technologies have provided the means to mass produce such compounds relatively economically. It is, however, becoming increasingly clear that if defined antigens and epitopes are to be developed as effective oral vaccines and for peptides to treat chronic diseases, such as diabetes novel approaches are required for both the site specific oral delivery and overcoming biological barriers *en route* to realise the clinical utility of any potential therapeutic system.

A large number of biologically active peptides and proteins have been introduced as drug entities and currently insulin, analgesic peptides (endorphins and their analogues), plasminogen activators, alpha and gamma interferons and other bioactive peptides can only be administered by frequent injections. Pain associated with injections and perceived disruption of life styles can cause poor acceptance of parenteral dosage forms by patients except those facing life threatening situations. A recent survey revealed that there were at least 150 peptide or protein drugs undergoing clinical trial in 1989 almost all of which could not be delivered orally (cited in Harris and Robinson, 1990).

There are many biochemical and cellular barriers which prevent the oral delivery of peptides, proteins or drugs with poor permeation characteristics such as aminoglycosides, cephalosporins and antifungals. The stomach has a low pH and is a protease containing environment. In the small intestine the pH is neutral but the total concentration of the proteases is in the order of 1mg/mL and contains detergent bile salts. At the cellular level there is the mucus barrier (approx. 400μm thick), brush border peptidases, tight junctions of limited permeability and epithelial and endothelial cell with cytosolic enzymes such as di/tri-peptidases, alkaline phosphatases and proteasomes. For the vast majority
of drugs the only established route of administration is by injection, a route which is not well accepted by patients particularly on chronic therapy. Thus drug delivery which in recent years has gained an equal footing with drug discovery is seen as the bottle neck representing the potential Achilles's heel of the biotechnology and academic efforts. Oral delivery of peptides and other gut-labile or poor permeating molecules is a tantalizing double edged sword. On the one hand it is the most desirable route of administration yet it is also the most hazardous to the health of the peptides. Lee notes that "a decade ago oral peptide delivery was considered a fantasy" (cited in Wallace and Lasker, 1993). Thus desirability and feasibility are at the opposite ends of the scale. Although the oral delivery may not be bioequivalent to invasive methods the inspite of advances made in this area (eg peptide-mimetics) the convenience to the patient in most cases will outweigh the demand for complete bioequivalence.

Consequently various strategies have been invoked in attempts to promote the intestinal absorption of such class of agents including the use of permeation enhancers, co-administration of protease inhibitors, lipid based formulations, bioadhesive polymers to increase the mucosal contact time and the generation of peptide-mimetics. The use of so-called "permeation enhancers" is popular which many have been found to improve oral peptide bioavailability. However enhancers suffer from non-specificity in action, propensity to elicit local irritation, irreversibility in action and the likelihood of the co-current enhancement of absorption of additional macromolecules such as antigens, environmental toxins, resident bacteria and digestive enzymes resulting in the possibility of hypersensitivity or pathological sequelae. It is sobering to remember that one of the first attempts to deliver insulin as in 1923 and the use of oral absorption promoter, saponin, was described as early as 1926 (cited in Davis, 1989). A multitude of permeation enhancers with differing modes of action have been employed (Aungst, 1988). Amongst these are bile salts which due to their relatively low toxicity and extensive mucosal absorption (approx. 18g/day in man) have been proposed as absorption enhancers. Aungst (1988), however,
could not demonstrate any hypoglycaemic effect of orally administered insulin in rats despite employing insulin at doses 5 times higher than administered by other routes (rectal, buccal nasal) with the co-application of sodium glycolate which also displays a modest inhibitory effect on luminal peptidases. Recently Sandoz reported that the oral bioavailability of Ocreotide, a somatostatin analogue, showed a modest increase from 1% to 3.5% with the application of a non-ionic surfactant. Such a well designed and properly conducted study employing cell cultures, animal models and following up with a Phase 1 study using human volunteers demonstrating a modest increase, and is important as it provides a realistic basis in the understanding of the problems associated with the oral delivery of peptides and proteins.

Co-administration of proteins with enzyme inhibitors has been shown to increase the oral bioavailability in some cases but as in the case of permeation enhancers the long-term administration of such agents must be investigated before this becomes an accepted practice. Okagawa et al (1994) has suggested that while some enzyme inhibitors alone increased the intestinal half-life of ebiratide, an undecapeptide adrenocorticotropic analogue, combination of such agents would be necessary for the complete protection of this peptide from the wide array of both luminal and brush-border enzymes. To date there are no known safe enzyme inhibitors that display non-specific inhibition of all the proteolytic enzymes in the intestine. A single peptide can be acted upon by a multitude of enzymes and one may conclude that protection of certain sites on the protein molecule or at a certain anatomical site may prove fruitless. Structural modification of polypeptides by either chemical or recombinant manipulation have improved stability and minimized enzymatic cleavage in some cases particularly for smaller molecules but for larger molecules such as human growth hormone where tertiary structure becomes important for biological activity modification can often mean failure to conserve the desired effect. Acyl-derivatives of Insulin have been synthesised to reduce enzymatic cleavage and to render them more lipophilic to promote their lymphatic
absorption (Asada et al., 1994). However the authors reported that these acyl
derivatives exhibited "adequate pharmacological activities" implying that this
was less than native insulin. Similar consideration apply to peptide-mimetics
which by definition are molecules "that no longer contain any peptide bonds and
have a molecular weight of less than 700kDa" (Moore, 1994). Most peptide-
mimetics discovered by random screening are receptor antagonists as
exemplified by the recently FDA approved angiotensin II mimetic, Losarten, with
the notable exception of methadone. Production of true mimetics, especially of
larger proteins seems a formidable task given the increased complexity of the
situation. Further problems associated with the mucosal, endothelial and blood-
brain absorption of such small hydrophillic molecules can be envisaged.
Although still in its infancy this approach holds promise given the rapid
advances made in computational chemistry, molecular modelling, NMR and
proteins crystallography. A further disadvantage of this approach is that
exclusively peptide and protein substitutes are generated by this method thus
excluding a large class of compounds which are also subject to enzymatic
degradation and poor mucosal penetration such as anti-sense oligonucleotides
and plasmid DNA for oral somatic gene therapy and mucosal vaccination.

The low availability of peptides is also attributed to too short a residence time
at the site of absorption. Bioadhesive formulations such as hydrogel or
microspheres either coated or encapsulated with the gut-labile agent have thus
attracted attention. Hexylcyanoacrylate nanoparticles have been shown to be
capable of enhancing the bioavailabilty of adsorbed vincamine relative to an
aqueous solution of the drug following oral administration to rabbits (Maincent
et al., 1986). It was hypothesised by the authors that this was not due to the
intestinal uptake of the particles but the increased residence or exposure to the
intestinal epithelium thus protecting it against luminal enzymatic degradation.
However previous attempts to promote oral absorption of Insulin by adsorption
to nanoparticles had proven less successful due to degradation by brush border
peptidases, cytosolic proteasome which displays trypsin, chymotrypsin and
carboxypeptidase activities and the recently discovered insulin degrading
enzyme (IDE) during the transit of the drug across the enterocyte. If other peptides are to be delivered by this method one also has to take into account the effects of first pass metabolism.

Redirection of peptide drugs through the lymphatic route has been investigated in order to avoid the destructive effects of first pass metabolism particularly as many of the current recombinants are bioactive peptides whose physiological target is the lymphatic system (interferons, immunosuppressives such as cyclosporin). Complexation of such drugs within microemulsions, lipid microspheres and in vehicles composed of agents resembling those in chylomicrons have been reported to promote their lymphatic absorption thus diverting them from the portal blood and mimicking the triglyceride pathway. Mixed micellar solutions formed in the duodenum with the aid of bile salts or the viscous isotropic phase upon the interaction of lipids with water (also known as the cubic crystalline phase) are both able to encapsulate GIT-labile drugs and exclude much of the water borne enzymatic activity. Both types of vesicles are then suggested to gain access to the villus lacteal after the endocytosis by the enterocyte (intact?) and excytosed as chylomicrons (ref). Herein the problem rests as the effectiveness of the selective lymphatic transfer of the system resides in its stability whilst traversing the enterocyte and interstitium. Cho and Flynn (1989) described a "water-in-oil microemulsion sprayed onto an inert carrier" system that was designed to present encapsulated Insulin to the epithelium with lipid components in proportions roughly to those within chylomicrons. In addition an undisclosed amount of the proteolytic inhibitor, aprotinin, was included in the formulation. Thus it was implied that the emulsion droplets would be absorbed intact such that substantial amounts of the administered dose would be delivered to the liver through attachments to chylomicrons and its remnants. However it is known that chylomicrons are formed by the re-synthesis of fatty acids in the Golgi-complex where several specific apolipo-proteins and lipids are added. These have a vital role in chylomicron transport and it is not clear how drugs can remain attached to a lipid particle through the various stages of metabolism and modification. Despite
this, blood glucose levels in 3 diabetic patients were successfully controlled for 6 months. However the data presented was very limited and bioavailability or total duration of blood glucose reductions were not reported. Furthermore insulin levels peaked quickly a phenomena inconstant with what is known about the fat absorption into the lymphatic system. Physiologically Insulin is secreted by the islets of Langerhans directly into portal capillaries thus it appears that the lipids in the formulation may have facilitate passage of Insulin as permeation enhancers rather than diverting it through the lacteals.

Preferential lymphatic uptake of peptides does not necessarily result in increased oral bioavailability. Yanagawa et al (1989) reported a 48-fold ineresae in the lymphatic absorption of cyclosporin in a mixed micellar solution but the absolute availability remained less than 0.05% The lymph itself contains appreciable amounts of enzymes of blood plasma. In human thoracic duct lymph Linder et al (1958) measured the content of aldolase, alkaline phosphatase and transaminase. Bruggeman (1975) could demonstrate pepsin-like activity after the micropuncture of canine gastric lymphatics, suggesting that lymphatics close to the intestinal secretory epithelium may carry enzymes from their cells of origin to the blood. However in general the rapid mixing of lymph from other sources reduces their concentration in major lymph trunks.

Rapid transit times and high proteolytic activity combined with slow absorption kinetics limits potential peptide uptake. Peptides and proteins ideally need to be delivered into a part of the intestine devoid of secreted digestive enzymes such as the colon which is drained by hepatic portal veins and lymphatics. Transit times in the colon often exceed 8h compared to 3h in the small intestine with little mixing involved. Thus the colon has attracted attention because of the perceived gentler environmental conditions. The targeted delivery of a formulation to the colon is feasible by either using coating agents/polymers that are susceptible to disruption to the colonic pH or bacterial enzymatic activity or with time dependent pulsatile systems. Kopecek et al (1991) strategy involved pH sensitive hydrogels which were able to protect the encapsulated peptides.
from gastric acids but swelled when reaching the higher pH of the colon thus increasing the permeability of the polymer and aiding drug release. The polymers, linked to fucosylamine residues, were able to bind to soluble colonic lectins that reside in the epithelium. However the absorption of peptides by the lymphatics of the colon is very limited further reduced by its lower surface area. Woodley (1991), however, dismisses the colon as a site of peptide absorption because of the high level of bacterial peptidases and that the peptide must traverse multiple membranes before entering the bloodstream where degradation *en route* can occur. Nevertheless the experiments of Kopecek and co-workers (1991) demonstrate elegantly that a combination of strategies are required in order to optimize oral peptide delivery.

The above illustrates some of the disadvantages associated with traditional use of such approaches given the known physiological behaviour of the gastrointestinal tract. Thus a combination of such approaches are required to overcome the mucosal barrier as all of the above approaches afford only a modest improvement of oral bioavailability because of their inability to protect protein and peptide drugs from proteases beyond the lumen during their transit across the enterocyte, interstitium, endothelial and hepatic cells. Since enzymes are ubiquitous complete absorption of a peptide/protein is an exception. Optimization and reproducibility of delivery is a more realistic objective rather than complete absorption. Unless a drug is prohibitively expensive to manufacture it matters more than 10% of the total dose consistently arrives at its intended destination than 90% was lost.

This thesis will examine the phenomenon of intestinal uptake of particulate matter with the view of exploiting these processes to increase the intestinal uptake of model sub-micron particles conjugated on the outer surfaces with ligand molecules that are known to bind to the intestinal epithelium and, in some cases, to induce their uptake through non-professional phagocytic cells such as the enterocyte. The oral administration of a drug or macromolecule
selectively encapsulated in a delivery system capable of protecting it from the ravages of the intestinal milieu, reducing the possibility of non-specific interaction with food or co-administered medication, allowing its absorption across the intestinal membrane without dispersion throughout the GIT and protecting it from cytosolic and first pass metabolism are the perceived advantages of oral nanoparticulate delivery systems that may open many interesting therapeutic possibilities (Table 1.1). Further, incorporation of antigens for oral vaccination has several advantages including not only the protection from degredation but the possible incorporation of cytokines to enhance immunogenicity and protection of distant mucosal tissues (McGhee and Kiyono, 1993). In addition a single dose of microspheres with programmed short and long biodegradation times may induce overlapping primary and long-lasting secondary immune responses, thus eliminating the need for booster doses (Eldridge et al., 1989).

Table 1.1 Perceived advantages of micro- and nano-particulate drug carriers

<table>
<thead>
<tr>
<th>Advantage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Relatively high payload of drug</td>
</tr>
<tr>
<td>Protection of drug and non-target tissues from each other</td>
</tr>
<tr>
<td>Controlled and sustained release of drug</td>
</tr>
<tr>
<td>Protection of drug from enzymatic degradation</td>
</tr>
<tr>
<td>Possible avoidance of food and drug interactions</td>
</tr>
<tr>
<td>Increased shelf-life and easier sterilisation</td>
</tr>
</tbody>
</table>

Adapted from O'Mullane et al (1987)
Microsphere vehicles composed of biodegradable polymers that are able to withstand intestinal degradation and during traffic through the enterocyte but are able to degrade in the systemic circulation upon the action of enzymes such as plasma esterases could achieve the desired result. This is the ideal case. The objective is to deliver orally peptides and other gut labile molecules such that their systemic value of 1-2% achieved by traditional oral drug delivery strategies is increased to the order of at least > 20%. However, only a small proportion of administered nanoparticulates are translocated to the systemic circulation. Thus the use of model polystyrene nanospheres in the studies to be described in this thesis is to increase the absorption of orally administered colloidal particles from a general level of 1-2% with use of bioadhesive ligands as bioadhesion significantly enhances intestinal particulate uptake as exemplified by non-motile enterobacteriaceae (reviewed in Trier, 1991). Reproducible and increased uptake of colloidal carriers could offer an alternative approach to the consistent systemic delivery of encapsulated drugs. In addition identification of the site and magnitude of uptake, particulate characteristics and the mechanistic aspects of systemically absorbed particles after intestinal administration will yield important information as a prelude to either the present or future use of biodegradable microspheres in both animal and human volunteer studies.

1.2 Historical perspectives of intestinal particulate translocation by the mammalian gastrointestinal tract

The GIT is commonly assumed to be an impenetrable barrier to the uptake of inert intraluminal macromolecular and particulate matter. O'Mullane et al (1987) concluded that the "transport of intact carriers across the GIT is restricted to exceptional and unusual circumstances" after their experiments had failed to demonstrate systemic delivery of nanoparticles after oral administration. This was also partially fuelled by the wide differences in the size and magnitude of particles absorbed, methods employed to evaluate the extent of luminal and systemic absorption and the mechanisms reportedly involved (Table 1.2)
Table 1.2 Factors affecting intestinal particulate translocation

<table>
<thead>
<tr>
<th>Category</th>
<th>Details</th>
</tr>
</thead>
<tbody>
<tr>
<td>Particle characteristics</td>
<td>size, charge and hydrophobicity</td>
</tr>
<tr>
<td>Size of dose administered</td>
<td>Hydrostatic and osmotic pressures</td>
</tr>
<tr>
<td>Length of administration</td>
<td>Chronic or single day</td>
</tr>
<tr>
<td>Core materials</td>
<td>polystyrene - non-immunogenic- liposaccharide capsules - auto-enhancement</td>
</tr>
<tr>
<td>Route of administration</td>
<td>Oral gavage, intragastric intubation etc. (Table 1.6)</td>
</tr>
<tr>
<td>Sampling times</td>
<td>gastric emptying</td>
</tr>
<tr>
<td>Particle free periods prior to sampling/sacrifice</td>
<td>competitive inhibition of administered particles</td>
</tr>
<tr>
<td>Environmental</td>
<td>slow GIT transit rate in diabetic rats, gut perforation in Crohn's disease</td>
</tr>
<tr>
<td>Gut status</td>
<td>within animal GIT, between species, Inflammation in Crohn's disease</td>
</tr>
<tr>
<td>Number and size of Peyer’s patches</td>
<td></td>
</tr>
<tr>
<td>Role of M-cells</td>
<td></td>
</tr>
<tr>
<td>Analysis of different regions of GIT</td>
<td></td>
</tr>
<tr>
<td>Method of Quantitation</td>
<td>(Table 1.6)</td>
</tr>
</tbody>
</table>
However numerous investigators past and present, including our laboratories, have consistently demonstrated that particulate matter can breach the intestinal barrier, the potential of which is an important phenomenon that has toxicological, immunological and pathological implications. More recently the application of the route for oral drug delivery using drug-encapsulated nanoparticles is being investigated. The other main criticism levelled against the intestinal uptake of particulates by a number of authors (Jenkins et al., 1994) relates to the fact that the tight GIT epithelium was designed to exclude intestinal particulate matter. The adoption of such negative approaches over the last decade in attempts to overcome biological barriers would have stunted development.

From Table 1.3 it can be seen that in addition to the size of particles translocated (from few nm to 150μm), widely differing surface characteristics of inert matter and their proposed route of absorption in various animal models has been reported over the last 150 years despite the action of gut motility and mucus (approx. 400μm thick) the latter which prevents the diffusion of luminal proteases which would otherwise damage the epithelial cells. This introduction is concerned with the evidence that supports or negates the concept of intestinal absorption of particulates, excluding liposomal and lipid microspheres or microemulsions which will not be discussed due to their inherent instability in luminal fluids, particularly bile acids which are excellent solvents for liposome-based products. Furthermore only the potential for uptake of particulates across the healthy or intact adult intestinal epithelium will be considered thus the effect of any pre-existing pathology or disease (eg Crohn's disease, ulcerative colitis etc) will not be discussed as this has been extensively reviewed in the literature and is beyond the scope of this thesis (Sanderson and Walker 1994). Nor will any consideration be given to the neonatal intestine which for a brief period of time (<1 month) displays an enhanced endocytic activity in transcytosing macromolecules for the acquisition of passive immunity.
For a long time carbohydrates were regarded as compounds completely devoid of biological function, serving merely as a source of energy for cells or as components of tissue secretions to provide protective materials for cells. Many biological recognition and adhesion processes involve interactions between carbohydrates and proteins. Cells, organisms and soluble components exhibit information about themselves in the form of exquisitely defined saccharide structures displayed in oligosaccharides present in glycolipid and glycoprotein components of biological membranes on their surfaces. The information is decoded by a group of highly stereospecific and specialised carbohydrate proteins, lectins, a structurally diverse class of glycoproteins present in a wide range of biological materials particularly plants but also bacteria, animal and human tissues, their only common features being the ability to bind carbohydrates specifically and reversibly. This has lead to the notion that, owing to their significant variability and ubiquitous presence in biological systems, carbohydrates represent a third biological code system in addition to nucleic acids and proteins.
Table 1.3 Some examples of intestinal absorption of inert particulate matter

<table>
<thead>
<tr>
<th>Year</th>
<th>Reference</th>
<th>Particle</th>
<th>Size (μm)</th>
<th>Magnitude of uptake^ (%)</th>
<th>Proposed route of uptake</th>
</tr>
</thead>
<tbody>
<tr>
<td>1844</td>
<td>Herbst</td>
<td>starch</td>
<td>10-100</td>
<td>n/d</td>
<td>n/d</td>
</tr>
<tr>
<td>1959</td>
<td>Barnett</td>
<td>azo dye</td>
<td>0.02-0.05</td>
<td>n/d</td>
<td>Enterocyte</td>
</tr>
<tr>
<td>1961</td>
<td>Sanders</td>
<td>latex</td>
<td>0.2</td>
<td>n/d</td>
<td>Enterocyte</td>
</tr>
<tr>
<td>1968</td>
<td>Volkheimer</td>
<td>various</td>
<td>7-150</td>
<td>n/d</td>
<td>Paracellular</td>
</tr>
<tr>
<td>1973</td>
<td>Pontrefact</td>
<td>asbestos</td>
<td>1-5</td>
<td>n/d</td>
<td>Enterocyte</td>
</tr>
<tr>
<td>1977</td>
<td>LeFevre</td>
<td>latex</td>
<td>0.2</td>
<td>n/d</td>
<td>Peyer's patch</td>
</tr>
<tr>
<td>1980</td>
<td>LeFevre</td>
<td>latex</td>
<td>6</td>
<td>n/d</td>
<td>Peyer's patch</td>
</tr>
<tr>
<td>1984</td>
<td>Nefzeger</td>
<td>acrylate</td>
<td>0.5</td>
<td>15</td>
<td>Enterocyte?</td>
</tr>
<tr>
<td>1988</td>
<td>Wells</td>
<td>latex</td>
<td>1</td>
<td>n/d</td>
<td>Macrophage</td>
</tr>
<tr>
<td>1989</td>
<td>Alpar</td>
<td>latex</td>
<td>1</td>
<td>39</td>
<td>n/d</td>
</tr>
<tr>
<td>1989</td>
<td>Eldridge</td>
<td>latex</td>
<td>1-15</td>
<td>n/d</td>
<td>Peyer's patch</td>
</tr>
<tr>
<td>1990</td>
<td>Damge</td>
<td>acrylate</td>
<td>0.3</td>
<td>n/d</td>
<td>Peyer's patch</td>
</tr>
<tr>
<td>1990</td>
<td>Jani</td>
<td>latex</td>
<td>0.05</td>
<td>18</td>
<td>Peyer's patch</td>
</tr>
<tr>
<td>1990</td>
<td>Childers</td>
<td>liposomes</td>
<td>0.6</td>
<td>n/d</td>
<td>M-cells</td>
</tr>
<tr>
<td>1990</td>
<td>Sass</td>
<td>latex</td>
<td>0.5</td>
<td>n/d</td>
<td>M-cells</td>
</tr>
<tr>
<td>1994</td>
<td>Jenkins</td>
<td>latex</td>
<td>1</td>
<td>1x10^-5</td>
<td>n/d</td>
</tr>
</tbody>
</table>

1. Appearance in extra-intestinal fluids ie. blood, lymph etc
Chronologically published reports on the intestinal uptake of particulates falls into 3 periods. Between 1840 and 1960 the demonstration of microbes and inert matter was interpreted as an abstract or an abnormal function of the intestine while some microbiologists acknowledged the possibility of systemic infection by the enteric bacteria (Fisher, 1931). As early as 1844 Gustav Herbst observed starch granules in the blood and lymph of a dog 3h after feeding a starch suspension. Until the turn of the century this was known as the "Herbst effect", the passage of solid undissolved particles from the intestinal lumen to the blood. Hirsch (1906) and Verzar (1911) confirmed the Herbst effect, demonstrating starch granules (> 10µm) in the blood and urine of dogs and humans following oral administration of flour. This early work summarising the intestinal absorption of "corpuscular" material has been reviewed by Verzar (1931).

The second period (1960-1980) yielded detailed information of specific intestinal structures, namely Peyer’s patches (described in detail in section 1.3.1), that were involved in the uptake of inert particulates. This was mainly due to the rapid advances in the development of optical and scanning electron microscopical technologies. During this period intestinal uptake of particulate matter gave rise to concerns of toxicological and immunological consequences of ingested particulates from diet or enviromental contamination breaching the intestinal epithelium. Nuclear fallout in the 1950’s, silicosis and asbestosis from mining were concerns through direct ingestion or, more probably from material swallowed after inhalation and clearance by the cilia from the lungs (Figure 1.1). The main proponents in this field were Bockman and Cooper (197300), LeFevre and co-workers (1970 - 1980) and Owen (1974 onwards). Volkheimer and colleagues in a series of publications from 1961 (reviewed in 1968) controversially demonstrated the regular appearance of a wide variety of large insoluble particles in the blood of human volunteers after peroral administration. These investigations aroused much of the recent interest in particulate ingestion.
Sources: Chemical Products and By-Products of Agricultural, Manufacturing, Food and Industrial Processes

Airborne Emission from Autos and Industry

Agricultural and Industrial Wastes

Food and Household Objects

Distribution

Soil washed by rain

Edible Plant Life

Poultry, Meat

B belt, Lakes, Oceans

Aquatic Life

Drinking Water

Oral Intake by Humans

INTESTINAL ABSORPTION

Feces

Blood

Health Effects

CNS

Reticulocytes

Kidney

Liver

Endocrine

Figure 1.1 Routes of particle ingestion
For a long time carbohydrates were regarded as compounds completely devoid of biological function, serving merely as a source of energy for cells or as components of tissue secretions to provide protective materials for cells. Many biological recognition and adhesion processes involve interactions between carbohydrates and proteins. Cells, organisms and soluble components exhibit information about themselves in the form of exquisitely defined saccharide structures displayed in oligosaccharides present in glycolipid and glycoprotein components of biological membranes on their surfaces. The information is decoded by a group of highly stereospecific and specialised carbohydrate proteins, lectins, a structurally diverse class of glycoproteins present in a wide range of biological materials particularly plants but also bacteria, animal and human tissues, their only common features being the ability to bind carbohydrates specifically and reversibly. This has lead to the notion that, owing to their significant variability and ubiquitous presence in biological systems, carbohydrates represent a third biological code system in addition to nucleic acids and proteins.

With increasing development and use of colloidal polymeric drug carriers during the eighties, investigators attempted to exploit, enhance or amplify the physiological phenomenon of intestinal particulate translocation particularly from the Peyer’s patches by optimising macroscopic characteristics (mainly alteration in size and surface characteristics) of both non- and biodegradable microspheres (Jani et al., 1990; Eldridge et al., 1989). The rationale behind the use of this route was the protection of the encapsulated material from the degrading milieu of the GIT both before and after translocation of microspheres across the intestinal epithelium and finally releasing the payload into the blood upon the action of systemic enzymes on the biodegradable carrier.
1.3 Site and mechanism of intestinal particulate translocation

Various terms have been applied to describe the uptake and transport of particulates across the intestinal epithelium due to the varying sizes of particles used in such studies. The most appropriate term to describe the passage of particulates across the intestine is probably translocation which has been used by many microbiologists (Berg et al., 1983) to describe the passage of bacteria from the GIT to mesenteric lymph nodes and subsequent dissemination to other distant organs. The term implies nothing about the mechanism of uptake and transport involved but simply signifies that penetration of the gut by particulates has occurred. Unless methodologies such as the use of appropriate endocytic, pinocytic or phagocytic inhibitors are employed in particulate uptake studies one cannot ascribe a particular mechanism with any degree of certainty. The term translocation will thus be used throughout this thesis unless investigators have conclusively described a mechanism. However it is pertinent here in the overall theme of the discussion that the sites of particulate penetration and possible mechanisms proposed to begin with a discussion of probably the most well known components of the GIT capable of and adapted to, translocating particulate matter, the Peyer’s patches.

1.3.1 Absorption of particulates across intestinal lymphoid tissue

A: Physiology of Peyer’s patches

Lymphoid tissue can be identified early in phylogeny in the gills and gut of species more advanced than cyclostomes (Marchalonis, 1984). Peyer’s patches (PP) are collections of sub-epithelial lymphoid follicles burgeoning amongst villi and distributed throughout the small intestines (Figure 1.2) from the pylorus to the ileocecal valves in a wide variety of animals including birds, reptiles and mammals (Kagnoff, 1981; Owen and Bhalla, 1983). Historically PP were of less of concern to physicians than to butchers who noted their thimble shaped
structures on the external surfaces of the intestines used as casings for sausages; nitrates in the preservatives coloured PP red and made them less marketable (Owen, 1987; Ten Cate, 1969). The intestinal lymphatics were most probably first studied by the Alexandrian school of medicine, Hippocrates considering PP as "intestinal glands" involved in reducing the "humidity" of the intestine (cited in Ten Cate, 1969). It was not until 1677 that Johann Conrad Peyer described the organs in greater morphological detail and correctly identified them as part of the lymphatic system that includes the spleen, thymus, lymph nodes, bronchial- and gut-associated lymphoid tissue (GALT) all of which have the same phyto- and ontogenetic origin. In mammals the GALT consists of a ring of lymphoid tissue encircling the oropharynx, PP in the small intestines, lymphoid nodules and clusters in the colonic mucosa lining the appendix and isolated lymphoid follicles that in total make up 25% of the all lymphoid tissue in man and produce the major secretory immune response (IgA). PP are located on the anti-mesenteric side of the gut and extend through the luminal epithelium, lamina propria and lamina submucosa.
Figure 1.2  Micrograph taken at low magnification showing a section of the human small intestine. Unlike in the rat the mucosa is thrown into transverse folds, the plica circlares PC covered with villi V. The mucosa MM is dived histologically into 3 layers: an epithelial lining, a supportive connective tissue lamina propria and immediately below a thin smooth muscle layer which produces local movement and folding of the layer. The vascular submucosa S is a layer of loose connective tissue that extends into the plica circulares and contains the larger blood vessels and lymphatics from which both types of capillaries arise into each villi. The inner circle CM and outer longitudinal LM muscle layers of the muscularis propria is the muscle wall proper and is the basis of continuous peristaltic activity. Larger lymphatics, blood vessels and nerves traverse through this layer originating from the adventitia the outer layer of connective tissue that conducts the mesenteric vessels and nerves. Where the adventitia is exposed to the abdominal cavity, as in the rat, it is referred to as the serosa S and is lined by a simple squamous epithelium called mesothelium. The most prominent feature of the small intestine is the presence of lymphoid aggregations within the lamina propria or more commonly known as the Peyer's patches PP. The M-cell that reside over the dome region cannot be identified using light microscopy. In smaller mammals such as the rat and mice the above layers are difficult to identify to their close proximity to each other and to their small width.
Although the base of the enclosed lymphoid follicles does not penetrate the muscularis mucosa they can extend extensively in the lamina propria and submucosa occupying a far greater volume than the luminal surface. Their distribution, number and size varies widely amongst species and individuals and is also age dependent (Table 1.4); in humans they can be identified as early as 24 weeks of gestation. In humans and rats PP occur with increasing frequency and larger sizes more distally in the small bowel. At birth human infants have about 100 to 250 PP and these increase in size after to exposure to the gut flora reaching a maximum of 300 at puberty. Numbers decrease with age (Corones, 1965). A similar trend was also reported in domestic mammals by Carlens in 1928 (cited in Rubas and Grass, 1991). Before puberty human PP are oval-shaped, usually 1-4 cm in width and 1-2 cm wide and increase in size with age, reaching 28 cm in length in some instances. On average human PP contain 10-60 follicles but have been noted to contain several hundred in the terminal ileum (980 in one patient). The size of the follicles does not vary greatly among species but the number of follicles aggregated within each patch increases with the size of the animal. The mouse for example has only 3 to 6 follicles per patch.

PP are not present in all animals. There are no reports of PP in some primates such as the Mandrillus leucophaceus and Papio annubis (Sonntag, 1922) and have not been observed in the Cynomolgus monkey (Rubas and Grass, 1991). In other animals such as the sheep, canine, rabbit and cattle there are two distinct types PP. The ileal PP is larger, with tightly packed follicles and functionally different from the jejunal PP, possibly representing the mammalian equivalent to the avian Bursa of Fabricius involved in the B lymphocyte production and priming, unlike in man where this occurs in the thymus. In contrast morphological studies in mice, rats and humans do not indicate the existence of these regional differences in PP. The above highlights the importance of choosing the correct animal model for experimentation on any aspect of PP function.
<table>
<thead>
<tr>
<th>Species</th>
<th>Age</th>
<th>Number</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>range</td>
</tr>
<tr>
<td>Bovine</td>
<td>1-10 yrs</td>
<td>22-45</td>
</tr>
<tr>
<td></td>
<td>10-14</td>
<td>14-36</td>
</tr>
<tr>
<td>Chicken</td>
<td>10 days</td>
<td>1-2</td>
</tr>
<tr>
<td></td>
<td>4 mths</td>
<td>5</td>
</tr>
<tr>
<td>Canine</td>
<td>0.5-1 yr</td>
<td>26-29</td>
</tr>
<tr>
<td>Horse</td>
<td>new born</td>
<td>245-320</td>
</tr>
<tr>
<td>Man</td>
<td>1-3 yrs</td>
<td>122-244</td>
</tr>
<tr>
<td></td>
<td>15-19</td>
<td>170-316</td>
</tr>
<tr>
<td></td>
<td>21-29</td>
<td>109-201</td>
</tr>
<tr>
<td></td>
<td>51-95</td>
<td>59-169</td>
</tr>
<tr>
<td>Mice</td>
<td>nd</td>
<td>6-10</td>
</tr>
<tr>
<td>dd-mice</td>
<td>60 days</td>
<td>6-12</td>
</tr>
<tr>
<td>c57 black mice</td>
<td>&gt; 1.5 yrs</td>
<td>4-12</td>
</tr>
<tr>
<td>C-mice</td>
<td>&gt; 1.5 yrs</td>
<td>4-13</td>
</tr>
<tr>
<td>Rabbit (NZ)</td>
<td>nd</td>
<td>2-10</td>
</tr>
<tr>
<td>Rabbit (Chinchilla)</td>
<td>nd</td>
<td>1-7</td>
</tr>
<tr>
<td>Rat (Wistar and S/D)</td>
<td>10 days</td>
<td>5-6</td>
</tr>
<tr>
<td></td>
<td>&lt; 1yr</td>
<td>15-18</td>
</tr>
<tr>
<td>Sheep</td>
<td>7 mths</td>
<td>24-40</td>
</tr>
<tr>
<td></td>
<td>adult</td>
<td>32-35</td>
</tr>
</tbody>
</table>

In general PP numbers are similar in the same species regardless of the strain and reach maxima at in young adults decreasing with age to a relatively constant number (Modified from Rubas and Grass, 1991)
The cyto-architecture of PP is comparable to that of the lymph nodes and is organised into follicular and parafollicular areas containing B and T lymphocytes respectively. A diagrammatic representation of a typical PP with its cellular components is shown in Figure 1.3. Although not well defined, each PP has an internal reticular framework supporting the whole structure but unlike lymph nodes is not surrounded by a capsule. The lymphoid cell components in PP have been analyzed in several species. PP host a wide variety of lymphocytes including B, T and dendritic cells representing 30% of the total cell population in the dome region. In humans T and B lymphocytes are found in clusters in PP at 14 weeks of gestation and increase in number thereafter. Follicles with germinal centres contain pre-B and mature B lymphocytes which actively differentiate as distinct from non-germinal B cells that are primarily located in the sub-epithelial zone (also called the dome region or mixed cell zone) and constitute 60-70% of the total PP lymphoid population. T lymphocytes predominate in the para- and interfollicular regions and are also present in the dome region and constitute 25-35% of lymphoid cells. A variety of accessory cells, defined as cells capable of antigen presentation to B and T lymphocytes form the remainder of PP cells. These include primarily macrophages, and to a lesser extent, stellate-shaped dendritic cells, the former containing dead lymphocytes and particulate material such as viruses and bacteria that have penetrated the epithelium overlying the dome region. The exact number of lymphoid cells varies widely among both species and strain of an animal and is also both age and immune status dependent. In humans biopsy specimens reveal a mean cell yield of $1.7 \times 10^8$ lymphocytes per gram of PP tissue (Rubas and Grass, 1991). Mucosal lymphoid tissue are sites of exceedingly active cell proliferation and lymphocyte trafficking reflecting the high concentration of blood capillary vessels and particularly lymphatic vessels compared to adjacent villi. This, combined with the great numbers of lymphoid cells, indicated to early immunologists that PP may play a more fundamental physiological role, as noted by Yoffey et al (1961) who first drew attention to the "high level of lymphocytopoietic activity as any part of the lympho-myeloid complex."


Figure 1.3 Diagrammatic representation of an ileal Peyer's patch in the rat. Peyer's patches lymphoid nodules are composed of three major components: follicular area (germinal centre, corona, and dome), interfollicular and parafollicular areas beneath villi, and dome epithelium. Curved arrows on the left show the influx of lymphocytes across postcapillary high endothelial venules, which mediate selective immigration of lymphocytes. M-cells are difficult to distinguish by light microscopy, and are inferred from the location of the groups of lymphoid cells in the follicle epithelium (small arrows). T, T-lymphocytes: B, B-lymphocytes: TBM, tingible body macrophage: m, muscularis (modified from Kato and Owen, 1994).
PP, tonsils and the appendix were considered vestigial organs with unknown function, important only as possible sites of suppuration. However several earlier observations indicated unusual phenomena associated with PP which were generally considered to be areas of weaknesses in the intestinal wall since they were first noted in hog intestine used as casings for sausages. Clinically PP were also notable areas of decreased tensile strength in the mucosal barrier especially as sites of intestinal penetration by bacteria such as *Salmonella typhi* as demonstrated by Remlinger (1897; cited in Owen, 1994) almost a century ago who observed swollen PP with such infection. Earlier in 1829 Louis had described intestinal perforation as a primary complication of typhoid fever reporting that the perforation was singular and located just above the caecum, which corresponds to the location of the major PP in humans (cited in Owen, 1993). This may also explain why PP are unusually few in the distal duodenum but more numerous and larger in the ileum. Slowing of intestinal contents above the ileocecal valve allows increased contact time between antigens and the mucosa, and that PP develop in response to this increased exposure. Although PP are well developed in fetal life it take few weeks after birth before activated lymphoid follicles appear (Bridges et al., 1956) and are further 2 years for a fully developed sIgA response (Mcdonald, 1992) reflecting once again the intimate relationship between the development of organized intestinal lymphoid tissue and exposure to intraluminal antigens.

The importance of PP in the actual induction of a humoral response was deduced only 20 years ago by surgical construction in rabbits of pairs of externalised intestinal loops which either had one PP or lacked one. Introduction of antigens into the lumen of one loop resulted in the appearance of SIgA in both loops over a similar time course but only if the immunised loops bore a PP. If the immunised loop lacked a PP, no response could be detected in the luminal secretions of either loop, irrespective of whether the immunised loop contained a PP (Robertson and Cebra, 1976).
Kumagai (1922) had noted that dead *Mycobacterium tuberculosis* fed to rabbits were taken up by lymphoid follicles in the PP and appendix, and the same process was seen with powdered erythrocytes, carmine and graphite particles suggesting that it was a general phenomenon and not a specific mechanism of bacterial invasion or bacterial motility. Further reports of inert material uptake by the PP in the human ileum, the avian Bursa of Fabricius and the rabbit appendix (Bockman and Cooper, 1973) focused attention on the epithelium covering the lymphoid follicles; these were subsequently found to contain epithelial cells specially adapted for uptake and translocation of both living and inert particulate material.

B: Uptake of particulates by the Follicle-associate epithelium - M-cells

One of the most critical aspects of PP concerns the overlying follicle-associated epithelium (FAE). Antigens breach PP for processing the FAE which thus replaces the afferent lymphatics of peripheral lymph nodes and epitomizes the principal difference between PP and lymph nodes. This characteristic is in keeping with the notion that the antigen is sampled directly from the gut lumen via the overlying epithelium. The efferent lymphatics then drain the absorbed particulate antigens to the mesenteric lymph nodes. Burrows (1931) investigating the pathology of poliomyelitis concluded "that the disease must enter by way of the gastro-intestinal lymphatics, PP and the solitary lymph follicles. Symptoms in the latter region are always present......from this portal of entry, the disease spreads to the contagious lymphatic tissues in the body."

The FAE has two characteristics compared with the adjacent villus epithelium that facilitates entrapment and translocation of antigens. the first is that the FAE in most species has fewer goblet cells that secrete mucus to entrap particles, impeding their attachment to the underlying epithelium (Bockmann and Cooper, 1973; Owen and Jones, 1974). This reduction in goblet cells allows greater contact between luminal contents and the FAE facilitating antigen sampling and
processing. The second and more crucially the more important differences between the 2 types of epithelium is the presence of specialised enterocytes termed M-cells, first described by Owen and Jones (1974) representing one of the most important finds in immunology in recent years. Such cells were first described by Schmedjte (1965) which he termed lymphoepithelial cells covering the dome-shaped lymphoid FAE in the rabbit appendix. Later Bockmann and Cooper (1973) discovered a similar enterocyte that could transport ferritin and India ink particles to underlying lymphoid tissue by pinocytosis in the chicken Bursa of Fabricius, mouse PP and rabbit appendix. In 1974 Owen and Jones found similar cells in the human ileal PP and appendix that engulfed one or more lymphocytes, macrophages or, rarely, polymorphonuclear leucocytes within an extracellular space termed the central hollow or pocket. The cell was called the "microfold cell" by its characteristics under SEM or "membranous cell" under TEM and were later designated as "M-cells" initially because of irregular microfolds over their luminal surface replacing microvilli found over enterocytes (Figure 1.4), but they are now thought as membranous as lymphocytes invaginating their basolateral surfaces compress them into attenuated bands. M-cell transport of limited amounts of luminal material for presentation to underlying lymphoid cells initiates a series of events leading to B-cell migration, differentiation and secretion of IgA antibodies over the entire intestinal lumen to remove adherent pathogens by immune exclusion. This peculiar function has been widely studied (Table 1.5). M-cells have been observed to take up inorganic material (Owen, 1977), viruses (Wolf et al., 1983; Scicinski et al., 1990) and various gram negative bacteria (reviewed in Kato and Owen, 1994) demonstrating the non-selectivity and size-independent uptake of widely different particulates.
Figure 1.4 Diagrammatic representation of M-cells. The basal surface of the M-cell forms an intraepithelial pocket containing lymphocytes or macrophages. M-cells transport macromolecules, particles and micro-organisms via clathrin coated pits, fluid phase endocytosis and phagocytosis involving extensions of cellular processes and reorganisation of submembrane actin filaments, resulting in the transport of foreign material to endosomal like vesicles in the apical cytoplasmic layer above the intraepithelial "pocket". The transcellular pathway is shortened by the deep invagination of the basolateral membrane that can approach the apical side to within 300nm. This favours the redirection of apical endosomal vesicles containing foreign material to this central hollow or pocket thus liberating enclosed particles, bacteria etc by exocytosis to immunocompetent cells. The particles can then be phagocytosed by PP or mesenteric lymph node macrophages, or evade capture and knead through the numerous lacteals present in the inter- or parafollicular areas which later drain to larger afferent lymphatics to the mesentery nodes. Antigen-sensitised IgA-committed B-cells leave the PP and migrate to distant glandular and mucosal sites where they terminal differentiate into polymeric IgA-producing plasma cells. Epithelial and glandular cells transport sIgA by receptor-mediated endocytosis, releasing sIgA into secretions thus preventing bacterial or antigen adherence by adsorbing onto their surfaces. Non-immunogenic inert particles such as latex nanospheres may not evoke this latter response.
Table 1.5 Substances and microorganisms taken up by M-cells.

**Particles and Macromolecules**

- Native and cationized ferritin
- Horseradish peroxidase
- Wheat germ agglutinin
- Cholera toxin
- Picibanil (OK-342)
- Gold core liposomes (750nm)
- Phosphatidyl choline liposomes
- Latex particles (500-750nm)

**Viruses**

- Reovirus type 1 and 3
- Poliovirus type 1
- Human immunodeficiency virus type 1 (HIV-1)
- Mouse mammary tumor virus

**Bacteria**

- *Vibrio cholerae*
- *Salmonella typhi and enteritidis*
- *Bacillus Camette-Guerin (BCG)*
- *Mycobacterium paratuberculosis*
- *Brucella abortus*
- *Streptococcus pyogenes*
- *Campylobacter jejuni*
- *Yersinia enterocolitica and tuberculosis*
- *Shigella flexneri*
- *E. coli RDEC-1 strain*
- *E. coli 0:124 K : 72 strain*

**Parasites**

- Cryptosporidium

Adapted from Kato and Owen (1994)
The very recent finding of M-cell transepithelial transport of *Streptococcus pneumoniae* R36a (Regoli et al, 1995), a gram negative bacteria confirms the long forgotten experiments of Kumagai (1922) of the general ability of the cell to interact with bacteria with very different kinds of cell walls. The fact that the *E. coli* RDEC-1 strain binds to the M-cell exclusively 4 hours after oral inoculation but to normal enterocytes only 24 hours later demonstrates the role of M-cells in antigen surveillance (Inman and Cantey, 1983). As the GIT is presented with the formidable task of differentiating pathogen from nutrient (eg whether to mount an immune response to surface coat proteins on enteroviruses against proteins derived from diet) and "self" from "non-self" (ie pathogens vs resident gut flora), the M-cells of the GALT can be considered to be the "AWACS" or early warning detection system of the GIT. As the GIT is relatively more "permeable" to particulates compared to, for example, skin, the role displayed by M-cells has created a long-standing need about the origin, regulation and, recently, the exploitation in the area of mucosal vaccination employing colloidal carriers. Since their discovery in the 70's a number of unique structural and cytoplasmic features have been elucidated in tandem with the trancytosis of widely differing sizes and nature of particulates ranging from bacteria, and viruses to inert material such as polystyrene and polylactide nanospheres (Sass et al., 1990)). M-cells shown diagrammatically in Figure 1.4 have a more open apical surface with less glycocalyx and mucus and more widely spaced irregular microvilli allowing the approach of particulates. M-cell expression of intermediate filaments in their cytoplasm and microfolds (predominately vimentin) compared to normal enterocytes, that is, more typical of cells of mesenchymal origin, reflects the active phagocytic activity and accommodation in shape to enfolded lymphocytes which can approach the apical membrane to within 300nm. Lymphocytes intrude the M-cell presumably in response to homing signals and come into contact with surveillance information in the form of macromolecules, particles and micro-organisms that have been translocated via clatharin-dependent pits and vesicles (Neutra et al., 1987), fluid-phase endocytosis in either coated or uncoated vesicles (Bockmann and Cooper, 1973; Owen, 1977) and phagocytosis involving extension of
cellular processes and reorganisation of submembrane actin assemblies, particularly with bacterium such as *Salmonella* (Bliska et al., 1992). The well developed microvesicular system and sparse lysosomes (16 fold reduction in volume fraction of dense bodies or lysosomal vesicles cf normal enterocytes) are morphological correlates of the transport function of the M-cells that serves to present absorbed antigens rapidly with little modification or degradation to the lymphoid cells beneath to allow their recognition and initiation of an immune response (Owen and Bhalla, 1986). This was shown by Keljo and Hamilton (1983) who observed that the transport of horseradish peroxidase across porcine jejunal epithelium containing a PP was 3 times greater than similar segments lacking one, and with significantly less degradation. Coupled to this, enzymatic histochemistry in the dome region of the PP (the highest concentration of M-cells) has revealed a lack of, or minor activity of, alkaline and acid phosphatase, succinate dehydrogenase, leucine aminopeptidase, lactase and sucrase, further reducing antigen degradation prior to their absorption by the FAE compared to neighbouring villi (Stiglmair-Herb et al., 1986) providing further evidence for the reduced enzymatic degradation of particulates and macromolecules during their trancytosis to underlying macrophages for the recognition and subsequent initiation of an immune response. The possible presence of endosomal hydrolases in M-cell transport vesicles has not yet been examined in detail but a recent study showed that apical vesicles are acidified (Allan et al., 1992). Most viruses and bacteria have been shown to be ultrastructurally unaltered during transport but this does not rule out limited enzymatic processing of their surface antigens as demonstrated very recently by Regoli et al. (1995). They demonstrated the formation of indentations in the outer wall of *Streptococcus Pneumonia* R36a during its rapid transport across the M-cell.

The FAE basement membrane overlying lymphoid follicles in rats and Owl monkeys has been found to be markedly porous in terms of the size and number of apertures (> 0.5μm) compared to adjacent villous cores (McGlugage et al., 1986). Such porosity facilitates the exocytosis of particles that have been
absorbed by the FAE and also the lymphocyte/macrophage traffic in both directions (Regoli et al., 1994). Allan and Trier (1991) demonstrated that intravenously injected sheep anti-laminin IgG-HRP conjugates labelled the basement membrane of murine blood capillaries in villi but not that of PP, although laminin was abundant in both. These differences in the permeability of the microcirculation to macromolecules underlying these epithelia may promote PP function by retaining the absorbed antigens in the PP interstitium, allowing their uptake by antigen presentation cells for initiation of an immune response or their removal by macrophages. This study provides further evidence that the lymphatic absorption of absorbed particles as lacteals are more permeable than blood capillaries.

Measurement of membrane potential, Cl− conductance and intracellular Cl−, K+, Na+ and H+ activities in the FAE of mice has distinguished two large populations of FAE having M-cell like or villous enterocyte-like properties. The presence of higher Cl− conductance, the inability to accumulate Cl− and the maintenance of low membrane potential compared to enterocytes has been suggested to aid the macropinocytosis of macromolecules and particles across the apical M-cell membrane (Cremaschi et al., 1990). Na+, K+-ATPase expression on the basolateral membrane, that maintains the Na+ electrochemical gradient energising the uptake of nutrients such as glucose and amino acids into the cells, is 20-60% lower in rabbit M-cells than neighbouring enterocytes (Mason et al., 1993). This indicates that the primary role of the FAE is the uptake of macromolecular antigens and particles with minimal involvement of active solute absorption since the expression of this membrane enzyme is related to their functional capacity for secondary active solute transport. This is consistent with earlier observations of rat FAE exhibiting reduced valine and other amino acids transporting capacity in sharp contrast to the prime function absorptive cells (Smith and Syme, 1982).

In a series of chronic oral feeding studies from 1970, Joel and LeFevre repeatedly demonstrated the accumulation of colloidal carbon and latex
microspheres in the PP of mice. Studies in which 2µm latex particles and colloidal carbon (20-50nm) were fed for 61 days demonstrated elegantly the prime function of PP since they contained the vast majority of particles compared to neighbouring villi but contributed only a small fraction of the intestinal weight (Joel et al., 1977). Furthermore the authors demonstrated a non-linear dependence on the concentrations ingested (i.e. a saturable limit was approached), that uptake was cumulative as particles could be found with difficulty after 2 days but with ease after 2 months feeding and this absorption took place in the germ-free mice i.e. secondary absorption following bacterial induced translocation since normal rats also demonstrated the same level of uptake indicative of an intrinsic property of PP. In a more recent study the authors showed the age-independent uptake of 1.8µm microspheres in young (24 days) and aged (18 months) rats showing the particle sampling ability of PP is maintained throughout life (LeFevre et al., 1989). More recently shorter chronic oral feeding studies (Jani et al., 1989, 1990: Eldridge et al., 1990) have confirmed LeFevre’s findings of PP being the main site of particle uptake, although all authors did not demonstrate directly the involvement of M-cell mediated uptake. The first direct demonstration of M-cell uptake of micron-sized polymeric microspheres for possible use in oral colloidal delivery was conducted only 5 years ago (Sass et al, 1990: Childers et al., 1990) where 0.5µm latex microspheres and liposomes were transported by M-cells. Since then there have been numerous ultrastructural studies both in orally graved rats and ligated intestinal loops containing PP showing not only M-cell transport of particles and liposomes (Jeurissen et al., 1987; Childers et al., 1990) but also bacteria and viruses (Table 1.5). Pappo and Ermak (1989, 1992, 1994) have conducted detailed morphological studies employing isolated rabbit intestinal loops. Fluorescent 600-750nm particles were internalised by M-cells with 10 minutes of intra-luminal application. The microspheres adhered to M-cells and were rapidly and synchronously trancytosed into the intraepithelial pocket, so that an estimated 5% of the injected particles were taken up in a single round of endocytosis. However M-cells comprise 50% of rabbit FAE cell population (Pappo et al, 1989) approximately 5 times greater than in murine models thus
it is not surprising that the order of uptake was one order greater in magnitude. However the use of animals subjected to trauma by obstructing intestinal segments under study with ligatures naturally increases the time of exposure of enclosed material to the epithelium and may initiate or enhance the uptake of unphysiological high concentrations, in the absence of peristalsis, the natural gut-clearance mechanism, of particulates (Rhodes and Karnovsky, 1971) ie. in order to facilitate detection of uptake that under physiological conditions would occur less frequently yet would be sufficient for the induction of an immunological response.

The acquisition of these structural and cellular modifications of M-cells is related to their origin. There is some controversy whether M-cells arise as a new cell type from the stem cells in the crypts encircling the PP (Bye et al., 1984; Scicinski et al., 1986) or by an immune related transformation of existing dome epithelial cells (Smith and Peacock, 1980; Smith et al., 1987). From studies in mouse PP Smith and Peacock (1980) have proposed that intra-epithelial lymphocytes induce the formation of M-cells from fully differentiated dome enterocytes since the GALT response to the to challenge by rotavirus, entertoxigenic E. coli and Salmonella typhi results in the increase in the number of intraepithelial lymphocytes and M-cells in FAE. (Popischil et al., 1986). The recent discovery that antigenic challenge by Salmonella led to the rapid formation of M-cells (within 24h of ingestion) throughout the FAE of germ-free mice PP (Savidge et al., 1991) re-enforces an earlier finding by the same investigators in the doubling of crypt-PP height and a 3 fold increase in the area occupied by M-cells after the transfer of specific-free pathogen (spf) mice to an animal house environment, the final value being similar to that recorded for long-term animal house animals. Additionally no M-cells were seen in the crypt region. This hypothesis has not been confirmed by a morphometric analysis performed in the same species. Scicinski et al. (1986) demonstrated that many normal FAE enterocytes in contact with intra-epithelial lymphocytes did not exhibit the characteristics features of M-cells ie one would have expected that the microvilli of those absorptive cells in contact with lymphocytes to be shorter.
and more widely spaced as compared with microvilli of cells with no contact with lymphoid cells. Furthermore no intermediate forms between M-cells and enterocytes could be detected such as gradual shortening of microvilli and disappearance of alkaline-phosphatase over normal enterocytes as M-cells have negligible apical enzyme activity. Finally the frequency of M-cells in spf mice was not lower than that in conventional mice while the number of lymphoid cells in the FAE of spf mice was reduced two fold. The recent discovery of vimentin cytoskeletal protein as a positive cell surface marker of M-cells in rabbit PP provides further support for the opposing view that M-cells arise directly from crypt stem cells as vimentin positive cells were found at the periphery of both rabbit PP and caecal lymphoid patch with no apparent contact with lymphoid cells and, more importantly, by their location appear to derive directly from undifferentiated crypt stem cells and not from mature columnar absorptive cells on the dome periphery (Jepson et al., 1992; Gebert et al., 1992). These latter observations are consistent with earlier studies conducted by Bye et al., 1984) who observed a spectrum of M-cells ranging from typical M-cells to immature-appearing M-cells along the PP dome base close to the mouth of the mouse crypts. These latter cells intermediate between undifferentiated crypt cells and mature M-cells displayed structural features, such as the lack of fully developed microvilli and a terminal filament web and the extensive absorption of cationised ferritin, but crucially lacked the lymphocyte-containing basolateral invagination characteristic of fully differentiated M-cells. However the appearance of H^3 labelled crypt cells as fully differentiated dome absorptive and M-cells takes 60h for migration whereas the appearance of fully formed M-cells after antigenic challenge is seen within 12-24h (Savidge et al., 1991). Thus it appears that accumulation of lymphoid cells in the vicinity of M-cells may be a phenomenon secondary to M-cell differentiation and that the sub-population of the epithelial cells at the base of the dome (ie. "immature-appearing" M-cells) is pre-determined or programmed to differentiate into M-cells upon the interactions of pathogenic strains of bacteria or by the products of lymphoid cells (eg lymphokines) which accumulate in response to antigenic challenge. Identification of factors that
mediate or induce the transformation of immature M-cells to mature M-cells, thus making it relatively more permeable and intracellular environment less hostile to foreign particles may provide an exciting method of overcoming or transforming a variety of biological membrane barriers.

A greater knowledge of the structure and function of M-cells may not only increase our understanding of the normal physiology of intestinal immune response and disorders (e.g., Crohn's disease, ulcerative colitis) but also provide a method of exploiting this route for the selective targeting of gut-labile drugs for oral administration if appreciable amounts of colloidal carriers can be directed to these structures. Even though M-cell activity is not clearly understood it appears that these cells are not involved in solute absorption as such small numbers of can hardly contribute to energy provision. The important part played by M-cells as initiators of early immune response and immune surveillance creates a long-standing need about the origin and regulation of M-cells and the factors that regulate the attachment and transport of widely differing sizes and nature of particles. Despite all the work that has been conducted many questions remain still unanswered. Similar to the lymphocyte in the 1950's the M-cell remains "Cellula plena mysterii". As fast as old problems are solved, new questions seem to arise.

1.3.2 Translocation of particles by enterocytes

Since the range of molecular sizes of macromolecules merges into the domain of particulate carriers it is not perhaps surprising that the uptake of small particles by enterocytes has been reported. The uptake of azo dye particles (20-40nm) by rat duodenal enterocytes through a fluid phase pinocytic mechanism was reported by Barnett in 1959 that were observed in membrane bound vesicles before finally appearing the intercellular spaces of the epithelium. The classical morphological study by Sanders and Ashworth (1961) demonstrated not only the uptake of 220nm latex particles by jejunal epithelial
cells but followed their route of translocation to the systemic circulation. Using TEM particles were seen to be carried through the cytoplasm in intact vesicles (phagolysosomes ?), discharged into the interstices of the lamina propria and gained access to submucosal lymphatics before finally appearing in the liver sinusoids and the space of Disse, all in the space of 2 hours after oral gavage. From their photographic evidence the precise mechanism of initial apical entry of the particles cannot be ascertained, although the epithelial cells do not display the characteristic features of M-cells such as the central hollow or sparse, irregular microvilli. Matsumo et al (1983) demonstrated the enterocyte-mediated absorption of Percoll nanospheres (PVP coated silica) of diameter 30nm, a small proportion reaching the mesentery nodes and entering the bloodstream before finally depositing in the thymus and liver. As before no attempt was made to quantify the extent of absorption. Toxicological studies have also shown the intestinal absorption of asbestos fibres (0.2-2μm) in rats (Pontrefract et al., 1973) and 1-5μM radioactively labelled resin particles in 6 week of calves (Payne et al., 1960) although no mention of lymphoid tissue uptake was mentioned in either study nor any detailed structural observations made. In the latter study it was noticeable that concentrated areas of uptake were the tonsils and pharynx, both notable areas of high content of lymphoid tissue which have been shown recently to contain M-cells. Hence the available evidence indicates that small particles can be taken up by enterocytes although the upper limit is subject to conjecture compared to the well established route of uptake by the M-cells, despite the presence of extrinsic barriers such as mucus and peristalsis.

The absorptive mechanisms involved in the initial site of entry of particulate matter in the nanosphere range are not well understood. In electron microscopy studies, the apical membrane is seen to vesiculate to form endosomes that bud off into the cytoplasm. On the inner aspects of the developing membrane compartments regular arrays of clathrin, a protein designed to form lattices around the membrane vesicles, is seen to appear. However not all macromolecular and particulate matter are endocytosed by this process as
exemplified by the clonic uptake of cationised ferritin in rats, mice and guinea pigs where both uncoated and coated vesicles were observed in both enterocytes and more surprisingly in goblet cells (Bockmann and Winborn, 1966). Maximally clathrin-dependent endocytosis is limited to 100nm yet many particles, particularly bacteria such as *Yersinia*, *Salmonella* and *Shigella* can penetrate and multiply within the lamia propria due to their constitutive or inducible expression of outer membrane proteins (Bliska et al., 1993). Such uptake is clathrin-independent and is characterised by the relatively large uptake particles (> 0.5µm) into phagosomes.

The mechanisms of enterocyte-mediated uptake are even more poorly understood than for M-cells. This omission partly arises from the lack of studies involving large particulates, more emphasis in recent years being placed on macromolecular uptake (ie 5-20nm) and M-cell mediated uptake. Phylogenetically it is not surprising that enterocytes are capable of particulate ingestion since the most primitive mechanism for ingestion of nutrients, or intracellular removal of xenobiotics, is the process of vesiculation or engulfment of particles. Although there is a scarcity of reports demonstrating enterocyte-mediated translocation of inert particles, the vast number of epithelial absorptive cells dwarf the minute level of M-cells, and as such represents an enormous area for potential absorption of inert particles if one can mimic the epithelial internalisation strategies utilised by bacteria and viruses (Chapter 5 of this thesis).

1.3.3 *Paracellular and macrophage mediated uptake of particles.*

There exist two further modes for particle translocation, paracellular and intestinal macrophage uptake, both of which have not been convincingly demonstrated and are difficult to understand given the known physiological behaviour of the intestinal tract. The uptake of fluorescent (1.09µm) and rhodamine (0.89µm) labelled microspheres across two separate ligated jejunal
loops devoid of grossly visible PP in mongrel dogs demonstrated that nearly all of the mesenteric node phagocytes contained particles of only one colour. This indicated that these phagocytes ingested the beads at the site only where the colours were separate (ie in the intestinal loops) and subsequently transported them to the draining lymph nodes (Wells et al., 1988) An additional experiment comprising of a single loop containing a mixture of the two microspheres showed, after 7 days implantation as before, macrophages containing multiple beads of both colours but rarely of the same colour. Although the loops were devoid of PPs the presence of isolated lymphoid follicles which contain M-cells and are capable of particulate uptake can not be discounted. The study of Joel et al. (1978) indicated that isolated lymphoid follicles were capable of particulate uptake. After carbon particles were ingested by mice for six months, isolated lymphoid follicles were identified as small black foci along the entire length of the GIT (150-220 per intestine) which were much more numerous than PPs (15-20 per intestine). In the study of Wells et al (1988) neither the initial site of particulate entry nor the initial site of microsphere ingestion by macrophages was identified. However, assuming that this intestinal segment had such abnormalities as increased inflammation or hydrostatic pressure that may have been partly responsible for particle uptake, then this would not be incompatible with a number of clinical situations such as diabetes and Crohn's disease (Urbanski et al., 1992) since nearly all studies conducted in the area of particulate uptake have focused on animals with normal intestinal function.

Phagocytic uptake of the protozoan *Giadia muris* by PP macrophages has been demonstrated in mice (Owen et al., 1981). Macrophages beneath the basal lamina in PP extended pseudopods into the inter-epithelial spaces to trap invading protozoa and enclosed them in phagolysosomes. Macrophages containing these organisms were surrounded by numerous lymphocytes suggesting that dendritic cells may not be the only type of antigen-presenting cells. The subsequent fate of the macrophages was unknown but this uptake mechanism may be similar to that reported by Wells et al (1988) since it seems likely that macrophages present in isolated lymphoid follicles are capable of
similar functions to those found in PP. As macrophages in M-cells can approach the apical membrane to within 300nm and form an important migration route for lymphocytes moving into the intestinal lumen (Regoli et al., 1994) it may be possible for such particulate capture, but would represent the least obvious method of exploitation for oral colloidal delivery.

When one considers the normal barrier functions of the epithelial tight junctions, then passage of particulates between absorptive cells seems even more unlikely than transepithelial passage. In normal circumstances, the zona occludens (tight junctions) of the epithelial cell membrane which begin just below the luminal surface and consist of small areas where the outer lamina of the opposing plasma membranes are fused to each other is thought to act as an effective barrier against the absorption of all but the smallest of molecules. However the paracellular transport of polycyanoacrylate nanocapsules (100-200nm) loaded with iodinised oil has been reported by Aprahmian et al (1987) that were identified in the intercellular spaces of the proximal jejunal cells by x-ray emission within 15 minutes of intraluminal administration. Nanocapsules were also found at the tips of the villi, thought to represent areas of desquamation of mature epithelial cells and responsible for the majority of the uptake. This would be analogous to the findings of Volkheimer and co-workers (1968) but the paracellular route is emphasised by the authors. The nanocapsules were prepared in the presence of 0.55% Pluronic F68 and the effect of this surfactant on the mucosal membrane cannot be ignored and may instead contribute to the enhanced paracellular uptake. This surfactant has been reported to cause damage to the gastric mucosae following perfusion of 1% solution and the damaged caused was reported to be comparable to the extensive degradation induced by sodium dodecylsulphate at 0.1% (Nadai et al., 1972).

More recently the paracellular absorption of metallic iron (5-30nm) was shown histologically in resin sections using TEM; however no evidence can be gained as to the initial site of entry of the metallic iron and from the photo-micrographs
it was noticeable that particles were distributed circumferentially around the tight junctions indicating that after trancellular absorption the particles moved laterally to the adjacent plasma membranes (McCullough et al., 1995). Furthermore there was a discontinuous deposition of the particles along the border not surprising since in addition to the tight junctions there exist two further types of barriers, zonula adherens and desmosomes.

Ferritin and adenovirus particles (both < 30nm) were located in the intercellular spaces between absorptive cells of rats in the study of Worthington and Syrotruk (1976) but these animals displayed breakdown of the junctional complexes as a result of long-term protein deficient diets in contrast to the study by Yamamoto (1982) who found that in addition to ferritin, HRP was also excluded from the tight junctions of normal rat jejunal enterocytes. Madara and Trier (1982) in a freeze fracture study further confirmed the intrinsic barrier nature of tight junctions by employing three macromolecular tracers (1.9, 12.3 and 40KDa) study that were all absorbed by the transcellular route; no movement via the paracellular pathway could be demonstrated. Although this barrier can be damaged in vitro by various treatments, alcohol (Draper et al., 1983) and various surfactants, it seems, unlikely that the passage of large particulates would be permitted.

1.3.4 Phenomena of Persorption

No discussion of intestinal particulate uptake would be complete without reference to Volkheimer and co-workers (1968) who have been criticised for their experimental observations but who have nevertheless ultimately stimulated much of the research being conducted at the present time in the oral delivery and absorption of colloidal material.
In a series of publications from 1961-75, Volkheimer and co-workers repeatedly described the passage of large particles in the micron range across the mammalian intestine by a mechanism termed persorption in both human and animal subjects. This phenomena had initially been reported in the 19th century (Herbst, 1844; Oesterlen, 1846; Marfels et al., 1854 cited in Seifert, 1983) and had been successfully demonstrated by Hirsch (1906) and Verzar (1911) at the turn of the century. Volkheimer reported the presence of a diverse group of particles including starch granules, plastic micropellets, crab shell particles, pollen, spores and iron-fillings with size ranging from 10μm to 150μm mainly in blood but also in such unlikely fluids as urine, cerebrospinal fluid and in the cord of newborn infants after such particles were administered to their mothers (Volkheimer et al., 1969). Volkheimer speculated that the intestinal passage of the particles resulted from the muscular activity of the muscularis mucosae by "kneading" through the tips of the villi at the desquamation zones, areas which are considered to represent barriers of reduced tensile strength due to the shedding of spent enterocytes. He coined the term "persorption" defining it as the paracellular passage of large non-deformable particles in the micron size range (particulary 7-70μm).

Volkheimer et al were also the first to investigate the effects of differing vehicles in which the particles were suspended, co-administered drugs, species and age dependency and quantitive aspects of particulate uptake. For example quantitation of particles from withdrawn venous blood contained 55 particles per 10mL after Volkheimer (1968) himself had ingested 200g of potato starch (2.4x10^9 particles). It was notable that agents that increased GIT motility and contractions such as caffeine and nicotine increased the uptake of particles, suggesting a muscular propulsion of the material across the epithelial barrier.

Since Volkheimer's early work many studies have been conducted on the intestinal transport of macromolecules and particulates using sophisticated analytical techniques that were not employed in Volkeimer's studies, but such paracellular transport has not been unequivocally demonstrated. Worthington
and Syrotruck (1971) were unable to demonstrate the passage of killed adenovirus type 5 (currently receiving considerable attention as a carrier in oral somatic gene and vaccination therapy) between cells of intact rat jejunum although it did occur in protein deprived rats due to the breakdown of junctional complexes. Furthermore the result of two long term studies with microcrystalline cellulose (> 20μm) in rats indicated that no observable transport of this material occurs (Steege et al., 1980) and Varga et al (1975), whilst studying the GIT transit rates of 10-20μm radiolabelled microspheres in rats, could not detect any radioactivity in either the washed intestine or systemic organs including blood and liver. Although starch granules have been photographed apparently within a villous that displayed an area of desquamation at its tip, the evidence of the penetration of large particulates is not convincing given the width of the paracellular route (10nm). The reported finding of particulates in portal and peripheral blood several hours after oral dosing is curious for a number of reasons as particles >7 μm are trapped by the pulmonary capillary beds by filtration and smaller particles removed by the mononuclear phagocytic systems, principally the kupffer cells given the well documented fate of intravenously injected particles. The presence of particles of intestinal origin in the CSF seems particulary startling since the blood-brain barrier has been described as an effective barrier to even small molecules. Volkheimer has also been criticised for his lack of controls employed in the studies however experiments were "carried out in animals after 24-36h fasting, in human volunteers after 3 days of starch-free diet" and as such cannot be disputed. Furthermore, identification of large particulates can be readily demonstrated using light microscopy which may have been the reason for the non-deployment of TEM although no conclusive photographic evidence for particulate penetration were included in the publications. Such large particulates (up to 150μm) were not observed in control experiments further corroborating their results.

The term persorption has been used broadly in the literature to describe the passage of particles across the intestinal mucosa without regards to the mechanism involved. Volkheimer described persorption as a passive process
that occurred at a very low frequency but was important in the accidental ingestion of large amounts of particulates, particularly relevant in recent years due to the Chernobyl nuclear fallout where radionuclide particles (1-100μm) were deposited over large distances. It is conceivable that particles may be in this vicinity at the right time to be pulled along the drag of the fluid into a relaxing villous that has just extruded some dying cells. If uptake by this route does occur there would be little scope for exploitation or control for the delivery of drugs in the light of the studies carried out in recent years.

1.4 Controversial aspects of intestinal particulate translocation

Not all efforts to demonstrate intestinal particulate translocation across the healthy adult epithelium have been successful. Previous sections of this thesis have shown that the route and the extent of absorption of particulate material is viewed with scepticism. This dichotomy pertains to the use of a relatively small molecule, PEG-4000 as a non-absorbable marker in assessing the integrity of the GIT epithelium (Mehvar and Shepard, 1992) whilst on the other hand there are a plethora of studies demonstrating the presence of particulates in the blood after peroral administration. In assessing particle translocation comparison of published data are encumbered by the variety of experimental designs employed, these varying notably in the use of animals of different species, age and sex, fasted or fed animals, different particle administration protocols, particle characteristics such as size, charge and hydrophobicity, length of particle and post-dosing ingestion period, sampling times and the non-standard selection of intestinal regions for analysis (Table 1.2). More crucially it is the variation in the recovery of particulate material and method of detection for administered samples that may intrinsically bias the data obtained (Table 1.6).
Table 1.6  Methods employed in the study of Intestinal particulate transport

<table>
<thead>
<tr>
<th>Routes of administration</th>
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</thead>
<tbody>
<tr>
<td><strong>In vivo:</strong></td>
</tr>
<tr>
<td>Orally in diet or drinking water</td>
</tr>
<tr>
<td>Orally by gavage</td>
</tr>
<tr>
<td>Orally by capsules</td>
</tr>
<tr>
<td>Perfusion of isolated intestinal loops</td>
</tr>
<tr>
<td>Intraluminal injection into isolated intestinal loops</td>
</tr>
<tr>
<td><strong>In vitro:</strong></td>
</tr>
<tr>
<td>Organ culture bath: Ussing Chambers</td>
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<tr>
<td>Cancer cell lines: CaCo-2 etc</td>
</tr>
<tr>
<td>Perfusion or injection into ex vivo intestinal loops</td>
</tr>
</tbody>
</table>

**Methods of analysing and quantifying model particulates**

*In vivo and in vitro*

Visualization and counting of particles in body fluids and sectioned tissues:

*Light and electron microscopy, Haemocytometer*

Fluorescence activated cell sorter, Coulter counter

Radiolabelled material: *Encapsulated tracer or core material, auto-radiography*

Extraction of model particles from tissues and quantification using:

*Dye extraction (fluorescence or spectrophotometry)*

*Gel permeation chromatography of cor material*

*High performance liquid chromatography of drug or tracer*
Juhlin (1958) was unable to demonstrate the passage of negatively charged methylmethacrylate particles (0.01-2μm) across the murine intestine despite reporting a method of quantitation that was capable of detecting 0.001% of the administered fluorescent particles. No information regarding background autofluorescence that may have interfered with the quantitation was given. Oligivie (1922) fed carmine particles to mice for 1 year and examined over 1000 slides for evidence of particle uptake with negative results. More recently Jenkins et al (1994) reported that the maximal uptake of intraluminally administered 150nm latex particles in rats was 1x10⁸ after 90 minutes concluding that the intestinal uptake of colloidal particles was solely limited for the purposes of oral vaccination. The study was poorly executed and the reasons for such a low quantitation, despite the use of "direct" assay method using FACS, can be grouped in problems associated with the efficient recovery of particles and the time given for particle translocation. Earlier Ebel (1990) using the same technique of flow cytometric analysis of orally gavaged 2.65μm latex microspheres in various tissue digested samples could only demonstrate maximally 6x10⁵% in a single PP, 3x10⁵% in the small intestine and 1x10⁴% in the spleen 48h after a single dose in pharmacokinetic based study in which mice were sacrificed 1, 2, 4 and 7 days later. However only 3 PP and a 3cm segment of the ileum were used for the quantitation, and the size of the latex particles employed in the study have now been shown to be too large for intestinal translocation (Jani et al., 1991, 1992). De Keyser et al (1989) has also reported that radiolabelled poly(diethylmethylidenemalonate) nanoparticles (200nm) were totally cleared from the mice GIT after oral gavage and only background radiation obtained from the reticuloendothelial organs. Despite the optimal size of particles employed, the surface may have been too polar for sufficient interaction with the mucosa, thus highlighting the need for optimising many particulate characteristics for successful bioadhesion and translocation.

At the other end of the spectrum high levels of systemic appearance of intestinally administered particles have been reported, one of which is credited to Alpar et al (1989) who showed 39% of 1.1μm latex particles in the systemic
circulation of rats 45 minutes the intragastric administration. The basis of the quantitation relied upon the examination of the number of particles in 100μL of blood drawn from the tail vein and extrapolated to the whole blood volume of the rat. In subsequent studies the same group reported that 35% of a dose of albumin microspheres (1-4μm) were circulating in the peripheral blood of rats 60 minutes after oral administration. Such claims require detailed examination and to be viewed with some caution particularly as albumin microspheres are known to disrupt in luminal fluids. The study also highlights the limitation of optical methods of quantitation of an extremely small sample of material with subsequent extrapolation to whole organ weight that is disproportionately larger to the original sample.

Alternative studies utilising radiolabelled particles have not been able to demonstrate unequivocally the translocation of intestinally administered particles usually due to problems of label stability. Nefzeger et al (1988) reported 10-15% absorption of radiolabelled polymethylmethacrylate nanoparticles in rats which, in the light of recent studies, probably consisted of low molecular weight components of the polymer (Le Ray et al., 1994 and chapter 2 of this thesis). Similarly Kreuter et al (1989) using polyhexylcyanoacrylate nanoparticles reported the small absorption of radioactivity but could not determine whether the radioactivity originated from the nanoparticles or its degradation products! Several independent studies conducted in our laboratories have demonstrated that the use of radiolabelled microspheres is an unreliable method of quantitation (Jani et al., 1990: chapter 2 of this thesis). Spelenhaur et al (1991) demonstrated that of the 20% of radiolabelled poly-lactide-co-glycolide nanoparticles (100nm) "crossing" the intestinal epithelium vast majority of the radioactivity was derived from water-soluble polymeric residues as evidenced by the large amount of radioactivity emanating from the bladder and urine. *In vitro* experiments had previously indicated that such particles degraded within 4h in simulated intestinal medium which obviously does not include esterases that are present in the intestinal epithelium.
Finally there are number of reports which are curious and difficult to explain when considering the known physiological phenomena on a rational basis. Steiner and Rosen (1990) reported that protenoid microspheres of less than 10μm can translocate readily through the stomach wall after oral administration. The proposed mechanism of the uptake for this feat has to date not been described. Pappo and Ermak described the uptake of 5% of an administered dose of latex microspheres in a "non-Physiological" closed gut loop system incorporating a single PP. Such a high level of uptake cannot be expected if the particles are able to transit normally through an un-obstructed intestine. Furthermore the authors recently claimed the selective targeting of PP with poly-lactide-co-glycolide microspheres, again in a closed gut loop system, contrary to most reports where such particles, in comparison to polystyrene microspheres, administered orally show little or no affinity to PP (Eldridge et al., 1990; Le ray et al., 1994). Confinement of any particulates in a ligated loop system incorporating a PP will undoubtedly increase the propensity for movement across the epithelium. Similarly Tomizawa et al (1994) could selectively target M-cells with phosphatidylserine liposomes after intra-duodenal application; obviously the authors knew the disruptive effect of gastric fluids on liposomes. Closed gut loop systems are useful for mechanistic studies in attempt to elucidate or corroborate a route of uptake. Unequivocal intestinal particulate translocation as normal sampling activity of the healthy GIT can only be demonstrated by peroral administration if such a route is to be used for the delivery of GIT-labile molecules and for mucosal vaccines. In conclusion, poorly accepted and scientifically unsubstantiated claims of uptake should not be allowed to detract from the mounting volume of sound scientific evidence demonstrating that particles can penetrate the GIT and that such behaviour can be modulated to some degree by particulate characteristics.
1.5 Strategies to increase the oral absorption of colloidal materials

Clearly as the size of the particles approach the domains of macromolecular dimensions increased intestinal absorption either by the PP of normal enterocytes is likely to occur. This inverse relationship between the size of the particles and intestinal absorption was convincingly demonstrated by the definitive study of Jani et al (1990) by oral gavaging rats for 10 days with polystyrene nanospheres and microparticles ranging from 50nm to 3μm and quantifying the extent of intestinal and systemic absorption using gel permeation chromatography after an exhaustive solvent extraction of tissue latex. Levels of particles reaching the blood, liver and spleen - a good indicator of particle having been absorbed and translocated - ranged from 18% for 50nm particles to 0.78% for 1μm microspheres taking into account the total rat blood volume. Systemic values of nanosphere absorption between this size range for 100, 300 and 500nm gave 12.6%, 8.6% and 1.89% respectively demonstrating the dramatic decrease in intestinal particulate absorption in a relatively narrow size range. Crucially no 3μm microspheres were detected or seen in histological examination in any region of the GIT, including PP or in the systemic organs thus defining the upper limit for intestinal transport. However this upper limit of uptake is subject to much debate. The need to determine the upper limit of particulate absorption relates to the volumetric capacity and hence the encapsulation efficiency of the desired drug molecule. It is tempting to use smaller particles but a simple calculation reveals that the volumetric capacity of a single 100nm hollow particle is some 2000 times lower than the volume occupied in a 1μm microcapsule representing a considerable loss in encapsulation despite the more extensive absorption of smaller yet poorly loaded nanocapsules. Eldridge et al (1990) reported that the upper limit of microspheres that can penetrate the PP and reach the systemic circulation was limited to microspheres of diameters >5μm whilst those which can breach the intestinal barrier but cannot reach the extra-intestinal tissues was limited to particles of <10μm. However the study relied upon the use of heterogeneously sized PLG microspheres and thus cannot accurately determine by optical
means in a finite number of tissue sections the relationship between microsphere size and its potential for absorption. The exclusion of larger particles from PP, the only site of absorption reported, and the upper limit of uptake was established through the use of coumarin-loaded microspheres with a range of diameters from 1 to 100μm, again presumably in mixed size population of microspheres. Examination of PP from mice demonstrated that while many microspheres less than 10μm were absorbed none greater than 10μm were seen in lymphoid tissue in agreement of earlier reports of the non-absorption of 15μm and 20μm particles by the PP (LeFevre et al., 1980; Varga, 1975) in mice and rats. Like Eldridge, LeFevre et al observed the presence of 5.7μm latex particles in the PP after chronic feeding for 77 days and in some instances to reach the mesentery node and penetrate as far as the lungs, although in the latter case particles may have been enclosed in mesenteric node macrophages that had migrated subsequently after microsphere ingestion. In a later study LeFevre et al (1989) reported the PP accumulation of 2μm rhodamine labelled latex microspheres in young and aged mice but insignificant amount of latex could be detected in the systemic organs thus contradicting the author’s previous work and in line with the studies of Jani et al. (1990, 1992) who used fluorescein labelled latex particles. This discrepancy may well be as a result of the difference in numbers of particles administered or the length of oral feeding but this is compensated by the more sensitive method of detection by Jani et al (1990) compared to the optical method employed by LeFevre et al (1989). However in a very recent study Lehr et al (1995) has shown that binding of commercially available nanoparticles of the same range depends upon the dye incorporated since fluorescein labelled nanospheres (50nm) adhered 5-fold more strongly to human cell lines than rhodamine labelled nanospheres of the same size. A more comprehensive study conducted by Eldridge et al (1990) illustrated the efficiency of microsphere absorption was found to correlate with the relative hydrophobicity of the microsphere wall material. A single dose of 10 different types of microspheres composed of biodegradable and biocompatible polymers indicated that the degree of PP accumulation of microspheres was proportional to their hydrophobicity with
polystyrene, polymethylmethacrylate and polyhydroxybutrate being more readily absorbed (1500 particles in 3 PP sections) compared to the more hydrophilic particles consisting of varying levels of lactic and glycolic acids (200-1000 particles/3 PP sections). As a class cellulosic microspheres were very poorly absorbed by the PP (<10 particles/3PP sections) reflecting their intrinsic polar surface nature. This is in agreement with a more recent study where polystyrene nanospheres (500nm) bound and subsequently transported by M-cells in ligated rabbit ileal loops by an order of magnitude greater than equivalently sized poly-DL-lactide-co-glycolide nanospheres (Jepson et al., 1995). The marked readiness with which particles of different surface physicochemical properties of comparable size can accumulate in PP had been noted earlier where carbon and latex particles more efficiently entered PP than asbestos, quartz and carmine particles that did not, reflecting the reduced wettability of the latter compounds (LeFevre et al., 1985).

For many years it has been widely accepted that passage of molecules across the intestinal epithelium, which constitutes the main barrier for oral drug absorption, increases with lipophilicity. Using two human cell lines Wils et al (1994) demonstrated that this concept may not be valid and that highly lipophilic compounds with octanol/water values above 3 demonstrated reduced transepithelial permeability coefficients and that a maximum was reached above which permeability decreased with rising lipophilicity. A similar situation may also apply to microsphere wall materials and although no systematic quantitative study of intestinal particulate uptake versus hydrophobicity has been conducted it is unlikely that the limited absorption of microspheres noted thus far can alone be increased by modification of surface properties. Use of microspheres composed of highly hydrophobic wall materials such as fluorocarbons may resolve this issue.

Charge is another surface property of potential importance in phagocytic and binding events. Both hydrophobicity and charge may be transiently altered by the adsorption of ambient molecules onto particle surfaces. Since the alimentary
canal represents an ever changing milieu with wide swings in pH and extraordinary assortments of surface active molecules, determinations of surface properties in situ in the gut will not be accomplished easily. Nevertheless Jani et al (1989) has shown a considerable reduction in the absorption of negatively charged carboxylate microspheres compared to non-ionised particles of the same size. To date no positively charged microspheres have been employed and it would be interesting to observe the binding and translocation of eg amidine coated microspheres since the intestinal epithelium displays a basal negative potential. Bockmann and Winborn (1966) showed the more avid transport by colonic epithelial cells of cationised ferritin over native ferritin.

The effect of the vehicle suspending the particles on intestinal absorption has not been directly tested, however studies by Alpar et al (1990) have indicated that latex microspheres dispersed in water are more readily absorbed than those suspended in an emulsion consisting of arachis oil and mixture of surfactants. In contrast Le Ray et al (1994) has shown that administration of 130nm PLG nanospheres with concentrated milk rather than water resulted in a modest yet faster rate of intestinal uptake than. This is suggestive of high lipid concentration promoting lymphotropism, although Ebel (1990) considers that presence of lipid components enhance particulate uptake either by increasing the GIT transit time thus allowing increased exposure to the mucosa for uptake to occur, or up-regulating M-cell activity given the highly adaptive nature of the cell whose role of immunesurveillance must increase with increased intralumimal load.

A more systematic study on the effect of dispersion medium on intestinal particulate uptake has shown that co-administration of non-toxic mixed micelles with colloidal inks (platinum ink, 10-40nm; Rottring ink, 100-800nm) and colloidal gold particles (5-40nm) promoted the uptake of particles from ligated colonic loops, accumulating in mesenteric and regional lymph nodes within 2h of application (Fukui et al, 1987). In the absence of mixed micelles no ink
coloration of the lymph nodes could be visualised, nor any gold particles be detected using atomic absorption spectroscopy. However the upper limit for particulate uptake was limited to 40nm and in all cases the absorption of colloidal material was enhanced 3-10 fold by mixed micelles. Interestingly more absorption occurred in the rectal area than the upper colonic region, the former displaying loosening of tight junctions in the epithelial layer.

1.6 Ligand mediated uptake of particulate matter

From the preceding sections it is clear that parameters such as size and surface nature of particulates are important determinants for efficient epithelial translocation, the degree of accumulation being proportional to the hydrophobicity of the material and reduction in particle diameters. However particle uptake involves intimate contact with the mucosa thus longer exposure or retention in the intestine would not only increase the probability of uptake but lead to a greater absorption. It is known that macromolecules, particulates and micro-organisms that are capable of binding to mucosal surfaces are generally more effective in eliciting mucosal responses (Russell-Jones and De Azipurura, 1987) and in some cases able to translocate the epithelium than those that do not adhere. Thus BSA-colloidal gold conjugates that does not bind to mucosal surfaces is transported at least 50 times less efficiently through rabbit M-cells than lectin-colloidal gold conjugates (Neutra et al. 1987) as are BSA-coated nanospheres (500nm) which exhibit a four-fold reduction in binding and a 20 fold reduction in uptake through mouse M-cells compared to slgA-coated particles (Porta et al., 1992) since slgA binding domains exist on the apical membrane of M-cells in most species.

Although the range of microorganisms and inert particles taken up is wide, the M-cells also exhibit selectivity of binding and uptake of certain components that have been isolated and used by some investigators for site-specific M-cell
delivery of colloidal particles. Pappo and Ermak (1991) showed that latex particles displaying surface adsorbed anti-M-cell monoclonal antibody 5B11 (IgM) adhered to and were translocated through rabbit M-cells 3-fold more efficiently than particles displaying IgM molecules of unrelated specificity or particles displaying their native surface chemistry. As the binding of the latter two controls was found to be similar this indicated absence of an IgM binding domains further supported by the continuing uncertainty whether M-cells actually display polymeric Ig or Fc receptors (Pappo and Ermak, 1988; Trier et al. 1992). Earlier Rubas et al (1988) had shown that the incorporation of reovirus sigma 1 protein, that is present on the outer surface of the virus and directs it M-cells, in lipid microspheres (200nm) resulted in a 20-40 fold increase traffic across the rabbit PP compared to uncoated vesicles. These finding raise the possibility for specifically targeting molecules or colloidal carriers to PP particuly for oral immunisation, but for subsequent booster doses or for repeated administration for chronic diseases, an intestinal sIgA response generated against such conjugates cannot be ruled out and may impair uses of immunogenic ligands. Furthermore such ligands have to be shown to be able to bind to M-cells of other species as M-cells are known to display altered binding specificity to bioadhesive macromolecules depending on the location of the PP within the same animal and particuly amongst species (Jepson et al., 1995). Thus *Ulex europeaus* I lectin binds with high affinity to ileal mouse M-cells but not to rat M-cells. As both Fc and Fab fragments of IgG (coupled to colloidal gold) display M-cell adherence despite the lack of Fc and poly Ig receptors leaves open the possibility that an M-cell surface lectin recognises ubiquitous oligosaccharides present on immunoglobulins. In addition for practical purposes the storage stability and retention of function of the ligands, not to mention the cost of production, are issues that have to be addressed.

To sample a wide variety of intraluminal particulates for immune-surveillance the M-cell has developed relatively non-selective mechanisms for binding and subsequent transepithelial transport. However it is seen from Table 1.3 that a
wide array of, predominately gram negative, bacteria and viruses adhere selectively or preferentially to M-cells of experimental animals. Thus the ability of M-cells to bind bacteria such as *Vibrio cholerae* results in the efficient sampling of this non-invasive pathogen, the close interaction between the bacterial outer membrane and the apical membrane inducing recruitment of submembrane actin filaments and resulting in phagocytic engulfment. However such processes of entry are seen in normal absorptive cells particularly in the entry of *Yersinia enterocolitica* and *Shigella flexneri* whose constitutive or inducible expression of specialised outer membrane proteins can transform normal enterocytes to behave like M-cells ie evoke their own uptake by inducing phagocytosis (Bliska et al., 1993). However selective M-cell adherence does not alone prove the existence of unique M-cell membrane receptors although it is tempting to postulate a common mechanism such as a lectin-carbohydrate recognition system which has evolved to allow the M-cell to sample entire subclasses of luminal organisms.

An important but yet an unanswered question is whether specific receptors exist on the surface of M-cells that mediate the selective attachment of bacteria. There is some evidence that the apical plasma membrane differs considerably from that of the adjacent enterocytes and it is likely that such specializations of the luminal surface facilitate specific attachment but to date identification of vital and bacterial components and that on the M-cell which mediate the binding have remained difficult to isolate. The subject remains an important research priority as bacterial and viral adhesins, once identified, could serve as competitive ligands for preventing corresponding bacterial infections. Coupled to bacterial epitopes they could be used for selective M-cell targeting of oral vaccines. Such targeting allows a substantial reduction in the amount of material required that needs to be administered and leads to the decrease in the amount either being lost or distributed over the entire epithelium. As peripheral immunisation induces poor mucosal immunity whereas oral immunisation offers the advantage that with some delivery systems can induce both mucosal and systemic immunity (Mestecky and Kiyono, 1993) site specific
targeting to inductive sites ie. M-cells which, in contrast to systemic lymphoid tissues, contain not only antigen-presenting cells but crucially a significant fraction of functional B-lymphocytes that are committed to the synthesis of sIgA antibodies will allow the employment of smaller dose of antigens.

However in terms of exploiting the M-cell route for the delivery of encapsulated GIT labile drugs the M-cells represent a minority cell type in the GIT, even where they are most numerous such as in the PP of rabbits and humans (larger GALT area) despite the M-cells considerable capacity of translocating adherent luminal particles. Furthermore the question of specific M-cell "receptors" is made more complex by the many routes of transport of different particles involved in the M-cell absorption. For example poliovirus is endocytosed via clathrin-coated pits (Scicinski et al., 1990), whereas reovirus is taken up in uncoated vesicles (Wolf et al., 1983) despite their similar size. Even different strains of the same organism have very different interactions. Among E.coli species the O:124K:72 strain is translocated by the Mcell but the O:124 RDEC-1 strain can only adhere to the M-cells. Reovirus-1 is taken up by M-cells but Reovirus is transported by both M-cells and columnar absorptive cells (Wolf et al., 1983). However such bacterial and viral interactions with the M-cells is dependent on the animal model employed and extrapolation to humans, in the clinical sense, is not always realised eg the study of Yersiniosis in humans is conducted in mouse models but not in rats or rabbits. Recently it has become clear that there are wide variations in ligand binding properties to intestinal M-cells depending on the animal model employed. Ulex europaeus agglutinin I binds selectively to mouse Intestinal M-cells but not in those in the rat despite their similar genetic background. Further this binding selectivity did not extend to mouse caecal M-cells demonstrating the marked regional and species variation in M-cell "receptor" expression (Jepson et al., 1995). Such variation in glycoconjugate expression may indicate differences in other surface properties such as charge and hydrophobicity that might influence the interaction of material with the epithelial surface. These observation demonstrate that caution must be taken in extrapolation of animal data on the
binding of colloidal matter between species and between GALT sites within a given species. This is particularly important when attempting to predict the effects of surface modifications on the targeting of microsphere preparations in man, where the ligand binding profile and apical properties of M-cells remained to be determined.

Preferential attachment to the M-cells may be due to the physical characteristics of their surface primarily in the increased accessibility due to the near-absent glycocalyx, lack of mucus in the immediate vicinity and sparse microvilli that normally constitute a significant barrier because of the size (1μm) and negative charge. In ultrastructural cholera toxin B-subunit which binds to specifically ubiquitous glycolipid receptor GM1 on both M-cells and enterocyte surfaces adhered only to M-cells when administered as coated colloidal gold particles (Neutra and Kraehenbuhl, 1994). Thus the association of the toxin to the particle inhibited its movement through the thick glycocalyx and prevented access to the microvillar membrane GM1 whereas the conjugate was more accessible to the receptor on the M-cell apical membrane. Since viruses and bacteria can also be considered as particulate polyvalent ligands they may also find their "receptors" more accessible on M-cells. This concept is supported by the observations that many bacterial pathogens that adhere rapidly to M-cells also succeed in forming tight attachment sites on normal absorptive cells after a lag period, the time taken for them to penetrate the mucus and underlying glycocalyx. Freter (1988) has shown that non-motile *Shigella flexneri* and latex particles can reach the enterocyte surface within 30min by moving along the natural lines of stress of the moving mucus.

Currently little is known about the efficiency of M-cell antigen sampling and more importantly apical membrane recycling. The observations of Pappo and Ermak (1989) that the number of 600-750nm latex particles applied to PP initially bind and are translocated within 10min of intraluminal introduction but the remaining free intra-luminal particle fail to adhere to the M-cells during the next 80min, suggests that particles are mobilised into PP in a synchronous
cycle lasting at least 90 min. This relative slow absorption is not conducive for targeting of colloidal material to the PP since they will be removed from the PP vicinity and detach from the luminal surface by gut peristalsis and mucus sloughing during this intervening period. It appears that the M-cell apical surface had been temporarily depleted of the components necessary for particle adherence, albeit these probably consist of low-affinity interaction with unmodified latex particles. Whether apical membrane molecules are replaced by *de novo* synthesis or by recycling of membrane microdomains that are initially used in particle engulfment is not known. It was assumed that latex binding and transport takes place synchronously in all M-cells. The limited capacity and efficiency of M-cell transport was highlighted by Porta et al. (1992) where only about half the total population of mouse M-cells could actually transport beads, similar to a more recent study where greater than 50% of mouse M-cells did not contain poly-lactide-co-glycolide particles (Ermak et al., 1995), and virtually none of these cells (< 3%) was able to bind and transport beads at the same time. A similar distinction has been seen previously when measuring the binding and endocytosis of Type 1 reovirus by M-cells in the mouse distal ileum (Wolf et al., 1984).
1.7 Objectives and rationale of thesis

Morfitt (1990) observed "infrequent and unpredictable by the swine lymphoglandular complex epithelium after installation of plain and fluorescin labeled beads, carbon particles, live and dead Treponema sp. bacteria, cholera toxin, cationised ferritin and a bovine serum albumin-colloidal gold conjugates into ligated colonic loops of anesthetised juvenile pigs". Coupled with the reduced number of M-cells and reduced surface area of PP and the fact that normal absorptive cells have essentially the same "receptors" on the apical membrane as M-cells but masked by mucus the intestinal epithelium represents a vast but yet an underated target for absorption of colloidal particles which, to date, no study has directly addressed. In a series of recent morphological studies it has been observed that native, unmodified micron sized latex particles are absorbed equally if not greater from the villous epithelium rather than the follicle-associated epithelium, although no mechanistic experiments for this translocation were conducted (Hazzard et al., 1995; Hodges et al., 1995). As the most abundant cell of the normal intestinal epithelium - the enterocyte - is also capable of receptor-mediated transcytosis in the apical to basolateral direction (Dix et al., 1990) and given that the total surface in the adult human gastrointestinal tract extends to 200-300m², the largest area of the body in contact with the external enviroment, an enormous potential exists for the absorption of particles similar to enteroinvasive bacteria mediated by their surface lectin-like adhesins.

In a wide variety of cells, the effeciency of endocytosis of extracellular material is greatly incresaed by prior concentration at the cell surface, either through binding to specific receptors or by adsorption to cell membrane glycoconjugates (Anderson and Kaplan, 1983). Animal cell surfaces, such as intestinal epithelial cells, are to large extent normally overlaid with carbohydrates which are involved in recognition functions for lectins (sugar binding proteins of non-immune origin), toxins, polypeptide hormones, antibodies, bacteria, parasites and viruses (Horowitz, 1977). Most, if not all, examples for specific bioadhesion
in the GIT rely on the lectin-sugar interactions with the notable exceptions of the active transport carrier mechanisms of di/tri-peptides and vitamin B12 and transferrin receptors and prodrugs that have been designed to exploit such routes of entry (Figure 1.5).

Figure 1.5 Schematic representation of protein carbohydrate interactions on the enterocyte surface. Plant and bacterial lectins bind to the carbohydrate containing glycoconjugates of the apical enterocyte surface. Conversely, integral endogenous membrane lectins may serve as receptors for glycosylated particles, bacteria or viruses.
For a long time carbohydrates were regarded as compounds completely devoid of biological function, serving merely as a source of energy for cells or as components of tissue secretions to provide protective materials for cells. Many biological recognition and adhesion processes involve interactions between carbohydrates and proteins. Cells, organisms and soluble components exhibit information about themselves in the form of exquisitely defined saccharide structures displayed in oligosaccharides present in glycolipid and glycoprotein components of biological membranes on their surfaces. The information is decoded by a group of highly stereospecific and specialised carbohydrate proteins, lectins, a structurally diverse class of glycoproteins present in a wide range of biological materials particularly plants but also bacteria, animal and human tissues, their only common features being the ability to bind carbohydrates specifically and reversibly. This has lead to the notion that, owing to their significant variability and ubiquitous presence in biological systems, carbohydrates represent a third biological code system in addition to nucleic acids and proteins.

The objectives for the present research were:

i) to promote the specific bioadhesion and epithelial translocation of nanospheres to ubiquitous cell surface glycoconjugates by the surface attachment of ligands involved in hapten-lectin interaction which in some instances are known to be internalised by enterocytes upon binding

and

ii) to investigate the potential of such ligands to aid the translocation of nanoparticles with reproducibility and reduced inter-subject variability, particularly important considering the narrow therapeutic indices of peptides and proteins.
The surface-coupled ligands were those that are widely used in nature, particularly by enteroinvasive bacteria for epithelial attachment and in some instances for internalisation such that the particles could be regarded as bacterial polyvalent ligands. The ligands consists of the following:

1. **Plant lectins** - The most intensively studied proteins capable of specific interactions with sugars are plant lectins which have a long and productive history. By virtue of their specific binding to epithelial cells, good resistance against proteolytic digestion, relative high bioavailability, compared to proteins of similar size, after oral administration to humans and experimental animals (Kilpatrick et al., 1985; Greer et al., 1988) and propensity to elicit receptor-mediated endocytosis, plant lectins have attracted attention as adjuvants to enhance the immune response to orally administered antigens and carrier proteins to increase the transport of unrelated antigens across the intestinal wall. Since lectins recognize and bind to terminal sugar residues of oligosaccharides, endocytosis is relatively uninfluenced by the size and composition of the aglycone component of the lectin that confers a measure of protease resistance either because it increases the rigidity of the protein or because the sugars sterically protect susceptible sites. Tomato lectin was chosen due to its non-toxicity upon epithelial binding (Kilpatrick et al., 1985) and its ability to be internalised by enterocytes (Naisbett and Woodley, 1994b).

2. **Intestinal endogenous lectin**: Lectins have not only been evidenced in plants but with increasing frequency in a variety of mammalian tissues (Barondes et al., 1992). The recent isolation of nine lactose-binding lectins from the rat intestinal epithelia (Oda et al., 1992), some of which were postulated to be secreted to the epithelial surface, prompted our investigation of employing lactose-conjugated nanospheres that may interact with such extracellularly localised and apically integregated lectins.
3. Bacterial outer membrane proteins: Bacterial adherence and colonisation of mucosal cell surfaces are generally mediated by carbohydrate attachment sites (Longhi et al., 1992) and as the outer epithelium of the intestinal mucosa is covered with a carbohydrate-rich layer composed of glycoproteins, mucopolysacharrides and glycolipids (Horowitz, 1977), it is no surprise that enterobacteriaceae have selected specific sequences of sacharrides as major binding sites for binding (Longhi et al., 1992). The adherence and internalisation of bacteria is widely regarded as an important perequisite for colonisation and virulence manifestation of the microorganism. Entry into the intracellular enviroment of the host is an essential step in the pathogenic lifestyle of a wide variety of microorganisms, since this may allow them to access more favourable niches in which they can survive, replicate or avoid host defences. In recent years it has become increasaingly evident that microbial entry into mammalian cells is the result of highly specific interactions of bacterial determinents with host cell surface receptors. The process has been termed microbial-directed endocytosis (Moulder, 1985) and often involves the subversion of existing host cell signal tranduction pathways (Baliska et al., 1993). In Yersinia pseudotuberculosis a single chromosomal genetic determinant (the inv gene) appears to be the main factor responsible for its entry into cultured mammalian cells (Isberg and Falkow, 1985; Isberg et al., 1987). The inv product has been charcterised as a large polypeptide of 103kDa localised in the outer membrane of the bacterium. When this gene is expressed in a noninvasive E. coli k-12 its product was translocated to the outer membrane as in Yersinia species, allowing the recombinant organism to enter cultured cells. The invasin carboxyl terminal-192 amino acids that is sufficiant and neccessary to confer invasiveness was isolated and coupled to nanospheres to assess its potential of augementing inert particle uptake.

These three appraisals are described in succeeding chapters in this thesis.
Chapter two - Experimental methods
2.1 Introduction

"You can't deliver it unless you can measure it" has been the response from one senior industrial research group scientist (Edgington, 1989) involved in the development of novel drug delivery systems. With so little of the basic physiology for biotech drug delivery understood, what emerges is the need for the multi-disciplinary research effort to explore the mechanisms of uptake, metabolism and excretion.

In spite of the accumulated evidence there is controversy over the nature and extent of the phenomenon of intestinal particulate translocation. One reason for this dichotomy, in addition to the differences in particulate characteristics reviewed earlier in chapter 1, are the different experimental protocols used to assess the extent of the phenomenon, approaches to the administration of test particulate substances (in vitro vs in vivo), sampling times and numerous other factors (Table 1.2) varying considerably in the literature. It is, however, primarily the methods of quantifying the extent of uptake, ranging from direct visualisation and counting of particulates in cleared tissue sections to exhaustive extraction and bulk analysis that is the most contentious issue since proof of the true extent of translocation is critical in determining the viability of the route. Uptake of the carrier must be commensurate with the required dose for activity of the encapsulated material. Thus the utility of this method of administration will be in question until some degree of control over uptake and biodistribution can be achieved.

The purpose of this chapter is to explain the experimental protocols employed in the studies described hereafter conducted with surface-modified latex nanospheres, the experimental methods employed and to review briefly some of the reported methods in an attempt to bring some degree of uniformity to the subject.
The absorptive function of the gut can be studied by numerous methods. Among the primary considerations in choosing an experimental technique to assess an aspect of the absorption processes are the following:

1. The test species to be used and mode of administration of test substance; must the study be conducted in humans or is there an appropriate experimental animal model?

2. The aspect of the absorption process of interest; overall absorption from the gut lumen to the systemic circulation vs process of transport across the apical or basolateral membrane of the enterocyte.

3. Which experimental or physiological variables should be controlled eg. presence of anaesthetic agents, sampling times.

2.2 Choice of appropriate animal model and mode of administration

Peyer's patches are not present in all animals. There are no reports of PP's in some primates such as the Mandrillus leucopaecus and Papio annubis (Sonntag, 1922) and have not been observed in the Cynomolagus monkey (Rubas, 1990) despite the extensive use of this species for immunological studies. In 1928 Carlens (cited in Rubas and Grass, 1991) reported on his detailed investigations on the morphology and ontogeny of intestinal lymphoid tissue of large domestic animals observing that the morphology and development of ileal PP's was different from the rest of PP's in the intestine. Recent studies in sheep (Reynolds et al., 1985), swine (Binns and License, 1985) and dogs (HogenEsch et al., 1987) have confirmed and expanded Carlens' original observations. The ileal PP is larger with tightly packed follicles and functionally different from the jejunal PP and may represent the avian-Bursa equivalent in these mammals involved in B-lymphocyte production and priming, which in humans occurs in the thymus. Fortunately in terms of
laboratory animals, mice, guinea-pigs and rats like man, with the exception of rabbits, do not possess such differences and thus are commonly used for immunological and particulate uptake experiments.

Throughout our extensive studies, both past and present, the rat has been employed mainly due to the similar numbers of M-cells present in their PP's (Savidge et al., 1990) ie. 10% of the total FAE population, and small intestinal transit time of approximately 3h (Varga et al, 1975), both of which correlate well with human intestinal physiology. In addition to the almost identical biochemistry to man, the ease of handling the animal (for oral gavage) is an important consideration for the safe and accurate delivery of the latex nanospheres in sharp contrast to rabbits and guinea-pigs where anaesthesia, which may effect gut motility, is invariably required.

Mode of administration

In all studies latex nanospheres were administered using a blunt-ended feeding needle (length = 6cm) at a dose of 12.5mg/Kg in a volume of 0.1mL. Fluorescent nanospheres of 500nm diameter were employed in all studies since these are inert, discrete and resistant to the actions of digestive and lysosomal enzymes but they have the additional advantage of being quickly and definitively recognised in tissues and sediments and more readily traced. Furthermore being non-immunogenic they are unlikely to cause their own uptake.

Among the various techniques for administering the test substance in an in vivo study this method has a number of advantages over incorporating the latex nanospheres in the drinking water or diet where accurate assessment of the amount ingested by the rat is less reliable and dependent upon the difference (LeFevre et al., 1990). Many studies have involved either intubating nanospheres into the stomach (Alpar et al., 1989) or direct administration into
the intestinal lumen which, although useful for mechanistic studies ie. site and mode of uptake, do not address pharmaceutical considerations such as the interaction of food or gastric emptying that may have an effect on the time it takes for particles to appear in the blood. Further, the direct administration of particulates to the intestinal lumen or into a segment of intestine that is closed by ligatures both proximally and distally not only requires the use of anaesthetic agents and compromises the normal gut motility but elevates the local concentration of the test substance to unphysiological levels that may reduce the integrity of the intestinal epithelium and knead particulates by hydrostatic pressure. The use of lipid microspheres and liposomes in such closures by-passes the known degrading effects of the stomach and jejunum and as such is a further step away from the clinical situation.

In order to evaluate the possibility that damage to the oesophageal wall due to the repeated gavaging manoeuvre might have been the cause for the direct particle entry into the mediastinum in chronic studies, control rats were gavaged with saline following the same protocol for ligand-coupled latex nanospheres. Upon sacrifice the oesophagus with the thymus, lungs and intact intestine attached was excised and exposed under the fluorescence microscope. The gavaging needle was inserted into the oesophagus and 0.5mL of a diluted suspension of 100nm and 300nm fluorescent nanospheres (1% w/v) was injected using higher pressure than the one applied for ligand-conjugated 500nm nanospheres. In no instance could we detect signs of leakage of the fluorescence into the mediastinal or outer connective tissues.

2.3 Methods for quantifying degree of absorption

In addition to the mode of administration the most critical aspect is the sampling procedure and the method of quantifying the extent or rate of absorption. Numerous methods for measuring absorption of particulates that include bacteria (Owen et al., 1986), polymer latex (LeFevre et al., 1978, 1989;
Jani et al., 1990) and other particulates (LeFevre et al., 1985) by the PP’s have been reported. Direct counting of particulates in tissue sections using microscopical techniques is a sensitive method but since particulates are not uniformly distributed within the organ eg follicles of PP’s the amount of tissue sectioning required for exhaustive particle counting is prohibitive. Confocal microscopy can alleviate some of the tedium by optical sectioning but the thickness of the tissue is the limiting factor especially with larger organs. LeFevre et al (1980) reported the appearance of latex-like particles that appeared in small numbers in many of the solubilised control tissues, serving as a reminder of the limitation of optical methods for the identification of small particles. At best optical methods can complement other techniques to be described and yield morphological detail on the route of uptake at a cellular level but can only provide a crude semi-quantitative method of measurement.

For a more accurate assessment of particulate translocation and deposition within organs macroscopic methods such as recovery of particles from chemically-digested tissue using gradient centrifugation (LeFevre et al., 1978) or filtration (LeFevre et al., 1989) can provide quantitative data from a large mass of tissue but both of these methods have drawbacks. The former cannot be utilised for small number of particles and the latter is unsatisfactory for particles that are degraded by strong acids (eg. polyvinyltoluene microspheres, poly[lactides]).

The use of radioactively labelled nanoparticles can obviate some of these problems as homogenisation of the tissue rather than digestion is only needed and the radioactive counts from a small representative sample is sufficient to extrapolate to the entire organ. In an initial attempt to develop a rapid method of quantifying the intestinal absorption of latex nanospheres, the procedure of Huh et al. (1974) was utilised to surface label 500nm polystyrene nanospheres with $^{125}$I. The method had the advantage that intact particles could be labelled in a relatively short time and the ease of removal of free label whereas traditional methods relied upon labelling the monomer followed by
polymerisation with the accompanying problems of latex purification, decrease in specific activity during the production and purification stage and handling and safety considerations.

2.4 $^{125}$I-labelled latex nanospheres to quantify intestinal translocation

Four mL of 500nm ($2 \times 10^9$ particles/mL) non-ionised fluorescein-labelled particles were added to 4mL of 0.01M NaI (carrier) containing 667µCi NaI$^{125}$, each addition preceded by bubbling nitrogen to remove air/oxygen. Two mL of the solution was sealed in a two 5mL amber glass vial under nitrogen. The ampoules were gamma-irradiated from a $^{60}$Co source (8 Mrad over 48h) (Huh et al., 1974). The contents of the ampoule was centrifuged (7000rpm on Biofuge 13, Heraeus) to remove unbound free iodine. This was repeated until radioactivity in the supernatant had reached a constant low value just above background usually after 4 washes.

2.4.1 Oral administration to animals of radiolabelled latex

Female Sprague-Dawley rats (n=4) (average weight 150g; 9-10 weeks old) were given a single dose of the radiolabelled-latex of 0.1mL (12.5mg/kg, 2.2µCi) by oral gavage. Animals were given free access to water throughout but starved overnight before dosing, food being resumed 2h later. The rats were kept in metabolic cages to facilitate the daily collection of urine and faeces for measurement of radioactivity and to prevent coprophagia. Animals were sacrificed using vaporised halothane and blood, systemic organs and the thyroid removed. The GIT was also excised and separated into Peyer's patches, colon and the remaining villous tissue.
2.4.2 Results and Discussion

After irradiation and removal of unbound radioactivity the percentage coupling of hot iodine to the surface of polystyrene surface was 4.2%, a value smaller than reported by Huh et al (1974) who obtained 10% binding to similarly sized beads. This is probably due to the shorter irradiation dose employed in our case. The formation of halogen intermediates and \( I_2 \) in irradiated iodide solutions have been relatively well characterised, the principal chemical reactions summarised below:

\[
\begin{align*}
H_2 & \rightarrow H, e_{(aq)}^-, OH \\
OH + I^- & \rightarrow I^+ + OH \\
I^- + I^- & \rightarrow I_2^- \\
I_2^- + I^- & \rightarrow I_2 \\
H + I_2^- & \rightarrow HI + I^- \\
H + I_2 & \rightarrow HI + I \\
e^- + I_2 & \rightarrow HI + I \\
\end{align*}
\]

Radical sites are presumably formed as a result of reaction by OH, e(aq)- and H at the particle surface: \( PS + (OH, e_{(aq)}^-, H) \rightarrow PS \)

Bonding of iodine at the surface then occurs through a reaction between the surface site and \( I, I_2^- \), or \( I_2 \) : \( PS + (I, I_2^-, I_2) \rightarrow PS--I \)

Specific reactions for surface formation are, of necessity, speculative but probably not greatly different from those observed with benzene and toluene (Huh et al, 1974).

Within 1h of dosing it became apparent that breakdown of the radioactive latex had occurred as evidenced by the extremely high radiation emanating from the urine. Since the latex cannot pass through the glomerular
membrane of the kidney tubules it was concluded that surface bound iodine had become detached while the traversing the GIT. After a single day's collection of urine 16 ± 3.46% of radioactivity of the original dose was detectable. On microscopic examination of the kidney and urine no microspheres were observable. Radioactivity readings of the thyroid, after correction for background, approached 10% and again no microspheres could be seen under fluorescence microscope. Thus no further attempt was made to calculate the "uptake" and deposition of particles in the remaining organs as the radioactivity emanating from the organs was not associated with the particles. Initially this was attributed to the high radiation dose employed to induce surface bonding of $^{125}$I to the beads but the same result was seen when a lower irradiation dose was used (4 Mrad over 20h).

The detachment of surface bound iodine was initially thought to be due to the surface erosion of the latex due to the high irradiation but scanning electron microscopy and light scattering analysis revealed that they were stable with regard to external surface, the particles undergoing no detectable change. In vitro studies with liver and gastrointestinal tissue homogenate prepared in Tyrode solution gave little (1%) evidence of cleavage (Figure 2.1); however the high levels of radioactivity from the thyroid and urine suggested breakdown of the complex. The result is similar to that reported by LeFevre et al (1977) who found 14-56% of the free label in the mice urine 1 day after the intragastric administration of styrene-butadiene copolymer latex particles (250nm), radioiodinated using chemically based method (Iodine monochloride). Although previous reports have described loss of radioactivity from $^{125}$I labelled polymers (Regoezzi, 1976) after their administration to animals, the cause for de-iodination has not been investigated. In the case of radioidinated latex the major factor is probably the lability of the C-I bond rather than the incomplete removal of unreacted $^{125}$I during latex labelling.
Figure 2.1 The influence of pH on the release of radioactivity from $^{125}$-labelled latex nanospheres incubated with supernatants derived from intestinal mucosa. Briefly, 1g of mucosal tissue was homogenised by hand using a teflon/glass potter homogenizer in an ice water bath. The homogenate in PBS (pH 7.4) was centrifuged at 27,000g to remove particulate and food debris. An aliquot of radioiodinated nanospheres (0.1mL) were incubated with supernatants at pH 7.4 (PBS) and the other adjusted to pH 8.0 (tyrode medium). At various time points 200μL of the mixture was removed (replaced with fresh buffer), filtered and diluted to 1mL for measurement of released radioactivity using a gamma counter. Since at later time points the brush border enzymes are probably inactive, the release of radioactivity is most likely to be due to the influence of pH, a small rise being sufficient to up-regulate the release as predicted by LeFevre et al (1977).
Although not all C-I bonds are unstable, the linkage under consideration, that of iodine to a saturated carbon atom is weak and subject to disruption in basic aqueous media such as the small intestine. The displacement of I by OH, the latter which is highly abundant in the small intestine, in alkyl iodides is a classic illustration of a bimolecular nucleophilic substitution reaction. Some workers have also reported the presence of enzyme systems in various mammalian tissues (Iodothyronine deiodinases, glutathione transferases) capable of removing iodide from aromatic rings without rupture of the benzene ring (Roche et al., 1952), and this may account for the overestimation in the intestinal absorption of extrinsically radio-labelled macromolecules which exceed estimates based on immunochemical methods (Skogh, 1982; Udall et al., 1980; Jones, 1977).

In contrast Pratten and Lloyd (1984) have reported the high stability of radioiodinated latex beads using the chloramine T method for bonding of iodine to unreduced double bonds that are present in sufficient numbers on the latex bead surface. The labelled beads were found to retain the label on storage at 4°C for 3 months and in tissue-culture medium for the duration of the experiments (12h).

It is unlikely that radiation induced damage may have led to the decreased chemical stability of the latex nanoparticles since several thousand Mrad of exposure are required to induce fracture of the main chains of polystyrene (Charlesby, 1960). Instead it appears that the method used to iodinate latex beads ranging from free-radical radiation induced surface bonding and emulsion polymerisation of labelled monomer (LeFevre et al., 1977) to chemical methods of labelling (Pratten and Lloyd, 1984) may be responsible for the complex stability.

However, our attempt to develop a rapid method of particle detection has highlighted the caution that needs to be exercised in determining the actual amount of intact carrier that is absorbed. Very few studies, with the exception
of Le Ray et al. (1994a,b), have focused on the stability of the bond between the radioactive label and the carrier itself generating situations where the marker or radiolabelled oligomers released by the nanoparticles, often by non-specific esterases highly abundant on the apical membrane of Peyer’s patches (Owen and Bhalla, 1983), are followed rather than the carrier themselves. As a result many discordant results concerning the oral absorption of nanoparticles have been reported ranging from 1-2% for 200nm \(^{14}\text{C}\)poly(hexyl cyanoacrylate) nanoparticles (Couvreur et al., 1986) and 270nm \(^{14}\text{C}\)poly(alkyl methacrylate) nanoparticles (Kukhan et al., 1989) to more than 20% for 100nm \(^{14}\text{C}\)poly(DL-lactide) nanoparticles (Spenlehauer et al., 1991) and 10% for 100nm \(^{14}\text{C}\)poly(methyl methacrylate) (Nefzgar et al., 1984) the latter of which could not determine whether the radioactivity was associated with the intact particles or its degradation products. To resolve this question Le Ray et al (1994) administered low-leaching 130nm \(^{14}\text{C}\)poly(DL-lactide) nanoparticles composed of improved end-chain-radiolabelled monomers to mice. The parallel administration of \(^{14}\text{C}\)acetic acid, the major metabolite of poly(hydroxyacids), allowed the accurate assessment of intestinal nanoparticulate uptake that was between 1.9 - 2.3% four hours after administration by gastric intubation.

Despite the problems associated with radiolabel leaching many studies continue to use this method of quantitation, generating more confusion in terms of the extent of intestinal particulate absorption. Use of microspheres that have the radiolabel incorporated in the core and coated with a thin layer of the composite polymeric material (Hinrichs et al., 1978) has been shown to reduce leaching rates less than 0.2% per day is an alternative, but formulation of biodegradable microspheres using this method may be difficult.

An alternative method for radio-labelling biodegradable microspheres is the incorporation and subsequent neutron activation of poorly-water soluble non-radioactive compounds of elements such as Samarium (Scholes et al., 1993), Erbium, Holmium (Parr et al., 1985) and barium. Prior to administration the
dosage form is exposed to a neutron beam that converts the stable isotope into a gamma-emitting radioisotope. Such neutron activation techniques developed at the University of Kentucky (Parr et al., 1985) for the development of alternative gamma scintigraphic investigations for the visualisation of the intestine have been recently employed using biodegradable microspheres, but leaching rates in vitro have exceeded 15% (Scholes et al, 1993).

A conclusion which seems evident from this study, and from the literature, is that experiments utilising radiolabelled microspheres in biological experiments should include a determination of the stability of the label. Measurement of radiolabel into the urine, or the thyroid for $^{125\text{I}}$ or $^{99\text{Tc}}$, is probably the simplest and most direct method for experiments in vivo.

Because of the instability of the label, radio-iodinated latex could not be used to quantitate latex particle absorption by the intestine. Biologically one of the advantages of using latex particles is the extreme inertness (in the metabolic sense) and non-immunogenicity. However at the same time the material has no easily utilisable chemical moieties to incorporate a stable radiolabel. Further there are no known chemical methods to quantitatively analyze polystyrene unless the polymer is pyrolysed to its monomer units (Jones and Moyses, 1961). Thus it was decided to revert to a physical method of identification and quantification of polystyrene chains using a method developed by Jani et al. (1990) employing gel permeation chromatography to effect the separation of the tissue-extracted latex.
2.5 Quantification of tissue-extracted polystyrene nanospheres by Gel Permeation Chromatography.

Steric exclusion liquid chromatography is a separation method whose historically significant publications date from the late 1950s and early 1960s. The subsequent development of this separation method and the successive recognition and discovery of its fundamental have also been reflected in the numerous names by which the method has been denoted up to the present time that include gel filtration (for water-soluble bio-polymers eg proteins, oligosacharrides), gel exclusion, molecular sieve and gel permeation chromatography (GPC; principally restricted to non-aqueous soluble synthetic polymers). Size-exclusion chromatography is a liquid chromatographic technique which separates molecules according to their size and can be divided into low-pressure (eg glass or burette-filled columns) and high performance size exclusion chromatography (closed systems operated at high pressures). This results in separations that commonly take 10-30min compared to the much longer (hours) separations of conventional size-exclusion chromatography.

In GPC separation of solution of the polymer containing a separating medium, usually beads of a porous crosslinked polystyrene-divinyl benzene gel. Typically the gel contains pores ranging in size from 6nm to $10^6$nm average diameter. The gel is completely immersed in the GPC solvent with the exclusion of any gases. When a polymer solution is passed through the column the polymer molecules will enter into those pores large enough to accept them. Consequently if the columns are packed with gel having distribution of pores, a separation of the polymer molecules in a mixture can be effected (mixed-bed columns). Very large molecules will only have a small number of pores available to them and will pass through the columns fairly rapidly. Very small molecules will thus enter most of the gel pores and will take proportionally longer to pass through the columns. Thus, in common with other column chromatographic techniques, solutes only move only when they
are in the mobile phase, the distribution of solute molecules between the mobile and stationary phases determining the average solute migration velocity. All forms of chromatography are, therefore, simply differential separation processes where sample components are selectively retained to different degrees by a stationary phase, which in the case of GPC is on the basis of average hydrodynamic radii. The concentration of the species in the mobile phase emerging from the columns is monitored continuously using a suitable detector, retention of the species in the columns reported as retention time, or less commonly, retention volume.

2.5.1 Experimental procedures

The tissue-latex extraction and subsequent separation and quantification by GPC to be described in the proceeding sections are essentially from methods developed in our laboratory (Jani et al., 1991; Jani, 1991) with minor modifications.

2.5.2 Extraction of polystyrene from tissues

Rats were typically fasted overnight (12-16h) and sacrificed using vaporised halothane. To minimize contamination, the peritoneal skin of each dead rat was removed prior to opening the abdomen. Surgical instruments were thoroughly cleaned between each autopsy. Blood samples (2mL) were directly taken via cardiac puncture before the removal of other organs. Small intestine with mesentery lymphatic network and lymph nodes, Peyer's patches, colon, liver, spleen, heart, kidney and lungs were analyzed. The excised small intestine and colon was flushed through with 10-20ml PBS to remove the luminal contents and non-absorbed polystyrene nanoparticles that might otherwise inflate the intestinal absorption readings. The Peyer's patches were then carefully excised as circular rings.
Dissected organs were frozen by liquid nitrogen and subjected to freeze drying for 48h, ensuring total removal of water that interferes in the organic extraction and which is incompatible with GPC columns. Each separate organ was ground to a fine powder in a mortar and the material transferred to a stoppered glass flask. 20mL of chloroform was added and the flask mechanically shaken overnight in a water bath at 30°C. The slurry of the ground powder was vacuum-filtered through a Whatman filter paper (catalogue number 541) using a Buchner funnel to collect the latex containing chloroform filtrate. After two further extraction-washes with chloroform, the filtrate was evaporated to dryness on a rotary evaporator (Heidolph WB2000, Germany) under reduced pressure at 60°C. The dry residue was resuspended in accurately measured volume (1mL) of the mobile phase (tetrahydofuran), filtered through a solvent-resistant 0.5μm filter (Millex-SR, Millipore, U.K.) and 100μL sample analyzed using GPC. At all times glass apparatus was employed and steps taken to prevent cross contamination of the latex samples between samples.

2.5.3 High-Performance Gel Permeation Chromatography

Tetrahydrofuran was used as solvent and for all mobile phase work. The low viscosity of THF permits high GPC resolution, relatively low back pressures (1000 psi) and swells organic gel packings facilitating its use in SEC separations. Two 300 x 7.5mm, 10μm mixed pore highly cross-linked spherical macroporous polystyrene-divinylbenzene matrix columns preceded by a 50 x 7.5mm guard column (PLgel, Polymer Laboratories, UK), a Rheodyne injector valve (Waters USA 7125) fitted with a 100μL sample loop formed the closed loop gel permeation components. A Waters 484 tunable UV absorbance detector (set at 254nm) was used for the detection of the eluted polystyrene in conjunction with a chart recorder/integrator (Hewlett Packard 3396A) to compute the area under curve measurements. A flow rate of 1ml/min was maintained (Waters 510 pump). To construct a calibration curve, freeze-dried
fluorescent nanospheres were dissolved in THF (1mL). A series of dilutions were made from this stock solution and 100μL injections made on to the GPC. The lowest limit of detection was calculated to be 0.1μg, indicating the sensitivity of the method.

2.5.4 **Recovery of polystyrene from tissue**

This was assessed by adding known amounts of suspension of fluorescent latex beads (10, 25 and 50μL of 2.5% w/v, 500nm) to homogenised rat livers (n=8; 5.75 ± 0.4g) and spleens (n=4; 0.63 ± 0.1 g). The liquidised organs were freeze-dried and extracted with chloroform and THF as above and the percentage recovery obtained. A mean value of 80% for the livers and 85% for the spleens were obtained; extraction values for the experiments described later were thus corrected by a constant factor throughout, the smaller organs (heart, lungs etc) using the spleen recovery value and the small intestine and liver uptake calculated using the spiked liver recovery value.

2.5.5 **Comments on the separation technique**

A typical chromatogram is shown in Figure 2.2. The extraction procedure is essentially an adaptation of techniques used commonly to extract lipids from animal tissues. As the hydrophobicity of latex (MW = 375,000) is similar to the extracted lipid material (mw=40,000) separation is most easily achieved using GPC on the basis of molecular size with two columns in tandem to effect acceptable separation/resolution of the peaks with respect to time. Although the total retention time calibrated using a very dilute solution of toluene in THF is 27 minutes, typically 35-60 minutes are required, depending on the sample, for a subsequent injection to be made, reflecting the adsorption of the lipid material onto the column packing. Since organs from four animals and triplicate readings for each organ were used to quantify the latex uptake/deposition, this resulted in the generation of only 1 data point per day for each organ examined.
Figure 2.2  Typical chromatographic peaks obtained after (A) fluorescent nanospheres dissolved in THF for calibration and (B) of tissue-derived latex using chloroform extraction. Peak B is that of a blood sample showing the presence of TL-conjugated nanospheres (chapter 3) in the systemic circulation after chronic oral dosing. Notice that the structure of polystyrene is relatively unaffected by the multiple extraction process as evidenced by the similarity in retention times.
Chronologically the work conducted with radioiodinated latex was the first to be carried out; the inexplicable urinary data led to the use of GPC for the analytical and quantitative determination of the polystyrene content of tissues and organs. Although the technique is slow and tedious, use of other techniques such as the spectrofluorometric measurement of fluorescein derived from the tissue-extracted latex particles (Juhlin, 1959) where background fluorescence is an interfering factor, has emphasised the reliability of the chromatographic method for assessing particle uptake in bulk-macerated tissue.
Chapter three
Lectin-induced oral uptake of latex nanospheres
3.1 Introduction

Boyd and Shapleigh (1954) introduced the term "lectin" (from the Latin legere, to pick, choose) to describe a class of proteins predominantly of plant origin that could agglutinate cells, first described by Stillmark (1888) after the observation that seed extract of castor bean, the lectin ricin, agglutinated erythrocytes and exhibited antibody-like sugar binding specificity. Subsequently the discovery of many similar substances in mammalian tissues, invertebrates, microorganism and cellular slime mould as well as plant tissues has prompted several attempts to reach a agreement as how to define a lectin (Goldstein et al., 1980; Kocourek and Horejsi, 1981). The nomenclature committee of the International Union of Biochemistry has defined a lectin simply as "a carbohydrate-binding of non-immune origin that agglutinates cells or precipitates polysacharrides or glycoconjugates" and is capable of specific recognition and reversible binding to carbohydrate moieties of complex glycoconjugates without altering the covalent structure of any of the recognised glycosyl ligands. This definition implies that lectins are multivalent, ie that they possess at least two sugar binding sites which enable them to agglutinate animal and plant cells and/or to precipitate polysacharrides, glycoproteins, peptidoglycans etc (Leiner et al, 1986). The sugar specificity of lectins can be divided into groups depending on their preferential binding to D-pyranose sugars namely glucose/mannose, fucose, N-acetyl glucosamine, galactose/N-acetyl galactosamine and sialic acid and is defined in terms of these monosaccharide(s) that inhibits lectin-induced agglutination or precipitation reactions. The emphasis on "non-immune origin" is included to distinguish lectins from anti-carbohydrate antibodies which may act as cell agglutinins. Furthermore many lectins are found in plants and bacteria that do not synthesize immunoglobulins.

In contrast to antibodies which show close structural homology, lectins are structurally diverse and vary in their molecular size, metal requirement in sugar binding, amino-acid composition and 3-dimensional structure, a reflection of
their diverse sources of origin. Despite their structural heterogeneity, lectins can be grouped into families with sequence homologies and common structural properties. The largest and best characterised family is that of Leguminous lectins. Two smaller families, also of plant lectins, are those from Gramineae (cereals) and Solanaceae (e.g. tomato, potato and thorn apple). As this comprises the vast majority of edible plants humans and animals are continually exposed to lectins. Their biological effect in humans is, however, not entirely known. Among animal lectins (collectively known as endogenous lectins to distinguish them from plant lectins) two distinct families have been recently identified; the C-type (Ca$^{2+}$-dependent) containing both membrane-bound and soluble lectins and the S-type (thiol dependent or soluble) consisting of lectins that share a specificity for $\beta$-galactosides. The existence of such homologous families demonstrates that these proteins have been conserved through evolution and argues strongly that they must have important function in nature. Although this function is not known with certainty it is generally believed that lectins serve as cell recognition molecules (Sharon and Lis, 1989).

Lectins are all glycoproteins of molecular weight ranging between 40-400 kDa and usually consist of two or more subunits, each subunit containing a single sugar binding site thus explaining the observed cell-agglutination reactions. Carbohydrate binding may involve several forces, mostly hydrophobic and hydrogen bonds; only rarely are electrostatic forces involved since most carbohydrates are devoid of electrical charge. As discussed by Quiocho (1986), the formation of carbohydrate-lectin complex involves the displacement of water molecules associated with the lectin polar groups and around the highly polar sugar with the establishment of hydrogen bonds; these latter bonds and the van der Waals' contacts are the dominant forces in binding stability. The existence of an additional hydrophobic site has been postulated to account for the binding to other non-glycosylated proteins (Roberts and Goldstein, 1983).
Although known for nearly a century, since the pioneering experiments of Ehrlich in his immunological studies employing the lectin abrin (cited by Sharon and Lis, 1972), no clear role for lectins in plant physiology has been established. Several functions for plant lectins have been postulated that include a defensive role in the protection of the plant from bacteria, fungi and insects, as storage proteins, stimulation of mitosis of embryonic cells and facilitation in rhizobial-legume symbiosis.

In the last two decades lectins have become the focus of intense interest as invaluable tools in analytical and preparative biochemistry, cellular biology and immunology, primarily due to their unique properties but also the recognition that virtually all cell surfaces are endowed with a glycocalyx, a carbohydrate coating composed of membrane glycoproteins, glycolipids, and glycosaminoglycans, collectively known as glycans or glycoconjugates. The carbohydrates residues of glycoconjugates encode a large repertoire of information per monomer unit, differing in the number and type of sugar residues, sequence of sugar moieties, type of anomeric linkage, presence or absence of branches and the type and number of sialic acids, thus contributing to structural microheterogeneity. In theory just 4 different monosaccharides can generate an astonishing 35,560 unique tetrasaccharides (Mody et al, 1995). Because of their structural variability, cell-surface carbohydrates encode unique signals that are recognised by polyvalent lectins in a complementary way to a ligand receptor interaction, resulting in numerous biological functions and application of lectins. These include utilisation as probes to explore cellular surfaces by binding through the carbohydrate portion of glycoproteins/lipids, following changes that occur in cell surfaces during physiological and pathological processes in cell differentiation to cancers, tracing of neuronal pathways, typing of blood cells and bacteria and for fraction of lymphocytes and bone marrow cells for bone marrow cell transplantation.
As lectins have been used widely in their purified forms in countless scientific studies and have been shown to possess potent in vitro and in vivo effects, and since almost all edible plants contain lectins and the intestinal epithelium is covered with carbohydrate moieties in the form of the glycocalyx and mucus, it is not surprising that significant attention in recent years has been given to their biological effects in vivo after oral consumption. The finding that many lectins, unlike most dietary proteins, are at least partially resistant to proteolytic degradation and to mild cooking (Nachbar et al., 1980) and, depending upon their carbohydrate specificity, not only recognise and bind to membrane glycans of epithelial cells but are subsequently endocytosed and appear in the systemic circulation has led a number of authors to investigate the possibility of utilising the potential of lectins as oral drug carriers particularly for gut-labile molecules. Experimental evidence has shown that 40-90% of the dietary intake of kidney beans (Phaseolus vulgaris) and tomato fruit (Lycopersicon esculentum) lectins can survive the passage through the GIT and be recovered in the faeces of experimental animals. Furthermore binding to the epithelium usually results in their rapid internalisation resulting in situations where 5-10% of kidney bean lectin has been reported to be absorbed orally (Greer et al., 1985) indicative of their relative high stability against the lysosomal apparatus.

Although this broadly supports the view that binding of lectins to epithelial glycans stabilises them against proteolytic breakdown the almost complete survival of Galanthus navalis (snowdrop) lectin, which does not bind, suggests that the glycoprotein structure of lectins confers rigidity to the molecule and makes them intrinsically resistant to proteolysis i.e. the presence of carbohydrates may reduce the protein's susceptibility to proteolysis by local shielding of protein surface regions (Pustzai, 1991). As a result a number of investigators have suggested that coupling of gut-labile molecules such as peptides and immunogens to lectins may not only protect the latter from intestinal peptidase action but also confer protection against the lysosomal system after endocytosis mediated by the lectin portion. However the vast
majority of plant lectins are toxic, ricin and phaseolus vulgaris being the most celebrated examples. The former inhibits protein synthesis due to binding to ribosomal apparatus involved in mRNA translation, and the latter exhibits a range of deleterious effects from effacement of brush border membrane and development of epithelial lesions to the hyperplasia of the GIT due to the redirection of polyamines from the blood to epithelial cells with the concomitant wastage of body component and loss in skeletal muscle.

In contrast solanaceous lectins (ie from tomato, tamirillo, potato, thorn apple) are not only unique in terms of their carbohydrate specificity (ie all bind to oligomers of N-acetylglucosamine) and close homology in chemical composition but are relatively non-toxic. In particular tomato lectin (*Lycopersicon esculentum*; TL) is an appealing candidate as an oral drug delivery carrier since it resists proteolytic digestion both in vitro (Nachbar et al, 1980) and in the mammalian alimentary canal, and binds to rat intestinal villi without deleterious effects (Kilpatrick et al, 1985) even after a concentrated lectin diet for 10 days. The widespread consumption of raw tomatoes provides additional circumstantial evidence of the low toxicity potential of tomato lectin of which the average ingestion by humans from tomato consumption has been estimated to range from 100-200mg per year (Nachbar et al, 1980).

*In vivo* studies utilising the potential use of TL for oral drug delivery as both an intestinal bioadhesive and a drug carrier have followed rapidly since the isolation of the lectin by Yeoman et al. (1978). Woodley and Naisbett (1994a) have reported the avid binding of TL to glycans on the enterocyte surface using an *in vitro* everted gut sac system which could be specifically inhibited by N-acetylglucosamine tetramer as the competing sugar. Additional studies demonstrated a significantly increased uptake of the lectin that was saturable and abolished at 4°C indicative of an absorptive endocytic mechanism of absorption. Upon oral administration to mice an immunologically detectable response against the entire lectin was mounted suggesting the absorption of the intact molecule. The authors theorised that covalent coupling of drugs to
TL may thus not only direct the drug towards an endocytic route of capture but deliver the entire TL-drug conjugate across the hostile milieu of the enterocyte.

With this in mind, and the fact that the oral absorption of sub-micron particles from the Peyer’s patches is low, of the order of 1-2%, we endeavoured to covalently couple TL to the surface of latex nanospheres in an attempt to redirect and enhance the intestinal absorption of colloidal particles via the non-lymphoid villous tissue that, as discussed in section 3.1 (Chapter 1), constitutes a much greater area for the potential absorption of nanoparticles compared to Peyer’s patches. Additionally, as discussed by Lehr et al. (1992) conclusions about the practical relevance of the cross-reactivity with mucus that was shown to reduce the binding between isolated pig enterocytes with TL-coated latex microspheres could only be possible after additional experiments in vivo. Finally a further aim of this study was to explore whether intestinal uptake of TL-coupled nanoparticles, by means of specific receptor-mediated interactions that permit binding directly onto the mucosal surface rather than onto mucus, could be made more reproducible and controllable, features important if the route is to be used for therapeutic drug delivery, incorporating protein and peptide drugs that have narrow therapeutic indices.

3.2 Materials and methods

3.2.1 Preparation of Tomato-lectin-nanosphere conjugates

Fluorescent carboxylated nanospheres (Polysciences Ltd, Northampton, UK 2.5 % w/w) of 500 nm diameter were covalently coupled to tomato lectin (TL). Briefly 4.5 mL were washed twice in 0.1M carbonate buffer (pH 9.6) by centrifugation (MSE 50M centrifuge 15000 rpm in 50 mL polycarbonate tubes followed by two washes in 0.1 M phosphate buffer to remove the storage buffer and any adsorbed surfactant/polymeric stabiliser. The microsphere pellet
was then resuspended by vortexing in 5.5 mL of phosphate buffer to which was added an equal volume of freshly prepared 2 % carbodiimide solution (dimethylaminopropyl-3-ethylcarbodiimide HCl). The mixture was then shaken gently for 3h to activate the carboxyl groups, a longer time leading to aggregation. The microsphere suspension was then centrifuged to remove the unreacted carbodiimide and further washed twice in 0.1 M borate buffer to remove any traces of carbodiimide which might otherwise cross-link the lectin molecules. To this 7 mL of borate buffer containing 2 mg of tomato lectin (Sigma, lyophilised, 60% purity by SDS) was added and the mixture gently shaken overnight at 8°C to allow coupling. Ethanolamine (1mL; 0.1M) was then added to block any remaining specific sites and the mixture shaken for a further 30 min at room temperature. The suspension was then centrifuged and the pellet resuspended in 10 mg/mL bovine serum albumin solution (BSA) for 30 min at room temperature to further block non-specific adsorption of cellular proteins. The microsphere suspension was centrifuged as before and resuspended to the original volume (4.5 mL) in distilled water. Control nanospheres consisted of tomato lectin nanospheres pre-incubated with 10mg of N-acetylchitotetraose (ie. the competing sugar) overnight to block the active sugar binding site.

3.2.2 Determination of the coupling efficiency of Tomato lectin to polystyrene nanospheres

A modified Lowrey method (Basinka T and Slomokowski, 1992) was employed to determine the percentage covalent coupling of TL to polystyrene nanospheres. The method has the advantage of directly determining the amount of protein attached to the nanospheres as compared to the determination of protein measurement of the protein solution after the incubation of nanospheres (ie. by difference) which can give inaccurate results. Briefly 200μL of lectin-microsphere conjugates were processed as normal protein solution (Lowrey et al, 1951). Upon centrifugation (Biofuge,
7000 rpm) the resultant supernatant was further clarified by filtration through a 0.2 μm filter (make, Millipore, U.K.) and the absorbance of the filtrate measured at 750 nm. Determination of triplicate readings against a standard BSA calibration curve gave a mean of 80 % coupling. The value of percentage coupling is probably higher due to the possible adsorption of the lectin to the filter membrane upon clarification of the supernatant.

3.2.3 Haemagglutination test for the retention of lectin activity

The presence of biologically active lectin after covalent coupling was assessed by lectin-induced agglutination of human erythrocytes. Two-fold serial dilutions of 100μL aliquots in PBS of the TL-conjugate suspension was made in a microtitre plate. 100 μL of a 2% "/₀ suspension of fresh human blood (washed once with Alsever’s solution followed by three washes in PBS) was then added to each well and incubated at 37°C for 30 min. Confirmation of activity was shown by comparing the value of the titre of dilution giving the last visibly detectable erythroagglutination against a positive control (TL solution) and three negative controls (PBS, uncoated nanospheres and lectin-conjugated nanospheres in the presence of N-acetyl tetrachitotetraose). The procedure was conducted in triplicate. Almost identical titre values for TL-conjugates were obtained compared to the tomato lectin test solution thus confirming lectin activity.

3.2.4 Oral administration to rats

Animals used in the experiments were all maintained, treated and housed according to the guidelines and regulations stipulated by the Animals Act 1987. A group of 5 female Wistar rats (average weight 180 g; 10 weeks) were
employed, of which 4 were used for quantitation and one for histological examination. Nanospheres were administered by oral gavage using a blunt-ended feeding needle, a dose of 0.1 mL (12.5 mg/Kg) being administered daily for 5 days. Rats were given free access to water but starved overnight before each dose, food being resumed 1 h later. A second group of rats were administered with an identical dose of Tomato lectin conjugated nanospheres blocked with N-acetylchitotetraose to test for specificity of epithelial bioadhesion. After the final dose all animals were fasted overnight in a microsphere free environment but allowed water to clear the gut of any unabsorbed nanospheres. Animals were kept in individual metabolic cages to prevent coprophagia and re-introduction of excreted nanospheres. Animals were sacrificed using vaporised halothane. Removal of organs and analysis of tissue extracted polymer was as described in chapter 2 (section 2.3).

3.2.5 Histological examination using cryostat tissue sections

As conventional histology employs organic solvents that solubilise latex nanospheres, cryostat sections were used in all experiments. Excised tissue was immediately frozen and embedded in a water-soluble cryostat medium (Tissue Tek II 4583 compound, U.S.A) on to a cylindrical metal chuck using isopentane-cooled liquid nitrogen. Throughout the sectioning and mounting procedures, the temperature was maintained at -30°C to -20°C. Tissues were cut at 5μm thickness except for the mesentery nodal tissue which were sectioned at 8-10μm because of the difficulty in thin sectioning of fatty tissue. Between sectioning, the knife was cleaned thoroughly using acetone to prevent cross contamination of nanospheres between tissues. The sections were viewed by fluorescence microscopy observed using Nikon Microphot FSA microscope equipped with a FITC filter.
3.3 Results

The intestinal uptake of polystyrene nanospheres on to which tomato lectin (TL) was covalently bound was quantified using the method discussed in section 2.3. Comparison of the absorption of TL-conjugated nanospheres with those inhibited with GluNac\(_4\) (= blocked nanospheres) reveals a 13 fold increase over blocked nanospheres in adsorption/absorption from the intestinal epithelium (Table 3.1). Approximately 15% of the dose is bound to the GIT epithelium similar to a previously determined value using tomato lectin solution (Kilpatrick et al., 1985). Substantial amounts of TL-conjugates remain bound to the intestinal epithelium, or at least are retained within the mucus for several hours in spite of gut peristalsis and mucus sloughing, factors that usually prevent non-specific adherence, since 13% of the dose adhering to the intestinal epithelium most probably derives from the final dose administered 24 h earlier.

**Figure 3.1** shows the accumulation of nanospheres within the mucus layer, possibly a prelude to absorption. As the mucus layer is several micrometers thick the penetration of nanospheres through this gel is the main barrier for oral particulate absorption; however non-motile bacteria such as *Shigella flexneri* are still able to penetrate mucus to invade the lamina propria, an achievement attributed to the movement along the natural lines of stress of the moving mucus (Freter, 1988). **Figure 3.2** demonstrates the passage of TL-conjugated nanospheres along the serosal layer, indicative of true absorption. Blocked nanospheres which may have been expected to absorbed from the Peyer's patches were, surprisingly, absorbed, albeit in smaller quantities, from the colon (**Figure 3.3**).
Table 3.1 Oral uptake of Tomato lectin-conjugated (TL) and blocked nanospheres (500 nm) as a function of total dose administered and organ weight.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>TL nanospheres (%)</th>
<th>TL nanospheres with N-acetylchitotetraose (%)</th>
<th>n/d</th>
<th>n/d</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intestine</td>
<td>12.8 ± 3.00</td>
<td>4.82 ± 2.10</td>
<td>n/d</td>
<td>n/d</td>
</tr>
<tr>
<td>Peyer's</td>
<td>0.89 ± 0.04</td>
<td>2.16 ± 1.21</td>
<td>n/d</td>
<td>n/d</td>
</tr>
<tr>
<td>Colon</td>
<td>0.90 ± 0.02</td>
<td>0.75 ± 0.01</td>
<td>1.11 ± 0.68</td>
<td>1.10 ± 0.67</td>
</tr>
<tr>
<td>Liver</td>
<td>2.64 ± 1.70</td>
<td>0.43 ± 0.28</td>
<td>n/d</td>
<td>n/d</td>
</tr>
<tr>
<td>Spleen</td>
<td>1.17 ± 0.80</td>
<td>2.88 ± 1.45</td>
<td>0.42 ± 0.38</td>
<td>0.74 ± 0.67</td>
</tr>
<tr>
<td>Blood¹</td>
<td>3.04 ± 0.64</td>
<td>1.82 ± 0.04</td>
<td>n/d</td>
<td>n/d</td>
</tr>
<tr>
<td>Heart</td>
<td>0.29 ± 0.03</td>
<td>0.45 ± 0.06</td>
<td>n/d</td>
<td>n/d</td>
</tr>
<tr>
<td>Kidney</td>
<td>0.67 ± 0.16</td>
<td>0.48 ± 0.20</td>
<td>n/d</td>
<td>n/d</td>
</tr>
<tr>
<td>Systemic²</td>
<td>23.0 ± 6.53</td>
<td>0.42 ± 0.38</td>
<td>n/d</td>
<td>n/d</td>
</tr>
</tbody>
</table>

¹ Blood sample (2mL) taken directly through the heart.
² Extrapolation to whole blood volume (12 mL for 200g rat)
Figure 3.1 Photomicrograph (x 30) showing the accumulation of lectin-conjugated nanospheres between villous tissue amongst the adherent mucus layer, prelude to absorption. In all photomicrographs the large white arrowhead denotes the direction of the intestinal lumen. Smaller arrowheads (either black or white for clarity depending on the background colour) are used to show the anatomical location of the nanospheres.

Figure 3.2 Photomicrograph (x 60) demonstrating the movement of lectin-conjugated nanospheres across two distinct histological layers, the submucosa and the serosal layer, in a cylindrical section of non-Peyer's patch tissue.
Figure 3.3 Photomicrograph (x 60) shows the percolation of blocked nanospheres in the muscularis externa and into the adventitia of the colon.

Low uptake in organised lymphoid tissue is probably related to the reduced availability of specific sugar residues complementary to the TL binding site over the M-cell apical membrane compared to the enterocytes, although this has to be confirmed by histological examination, and also the fact that the most abundant cell in the intestine is the enterocyte, compared to the M-cell population which constitutes less than 1% of the entire intestinal cells. Figure 3.4 shows a virtual absence of lectin-conjugated nanospheres in the Peyer's patch domes in contrast to blocked nanospheres which are intimately associated with the blind-ended lymphatics (Figure 3.5) demonstrating the redirection of particles bearing lectins through the villous tissue. Further confirmation is demonstrated in Figure 3.6 where TL nanospheres are only seen in the serosal layer directly beneath the villous side of a colon section bearing a caecal lymphoid patch.
Figure 3.4 Photomicrograph (x 40) from a section of Peyer's patch demonstrating the relative absence of lectin-conjugated nanospheres in the dome region.

Figure 3.5 Photomicrograph (x 45) of blocked nanospheres showing intimate association with lymphatics in the follicle region of the Peyer's patch prelude to systemic absorption via the mesentery modes. Lectin-conjugated nanospheres in contrast are absent from this region (figure 5) probably due to the lack of glycoconjugates of the glycocalyx which is more abundant over villous tissue compared to Peyer's patch, thus preventing absorption from this route.
Figure 3.6 Lectin-conjugated nanospheres in the serosal layer immediately below the villi of a cylindrical section of a caecal Peyer’s patch. Follicle region of the same section (ie Lymphoid tissue) revealed no nanospheres indicating the absence of lectin binding sites over the follicle-associated epithelium (x 180). The whole circular section can only be viewed under low magnification but the resolution is too low to observe any fluorescence (due to the size of the nanospheres) in a particular location.

Systemic appearance of intestinally absorbed TL-conjugated nanospheres

It is evident from Table 3.1 (and Figures 3.7 - 3.10) that after epithelial internalization of TL-conjugated nanospheres, they able to traverse the enterocyte basement membrane, enter the lymphatics in the submucosa (Figure 3.7) and translocate to various organs once the lymph drains into the right sub-clavian vein. The spleen and blood (2.0 mL sample) show the greatest uptake on the basis of organ weight but if the blood value is extrapolated to include the whole blood volume of a rat (12 mL for 190 g rats from Baker et al., 1979) the accumulation after 5 days rises to 18% giving a total systemic uptake of 23% (ie including the systemic organs), an almost 50 fold increase in uptake compared to the uptake of blocked nanospheres. In contrast the systemic appearance of blocked nanospheres was solely limited
to the spleen and no nanospheres could be detected in the liver. Figures 3.8 and 3.9 demonstrate low levels of accumulation of nanospheres in the liver centrilobular vein amongst the dried erythrocytes in sharp contrast to blocked nanospheres where none were seen in any part of the liver. Splenic uptake is higher on the basis of organ weight due to its slow percolating microcirculation and parenchyma rich in lymphatic tissue. Clusters of TL-conjugated nanospheres are seen in the splenic reticular channels (Figure 3.10). The presence and the alignment of 500nm TL-conjugates in a uniform arrangement along the trabeculae has been also observed with 1μm plain latex microspheres (Jani and Florence, 1992) and confirms the analytical data that, in addition to the liver, substantial amount of the dose is accumulated here. This may reflect the size of fenestrations between the trabeculae and the reticular spaces trapping the 500nm nanospheres (Weiss, 1988), since smaller sized nanospheres, although systemically absorbed to a significant extent, are not readily seen in histological sections (Jani and Florence, 1992). The observation is important in understanding the fate of intestinally absorbed colloidal drug carrier systems. The low amount of latex in the heart and kidneys is probably due to the retention of TL-nanospheres in the coagulated blood of the capillaries upon sacrifice. Bone marrow or cerebral tissues were not examined.
Figure 3.7 Concentrated areas of nanospheres are clearly visible at the junction of three medullary cords of a mesentery node. The nanospheres travel along distinct pathways of the reticular fibres as shown by the arrowheads converging into a central hollow indicating that the route of intestinally absorbed nanospheres to the systemic circulation is via the villous lacteals that have incomplete basement membranes and wider fenestrations rather than villous blood capillaries. (x 80).

Figure 3.8 Clusters of lectin-conjugated nanospheres accumulated in the centrilobular vein of a liver hepatocyte amongst the dehydrated erythrocytes, indicative of true systemic translocation of orally administered 500 nm particles (x 30)
Figure 3.9 A magnified photomicrograph of Figure 3.9 (x 60).

Figure 3.10 Deposition of lectin-conjugated nanospheres in the reticular channels of the spleen (x 45).
3.4 Discussion: Implications for oral drug delivery

Several authors have discussed the possibility of targeting Peyer’s patches by optimising carrier microsphere surface characteristics such as hydrophobicity (Eldridge et al., 1989). There is some presumption that the degree of accumulation of nanoparticles is directly proportional to their surface hydrophobicity, but Wils et al (1994) has shown that increasing lipophilicity of drugs may lead to their decreased transepithelial permeability. The effect of particle charge on the magnitude of absorption remains debatable, previous work in this laboratory finding considerable reduction in absorption of negatively charged polystyrene particles compared with non-ionised surfaces (Jani et al., 1989) whereas uptake of negatively charged liposomes particularly through the Peyer’s patches has recently been demonstrated (Tomizawa et al., 1993) albeit from an enclosed loop encompassing a Peyer’s patch.

One approach to overcome non-specificity of interaction is to exploit a receptor mediated process within the GIT such as the vitamin B\textsubscript{12} receptor (Russell-Jones and De Azipurua, 1988), endogenous integral membrane lectins (Hussain and Florence, 1985; Rihova et al., 1982) and alpha-beta integrin receptors exploited by certain bacteria (Hussain and Florence, 1985; Tran van Nhieu and Isberg, 1994). In an elegant study Pappo et al (1990) demonstrated that latex nanospheres displaying surface adsorbed anti-M-cell antibody 5B11 (IgM) adhered to and were absorbed by rabbit M-cells 3-fold more efficiently than particles displaying IgM of an unrelated specificity or particles displaying their native surface chemistry. The sigma (σ1) outer protein of Reovirus 1, which selectively invades Peyer’s patches, when incorporated in liposomes resulted in a 20 fold uptake through the Peyer’s patches compared to villous tissue but the possibility of an intestinal IgA antibody response generated against these ligands cannot be ruled out particulary on repeated administration (Rubas et al., 1990). Attempts to exploit a receptor mediated pathway, initiated by Biotech Australia employing cyanocobalamin-conjugated drugs may be limited by their low uptake as Vitamin B\textsubscript{12} absorption peaks at
1-2 μg per day regardless of the dose limiting this system to those drugs that require only nano-molar quantities for pharmacological activity. The proposal to employ nanospheres, conjugating encapsulated proteins to Vitamin B₁₂, would require a B₁₂ depleted diet to ensure proper dosing.

Although recent evidence suggests that Peyer's patches are the major sites of colloidal uptake via specialised M-cells (Sass et al., 1990; Jepson et al., 1993) due to the reduced brush-border filaments and mucus coverage and to the unique characteristics of the absorptive cells of the FAE (enhanced endocytic activity), the present study reveals a 14-fold increase in the adsorption/absorption of TL-nanospheres through the non-lymphoid villous epithelium compared to the FAE. The mapping of lectin binding sites over the rat M-cells and enterocytes by Owen and Bhalla (1983) reported the equal binding of 3 different lectins including wheat germ agglutinin (WGA) which has a similar binding specificity to TL (ie. poly-N-acetyl glucosamine residues) thus lending some support for our findings that TL binding sites are over M-cells are smaller in numbers compared to non-lymphoid tissue that exhibits a relatively large surface area, and from where much of the absorption is seen to occur. Although both types of cells may share common glycoconjugates, different steric arrangements or groupings of sugar residues may exist. However the use of lectins has not revealed carbohydrate sites unique to the M-cell surface of rat Peyer's patches in contrast to mouse and rabbit M-cells where much of the investigations has centred in recent studies. Reduced TL-binding sites over the FAE thus directs the intestinal absorption through the non-lymphoid tissue where abundant glycoconjugates containing N-acetylgalucosamine sequences are present (Naisbett and Woodley, 1994b).

In addition to the endocytic activity of the M-cells, reduction of mucus coverage over the Peyer's patches has been suggested by many authors as being an equally important determinant for the selective uptake of particulate material. Early microbiologists considered mucus gel to be "particle and macromolecule proof coating for cell surfaces" through which "bacteria must
bore a channel" by means of special virulence mechanisms (Edwards, 1978).

In contrast, studies by Freter (1987) have shown that non-motile bacteria can penetrate mucus within 15 minutes after incubation of intestinal slices in bacterial suspensions or when bacteria are injected into intestinal loops of mice and rabbits. Furthermore yeast cells or inert latex particles in the size range of bacteria also penetrated mucus gel, the rate of penetration being inversely related to their size, albeit at a slower rate. It was suggested that particles penetrated along the lines of stress of the moving mucus which are created when mucus is extruded by goblet cells. Such lines of stress may be expected to parallel the flow of the mucus thereby forming channels for particulate penetration that lead from the lumen to the epithelium.

According to Walker (1987) the preferred route of uptake of low concentrations of luminal antigens is through the M-cells, but at higher concentrations the enterocytes are also involved. Furthermore Sternson pointed out that Peyer's patch uptake is compromised by the limited efficiency and capacity of the pathway. Pappo and Ermak confirmed Sternson's hypothesis showing that only 5% of the total dose of 500 nm latex particles placed in rabbit ligated loops containing Peyer's patches were taken up in a single round of endocytosis and no additional nanospheres adhered to the M-cell membrane during the next 90 min. Porta et al (1992) further showed that <3% of M-cells could both adhere and internalize particles and that only half of the mouse M-cell population had endocytic capability. This indicates, in line with our results, that the importance attached to the Peyer's patches as the principal site of particulate absorption may have been over-emphasised, and that normal epithelial cells can also be induced, with appropriate ligands such as plant lectins and bacterial adhesins (Hussain and Florence, 1995; chapter 5), to absorb particulate matter.

Many mammalian cells are capable of receptor mediated endocytosis even after the neonatal stage and although the M-cells are generally believed to be the main portal of entry of particulates, the most abundant cell in the intestine
is the enterocyte. O’Hagan (1990) states that the upper diameter for enterocyte uptake appears to be 30 nm, but Sanders and Ashworth (1961) demonstrated in rat jejunal epithelial cells the presence of 220 nm latex which subsequently translocated to the liver sinusoids. In a wide variety of cells, efficiency of endocytosis of extracellular materials is enhanced by prior concentration at the cell surface, due either, to binding to specific receptors or by adsorption to cell membrane glycoconjugates. Though the nanosphere is several orders larger than TL, ligand density is an important determinant for internalization; phagocytosis of nanospheres by the epithelial cell may be induced by ligand (approximately 6000 TL molecules per microsphere) clustering on the apical epithelium resulting in the generation of secondary signals to the epithelial cytoskeleton and resulting in the circumferential coating of the apical membrane around the microsphere ending in the inclusion of the nanospheres in a phagosome (Tran van Nhieu and Isberg, 1994). Although this may explain the ability of TL to induce microsphere absorption through the epithelium and subsequent appearance in the mesentery nodes and blood, the existence of “lectin-receptors” has not conclusively been demonstrated. Lectins will bind to any glycoconjugates presented to them provided they contain complementary binding sites. Blotting experiments with electrophoretically separated rat small intestinal brush-border proteins (Naisbett and Woodley, 1994b) demonstrated specific TL binding to many high molecular weight components.

The high circulating levels in the blood (19% or $3 \times 10^{10}$ particles) indicate true intestinal absorption and the apparent inability of the reticuloendothelial system to sequester the majority of the nanospheres indicates that opsonisation of colloidal particles, is in some manner, prevented. This may be due to the charged surface or to remnants of TL still present on the nanospheres which may act similarly to bacterial capsular polysaccharides in exhibiting anti-phagocytic activity. Although requiring further histological studies, it appears unlikely that the nanospheres in the blood are contained within circulating macrophages due to the low numbers of such cells in the rat blood ($<10^3$ per
mL of blood). Furthermore, quantification of TL-conjugated nanospheres in isolated mesentery nodal tissue could not demonstrate any deposition where vast majority of macrophages reside (although histological examination revealed low numbers of particles; Figure 3.7). Since the translocation of epithelial-absorbed particulates is believed to occur via lymphatic tissue rather than endothelial-walled blood vessels (due to large gaps in the wall of the former), the low levels of TL-conjugated nanospheres in nodal tissue indicates that either phagocytosis by lymph-associated macrophages appears to be inhibited by some unknown process or nanospheres may gain direct entry into the bloodstream (for this reason quantification of mesentery nodal tissue was done in conjunction with rest of the non-lymphoid, or villous, intestinal tissue in subsequent studies).

TL has been shown to exert immunosuppressive effects on human T-lymphocytes in vitro (Kilpatrick et al., 1987), a property reported for very few other dietary lectins. The nature for this anti-mitogenic property (ie. suppression of T-lymphocyte transformation/proliferation) is currently not known, however it has been suggested that TL may bind to growth factors produced by the stimulated lymphocytes themselves, such as interleukins, or might prevent the production of other lymphokines/monokines indirectly as a consequence of binding to lymphocyte receptors or receptors on antigen presenting cells. As lymphokine-activated macrophages exhibit a variety of responses, including increased phagocytic and bactericidal activity, it is possible that some active nanosphere associated TL may confer increased resistance to phagocytosis due to T-cell inhibition.

Further, data from intravenously injected latex particles demonstrate that unmodified sub-micron latex particles are removed by the RES within 5-10 min, mainly by the Kupffer cells of the liver (60-90%) and to a lesser extent by the splenic red-pulp macrophages and reticular cells. As a consequence less than 1% of the injected dose remains in the blood circulation after 15-30 minutes. Since most mammalian cells carry a net negative charge, electrostatic repulsion
between surfaces of like charge may be the mechanism by which the carboxylate surface groups of the nanospheres could introduce a repulsive force between the particles and phagocytic cells. Very recently Ayhan et al (1995) reported the "pronounced drop" in the number of carboxylate labelled latex microspheres phagocytosed by negatively charged rat peritoneal macrophages whereas maximum phagocytosis was observed with positively charged dimethylamino latex nanospheres. Thus the presence of charged group such as uronic acids or phosphate groups found in many microbial capsules are one of the bacterial virulence mechanisms designed to confer resistance to phagocytosis. Similar work with liposomes has shown that increasing the negative surface potential by altering phosphatide content has a strong suppressive effect by mouse peritoneal macrophages even when the liposomes are opsonised by complement (Roerdink et al., 1983). This suggests that opsinisation and strong surface negative charge can be opposing forces that work actively against each other. In contrast other workers have found that negative liposomes compared with neutral or positive liposomes show a faster rate of clearance and a higher liver and spleen uptake (Senior et al., 1985). Similar results were reported by Wilkins and Meyers (1966) after injection negatively charged gum-arabic coated latex nanospheres. Clearly the connection between phagocytosis, organ distribution, and surface potential is far from simple, and concomittant changes in other surface properties, such as differences in surface hydrophobicity, may override any effects produced by variations in surface charge.

Yet another minor possibility exists in that TL-conjugated nanospheres may have adsorbed components of the tissues or their contents whilst being absorbed and translocating across the intestine. This may predispose the particles from not being recognised as "foreign or non-self" in immunological terms thus avoiding sequestration by the mononuclear phagocytic system.

Rypacek (1992) suggests that non-degradable polymeric material, that is larger than the limit of glomerular filtration, is eventually stored in the secondary
lysosomes of cells of the RES. Subsequent redistribution amongst such cells, as a consequence of cell turnover or excytosis and recapture of the released material by other cells, may result in the excretion of the material in the lung by aveolar macrophages which, according to some investigators, originate from the liver or spleen. However, in both single and chronic studies, no nanopsheres could be detected in aveolar tissue upon quantification or after histological examination, suggesting once again that nanospheres may be freely circulating in the blood.

The apparent non-sequestration of TL-conjugated nanospheres by the mononuclear phagocytic system is advantageous in biomedical research as removal from the bloodstream will limit the possibilities of targeting other systemic organs such as the bone marrow or prevent the sustained release of encapsulated material in the systemic circulation. Avoidance of opsonisation will also prevent capillary blockage, especially as indications exist that opsinincoating of foreign particles can result in particle aggregation (Saba et al., 1967). Many investigators, researching the area of intestinal particle absorption for use as oral drug carriers, appear to be more concerned about the phenomena itself rather than the subsequent events proceeding absorption. To the best of our knowledge, this appears to be the first time that orally applied nanospheres are able to avoid RES capture; the factors responsible for this phenomena remain to be elucidated.

Although numerous investigators have suggested the use of TL as a bioadhesive component of drug delivery systems (Lehr et al., 1992; Naisbett and Woodley, 1994a) the in vivo data in this paper represents the first test of the consequences of the incorporation of this lectin on to a delivery system. TL does not disrupt the integrity of the intestinal epithelium in contrast to the red Kidney bean (Phaseoleus vulgaris) lectin and wheat gem agglutinin. Although no long term toxicity studies have been conducted with TL, it is unlikely from circumstantial evidence ie. widespread consumption that it will have any deleterious effects. In a very recent study Naisbett and Woodley
(1994d) demonstrated that feeding microgrammes quantities of TL to mice in vivo elicited high specific serum IgG responses and, to a lesser extent, specific intestinal IgA responses concluding that while this may limit the use of TL as an oral drug carrier for chronic delivery, the possibility is raised of using TL as an adjuvant to promote immunisation by the oral route. However this extrapolation is based on a single feed of the lectin to mice and serum IgG response to TL, that is not present in the normal diet of rodents. It is not, therefore, surprising that this may be considered as priming of the immune system. Virtually all proteins examined have been found to induce some oral tolerance, although some controversy exists over whether this applies to proteins that can bind and translocate the intestinal mucosa (Mowat, 1994).

Continuous feeding of TL to mice may lead to a state of oral systemic immunological tolerance (as in humans where tomato fruits are consumed raw or lightly cooked worldwide from weaning to throughout life), preventing the induction of active immunity when the offending antigen is encountered on subsequent occasions. This phenomenon can be illustrated readily by feeding a protein before immunizing the animal via an immunogenic route where it is found that antigen-fed animals have almost complete suppression to the challenge immunogen (Mowat, 1994). The diet of all animals consists of a complex mixture of animal and vegetable products, most of which are non SELF in immunological terms and, hence, are potentially antigenic. The GALT are the largest in the body containing a wide array of effector mechanisms to counteract continual bombardment of potential antigens. Mobilization of this powerful battery of responses against food antigens would be undesirable partly because it would limit their uptake metabolic usefulness but, more importantly, because hypersensitivity to foods can produce intestinal pathology, as typified by coeliac disease and other food sensitive enteropathies. Further, normal humans have little or no intestinal IgA antibody against food antigens (O'Mahony et al., 1994). Work with OVA has shown that, although tolerance of serum antibody IgG antibody responses had almost disappeared by six months after a single feed of OVA, the delayed type
hypersensitivity tolerance remains essentially intact up to at least 18 months (Strobel and Ferguson, 1987). As the average lifespan of a laboratory rodent is around 2 years this highlights the potency and stability of this immunoregulatory phenomenon. In addition several investigators using both protein and non-protein antigens have shown that intestinal IgA production is tolerized by feeding antigens (Mowat, 1994) consistent with the apparent absence of IgA antibodies against food antigens in normal individuals and indicates that local IgA production is probably regulated in the same way as systemic immunity in orally tolerant animals. Thus, the suggestion by Naisbett and Woodley (1994b) that multiple administration of TL-coupled macromolecules may be disadvantageous for drug delivery, does not appear to be borne out by our results, particularly given that the main mechanism by which particle translocation is inhibited, as exemplified by bacteria, is the complexation of secreted IgA with the invading particle. Further, the following section will reveal that a single dose of TL-coupled nanoparticles fails to elicit the high uptake observed with multiple dosing.
3.5 Single-dose administration of TL-conjugated nanospheres

Initially the experimental design utilised chronic administration rather than single-dose administration because intestinal uptake of particles appears to be cumulative and is most easily demonstrated by long-term experiments. It was therefore of interest to quantify the uptake of TL-conjugated nanospheres following a single dose to observe the ability of TL to induce intestinal particulate uptake. From Table 3.2 it seen that, in sharp contrast to data obtained following chronic administration for 5 days, very little intestinal absorption is seen, only 1 in 4 animals tested demonstrating slight particulate translocation as evidenced by the appearance of only 2% of the dose administered in the systemic circulation.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Uptake</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Gastrointestinal</em></td>
<td></td>
</tr>
<tr>
<td>Peyer’s patch</td>
<td>n/d</td>
</tr>
<tr>
<td>Intestine</td>
<td>n/d</td>
</tr>
<tr>
<td>Colon</td>
<td>n/d</td>
</tr>
<tr>
<td><em>Systemic</em></td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td>n/d</td>
</tr>
<tr>
<td>Spleen</td>
<td>n/d</td>
</tr>
<tr>
<td>Blood (12mL)¹</td>
<td>2.01 n=1</td>
</tr>
<tr>
<td>Total systemic</td>
<td>2.01 n=1</td>
</tr>
</tbody>
</table>

n=4, n/d= not detected

1. Blood sample (2mL) taken via cardiac puncture; value extrapolated to whole blood volume (12mL for 200g rat)
This result has profound implications for the use of tomato lectin both as a bioadhesive and as an agent promoting the endocytosis of colloidal particles and coupled drugs. The reason for this large discrepancy in data concerning the intestinal uptake of particles between single day and a multiple dosing regime is not immediately apparent without additional in vivo experiments. Two possible explanations exist; Firstly that multiple dosing of TL-conjugates may have resulted in the expression of additional "lectin-receptors" due to constant "antigenic" stimulation of the gut. A similar result has been described by Pustzai and co-workers (1990) who have shown that lectins such as GNA (Galanthus nivalis) which did not bind to the rat epithelium on initial exposure (1 hour after instillation in an intestinal loop) became highly reactive with the brush border membrane after continual exposure lasting 10 days, accompanied with extensive endocytosis, mirroring changes in gut receptor expression it had induced during the course of the experiment. Initial binding was seen 5-6 days into the experiment which is similar to the duration of our multiple-dosing study with TL-conjugated nanospheres.

It is possible that rather than the expression of additional lectin "receptors" TL may have bound to pre-existing receptors for endogenous ligands. Plant lectins such as Con A and WGA have been shown to activate the constitutive tyrosine kinase activity of the epidermal growth factor receptor, isolated from the rat liver, to a similar extent as the measured enhancement induced by the native ligand (EGF). Incubation with the respective sugars abolished the effect confirming that the response was mediated via carbohydrate biosignalling (Zeng et al., 1995). EGFR is also present on the intestinal epithelium and is responsible for the proliferation and maturation of epithelial cells (Chabot et al., 1987). Ryder et al. (1992) demonstrated that the incubation of Peanut agglutinin to biopsy specimens of normal colonic mucosa caused statistically significant increases in crypt cell production. Several lines of evidence have suggested that the EGFR is a glycoprotein with N-glycosidically linked oligosacharrides and the stimulatory effects of plant lectins may represent indirect pertubations of the EGF binding site on the glycoprotein moiety raising
the possibility that the carbohydrate chains are receptors for other endogenous regulators, distinct from EGF, which modulate the cellular response to the growth factor (Feizi and Childs, 1985). This raises the possibility that TL may have increased the production or maturation of immature crypt epithelial cells, which is known to take 4-6 days, thus resulting in a greater number of enterocytes capable of translocating nanospheres. Histological examination of intestinal tissues following single and multiple dosing with TL-conjugated nanospheres did not display hyperplasia of the gut, that has been observed with lectins such as WGA and PHA, at both the macro- and microscopic level but one cannot exclude the possibility of an increased number of enterocytes without additional in vitro experiments such as the level of chromosomal incorporation of H3-thymidine into crypt cells. Pustzai and co-workers (1995) have shown that by speeding up cellular turnover many diverse lectins which possess growth factor activity can increase the proportion of immature epithelial cells and lead to an increase in cytoplasmic/membrane components with additional terminal glycosyl groups.

Ryder et al. (1992) have suggested that plant lectins may instead bind to membrane glycoproteins, rather than EGFR, that have growth regulating activity whereas Feizi and Childs (1985) have proposed an alternative scheme by which lectins bound to EGF/EGFR complexes form a macromolecular unit with other adjacent membrane bound glycans, that may be linked to other extra- or intracellular signalling systems, thus modulating the expression of EGF. The mechanism by which an adaptive transient change induced by TL on the ability of the intestine to absorb large particles is most probably linked in some manner to the continual deposition and binding to the intestinal epithelium. Thus after a single dose of TL-conjugated nanospheres no bioadhesion is observed concomittant with the absence of nanospheres in the blood, in stark contrast to the multiple dosing regime where almost 14% of the dose is either adsorbed or within the intestinal tissues with a further 24% in the systemic circulation or vital organs. In lectinology it is well known that clustering of lectins onto the extracellular cell surface is an obligatory step for
triggering off a cellular response ie. highly dose dependent and that virtually all cell systems exposed to plant lectins respond with an increased level of intracellular Ca2+. Northover et al. (1994) demonstrated that the binding of WGA and Con A to endothelial cells increased the leakage of injected colloidal carbon into the walls of microvessels in the villi of rat small intestine and was reversible in the presence of the inhibitory sugar. A 10-fold reduction of WGA failed to induce the leakage. The authors suggested that the leakage was due to the multivalent intercations of "receptors" resulting in the formation of cross-bridges, a phenomenon which can occur because of the relatively slow kinetics of carbohydrate binding (Lis and Sharon, 1986). Since the effects were inhibitable by a protein Kinase C inhibitor it was suggested that cross-linking caused the receptors to "bend" as though they had been stimulated by an endogenous ligand. This would lead to stimulation of the inositol phospholipid pathway, resulting in the formation of inositol triphosphate and diacylglycerol (DAG), the former releasing intracellular Ca2+ and the latter activating PKC. Furthermore, DAG perturbs bilayer structure (Petty, 1993). These results suggest that direct binding of lectins to receptors for endogenous ligands may not be responsible for the various phenomena observed and that binding to oligosacharrides in close proximity to receptors may effect the microenvironment thus mimicking the effects of endogenous ligands. That activation of protein kinases and increases in intracellular levels of Ca2+ is inextricably linked to cell surface receptors and cell signalling, and has been shown to interact closely with the cytoskeleton, strongly suggests that TL in sufficient extracellular levels not only binds to glycans in contact with the cytoskeleton but, due to cross-linking of these glycans, is able to mobilise the cell machinery involved in endocytic processes. The fact that minimal amount of intestinal bioadhesion and subsequent translocation into the systemic circulation was seen with blocked TL-conjugated nanospheres after multiple dosing would seem to confirm the above premise. Woodley and Naisbett (1994b) have described the mechanism of TL uptake by enterocytes to be absorptive endocytosis since low temperature (4°C) and use of metabolic and cytoskeletal inhibitors drastically reduces internalisation indicating the
requirement of energy and cytoskeletal elements for the movement and fusion of apical membranes and vesicles. Binding of TL to extracellular glycans therefore must link the signal transduction to the cytoskeleton that results in actin reorganisation and polymerisation for the production of an endocytic vesicle. Appealing as it is, this mode of ligand mediated entry of inert particles by epithelial cells has not been directly tested. Further Woodley and Naisbett (1991a) have shown that TL binding is mediated through brush border membranous glycoproteins in the 116-160 kDa molecular mass range that strongly corresponds to intestinal brush border digestive enzymes which are known to be complex glycoproteins and certainly not involved cytoskeletal rearrangements. However the uptake of many plant lectins via clatharin coated vesicles by a variety of non-professional phagocytic cells does leave open the possibility of an endocytic mode of internalisation of TL-conjugated nanospheres particularly as integrin receptors (membrane spanning glycoproteins present on the enterocyte in small numbers), that are involved in cytoskeletal rearrangements, are exploited by many pathogenic bacteria for epithelial internalisation (Chapter 5 of this thesis). However no consensus viewpoint for phagocytic membrane signalling and internalisation has emerged, detailed consideration of which is beyond the scope of this thesis. As vast majority of the mechanistic studies have been conducted with macrophages-mediated antibody-dependent phagocytosis that has shown to involve complex pleitropic interactions between the products of membrane and soluble protein kinases with a variety of cellular microfilaments, it will be highly speculative to transfer information derived from such systems to explain the mode of epithelial internalisation of TL-conjugated nanospheres.

An alternative explanation is that TL, with repeated administration, may have displaced endogenous/ exogenous N-acetylglucosamine-specific lectins or other ligands from the receptors of both villi and crypt which contain terminal N-acetylglucosamine residues. It is unlikely, however, that resident bacteria that are attached to the mucosal epithelium, commonly in the form of surface lectins on filamentous assemblies of protein subunits known as fimbriae, would
have been displaced since vast majority of enteric bacteria possess type-1 fimbriae which bind mannosides, the optimum receptor corresponding to a trisaccharide (Sharon, 1987). Although experimental data is lacking, there are no known reports of intestinal bacteria utilising type G fimbriae, which bind to N-acetylglucosamine residues; of the two that exist both are involved in pyelonephritogenic infections (Rhen et al., 1986). Although some "enteric" strains of bacteria utilise N-acetylglucosamine for attachment to mucosal membranes (Streptococcus salivarius and Actinomyces spp.) they are almost exclusively confined to the oral cavity and responsible for dental caries (Mirelman, 1986). Further they do not exhibit exclusive monosaccharide specificity and frequently bind to galactose and lactose residues, possibly indicating the multiple expression of adhesions on such single bacteria for efficient attachment to mucosal membranes.

It is also unlikely that the higher uptake seen after 5 day dosing is the result of TL-induced epithelial damage as observed with numerous other plant lectins. Kilpatrick et al. (1985) has shown the absence of any deleterious effects on intestinal villi or epithelial integrity after administering a TL concentrated diet to rats for 10 days. Our own histological evidence supports the reported observations (Figure 3.11). In contrast lectin-induced epithelial lesions, when it does occur, takes the appearance of dislocated villi and "loosening" of the inter-villi distance.

Thus it is unclear whether the high intestinal translocation of particles observed in the chronic dosing studies is due to the inducement of receptor expression by TL or as a result of alteration in membrane components. However it is unlikely that this high uptake is simply due to chronic deposition and accumulation of particles at the mucosal surface as there is continual shedding of epithelial cells into the lumen, rapid turnover of mucus and peristaltic waves, particularly the post-prandial wave, all of which serve to dislodge adsorbed particles and form the non-specific part of the intestinal defence system. Furthermore studies in this laboratory have shown only a
defence system. Furthermore studies in this laboratory have shown only a minimal amount (1.89%) of true translocation of 500nm un-modified latex particles following continuous administration for 10 days (Jani et al, 1991). Since lectins react with any cell surface glycosyl moiety that is exposed and complementary to them it is doubtful whether specific "lectin receptors" exist and that adaptive changes seen in intestinal function, such as the suppression in modulatory activity of membrane-bound enzymes by the presence of concanavolin A in the diet of experimental animals (Erickson et al., 1985), may be due to membrane perturbation.

Figure 3.11 Photomicrograph of an ileal section taken after 5 days continuous dosing with tomato lectin solution using the same quantity of lectin as with TL-conjugates shown in previous photomicrographs. Notice the tight arrangement of the villi which is a characteristic of a healthy epithelium (stained with toluidine blue, x 80)
In principle, under defined conditions it is possible to detect the biological results of such macromolecular-"receptor" intercations. Unfortunately, due to the intrinsic complexity of the intact cell surface, the molecular mechanisms and the events proceeding ligand binding, are still rather obscure leading many investigators to focus on studying the dynamic properties of glycolipids and/or glycoproteins as mobile receptors by incorporating them into phospholipid membranes/vesicles. Glycophorin-phospholipid-reconstituted systems have been shown to be viable systems for the study of lectin-glycoconjugate interactions that occur at the cell surface (Taraschi et al, 1982). Glycophorin, the major integral sialoglycoprotein of the human erythrocyte membrane is known to contain receptors for several plant lectins due to its high content of sialic acid and N-acetylglucosamine residues. Use of such systems have revealed that lateral phase separation or local clustering of glycoconjugates induced by lectin-glycolipid/protein intercations can result in a substantial reorganisation of membrane components. Taraschi et al. (1982) have shown that when reconstituted with phosphatidylcholine that, in isolation, adopts a bilayer structure, glycophorin-containing bilayers display no reorganisation of the membrane phospholipid upon the addition of WGA. However when reconstituted with phosphatidylethanolamine, an unsaturated phospholipid which in isolation can adopt non-bilayer structures, the addition of WGA resulted in the destabilisation of the bilayer and the drastic reorganisation of the phospholipid from a bilayer to a non-bilayer, hexagonal HII phase. In the latter case the cross-linking of the sugar containing head groups of glycophorin within the individual vesicles by WGA results in the aggregation of the protein into "patches" thus removing the extensive bilayer stabilising effect of the previously randomised protein molecules. As a result, large domains of lipid are freed from the influence of the protein and revert to the hexagonal HII phase, that would ordinarily occur in isolation.

Although vastly dissimilar systems, information from glycoconjugate-phospholipid-reconstituted systems may provide a basis from which a plausible interpretation of the higher intestinal uptake of TL-conjugated nanospheres
observed after chronic dosing can be formulated particularly since glycophorin is a potent inhibitor of TL (Nachbar et al., 1980). Continual deposition and binding of TL-nanospheres to the rat small intestinal epithelium results in a situation where sufficient numbers of TL molecules are present to cross-link membrane stabilising glycoproteins leading to the transformation of the brush-border lipid bilayer to a non-bilayer formation, the latter which has been implicated in endocytosis. Given that there are $1 \times 10^{16}$ TL molecules on each latex sphere, that lectin-mediated events are characterised by their dependency on extracellular concentration and the ubiquitous presence of N-acetylglucosamine residues in apical epithelial glycans (Forstner and Wherrett, 1972) it is not unreasonable to speculate that the threshold level of TL molecules necessary to trigger membrane skeletal transformations occurs several days into the chronic administration schedule, since very little binding and translocation was seen after a single day’s administration.

In conclusion any one of these lectin-induced effects, both in the experiments described in this thesis and of those reported in the literature, which is inhibited by the respective sugar molecules is presumptive for the presence of that mono/oligosaccharide or related sugars at the cell surface; however unequivocal proof requires isolation and chemical analysis of the surface "receptor", a procedure which has only been performed in only a few instances. While some aspects of the above models, particularly the mode of particulate internalisation and the apparent intestinal adaptations that are evoked by TL are speculative, they are not inconsistent with the physical and functional properties of the glycolipid/protein components of biomembranes.

The recent interest in the application of lectins in the context of oral drug delivery with the aim of improving the oral bioavailability of poorly absorbed drugs stems from the observation that prolonged and/or intensified contact to the intestinal mucosa leads to an elevated appearance of the coupled drugs in the systemic circulation. Morgan et al. (1991) reported that linking the peptide B-chain of insulin to polymers such as HPMA [N-(2-hydroxypropyl)
methacrylamide] led to a decrease in B-chain degradation from the intestinal brush border enzymes suggesting that coupling may not only protect the labile drug from local destruction at the site of adherence but possibly within the absorbing tissue prior to recaging the systemic circulation. Studies conducted by Lehr and co-workers have shown that application of TL and *Phaseolus vulgaris* isolectins to the apical side of Caco-2 cell monolayers "showed only a marginally, if any, enhanced transcytosis, in spite of specific binding and partial internalisation" and that such transport "must have occurred predominately by fluid-phase rather than absorptive transcytosis." Further, Kilpatrick et al. (1985) estimated that only 2.3% of TL appeared in the blood after the author himself had consumed tomato juice spiked with iodinated TL.

These results raise serious doubts as to the applicability of lectins for use as intestinal drug carriers for poorly absorbed molecules or for the delivery of immunogens for oral immunisation, particularly as our data indicates that binding and uptake of TL-conjugated nanospheres by the mucosa occurs by the possible induction of "lectin-receptors" after repeated dosing (antigenic stimulation ?) to animals.

### 3.6 Conclusion

This study has demonstrated the use of TL as an example of non-toxic mucoadhesive agent that can greatly enhance the intestinal absorption of colloidal particles only upon multiple dosing. Further, it is seen that systemic appearance of nanospheres can be enhanced significantly with the use of tomato lectin demonstrating not only the permeability of the conjugates through the mucus but the absence of significant impedance of intestinal uptake due to lectin interaction with mucus. In addition and more importantly we have demonstrated that tomato lectin redirects the internalization of the nanospheres through the villous tissue, not entirely surprising considering the ubiquitous presence of N-acetylglucosamine residues on the surface of
epithelium throughout the GIT. Since the non-lymphoid intestinal epithelium is
greater in surface area compared to M-cells the use of mucoadhesive ligands,
which posses binding sites present in large numbers in the entire intestine,
offers a vast area of absorption of colloidal particles and not just restricted to
Peyer’s patches.

Several questions remain unanswered such as the effect of interaction of the
particles and lectins with food and commensal gut flora and its subsequent
effect on local gut ecology. As discussed by Lehr et al. (1992), in addition to
the possible toxicity, the applications of many other lectins for the promotion
of bioadhesion or as intestinal drug carriers appears a priori to be limited by
the possible interactions with common monosacharides in food carbohydrates.
However specific food intercations with TL can be expected to be less
pronounced as poly-(N-acetyl glucosamine) residues are relatively scarce in
foods partly due to digestion by saccharidases but also the reduced likelehood
of encountering such sequences in consumed oligosacharrides. Further it is
known from that N-acetylglucosamine itself (ie the monomer), that is obviously
more abundant in food, than its polymers, does not inhibit the TL-induced
aggregation of erythrocytes (Inhibitory ratio of GluNac₄ : GluNac = 6000 : 1,
from Nachbar et al., 1980).

The demonstration by Woodley and Naisbett (1994a) of TL binding to high
molecular weight glycoproteins that correspond to brush border enzymes may
be advantageous for TL-coupled gut-labile molecules as lectins of similar
binding specificity such as WGA are able to suppress aminopeptidase activity,
an enzyme that may cleave and degrade the coupled molecule. However,
binding of plant lectins to the rat brush border membranes can induce variable
changes in the activity of several membrane associated hydrolases particulary
aminopetidases (Erickson et al., 1985) and are able to release some
membrane bound enzymes, such as amylase and lipase, into the lumen
(Sandholm and Scott, 1979). Utal et al (1990) have further shown that the in
vitro absorption of nutrients such as glucose, luecine and Ca²⁺ by ligated
intestinal loops was significantly decreased in the presence of vegetal lectins. The mucotractive effect of some lectins may in part account for this reduction of nutrient absorption but will also be a handicap for the diffusion and absorption of lectin coupled drugs and particularly nanospheres as mucus is the main barrier for particulate absorption. Since assimilation of food substances is dependent upon their prior intestinal degradation, interference in the digestion and absorption of vital nutrients will have serious consequences on health. The use of lectin coupled oral delivery systems, in which the the amount of lectin ingested will most probably exceed that would normally be consumed by the patient, will need to focus on the effects of high concentrations of "non-toxic" fruit lectins on the overall health, particularly with repeated administration. However the potential actions of lectins are not always deleterious. Freed (1985) has suggested that they may be beneficial in the management of diabetes mellitus, since apart from slowing down absorption of carbohydrates, some food lectins have insulinomimetic effects and insulin sparing properties. As demonstrated by Pustzai et al (1995) dietary lectins can also induce changes in glycosylation patterns of the intestinal epithelium leading to either the appearance of new or the removal of existing glycosyl structures and consequently changes in the binding potential or brush border enzymes or indigenous bacteria. Lectin-induced dysbacteriosis, which is unlikely in the case of TL as discussed earlier, can lead to major shifts in the bacterial flora from one part of the intestine to another.

The above serves as a reminder that the enthusiasm displayed by a number of authors in recent years regarding the application of lectins as components of oral drug carrier systems needs to be counterbalanced by further investigations into the possible deleterious effects of lectins in intestinal physiology. This is borne out by the results of the vastly different rates of uptake of TL-conjugated particles presented in this thesis, and whether the adaptive change induced by TL on epithelial function is beneficial for the purposes of enhancing nanoparticulate absorption remains to be seen.
Considerations such as the immunosuppressive effects on T-lymphocytes and low isolation yields [only few mg of TL are obtainable from several Kg of tomato fruit (Lehr et al., 1992)] may well limit the application of TL unless it can be cloned and expressed in bacterial vectors in high yield. Despite this TL possesses some useful pharmaceutical features that may make it an interesting candidate for drug delivery purposes. Nachbar and Oppenheim (1992) have reported the remarkably high stability of TL in both aqueous and dry state. When lyophilised the lectin is stable indefinitely (at least 3 years), stable in PBS at -20°C for up to 6 months, 4°C for several weeks and at room temperature for a week. Further it is relatively resistant to heat denaturation at 100°C in its crude state, 1 hour of exposure to pH 1.5 decreases its activity by only 25% and no change is observed over the pH range 4-9.
Chapter four

Oral uptake of glycosylated latex nanospheres
4.1 Introduction

Sugar chains of glycoproteins and glycolipids are a predominant feature of the surface of all cells. It has been known for many years that the plasma and apical membranes are, to varying degrees, coated on their external surface with a layer of carbohydrate-rich material, collectively known as the glycocalyx, which mainly consists of oligosaccharide chains covalently linked to membrane proteins and lipids. It has been estimated that for a single erythrocyte, the concentration of hexoses in the glycocalyx is in the order of 0.52M and the concentration of hydroxyl groups is ~3M (Jones, 1994). The use of exogenous plant lectins for over a hundred years as probes to detect defined carbohydrate sequences on mammalian cell surfaces, as well as the strategically favourable placement of oligosaccharide chains of membrane glycans on the plasma face of the cell, prompts the question as to their existence and function.

It is reasonable to question or hypothetically attribute some fundamental physiological relevance for this stereospecific behaviour. Clinically relevant and scientifically important are the observations that aberration from the normal route of cell function or development are not only linked to changes in the carbohydrate portion of secreted or cellular glycoconjugates but in many cases are manifested by the incorporation of novel or altered glycoconjugates at the cell surface, for example the expression of modified Lewis series of blood-group antigens in the epithelial portion of colonic adenocarcinomas.

Carbohydrates are structurally complex and diverse molecules that have profound and pervasive influence on life processes. There are number of important biological phenomena which depend on carbohydrate-protein interactions. For example, heparin, a sulphated polysaccharide plays an important role in the blood coagulation system. Delivery of newly synthesised lysosomal enzymes from the Golgi apparatus to lysosomal vesicles requires the recognition of the mannose-6-phosphate residues on these enzymes by a specific receptor. In the preimmune defence system of the human body a
mannose binding protein interacts with antigens through their surface carbohydrates and promotes opsonization or initiates complement fixation (Reviewed in Lee and Lee, 1995). Thus increasingly, carbohydrate-protein interactions are viewed as important mechanisms for the transfer of biological information between cells. In addition to the large number of phenomenological examples such as those cited above, there are two further fundamental reasons for promoting carbohydrate-protein interactions as means of cellular communication. As discussed above, it is now well established that most cells are covered with carbohydrates and these determinants as well as those on circulating glycoproteins are modulated depending on the physiological status, such as developmental processes and oncological transformation. Secondly, unlike biological polymers such as oligonucleotides and proteins, carbohydrates, due to the presence of multiple functional groups (commonly hydroxyl moieties) on each monomeric unit, are capable of forming many different combinatorial structures from a relatively small number of hexoses. Thus each structure can potentially carry a specific biological message, thus widening the spectrum of reactivity that is possible from a limited number of monomers (Drickamer and Taylor, 1993).

Since for many cells it is the dense coating of carbohydrate that the cell presents to the extracellular medium, and that numerous cellular processes including growth, morphology, motility and differentiation are partially controlled by the extracellular signals (in the form of glycosylated ligands) that must be received at the cell surface, it is therefore perhaps not surprising that in ensuing years, since the discovery by Ashwell and Morell (1974) of the asialoglycoprotein receptor at the surface of liver parenchymal cells that mediates the serum clearance of exposed galactose/N-acetylglactosamine bearing glycoproteins, a large number of carbohydrate-recognition domains (CRD), more commonly known as animal or endogenous lectins, have been isolated with increasing frequency, and in many cases have been ascribed roles in biological recognition.
Early classification of endogenous lectins was based on the method of their isolation from animal tissue—soluble lectins, which were extracted with simple aqueous media often fortified with complementary sugars that may dissociate them from membrane bound glycans, and membrane or integral lectins that require detergents for solubilisation. Soluble lectins are thought to play a role in secretion or organisation of extracellular glycoconjugates while the membrane lectins have been clearly demonstrated to be involved in the translocation of extracellular glycoconjugates into the cell as exemplified by hepatic and macrophage asialoglycoprotein receptors (Li et al, 1980). More recently, endogenous lectins have been grouped into classes based on the nature of their carbohydrate ligands, the biological processes in which they participate and their dependence on divalent cations for activity in addition to their cellular localisation.

As the primary structures of more than 100 animal lectins (from widely divergent species) have been determined, ranging from the human lung (Hirabayshi et al., 1989) to more obscure sources such as the alligator hepatic lectin (Lee et al., 1994), classification is now based on the polypeptide motif present in each group that is conserved among the lectins to which the carbohydrate-binding activity can be ascribed. Comparison of these CRD reveals that animal lectins can be classified into 4 groups, of which the first two are dominant: C-type lectins, S-type lectins (now called galectins), P-type lectins and others. P-type CRD's so far constitute a small group of animal lectins that bind mannose-6-phosphate as the primary ligand. The C-type CRD's derive their name from the requirement of calcium ions for their binding activity. In addition they are all extracellular (ie membrane bound) although they bind a diversity of sugars. C-type lectins are found widely in the animal kingdom, including humans predominately in the serum, extracellular matrix and membranes. One of the most diverse groups of C-type lectins is the category of type II transmembrane receptors which mediate glycoprotein endocytosis and degradation and includes mammalian asialoglycoprotein receptors, macrophage mannose and galactose receptors (Ng and Hew, 1982) and several lymphocyte receptors (Drickamer et al., 1993).
In contrast galectins (so-called S-type lectins because of their cytosolic localisation, i.e. soluble with a dependency on thiols as reducing agents for the expression of their full binding activity) display no requirement for divalent cations and bind almost exclusively to β-galactosides such as lactose and N-acetyl-lactosamine. Like C-type lectins, galectins are expressed with distinct but overlapping distributions in mammalian tissues. Communication about galectins has been impeded by the lack of a generally accepted nomenclature, which has led to the proliferation of names as each lectin is rediscovered in different contexts. In light of new structural information and a recent initiative by recognised leaders in the field (Barondes et al., 1994) to reorganise the taxonomy and to homogenize the terminology of galectins, a consensus on naming has recently been reached which is based on their molecular weights and the number of β-galactoside CRD’s within the folded globular structure. Presently four mammalian galectins have been well characterised. Thus galectin-3, which is particularly abundant in activated macrophages and epithelial cells, is the name used for the same lectin that has been described in human, bovine and rat tissues as L-29 (rat lung), Mac-2 (murine macrophage cell surface marker) and IgEBP (rat intestinal immunoglobulin-E binding protein).

Of the earliest galectins isolated, and of particular interest in relation to the intestinal epithelium, are two lactose-binding lectins in the adult chicken intestine designated CLL I and II (Beyer et al., 1982). CLL II is by far the more abundant of the two and is found on the surface of the intestinal epithelium. Mucin is a strong inhibitor of haemagglutination by the lectin suggesting that it may play a role in the organisation and stabilisation of the intestinal surface of the mucus. The lectin was able to cross-link mucin with membrane bound glycans, thus aiding the maintenance of an efficient mucus coat on the intestinal epithelia. Furthermore CLL II was shown to endocytose glycoproteins into the interior of the cells.

These early observations that galectins, normally confined to the cytosol, can be secreted onto the cell surface and be involved in endocytosis of extracellular
material were later confirmed following the isolation of nine galectins from the rat intestinal (RI) epithelium (Leffler et al., 1989) designated as RI-A, B etc. RI-F (galectin-3) has now recently been detected at the cell surface of enterocytes, and a closely related homologue is able to bind immunoglobulin-E (IgEBP, galectin-3) in the rat intestine (Brassart et al., 1992). The most abundant (and exclusive) rat intestinal lectin RI-H (galectin-4), a proteolytic fragment of much larger intestinal lectin called L-36, has been predicted (Oda et al, 1994) to accumulate at the epithelial surface but no direct evidence to date has emerged. As with other members of the galectin family, this abundant protein would be expected to be concentrated in the cytosol because its cDNA does not predict the existence of cleavable signal peptide or a transmembrane domain. However both CLL-II are RI-F galectins are able to accumulate on extracellular surfaces despite the lack of an encoding signal in their cDNA.

The authors suggested that as L-36 (galectin-4) is so highly expressed in full-length form in the rat small and large intestine, and since secretory products of mucosal surfaces contain glycoconjugates which may be excellent ligands for lactose-specific lectins (Beyer et al., 1982), it is possible that L-36 is also secreted allowing interactions with extracellular glycoconjugates. The subfamilies of galectin-1 and 3 are abundant not only in the cytosol but also extracellularly and there is evidence that these lectins are externalised by atypical secretory mechanisms (Cooper et al., 1994). Non-classical secretions of cytosolic proteins that play extracellular roles have been demonstrated for interleukin-1β and other proteins (Kuchler, 1993).

In addition to the galectins, some C-type lectins that correspond to the hepatic asialoglycoprotein receptor minor subunits have been shown to be expressed at the apical surface of the rat intestinal epithelia (Hu et al., 1991). Since epithelial glycoconjugates containing terminal or penultimate lactosyl residues may play a role in cellular interactions by serving as complementary ligands, these recent discoveries of endogenous lactose-binding proteins in rat epithelial tissue prompted our investigation of the use of glycosylated latex nanospheres to
secure epithelial bioadhesion and/or uptake through interaction with such lectins which, as discussed, may be secreted and incorporated into the cell membrane.

Immobilisation of short ligands onto the surface of nanospheres has some advantages over immobilisation of large polymeric material such as tomato lectin. These advantages include the attachment of the ligand at high density onto the surface of colloidal materials, the reduced possibility of steric hindrance and the ease with which such small structures can be prepared and isolated in abundance. Further pharmaceutical considerations such as the long-term storage may be feasible as simple structures have sufficient stability against pH and temperature.

4.2 Experimental methods

4.2.1 Preparation of lactose conjugated nanospheres

Mono-dispersed carboxylated microspheres (500nm, 2.5% \(\%\)) with covalently linked fluorescein were covalently coupled to p-nitrophenyl lactose (Sigma, UK) using Woodward's reagent K (N-ethyl-5-phenyl-isoxozolium 3' sulphonate, Lancaster chemicals, UK) according to the method of Seyfried-Williams and McLaughlin (1983). Briefly 3mL of nanospheres were incubated and rotated with 15mg of Woodward's reagent K at room temperature for 15 minutes, the latter reacting with the carboxylate groups to yield active esters (Woodward and Olofson, 1961). Excess Woodward's reagent K was removed by centrifugation. Activated nanospheres were resuspended in PBS and 50mg of p-nitrophenyl \(\beta\)-D-lactopyranoside added and the mixture rotated at room temperature for 2h. Nanospheres were resuspended in PBS. To test for specificity of intestinal bioadhesion, control nanospheres were prepared by incubating lactose-conjugated nanospheres (1 mL) overnight with 20mg of a plant lactose binding lectin (Erythrina cristagalli, Vector Laboratories, UK).
4.2.2 Estimation of glycoside coupling efficiency using an enzyme assay

A method for determining the amount of lactose/D-galactose in foodstuffs (Lactose detection kit, Boehringer Mannheim Diagnostics, Germany) was adapted to estimate the amount of p-nitrophenyl lactose bound to the esterified nanospheres. The following procedure was conducted in triplicate. A 100μL aliquot of lactose conjugated nanospheres was incubated with 200μL of nicotinamide-adenine dinucleotide (NAD) in citrate buffer (pH 6.6) and 50μL of β-galactoside enzyme (60 units/mL) for 15 minutes at room temperature in a centrifuge microtube (1.5mL, 0.22μM filter, Ultrafree C3, Millipore, UK).

Lactose is hydrolysed to D-glucose and D-galactose in the presence of this enzyme as shown below:

\[
\text{Nanosphere-P-nitrophenyl-D-glucopyranose-β-D-galactopyranose} \rightarrow \text{Nanosphere-P-nitrophenyl-D-glucopyranose + β-D-galactopyranose}
\]

Removal of nanospheres via centrifugation (4000g, Biofuge) allows the pellet of beads (with the attached P-nitrophenyl-D-glucose) to accumulate in the microtube, that can be discarded, thus allowing the D-galactose containing supernatant to be separated in the Eppendorf tube. Adjustment of the latter sugar containing solution to pH 8.6 (1mL of potassium diphosphate buffer) followed by the addition of a second enzyme, galactose dehydrogenase (50μL, 9 units/mL) allows the oxidation of D-galactose to galactonic acid as shown below:

\[
\text{D-galactose + NAD} \rightarrow \text{D-galactonic acid + NADH + H}
\]
After incubation for 25 minutes at room temperature (cessation of reaction), the increase in NADH is measured by its absorbance at 340nm in a 4mL plastic cuvette in a final volume of 3.3mL. The amount of NADH formed in the reaction is stoichiometric with the amount of lactose on the surface of the nanospheres. A blank and several lactose standard solutions processed as above allowed the construction of a calibration curve from which a mean reading of 75% coupling of p-nitrophenyl-lactose onto nanospheres was obtained after triplicate determinations.

4.2.3 Determination of the retention of activity of surface bound lactose

Unlike the heamagglutination reaction for lectins, no suitable test exits to determine the retention of the surface bound sugar following conjugation to carboxylated nanospheres. A double labelling method was developed that relied upon the electron microscopic visualisation of surface bound lactose. 100μL of lactose-conjugates, plant lectin-blocked, activated and carboxylated nanospheres were incubated with biotinylated *Erythrina cristagalli* in 10mM Hepes Buffer (0.15M NaCl, 0.1mM Ca2+, pH 7.5) lectin to a final concentration of 50μL/mL for 1h at room temperature. Unbound lectin was filtered by centrifugation as described above and washed a further twice with the same buffer to ensure complete removal. Avidin D-ferritin (Vector Laboratories) conjugate was then added to a final concentration of 170μg/mL to the resuspended nanosphere pellet in PBS (pH 7.8) and incubated overnight to allow for coupling. Unbound ferritin conjugate was removed by centrifugation, washed twice in PBS and the pellet transferred through a series of descending ethanol-water mixtures for dehydration followed by two washes in 100% absolute alcohol. The pellet was embedded in Epon/araldite resin, trimmed and 50-80nm unstained sections examined under a Joel 1200EX transmission electron microscope operated at 80KeV. The results are depicted as photomicrographs in Figures 4.1 and 4.2.
Figure 4.1  Avidin-ferritin label is concentrated heavily around the active lactose conjugated nanospheres. The deformation in nanosphere shape is due to the high temperature at which the Epon-Araldite resin and particles (70°C) are incubated to allow the resin to embed the particles before sectioning. A high surface density of lactose residues were immobilised to ensure that possible binding of the particles to the epithelium was not inhibited by the adsorption of non-specific components from the intestinal milieu.

Figure 4.2  Lactose-conjugated nanospheres pre-incubated with lectin from *Erythrina cristagalli* show a marked reduced binding of the avidin-ferritin complex. Complete blockage of all of the active lactose binding sites is possible only with the use of equimolar amounts of the plant lectin (~300mg) that is prohibitively expensive and may alter the surface nature of the nanospheres.
4.2.4 Dosing regime

Two groups of 4 female Sprague-Dawley rats (10 weeks, 200 g) were administered a dose of 0.1 mL of lactose conjugated and blocked nanospheres daily for 5 days (12.5 mg/Kg) by oral gavage with a blunt ended feeding needle. Rats were given free access to water but fasted overnight, food being resumed 1h after dosing. After the final dose rats were fasted overnight in a nanosphere-free environment but allowed water to clear the gut of any adsorbed microspheres. The removal of organs and quantification of tissue extracted latex by gel permeation chromatography was accomplished according to the method as described in section 2.5 of this thesis. Animals were kept in individual metabolic cages to prevent coprophagia and re-introduction of excreted microspheres. For comparison with oral uptake of unmodified non-carboxylated (500nm), previous data from this laboratory using plain microspheres and identical method of quantification is also shown.

4.3 Results and Discussion

The existence of endogenous lectins which recognise carbohydrate epitopes present on other membrane glycolipids and glycoproteins implies a clear role for them in biosignalling. The recent identification of lactose binding lectins in the mammalian intestine has not only presented novel opportunities for bioadhesive drug delivery systems and selective targeting of colonic carcinomas but also as putative biochemical markers of neoplastic progression in intestinal cancers (Lotan et al, 1994). Although many of the identified S-Lac lectins are concentrated in the epithelial cytosol, two mammalian galectins designated L-14 and L-29 are secreted extracellularly by an atypical mechanism. L-36, exclusively found in the intestine, is highly abundant in the rat mucosa As secretory products of mucosal surfaces contains glycoconjugates which may be suitable candidates
for β-galactoside specific lectins, it is possible that this lectin is also secreted and integrated into the luminal epithelial membrane allowing extracellular interactions with glycoconjugates. The finding that the bioadhesion and absorption of lactose conjugated microspheres was universally low throughout the entire intestine (Table 4.1) indicates either competitive inhibition by food components such as complex carbohydrates or even simple sugars from the rat diet feed or endogenous glycoconjugates such as mucin or the non-secretion of cytosolic galectins. While the results must be considered to be preliminary, they do not offer much hope for solid oral glycosylated systems. Histological examination revealed the virtual absence of nanospheres in all tissues examined. This has reaffirmed the protocol for all studies described in this thesis, conducting parallel nanosphere quantitation and microscopy experiments to ensure that time-consuming microscopy is done only when the quantitative data indicate the presence of nanospheres.

Lactose conjugated microspheres inhibited with the lectin from *Erythrina cristagalli* would thus be expected to show a higher non-specific bioadhesion and to behave similarly to plain unmodified nanospheres of the same size. However this was not realised probably due in part to the fact that equimolar concentration of the plant lectin was required for the complete blockage of lactose molecules (Figure 4.2) and that it exhibits broad galactose binding specificity (Iglesias et al, 1982). Nevertheless compared to both lactose-conjugated and plain nanospheres, small quantities of blocked nanospheres were present in the systemic circulation as *Erythrina cristagalli* lectin afforded partial protection from hepatic capture via hepatic β-galactoside binding lectins.
Table 4.1 Oral uptake of lactose-conjugated, inhibited and plain microspheres as a function of total dose administered (%)

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Lactose</th>
<th>Inhibited</th>
<th>Plain¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intestine</td>
<td>0.1</td>
<td>n/d</td>
<td>4.28 ± 0.52</td>
</tr>
<tr>
<td>Peyer's</td>
<td>&lt;0.01</td>
<td>n/d</td>
<td></td>
</tr>
<tr>
<td>Colon</td>
<td>n/d</td>
<td>0.06</td>
<td>6.53 ± 0.75</td>
</tr>
<tr>
<td>Liver</td>
<td>&lt;0.01</td>
<td>n/d</td>
<td>1.38 ± 0.35</td>
</tr>
<tr>
<td>Spleen</td>
<td>n/d</td>
<td>0.1</td>
<td>0.51 ± 0.03</td>
</tr>
<tr>
<td>Blood²</td>
<td>n/d</td>
<td>0.63</td>
<td>n/d</td>
</tr>
<tr>
<td>Systemic³</td>
<td>n/d</td>
<td>3.4 ± 0.52</td>
<td>1.89 ± 0.4</td>
</tr>
</tbody>
</table>

n=4  n/d= not detectable

1. Data from Jani et al (1990) using plain microspheres (500nm) and chronic administration for 10 days. Whole intestine used for quantitation, histological examination indicating the Peyer's patches to be highly enriched with microspheres compared to villous tissue.
2. Blood sample (2mL) taken via cardiac puncture.
3. Includes extrapolation to whole blood volume (12 mL for 200g rat)

Recent studies (Inhora and Raz, 1994) have shown that pectin, a vegetal polysaccharide rich in β-galactoside residues that is present in the rat diet feed (1.5%, Special Diet Services, Hull, UK), binds to galectins with such high affinity that it has been considered as an anti-metastatic agent to prevent the colonisation of cancerous cells, some of which express high levels of galectins after oncogenic transformation, to other body compartments. Even small amounts of pectin in the diet feed could have been responsible to some extent in preventing the binding of glycosylated nanospheres to endogenous cell surface lectins due to competitive inhibition.
Although lactose was chosen as the glycoligand, since galectins have preferential affinities for lactose containing macromolecules over the terminal monosaccharide (D-galactose), it has recently become evident that intestinal galectins display differential binding affinities with lactosyl containing moieties (Oda et al, 1993). Generally, binding to N-acetyllactosamine is 4-10 times stronger than to lactose (Ahmed and Vasta, 1994), and branched lactosyl containing glycans demonstrate even higher affinities, probably due to such oligosaccharides having the correct spacing of β-galactose residues. If such galectins are incorporated in the rat apical epithelia, it is possible that lactose conjugated nanospheres cannot effectively compete with and displace endogenous ligands (eg from mucin, glycocalyx) with intestinal galectins. Surface attachment of branched carbohydrate structures with β-galactoside residues to nanospheres may thus display stronger bioadhesion with intestinal galectins although steric hinderance may introduce additional complications, thus leading to our choice of a low molecular weight ligand.

Since for many targeting vehicles cell surface binding alone may be insufficient to trigger internalisation, increasing the surface density of carbohydrate ligands may provide multiple, albeit low affinity, interactions for the promotion of endocytosis. Adachi et al. (1994) have recently described the relationship between surface density effect of poly(lactose)-conjugated nanoparticles upon cellular binding and internalisation in cultured hepatocytes. Increasing the surface density led to rapid internalisation of particles by receptor mediated endocytosis. Binding of multiple sugar residues is apparently a requisite for generating strong interactions in endogenous lectins. This finding has added importance since endogenous lectins exist in clusters or aggregates enabling them to exhibit very strong binding forces with multivalent ligands. The determination of the efficiency of coupling p-nitrophenyl lactose to nanospheres using Woodward’s reagent K
revealed that 75% of the sugar was covalently surface bound. As this represents an appreciably high surface loading it may be that galectins expressed in the intestine are more widely spaced leading to the so-called low "glycoside cluster effect." This has been demonstrated for IgE-binding galectin in the rat intestine where the Brassart et al (1992) reported that "brush borders were not constantly labelled" with immunofluorescent Immunoglobulin E indicative of the sparse membrane incorporation of cytosolic generated galectins.

5.4 Conclusions

Without further additional \textit{in vitro} and \textit{in vivo} experiments it is difficult even to make tentative suggestions concerning the non-realization of either bioadhesion or subsequent epithelial translocation of lactose-conjugated model latex nanospheres that may have been expected to occur either by electrostatic interactions via hydrogen bonding of free hydroxyl groups on lactose or through binding to intestinal galactoside-binding lectins. The \textit{in vitro} experiments described earlier clearly demonstrated that lactose was bound to the surface of nanospheres with high density and covalent coupling did not alter the ability of the hexose to bind to carbohydrate-binding molecules (Figure 4.1). Furthermore as very low levels of the enzyme lactase are present in the intestine of adult rats, the likelihood of lactose molecules becoming detached is very low (Maiuri et al., 1992). The fact that partially inhibited lactose-conjugated nanospheres (ie. blocked with \textit{Erythrina cristagalli} lectin) were more readily trancytosed into the systemic circulation compared to free lactose conjugated particles may indicate the importance of physical and dietary factors in oral glycosylated systems.
In addition our results may imply the absence or non-secretion of cytosolic lactose binding lectins to the extracellular surface of the enterocytes. Although in some systems roles for membrane endogenous lectins is evident (e.g. selectin-mediated homing, attachment and migration of leucocytes to the endothelium) there is little consensus concerning the function of intestinal galectins both at the apical level, if they are secreted, or in the cellular compartment itself where they reside in great abundance. It has been proposed that intestinal lectins may bind the mucin, which is composed of β-galactoside residues, and organise it for secretion or after the complex has been secreted as seen with 14kDa chicken galectin which is secreted into the lumen (Beyer and Barondes, 1982). The situation is made more complex by fact that the possible secretion of mammalian intestinal galectins onto the extracellular surface is known largely by inference (Oda et al., 1993) by comparison with other members of the galectin family that all have certain conserved structural features. For example the rat lung galectin-3 (L-29) which has been shown to be secreted onto the alveolar surface (Cherayil et al., 1989) displays an almost identical structural homology with the rat intestinal immunoglobulin E binding protein and the rat intestinal lectin-F, the secretion of both of which has only been inconclusively demonstrated (Brassart et al., 1992). Thus the prediction of the secretion of L-36, the most abundant intestinal galectin, by Oda et al (1993), and our assumption of this event occurring does not appear to be borne out by the results presented here.

The low intestinal bioadhesion observed with both types of nanospheres compared to plain nanospheres may be due also to the highly hydrophilic and polar nature of the hydroxyl groups of the bound lactose compared to the known hydrophobic surface of plain, unmodified nanospheres. This low epithelial bioadhesion is in contrast to several workers who have shown (Kopecek et al., 1992) using soluble glycosylated polymeric materials such as N-[hydroxypropyl]
methylacrylamide conjugated to fucosylamine residues a direct proportional increase in bioadhesion with the content of pendant saccharide moieties to guinea-pig everted colonic sacs, indicating the presence of fucose-binding endogenous lectins on the apical membrane of colonic enterocytes. Although no attempt was made to quantify any absorption, the studies indicated possibilities for site-specific delivery to colonic adenocarcinomas (where there is an increased surface expression of endogenous galectins) of carbohydrate derivatised-anti-tumour agents, or biochemical markers to follow the progression of premalignant colorectal cancers to invasive metastatic tumours (Schoepner et al., 1995; Rushfeldt and Smedsrod, 1993).

Perspectives for future work

The precise physiological function of CRD's have yet to be identified. An understanding of the functions of endogenous lectins depends, ultimately, on the identification of the specific glycoconjugates with which they actually associate in tissues in which they are found. This could lead to the modification or synthesis of potent carbohydrate ligands that may interact with intestinal galectins with much higher specificities and affinities than simple structures such as lactose thus affording the possibility of epithelial internalisation. Further, in vitro experiments have to clearly demonstrate the surface presence of galectins on normal tissue both within experimental animals and in humans, and in vivo studies to ascertain the likelihood of administering glycosylated polymers/colloidal materials with a polysaccharide-depleted diet. Further work is required to elucidate such factors or combinations thereof.
Chapter 5

Utilising Bacterial Mechanisms of Epithelial Cell Entry:

Invasin-induced oral uptake of latex nanospheres
5.1 Introduction

Biotechnological alternatives to permeation enhancers may develop from research on enteroinvasive bacterial surface proteins. Facultative intracellular bacteria such as *Salmonella*, *Shigella* and *Yersinia* are able to penetrate epithelia and become internalised by normally non-phagocytic mammalian cells through either inducible or constitutive expression of surface proteins. Once exposed to the epithelia, the proteins serve as a key - unlocking an endocytic pathway. Thus mimicking these natural proteins may provide another, more selective and efficient mechanism for delivery of colloidal materials, and hence biotech drugs across a variety of biological membranes.

Adherence and invasion into host epithelial cells is an important virulence factor for many pathogenic bacteria and viruses. Both bacterial and host determinants are needed to facilitate adherence by providing ligands and their corresponding receptors to avoid the mechanical host defence mechanisms of ciliary movement, secretion and the flow of intestinal chyme to remain associated to the mucosa prior to internalisation. In addition, bacteria have been shown to transduce an invasion signal across the epithelia to induce the host cytoskeletal rearrangements necessary for bacterial internalisation. Reaching the intracellular compartment constitutes an essential feature of their pathogenic lifestyle since this environment may protect the bacteria from the host's immune system and many chemotherapeutic agents, provide them with nutrients to ensure their growth, or simply allow them to gain access to deeper sterile tissues such as the reticuloendothelial system.

While invading bacteria often differ in their invasion determinants, the underlying principle used to enter eukaryotic cells is essentially similar: the bacteria excrete, or present, ligands that are recognised by host cell surface receptors and mediate a series of biochemical events that elicit the uptake of the attached bacterium. Two major strategies for entry into mammalian cells have been investigated in detail (Isberg and Tran Van Nhieu, 1994). Strategy
I is very similar to that for ingestion of particles by phagocytic cells such as macrophages. This strategy, often referred to as the "Trojan horse" technique, only requires that the appropriate receptors bind a bacterial-coded ligand that mimics a benign host protein. An important property of strategy I is that it is exclusive i.e. a single bacterium binds and promotes its own entry, without facilitating entry of other bacteria in the immediate vicinity. An example of this mode of entry is illustrated by the enteropathogenic Yersinia where different members of the β1 integrin receptor family bind to Invasin, an outer membrane protein that mediates adherence and internalisation into epithelial cells (Isberg and Leong, 1990).

The second strategy results from the active communication between the bacterium and the host cell that results in the reshaping of the host cell surface, and targeting of the pathogen to a morphologically distinct intracellular compartment. This strategy, also known as "membrane ruffling", is inclusive because a single bacterium, such as Salmonella, expresses factors that facilitate the passive entry of bacteria or other particulates in the population (Falkow et al, 1993). Provided the ligands can be isolated and characterised, particularly the delineation of short sequences responsible for the events, both strategies may be exploited in biotech drug delivery across a variety of endothelial and epithelial biological barriers.

Genetic and molecular manipulations have revealed many important features of the entry process of the so-called "Trojan Horse" strategy. As an example of this approach, the enteric pathogen Yersinia enterocolitica, responsible for diseases ranging from mild gastroenteritis to life-threatening septicemia, has been used as a model system for bacterial penetration in an attempt to identify novel chemotherapeutic agents. Such analysis has revealed that enteropathogenic Yersinia encodes at least three gene products, two chromosomal and one plasmid derived, that are each individually capable of promoting uptake into cultured mammalian cells (Isberg, 1989), the most efficient of which is Invasin, the product of the chromosomal inv gene, and the
first to be identified (Isberg and Falkow, 1985). Subsequently two further gene products were isolated, the \textit{ail} and the plasmid encoded \textit{yadA} proteins based on their ability to promote a low level of uptake of \textit{Yersinia tuberculosis} in the absence of invasin expression. Although several invasion factors exist, the highest level of penetration into cultured mammalian cells is promoted by invasin, which, when expressed in non-invasive bacteria or coated on metabolically inert nanospheres, is alone sufficient to confer invasive ability into cultured cells.

Invasin is a 986-amino acid outer membrane protein (Figure 5.1) isolated from both \textit{Yersinia tuberculosis} and \textit{Yersinia enterocolitica}. Even innocuous bacteria, such as noninvasive \textit{E. coli} that express invasin on their surface can efficiently attach to and become internalised by cultured mammalian cells (Isberg and Falkow, 1985). Furthermore micron-sized latex beads coated with invasin coated membranes bind to and are internalised by cultured cells (Young et al., 1992). Analysis of truncated derivatives of this protein has shown that the carboxyl-terminal 192 amino acids are necessary and sufficient to promote attachment and entry (Leong et al., 1990) and latex beads coated with this terminal region are efficiently internalised by mammalian cells (Isberg and Tran van Nhieu, 1994). The invasion process must obviously involve coordinated action between the cell receptor recognised by invasin and the cell cytoskeleton. This is consistent with the finding that multiple members of the \( \beta \), chain integrin family of cell adhesion molecules act as invasin receptors (Isberg and Leong, 1990). Further, polymerisation of actin into microfilaments and accumulation of actin-binding proteins are the key factors of this process, reminiscent of macrophage internalisation of particulate material; thus cytochalasin D, which blocks the polymerisation of actin, blocks \textit{Yersinia} entry into epithelial cells.
A. Shaded region contains the carboxyl terminal 192 amino acid cell binding domain necessary and sufficient for bacterial entry.

B. Model for Invasin structure in the outer membrane of Yersinia. The cell binding domain from amino acid 794 is denoted by the thick line which is the region isolated and coupled to the polystyrene nanospheres.
Once Invasin binds to the appropriate cell receptor, the internalisation proceeds almost automatically without requiring any other bacterial factors, as shown by the efficient uptake of latex beads coated with purified invasin derivatives by cultured mammalian cells (Isberg, 1991). This is in sharp contrast to tomato lectin where high uptake of coupled nanospheres was only apparent after chronic dosing in animals, and little or no uptake was detectable after a single day’s administration. Although vastly dissimilar systems (ie. cultured cells vs in vivo), the use of invasin (unlike tomato lectin), that has been shown clearly to mimic host receptor ligands in utilising the cellular machinery that is capable of initiating cytoskeletal events necessary for internalisation, may prove to be a more fruitful approach in unlocking the endocytic potential of the epithelial cell. Coupled with the fact that none of the above mentioned reports (ie. E. coli expressing invasin, uptake of invasin coated latex beads) involved animal models, it was of interest to assess the potential of the carboxyl-terminal 192 amino acids of invasin of enhancing the translocation of orally gavaged invasin coupled nanospheres in vivo. Finally the relative simplicity of invasin-mediated internalisation (ie. the attachment and uptake is mediated by the same ligand unlike the epithelial entry of bacteria such as Salmonella in which two distinct ligands are used, one for attachment and another to initiate a signal transduction cascade that promotes the cytoskeletal rearrangements [Falkow et al.,1992]) suggests that it might be exploited to introduce macromolecules or colloidal materials into a variety of mammalian cells for research and therapeutic purposes.
5.2 Experimental Procedures

5.2.1 Isolation and purification of Carboxyl-terminal 192 amino acid truncated derivative of Invasin

Maltose-binding protein (MBP) and the fusion protein containing the C-terminal 192 amino acids of invasin were produced in an *E.coli* strain using the p-MAL-p2 plasmid fusion system. These recombinant bacterial strains expressing either the MBP or the fusion protein composed of MBP coupled to the carboxyl-terminal 192 amino acids fragment, that is alone sufficient to confer the invasive phenotype, were constructed by Dr John Tite and colleagues at the Wellcome Research Laboratories (Kent, UK), and was a generous gift which allowed the experiments described herein to be conducted (Brett et al., 1993).

Briefly, the pMAL-2 plasmid vectors (Figure 5.2) provide a method for expressing and purifying a protein produced from a cloned gene (polylinker) that is inserted downstream from the *malE* gene of *E. coli* which encodes a MBP, resulting in the expression of an MBP fusion protein. The signal peptide on the pre-MBP directs fusion proteins to the periplasm, thus allowing the proper folding and disulphide bond formation (which incidently is crucial for the biological activity of Inv-192 fragment [Leong and Isberg, 1993]) to take place in the periplasm of *E. coli*, as well as allowing the purification of the fusion protein using MBP's affinity for maltose/amylose immobilised on an affinity chromatographic column. Thus pMAL-p2 is the vector of choice if the protein of interest is the extracellular domain of a transmembrane protein.
The gene encoding for the Invasin-192 amino acids is inserted between the restriction sites *maIE* (MBP) and *lacZα* (Brett et al., 1993). Between the *maIE* sequence and the polylinker there is a spacer sequence encoding for 10 asparagine residues. This spacer insulates MBP from the protein of interest, increasing the chances that a particular fusion will bind tightly to the amylose resin. The vector also encodes a sequence coding for the recognition site for protease factor Xa, allowing the protein of interest to be cleaved from MBP after purification on an amylose column (Figure 5.3).
E. coli cells harbouring the plasmids encoding for either MBP or the fusion protein were grown separately overnight at 37°C in Luria broth (500 mL) containing 100μg/mL ampicillin (Figure 5.3). Growth was monitored to $A_{600} = 0.5 (\sim 2 \times 10^8)$ and the gratuitous inducer Isopropyl thiogalactoside (Sigma, UK) added to a final concentration of 1mM to induce the lac promoter. Incubation was continued at 37°C for a further 3h after which the cells were pelleted (4000g for 20min at 4°C), the supernatant discarded and the cell pellet resuspended in sterile column buffer (25 mL of 20mM Tris pH 7.4) containing the protease inhibitors phenyl methyl-sulphonyl fluoride (150μg/mL), aprotinin (0.03 units/mL) and 1mM EDTA.

The following steps were all conducted at 4°C to minimise the possibility of degradation. Resuspended cells were lysed using a Branson sonicator (50% amplitude, 3 x 15s pulses) on an ice-water bath. After clarification of the lysate/insoluble fraction (12000g for 40 min; Dupont) the supernatant containing either MBP or MBP-Inv 192 loaded onto a custom-made 25mL cross-linked amylose column (2.5cm diameter; New England BioLabs, UK) and the column washed with 100ml of sterile column buffer containing protease inhibitors. MBP and the hybrid protein were eluted with column buffer containing 10mM maltose. Twenty fractions of 3mL each were collected and fractions 5-15 containing the highest amount of protein (as determined by assaying with the Bradford protein assay) were pooled, concentrated to 1mL volume using micro-ultrafiltration system (Model 8MC stirred cell, Amicon Ltd., UK) using a low protein-binding ultra-filter membrane (25mm, YM10, Amicon) with 10kDa molecular weight cut-off. Typically between 10-20mg of each protein could be purified from 500mL of cultured cells as estimated by the Lowrey assay (Lowrey et al, 1951). Expression of both proteins was confirmed using SDS-Page analysis (Figure 5.4 for further description).
Figure 5.3 Schematic representation of expression and purification of fusion proteins using the pMAL-p2 vector. The MBP moiety of the fusion protein allows its isolation in an efficient one step procedure on a starch column. The gene of interest is cloned to 3' to the malE gene, expressed, cells lysed and the extract poured over column of amylose resin. The MBP fusion protein binds to the column and the remaining proteins in the cell extract are washed through the column. The fusion protein is eluted with free maltose, and if desired cleaved with factor Xa to separate MBP from the protein of interest. Invasin-192 was not cleaved from MBP as the hybrid protein is more stable than invasin-192 alone (Leong et al., 1990)
Figure 5.4  SDS-Page analysis of purified MBP and MBP-Inv-192 Fusion proteins (12.5% acrylamide for resolving gel, run at 25mV). The predicted molecular mass for MBP and MBP-invasin are 45kDa and 63kDa respectively (Leong et al., 1990)
5.2.2 Coupling of MBP-Inv-192 fusion protein onto Nanospheres

Purified proteins were coupled to 500nm fluorescent carboxylated nanospheres using water-soluble carbodiimide as described earlier for tomato lectin. Coupled nanospheres were incubated with BSA to prevent non-specific adsorption. The coupling reaction for MBP-invasin-192 is depicted in Figure 5.5; peptide conjugation to nanospheres can occur either through the smaller invasin-192 moiety or within the carrier molecule (MBP) via their basic amino acid constituents which contain a secondary free amine group (lysine, glutamine and arginine). Thus multiple conjugation points are possible. Covalent coupling directly of nanospheres to the terminal amino acid (isoleucine) that displays the carboxyl group, and which is critical for integrin binding (Isberg et al., 1993) is highly unlikely, although intra-molecular coupling that would most probably result in a biologically inactive molecule cannot be totally excluded.

Unfortunately, unlike tomato lectin or lactose coupled nanospheres, no simple/suitable test exits to confirm the retention of biological activity of the conjugated truncated invasin derivative, apart from performing in vivo receptor binding studies (eg Scratchard analysis) using purified β1-integrin molecules coupled with fluorescent antibodies, or the observation that such nanosphere conjugates can actually translocate cultured mammalian cell lines which has been described with microspheres coated with such derivatives (Isberg, 1994). The fact that β1-integrin receptors from the human placenta could be purified with high yield using invasin-agarose affinity chromatography, in which truncated invasin derivatives (MBP-Inv-479) were covalently crosslinked to agarose beads, strongly indicates the retention of biological activity following immobilisation on to inert supports (Tran Van Nhieu and Isberg, 1991).
The peptide bond may form in one of places along the hybrid protein either within MBP or with the invasin moiety. It is likely that several such bonds are formed with basic amino acids that have a free secondary amino group resulting in the "wrapping" of the fusion protein around the nanosphere. This may confer stability against proteolytic attack.
Since conducting the latter experiment would constitute a repeat of previous published studies, and the fact that cultured colonic mammalian cell lines may lead to misleading interpretations (ie may under or, more likely, over express integrin molecules and other determinants) due to their cancerous nature, the decision was taken to directly administer coupled nanospheres to rats soon after the coupling reaction.

In addition to MBP coupled nanospheres as a control, a further control consisting of MBP-Inv-192 nanospheres blocked with 2% porcine mucin was employed. Comparison of the ability of simple and complex carbohydrates to inhibit bacterial invasion of \textit{E. coli} expressing invasin on its outer membranes has indicated that mucin was by far the most effective competitive inhibitor of invasin (Lonhgi et al., 1992).

5.2.3 Administration, extraction and quantification of nanospheres

A single dose (0.1mL or 12.5mg/Kg) was given orally to a group of 4 rats which were sacrificed 24h later. Quantification of tissue extracted latex from excised systemic organs and intestinal regions using gel permeation chromatography was accomplished as described previously (Chapter 2).

5.3 Results and discussion

After a single dose 13% of MBP-Inv-192 conjugates (thereafter referred to as invasin) were found in the systemic circulation compared to 2 controls, MBP-conjugates and invasin conjugates blocked with mucin, which both exhibited < 2\% (n=1) in the blood (Table 5.1). The results here are in line with previous reports in which \textit{E.coli} expressing invasin on its outer surface (Isberg and Falkow, 1985) or coated with \textit{Yersinia} membranes and inert particles coated
with invasin containing membranes (Young et al., 1992) or MBP-Invasin-192 fusion protein (Tran Van Nhieu and Isberg, 1994) all efficiently entered human cell lines. However the results here represent the first test of Invasin's capability of enhancing in vivo the trancytosis of nanospheres across the epithelial barrier. It also demonstrates and confirms previous findings (Young et al., 1992) that other bacterial components are not necessary for internalisation to succeed, nor does it require energy derived from the metabolic activity of the microbe. Furthermore the results also show that i) large peptide fragments can be stabilised on the surfaces of nanospheres upon covalent attachment, ii) the MBP moiety does not appear to be involved in the epithelial entry process of nanospheres despite its possible lectin-like activity, and they confirming previous reports that the C-192 terminal of invasin per se can induce particulate uptake.

Table 5.1. Oral uptake of Polystyrene nanospheres (500nm) conjugated bacterial ligands (% of orally administered dose)

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Invasin</th>
<th>Invasin + Mucin</th>
<th>Maltose Binding Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Gastrointestinal Uptake</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peyer's</td>
<td>n/d</td>
<td>n/d</td>
<td>0.25 n=1</td>
</tr>
<tr>
<td>Intestine</td>
<td>n/d</td>
<td>6.42 n=1</td>
<td>n/d</td>
</tr>
<tr>
<td>Colon</td>
<td>n/d</td>
<td>n/d</td>
<td>0.3 n=1</td>
</tr>
<tr>
<td>Total</td>
<td>n/d</td>
<td>6.42 n=1</td>
<td>0.55 n=1</td>
</tr>
<tr>
<td></td>
<td>Systemic Uptake</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td>n/d</td>
<td>n/d</td>
<td>n/d</td>
</tr>
<tr>
<td>Spleen</td>
<td>n/d</td>
<td>n/d</td>
<td>n/d</td>
</tr>
<tr>
<td>Blood(^1)</td>
<td>1.47±0.9</td>
<td>0.31 n=1</td>
<td>0.24 n=1</td>
</tr>
<tr>
<td>Blood(^2)</td>
<td>13.0±5.3</td>
<td>1.98 n=1</td>
<td>2.01 n=1</td>
</tr>
<tr>
<td>Total</td>
<td>13.0±5.3</td>
<td>1.98 n=1</td>
<td>2.01 n=1</td>
</tr>
</tbody>
</table>

\(N=4\) except where indicated n/d = not detectable by GPC  \(^*P < 0.05\)

1. Approximately 2mL blood taken directly via cardiac puncture
2. Extrapolation to whole blood volume (12.8mL for 200g rat, or 64mL/Kg)
The site of intestinal uptake of the nanospheres was less clear; from previous reports *E. coli* either expressing or coated with invasin are internalised immediately upon binding to apical epithelial membranes of cultured cells, as are latex 1μM nanospheres coated with MBP-Inv-192 fusion protein via β-1 integrin receptors (Tran van Nhieu and Isberg, 1994). This may explain the lack of detection of invasin conjugates from both the large and small intestine due to the rapid ingestion and clearance over the 24 hour period prior to sacrificing of the rats. Despite the fact that the levels of absorption of invasin- and MBP conjugated nanospheres by the intestine were barely detectable using GPC, histological evidence presented in Figures 5.6 - 5.9 show clearly the great abundance of invasin-conjugated nanospheres deposited in the serosal layer of distal ileum compared to the sparse appearance of MBP-conjugates and blocked nanospheres in the same region (Figures 5.10 and 5.11). These histological observations are in line with the quantified systemic data strongly indicating that invasin conjugates are able to enter the epithelial layer with some ease, and that control nanospheres solely coupled to the carrier itself are unable to bind or translocate the epithelia despite the presence of a carbohydrate-binding moiety. Further, the latter result suggests that:

i) inspite of the possible lectin-like activity of MBP, there appears to be a deficiency of maltose residues on the apical enterocyte membrane. The lack of such residues/sequences has also been previously reported by other workers probing the rat intestinal epithelium with glucose/ mannose binding lectins (Pustzai et al., 1990; Etzler, 1987).

ii) the translocation of invasin-conjugates is due entirely to the truncated invasin derivative. This is further supported by the low systemic appearance of invasin-conjugates preincubated with mucin, a highly sialylated polymeric glycoprotein that has been previously shown to be a strong competitive inhibitor of the entire invasin molecule (Longhi et al., 1992). The high level of intestinal adsorption associated with blocked Invasin conjugated nanospheres
by a single rat (Table 5.1) may either be due to the incomplete removal of intestinal contents during excision of this organ (since very few nanospheres were seen in intestinal serosal layers), or it may be the reason why the same animal gave rise to the low systemic uptake of blocked nanospheres (2.04%, n=1). From this result one would expect that invasin-coupled nanospheres should also interact with the rat mucin, yet 13% of the administered dose is found circulating in the blood. The reason for this anomaly is presently unclear but it may be due to the differing properties of the two mucins or the fact that some of the administered dose is able to escape and reach the apical enterocyte surface.

Figure 5.6 Photomicrograph (taken under UV-light) of invasin-coupled nanospheres abundantly deposited in the serosal layer of the distal ileum (x 100)

Figure 5.7 Photomicrograph of the same region as depicted above but from a different section of the distal portion of the ileum. Normal light allows clear identification of intestinal histological tissues, the nanospheres accumulating in the serosa outside the thick layer of muscular tissue (x 100)
Figure 5.8 Photomicrograph of figure 5.7 above but at a lower magnification (x 40) thus allowing the entire cryostat tissue to be seen. Notice the mass of microspheres in the lumen adsorbed onto the food components/chyme, and the radial diffusion of particles along the villi (white arrows).

Figure 5.9 In contrast to the above only sparse appearance of MBP-coupled nanospheres can be seen (x 125) in the distal ileum.
Figure 5.10 Blocked nanospheres (ie MBP-inv-192 particles pre-incubated with mucin) display similar level of appearance in the serosal layer of the distal ileum as nanospheres conjugated to the carrier protein as shown in the Figure 5.9 (x 125).

Further, as seen with TL-conjugated nanospheres, in vitro experiments revealed extensive cross-reactivity with porcine mucin (Lehr et al., 1992) yet the in vivo work presented in chapter 3 demonstrated the appreciable systemic absorption of TL-conjugated nanospheres. This last statement suitably illustrates the basis for all of the work that has been conducted in the course of the studies presented up to this juncture of directly challenging a system in animal models since, in many cases in vitro studies may prematurely terminate or preclude further experimentation.

iii) in conjunction with i) and ii), the high level of systemic uptake observed with MBP-Invasin nanosphere conjugates is unlikely to be due to the immunogenicity of the bacterially derived protein.

iv) large peptide fragments can be stabilised on the surface of inert supports. Several studies have demonstrated that molecules prone to enzyme inactivation can be made more resistant to proteolytic degradation by covalent coupling to polymeric material or even endogenous ligands. Thus the coupling
of insulin B chain to soluble HPMA polymers afforded some protection against intestinal degradation from brush border enzymes (Morgan et al., 1991). Further the adsorption of gut-labile vincamine onto poly(hexyl cyanoacryalte) nanoparticles increased the relative oral bioavailability to 162% in comparison to the solution of the drug (Maincent at al., 1985). More recently studies by Chavanay and co-workers (1994) have shown that oligonucleotides, that display even faster rates of degradation than proteins, when bound to poly(hexyl cyanoacrylate) nanoparticles were “totally protected against enzyme degradation even with 5h incubation with the enzyme.” The half-life of bound oligonucleotides (1200min) increased over 600 fold compared to free oligonucleotides in solution (2min) when incubated with exonucleases and, remarkably, was not modified by a 20-fold increase in the enzymatic activity of the medium.

Although, generally, throughout the studies described in this thesis there has been a direct correlation between the levels of nanospheres seen in tissue sections and the magnitude of uptake in such tissues as assessed by quantification of tissue extracted latex by GPC, the uncoupling observed here with Invasin-conjugated nanospheres serves as a reminder once again the limitations of using optical methods (ie counting of particles by histological examination) as the sole method of quantifying intestinal particulate absorption. Histological examination of cryostat tissue sections from different regions of the intestine (ie. jejunum, proximal and distal ileum as well as PP’s from such regions), revealed varying levels of the distribution, binding and true translocation to the serosal layers of Invasin-conjugated nanospheres, with the distal ileum being the most heavily deposited region (Figure 5.6) and the proximal ileum/duodenum the least. With tomato lectin-conjugated nanospheres, however, there appeared to be a broad, uniform absorption throughout the intestine, although the ease with which nanospheres in the distal ileum and colon could be found was greater compared to tissue sections derived from the proximal regions. However multiple dosing may have caused intestinal redistribution of TL-conjugated nanospheres from their initial site of
absorption. Examination of haematoxylin and eosin stained tissue sections, as well as unstained tissues without UV light, indicated no apparent damage that may have resulted in such selective congregation of invasin conjugates in the distal portion of the intestine (Figures 5.12 and 5.13). However it is well established that virulent *Yersinia* strains harbouring the genes that encode for invasin preferentially colonize the distal regions of the intestine, particularly the lymphoid tissue that is particularly abundant around the ileocecal junction (Hanski et al., 1989), and therefore what may appear to be rather a surprising finding may in part be explained by the tropism that invasin displays for the distal ileal tissue (Pepe and Miller, 1994), thus directing such site-specific absorption of invasin-conjugated nanospheres. The non-appearance of invasin conjugates (Figure 5.13) in Peyer's patches, which display an even greater clearance of absorbed inert material, is probably related to the low population of enterocytes and M-cells comprising the overlying epithelium.

*Figure 5.11* Photomicrograph of haematoxylin/eosin stained (method described by Stevens et al, 1990) distal ileal tissue from rats dosed with active invasin coupled nanospheres. Notice the PP characterised by the two dense follicular regions with overhanging and intermediate villi (x 40)
If man is big
for the most effective
competition in habitat
of invasion, why was
it used.
Stability of invasion
in the milestone
tract
host city
Figure 5.12 Higher magnification of photomicrograph (x 100) depicted in Figure 5.11 before staining. No apparent damage is observable, such as loosening of villi and cryptic regions. Comparison of the photomicrographs also demonstrates how staining abolishes, or more correctly quenches fluorescence emanating from the particles.

Figure 5.13 The relative absence of invasin-192 coupled nanospheres in the PP at the ileocecal junction is seen in photomicrograph under UV-light.
The complete absence of all nanospheres from the reticuloendothelial system and other systemic organs particularly with invasasin conjugates demonstrates that orally absorbed nanospheres coated with (glyco)proteins behave in a similar manner to intravenously injected particles (eg Pegylated liposomes, poloxomer coated nanospheres) designed to avoid the liver and spleen. However since the same event was seen with TL-conjugated nanospheres it would appear that the negatively charged surface of nanospheres may actually be responsible for displaying this apparent anti-phagocytic behaviour, since this is the only common factor between the two widely differing (in terms of the coupled ligand) conjugates. This is further supported by studies conducted in our laboratories that have shown that non-ionised latex nanospheres displaying their native chemistry and of the same size as those used here are, by contrast, only found in the spleen and liver (Jani et al., 1990).

5.4  Comparison of the efficiency of bacterial and plant ligands in enhancing nanosphere translocation across epithelia

From Table 5.2 the efficiency with which Invasin promotes the intestinal absorption and systemic appearance of intestinally applied nanospheres compared to tomato lectin is immediately apparent. Figures 5.6 and 5.10 depicting the distal ileum (of invasin and MBP dosed animals) confirm the correlation between the systemic appearance of nanospheres and the rate of absorption/deposition in the serosal layer, despite the fact that in neither case could the levels of absorption be quantified by GPC. This may be due to the relative inefficiency of tomato lectin augmenting nanosphere translocation throughout the intestine versus the apparent site-specific, yet proficient, absorption of invasin-conjugates.
Table 5.2. Comparison between the efficiency of bacterial and plant bioadhesive ligands in augmenting the oral absorption of latex 500nm nanospheres (% of oral dose administered)

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Invasin</th>
<th>Tomato lectin</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Gastrointestinal Uptake</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peyer's</td>
<td>n/d</td>
<td>n/d</td>
</tr>
<tr>
<td>Intestine</td>
<td>n/d</td>
<td>n/d</td>
</tr>
<tr>
<td>Colon</td>
<td>n/d</td>
<td>n/d</td>
</tr>
<tr>
<td>Total</td>
<td>n/d</td>
<td>n/d</td>
</tr>
<tr>
<td><strong>Systemic Uptake</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td>n/d</td>
<td>n/d</td>
</tr>
<tr>
<td>Spleen</td>
<td>n/d</td>
<td>n/d</td>
</tr>
<tr>
<td>Blood^1</td>
<td>1.47±0.9</td>
<td>0.24 n=1</td>
</tr>
<tr>
<td>Blood^2</td>
<td>13.0±5.3</td>
<td>2.01 n=1</td>
</tr>
<tr>
<td>Total</td>
<td>13.0±5.3</td>
<td>2.01 ^ n=1</td>
</tr>
</tbody>
</table>

n=4 except where indicated  n/d = not detectable  ^P < 0.05

1. Approximately 2mL blood taken directly via cardiac puncture
2. Extrapolation to whole blood volume (12.8mL for 200g rat, or 64mL/Kg)

The difference between the two ligands is obviously related to the complementary binding structures in the intestinal epithelia, and the fact that invasin is primarily synthesised by virulent *Yersinia* to overcome the epithelia barrier to continue its lifecycle in mammalian hosts whereas the function of tomato lectin even within the tomato fruit is still unknown. Although both ligands are able to attach to the extracellular surface of epithelia, mere binding alone does not necessarily lead to internalisation. Such uncoupling between surface binding and transcytosis has been reported for tomato lectin by Lehr and Lee (1993) where only a marginal enhancement of absorption by cultured cells occurred. In contrast, invasin upon binding to integrins is able to transduce a signal across the membrane that results in substantial reorganisation of the cytoskeleton, and which can be blocked by cytochalasin 174
D indicating the role of actin and actin-associated proteins, talin and filamin, in the internalisation process (Young et al., 1992). However as discussed by Tran van Nhieu and Isberg (1994) the exact nature of the signal transduction mechanism promoted by invasin still remains a "black box". However the extremely high affinity with which invasin binds to β1 integrins, compared to natural β1 ligands, has been shown to be the primary characteristic that allows this protein to promote efficient particulate internalisation (Tran van Nhieu and Isberg, 1991) since bacteria coated with a variety of integrin ligands such as fibronectin, which bind integrins 200 fold less strongly than invasin, usually adhere to host cells without subsequent internalisation (Van de Water et al., 1983). A similar situation with plant lectins has been described by Pustzai and co-workers (1994) where lectins such as Phaseolus vulgaris and Sambucus nigra II which bind strongly with the intestinal epithelia are rapidly internalised compared to moderately binding lectins such as tomato lectin or wheat germ agglutinin which largely remain on the surface. This may be the result of the former lectins displaying complex oligosacharride binding patterns (Kornfield, 1983) compared to the latter that only bind short sequences of sugars. Binding large number of sugar residues statistically increases the chance that these lectins will be able to bind critical sugar residues that may be part of a receptor sequence (Fezai and Childs, 1985). This is also appears to be the case with the hybrid MBP protein containing the C-terminal 192 amino acids of invasin since smaller truncated derivatives are unable (eg MBP-inv-61) even to bind to cultured Hep-2 cells (Leong et al, 1990) suggesting that noncontagious residues in the terminal 76-amino acid loop of invasin are involved in binding (Figure 5.1B). Therefore it seems probable that binding molecules that is able to "grip" the receptor involving a large number of its constituent monomers are able to transduce a signal necessary for subsequent events. Tighter binding thus appears to bring about a conformational change in the receptor (a euphonious slang phrase adored by biochemists for describing inexplicable biological processes ! [personal communication, W.A. Gibbons, 1987]) enabling the mobilisation of cytoskeletal apparatus for internalisation. This requirement is met by the slow off-rate of
the integrin-invasin complex (dissociation $t_{1/2} = 20$ min) which disfavours dissociation (Tran Van Nhieu and Isberg, 1991) and ensures that a enough contacts will be established between receptors and invasin around the circumference of ligand bearing particulates to complete the phagocytic process, as proposed for the zipper model of phagocytosis (Silverstein, 1995). In contrast plant lectins usually bind comparatively slowly, and under solution conditions it can take minutes or even hours for an equilibrium to be established (Cummings, 1994). Only lectins that contain secondary binding sites ie. hydrophobic binding site (eg. Phaseolus vulgaris and bacterial fimbrial lectins), that are distinct from the sugar binding region, display similar dissociation constants to invasin-like molecules in the nanomolar range for the appropriate multivalent ligand (Rini, 1995).

5.5 Conclusions and perspectives for future work

This study demonstrates that a unique bacterial protein that is involved in the epithelial translocation of Yersinia is able to retain its functionality upon conjugation to latex nanospheres and induce their epithelial absorption. As the enterocyte is the most abundant cell in the intestine it is logical to target this vast area for absorption of biodegradable colloidal carriers incorporating gastrointestinal-labile molecules, thereby affording protection of the latter against the degrading milieu of the intestine. The magnitude of absorption of invasin-conjugated nanospheres after a single day's administration (13 ± 5%) is considerably less than reported by Alpar et al (1990) who demonstrated the systemic appearance of unmodified micron-sized latex particles in the region of 40% of the administered dose within 1h of dosing. Although there are inherent differences between the two studies (surface chemistry, size of particles), most notably in the method used to quantity the systemic absorption, they do however indicate that, paradoxically, greater absorption than that observed with gut-labile molecules such as peptides and proteins, or even nondegradable polymers (eg. PVP markers) employed to assess gut permeability, is real possibility. While the optical counting method of Alpar et
al. (1990) could be criticised in terms of its use of extrapolating a small quantity of blood (0.1mL) to estimate the total systemic absorption of microspheres, the value that we have obtained here using 2mL of blood bears considerable resemblance to the study performed by Bolin et al. (1982) where as many as 20% of the added bacteria encoding invasin are internalised by a monolayer of cultured cells. Despite our use of two controls to determine the specificity of uptake, further studies employing a greater number of animals are required using specific inhibitors such as the recently discovered disintegrin molecules, members of cell adhesion inhibitory peptides derived from viper venoms (Gould, 1990). This is particularly important since any model for describing the invasin-promoted bacterial or particulate internalisation must take into account two potentially contradictory phenomena. In particular, it must explain why the invasin-bearing determinant binds a receptor that is usually bound to the extracellular matrix (ie basolateral side of the epithelial cell) and thus is not readily accessible to invasin. Furthermore it must take into account how a receptor that is not actively endocytosed interacts with the endocytic machinery as a prerequisite for uptake.

The answer to the first question is presumably related to the high affinity binding that invasin displays for β1 integrin receptors. As suggested by Isberg (1992) "one possibility is that increased affinity of invasin for integrin makes it more effective at recruiting a sufficient number of integrin molecules to the potential entry site, which for reasons we still don't understand is an important first step in phagocytosis." Thus invasin allows internalisation by cells that have limiting amount of receptors since it can sequester sufficient receptors for uptake (Figure 5.14). The fact that invasin can bind to multiple members of the integrin family would also support this concept ie. to utilise as many host cell receptors that are linked intimately to the endocytic apparatus of the cell. Further work by Longhi et al. (1992) suggests that in addition to integrin receptors, branched membrane glycoproteins and glycolipids containing sialic acids and N-acetyl glucosamine residues play an important role in the invasive capacity of the E.coli strain expressing the invasin protein.
Figure 5.14 Proposed mechanism of integrin-mediated internalisation of Invasin-coupled nanospheres into epithelial cells. High affinity binding by invasin ($t_{1/2} = 20$ min) to integrin receptors allows efficient competition with bound extracellular proteins. The tighter binding allows sequestration of additional integrin receptors that results in circumferential binding or "zippering" to form a vacuole. Nanospheres are then internalised into a membrane-bound inclusion by an actin/myosin dependent mechanism similar to phagocytosis. The entire sequence of events resembles the movement of a cultured cell whose filodopia seek out new surfaces, pulling the leading edge of the particle forward (adapted from Tran Van Nhieu and Isberg, 1995)
Whether these determinants are part of a novel receptor yet to be identified remains to be seen. Work conducted using colonic adenocarcinoma cell lines expressing small intestinal epithelial characteristics has shown that integrin receptors are present only at the margins of fully differentiated enterocytes (ie cell junctions) but are intensively expressed by the proliferative undifferentiated cells, entirely covering the cell surface (Coconnier et al., 1994). Indeed the entry of *Yersinia tuberculosis* strains expressing invasin is actually inhibited by epithelial cell differentiation, indicating that entry is only possible via immature enterocytes in the crypt region. This may advantageous in the field of somatic gene therapy for inherited intestinal disorders such as cystic fibrosis since it may be possible to deliver encapsulated oligonucleotides with increased efficiency to intestinal stem cells, the logical target for gene transfer, that reside in the cryptic region. The use of colloidal carriers coated with invasin-like molecules may overcome the problems of poor permeation and inflammation associated with genetically engineered retroviruses that have so far been unsuccessfully used for this corrective approach (Sandberg et al., 1994).

The integrin receptor superfamily is a common target for the binding of microbial pathogens. These receptors, involved in binding various basal membrane proteins, such as fibronectin, collagen and laminin, are composed of \( \alpha \) and \( \beta \) transmembrane subunits selected from among 16 \( \alpha \) and 8 \( \beta \) subunits that heterodimerize to produce more than 20 different receptors. Alternative splicing of each subunit add further complexity (Hynes, 1987). The tight association with the cytoskeleton and the fact that extracellular signals can be transduced through integrins to promote tyrosine phosphorylation of membrane proteins, actin polymerisation and cytoskeletal rearrangements are all potentially advantageous to the invading microorganism (Figure 5.15). It is therefore not surprising that intracellular parasites have selected these receptors, despite their low numbers, and have capitalised on the associated endocytic machinery to gain entry into eukaryotic cells (Finlay, 1990). For example the obligate intracellular parasite *Leishmania* that survives within
macrophages, gains internalisation into host macrophages by binding directly to the complement receptor (CR3), an integrin, using its surface glycoprotein gp36 (Russell et al., 1989). A similar situation also exists for Bordetella pertussis, the causative agent of whooping cough, that is able to gain entry into the epithelial cells in the upper respiratory tract and monocytes using its filamentous haemaglutinin that is expressed on its outer membrane (Relman et al., 1989). Though much is known about the extracellular interactions between integrins and their ligands, significantly less is known about the intracellular biochemical pathways that leads to the mobilisation of the endocytic apparatus, the complexity of which precludes its detailed discussion here (Clarke and Brugge, 1995).
Surface exposed Invasin upon binding to the integrin receptor generates tyrosine phosphorylation that stimulates cytoskeletal rearrangements. This secondary signal, yet to be fully elucidated, modifies cytoplasmic proteins (talin?) forming bridges between integrins and actin filaments by α-actinin (redrawn from Finlay, 1990)
Although the vast majority of the work conducted with ligand-integrin interactions by microbiologists is concerned with gaining more detailed information in the design of new antibiotics to combat infection by intracellular bacteria, which are notoriously difficult to treat, it may also pave the way forward for researchers in the science of drug delivery to mimic such bacterial strategies. As no new unique component of the intestine has been identified, apart from the Peyer’s patches, that mediates particulate absorption, research in the area of bacterial cell entry will not only yield novel potential ligands in overcoming biological barriers but more information on the untapped endocytic potential of our own epithelial cells for the design of novel strategies to circumvent epithelial membrane barriers.
Chapter six

Conclusions, practical considerations and future perspectives
6.1 Use of Ligands for promoting epithelial nanoparticle internalisation

The series of experiments conducted and described in this thesis have shown, that keeping all other parameters constant, the magnitude of epithelial translocation of nanospheres is significantly altered by the surface nature of the particulates, or more specifically the endocytic capability of the surface coupled ligand. Quite apart from the histological and quantitative evidence so far presented, the ability to modulate and exert control over the epithelial translocation process, from the dramatic increases seen with invasin to the inadvertent decrease witnessed with lactose ligand, argues strongly for the existence of the phenomenon that many critics might consider in medieval terms such as "black magic". Thus after 5 days continuous dosing, the systemic appearance of TL-coupled nanospheres approaches 30% of the administered dose while only 0.1% of similarly sized nanospheres displaying lactose on their exterior are retained solely on the intestinal epithelium. The large difference is reflected by the confirmed existence of binding structures on the intestinal epithelium for TL since pre-incubation with inhibiting sugar markedly reduced binding and translocation, correlating well with other studies performed using TL (Lehr et al., 1992; Naisbett and Woodley, 1994a,b), while for lactose the secretion of cytosolic lactose-binding lectins to the apical surface of the epithelium has only been postulated to occur (Oda et al., 1991). A further aspect which remains to be studied for many of the endogenous lectins already discovered is the identification of their natural ligands to decipher the functions of glycosylation in mammalian cells.

Even more impressive intestinal absorption following a single dose was seen with invasin-coupled nanospheres, for which the role of the ligand in promoting efficient epithelial translocation and the its binding to complementary receptors has been clearly shown and fully characterised (as reviewed by Tran Van Nhieu and Isberg, 1994), compared to the much touted TL that demonstrated, surprisingly, a poor potential for augmenting nanosphere absorption in vivo, confirming the recently raised doubts of Lehr and Lee (1992) who could only demonstrate a marginal, if any, transcytosis of the lectin itself in vitro. While
invasin is able to bind to receptors that are intimately linked to the cytoskeletal apparatus that controls the endocytic machinery of the epithelial cell and able to transduce a signal to "kick-start" the internalisation process, it appears that TL merely adheres to the intestinal epithelium, only to be washed away either by peristalsis, mucus turnover and/or enterocyte erosion. Perhaps the number of contacts made by TL with membrane glycans is insufficient, the affinity of the interaction is too low, or the large number of glycoproteins from the mucin effectively compete with the binding of TL to the glycocalyx, since continual deposition by multiple dosing results in significant intestinal absorption. Whether this is due to membrane destabilisation or the expression of novel receptors by the accumulation of TL at the apical surface remains to be seen.

Furthermore, as discussed in chapter 5, invasin is a ligand designed specifically by bacteria to subvert host receptors apparently designed for other purposes, while the functions of lectins in plant physiology remains still poorly understood. The promotion of the use of lectins as oral drug carriers stems largely from the observational step that such agents can adhere to epithelial surfaces and that in some instances they are able to be internalised by the epithelia (Naisbett and Woodley, 1994b; Pustzai, 1990) with scant mention concerning their long-term effects on intestinal physiology or on the indigenous bacterial flora involved in symbiotic functions; however, one should take a cue from enteroinvasive bacteria of the ability of many obligate intracellular pathogens to exploit intestinal receptors designed for entirely different purposes, and of the dual capacities of many receptors to mediate both endocytosis and, in the case of integrins, cell adhesion. Furthermore, the identification of a ligand that is truly able to be transcytosed is a priority as it should not escape the fact that the translocation of unmodified particles across the epithelia is a relatively inefficient process with infrequent occurrence that is largely limited to the PP as part of the GIT immune surveillance. In addition, one would expect that if this phenomenon were the norm, examination of mesenteric lymph nodes would reveal a burden of particulate matter. Gross et al (1974) emphasized that even in workers occupationally exposed to particulate matter such as coal and silica dusts, there
is no involvement of mesenteric lymph nodes. Earlier still, Trier and Rubin (1965) after studying the ultrastructural abnormalities associated with the malabsorption syndrome of Whipple's disease that is characterised by variable alterations in villous architecture and epithelial cell ultrastructure, concluded, that even in these extreme conditions, the mucosal barrier retained its integrity.

Ligands such as invasin, that are necessary virulence factors of intracellular bacteria, may be one solution to promote the intestinal uptake of nanoparticles from a low basal level of around 1% to regions of 20% to confer any degree of feasibility of utilising colloidal systems as oral peptide carriers. While work has shown that sub-100nm latex particles displaying their native chemistry can be absorbed significantly to a level approaching 18.5% (re-calculated on the basis that a 200g rat has 13mL blood) of the administered dose with chronic feeding for 10 days (Jani et al., 1990), the encapsulation efficiency of peptide/proteins, which still remain relatively expensive to produce despite the advances in the biotechnology sectors, is likely to be very low. This was primarily the reason why studies were carried out with 500nm nanospheres, in an attempt to achieve an acceptable balance between the potentially high encapsulation efficiency with sufficient intestinal uptake. Further, the size of nanospheres employed corresponds to the juncture at which maximally sized passive pinocytic vesicles are formed (clathrin-dependent) and the formation of smallest active phagocytic (clathrin-independent) vesicles that can be triggered (Rabinovitch, 1995).

Invasin is an excellent example of an atypical ligand that is both capable of binding and internalising the attached particulate, since not all ligands (eg the uncoupling seen with TL), even bacterial in origin, are able to display such functionality within the same domain or even within the molecule. While invasin is the product of single genetic locus (Isberg and Falkow, 1985) termed inv, the molecular basis of the entry of other microorganisms such as Shigellae and Salmonellae are much more complex. In Shigella species more than 30 proteins encoded in a large virulence-associated plasmid are required for the internalisation into host cells (Sansonetti, 1992). Likewise, Salmonella species
use distinct determinants for attachment and invasion since many noninvasive mutants display wild-type levels of attachment. Further, the genetic factors necessary for entry into epithelial cells encompass numerous loci distributed around the chromosome, suggesting a complex and multifactorial process (Groisman and Ochman, 1993).

As mentioned throughout, opinions differ on whether particulate uptake is a significant physiological process, the wide range of mechanisms (Chapter 1 of this thesis) proposed to explain the phenomena revealing the general uncertainty in the field. Receptor-mediated endocytosis of particulate material, as shown with invasin, is without doubt the most direct, efficient and specific mechanism by which colloidal material can be transferred across the epithelial membrane. A further advantage of invasin-like ligands is the fact that they promote exclusively the internalisation of the attached bacterium/particle ie. upon uptake a tight phagosome is formed around the bound particle that excludes the presence of other particulates in the immediate vicinity. This membrane annealing property is another advantage of such ligands. On the other hand ligands synthesised by Salmonellae and Shigellae are hampered by the fact that they are inclusive, since after signal transduction a membrane ruffling events occurs that either traps multiple bacteria in a spacious vacuole, or allows uptake of bound bacteria induced by distant entry-competent bacteria (Figure 6.1). In this context they resemble permeation enhancers in the non-selectivity of uptake that may allow the epithelial entry of adsorbed bacteria, viruses and intestinal enzymes with deleterious consequences. For phagocytic entry, there is an absolute requirement of actin polymerisation in the host cell as exemplified by bacterial-mediated translocation into enterocytes (Young et al., 1992) and the macrophage mediated removal of opsonised particulate antigens (reviewed in Silverstein, 1995). Critical to the realisation of employing this pathway is the identification of receptors on the apical enterocyte membrane that are linked with the cytoskeletal apparatus associated with the endocytic machinery. Fortunately thanks to microbiologists, one set of receptors, namely integrins, exploited by a wide range of bacteria have been isolated.
Figure 6.1 Two strategies for internalisation of bacterial pathogens by mammalian cells that may be utilised in drug delivery technologies (modified from Tran Van Nhieu and Isberg, 1994)

A. Exclusive or "Trojan Horse" strategy as exemplified by invasin-mediated uptake of inert particles or enteropathogenic Yersinia. The particle [in black] adheres to integrin receptors initiating circumferential binding about the surface of the organism. Nearby particles [in white] that do not display invasin-like molecule is unable to bind to mammalian cell, remaining extracellularly localised.

B. Inclusive or non-specific strategy as exemplified by Salmonella or Shigella species. Bacteria or particles expressing "membrane ruffling" ligands allows neighbouring bacteria or particulates not expressing such ligands to gain entry into the mammalian cell. This is thought to be due to the induction of large ruffles on the apical membrane leading to either "trapping" of nearby entry defective particulates or due to intracellular signalling to the site of binding of the other particles/bacteria. This strategy of entry across biological membranes resembles the mechanism of action of permeation enhancers and may suffer from similar disadvantages.
In order to achieve effective receptor-mediated transcytosis of nanoparticles there are at least three criteria a specific receptor must possess in order to be considered as potential transport carrier, as exemplified by the hepatic LDL receptor. First, the receptor should be exposed on the apical surface of the enterocyte where the nanoparticles will be freely accessible. Second, after internalisation the receptor should be delivered to the opposite surface ie basolateral membrane rather than be recycled to the original surface or degraded in lysosomes. Finally after transcytosis, the receptor should be retrieved to the original apical surface in order to continue the transport process. To date, no epithelial receptor, including integrins, have fulfilled these criteria.

However the ligands, including the carboxyl-terminal invasin fragment, are far from ideal in the context of receptor-mediated drug delivery. While invasin may be a useful experimental tool it is extremely unlikely that it could ever be considered as potential ligand despite the fact that bacterial vectors exist, such as those employed in the isolation of MBP-inv-192, to generate large quantities. Firstly, the isolated hybrid protein cannot be cleaved since it has been previously noted that, in spite of the stringent precautions taken, such as the maintenance of protease deficient media, the cleaved Inv-192 fragment is unstable (Leong et al., 1990). Secondly even if the fragment can be expressed in a protease-deficient strain, chemical (eg deamination, isomerisation, disulphide exchange etc) and physical (eg. aggregation) instability of large peptide segments are complicated problems commonly encountered during protein processing, formulation and storage (Li et al., 1995). It was for this reason, therefore, protease resistant ligands such as TL or simple structures such as lactose were considered prior to the experimentation with invasin. In this regard, lactose as a ligand was particularly appealing as endocytosis of glycosylated latex nanospheres had been shown to be relatively uninfluenced by the size and composition of the aglycone (which in this case was the nanosphere itself), since endogenous lectins mainly require the recognition and binding of the terminal sugar residues of oligosaccharides (Adachi et al., 1994) to activate the internalisation process.
An ideal ligand would therefore have to possess properties that not only include the inclusion of both binding and internalisation determinants in the same domain but display a relatively small structure resistant to chemical, physical and enzymic instabilities. The recent advances in design and synthesis of peptidomimetics coupled with the screening of vast collections of small organic molecules by combinatorial methods may greatly aid in identifying highly active individual invasin-like molecules with smaller structures. Studies with invasin, for example, indicate conflicting pictures in this approach. The retention of biological activity of the inv-192 fragment is critically dependent on the maintenance of a disulphide loop in this portion (Figure 5.1), in addition to the presence of a free carboxyl group at the extreme terminus of the fragment. But it remains unclear what specific sequences in this 192 amino acid binding domain are responsible for high affinity binding to the Integrin (fibronectin) receptor. Invasin and fibronectin bind integrin αβ1 at mutually exclusive sites with different affinities, the binding of both of which is inhibited by RGD peptides (Arg-Gly-Asp) that constitutes the active binding site of fibronectin (Tran Van Nhieu and Isberg, 1991). RGD is a common motif in a variety of integrin binding ligands that alone is capable of displaying partial or in some instances full biological activity (Ruoslacht and Piersbacher, 1987). However Inv-192 contains no RGD sequence, nor any sequence that closely resembles RGD, leaving open the possibility that invasin mimics the RGD structure using a related sequence. By generating point mutants of invasin that binds integrins, Leong et al. (1995) have very recently have identified an 11 amino acid sequence containing a critical aspartate residue that is required for receptor recognition since even a conservative glutamate substitution abolished bacterial penetration. It would be of considerable interest to employ either this sequence or RGD trimeric peptide as integrin recognition elements/ligands to promote the epithelial internalisation of nanospheres. Preliminary work using this approach in the field of haematology has shown some encouraging signs (Kasuya et al., 1993). The covalent coupling of the tetrapeptide, RGDS (Arg-Gly-Asp-Ser), that is one of the active sites of cell adhesive proteins such as fibronectin and fibrinogen, onto latex microspheres was not only able to bind to platelets but allowed the induction of
platelet activation resulting in the formation of platelet aggregation that is a requisite step in blood coagulation.

6.2 Variability of uptake, route of absorption and fate

Wide variations reported in the literature concerning the uptake of intestinally applied particulates is probably the most contentious issue in the field that continues to fuel controversy, and hampers its acceptance. The literature reviewed in chapter 1 (section 1.3) revealed many factors and sources that could account for this variability. While critics have been pre-occupied with the very existence of the phenomenon, it has escaped the attention of many that such variations in uptake are largely dependent on the surface chemistries of nanoparticles, as exemplified by the data thus far presented. Further it is difficult to compare studies with wide variations in the experimental protocols themselves. The use of ligands binding to complementary determinants on the apical enterocyte membrane (TL) or exploiting a receptor-mediated process (invasin) was anticipated to reduce the influence of the various aspects of gastrointestinal physiology which can vary widely within individual animals (eg. pH, meal intake, motility, bile output etc), and was the prime motivation for this approach in an attempt to both increase absorption and to reduce variability of uptake. In this regard some success was achieved since variations in the systemic appearance of nanospheres (SD = 5.3% for invasin, 7.5% for TL after 5 days) was considerably less than variations in mucus thickness (the main extrinsic barrier) reported in the literature, where differences in the mean thickness of the mucus layer between samples from the same rat (ie. proximal and distal to the pylorus) and overall mean thickness between rats of duodenal sections ranged up to twofold in values (McQueen et al., 1983). In addition, the authors further reported 10-fold variations in mucus gel thickness at different locations from the same section, and "in extreme cases values of 5 and 500μm were recorded from the same tissue sample." These substantial local variations may also explain, in addition to diffusion of nanospheres through the mucus, how particulates are able to gain access to the epithelial surface.
Intra- and inter-subject variability is an inherent feature, and a major "headache" for regulatory authorities, in biological studies but the variations in the systemic appearance of ligand-coupled nanospheres and of unmodified microspheres of different diameters (Jani et al., 1990) is considerably less than many classes of clinically used drugs such as antifungals, cyclosporins, hypoglycaemics (and may also explain why virtually all drug delivery conferences each year have satellite symposiums and workshops devoted to the absorption of highly variable drugs and drug formulations!). For example the oral bioavailability of metformin (MW = 176 Da) ranges from 30% to 50% which is problematic for diabetics and practitioners in the control, to a modest degree of certainty, of the level of glucose in the blood. The fact that numerous in vivo studies conducted in this laboratory over the last few years indicating low variations in nanosphere translocation is a promising feature for oral colloidal drug delivery systems as they were primarily investigated for delivering peptides and protein drugs, compounds with narrow therapeutic indices.

6.3 Route of nanosphere absorption and fate of systemically absorbed nanospheres

Despite our use of fluorescently labelled nanospheres that are normally employed in tracer studies, it was difficult to ascertain the exact route of particle translocation after the nanospheres had been internalised by the enterocytes, warranting the need for additional time-course experiments. Such studies carried out over 30 years ago with 200nm latex nanospheres demonstrated clearly the role of normal enterocytes in the internalisation of particles and their subsequent appearance in villous lacteals, suggesting the lymphatic system as the route of translocation to the blood (Sanders and Ashworth, 1961). Further, nanospheres were seen in portal blood vessels, similar to the appearance of TL-conjugated nanospheres (chapter 3, Figure 3.8), indicating that nanoparticles are able to escape macrophages that are located in abundance in the mesenteric lymph nodes. More recent studies however have indicated that following a single
dose sub-micron nanoparticles are taken up preferentially by PP’s within 1-2h following administration (Jani et al., 1992). Whatever the mechanism, it is unclear how particles are able to gain entry into mesenteric lymphatics and endothelial-walled vessels in the subepithelial complex meshwork composed of connective and muscle tissues of the serosa, lamina propria, etc. One possibility is that after particles are excytosed at the epithelial basolateral membrane they gain entry into "pre-lymphatics", vessels of non-endothelised channels, that are located in the gel-like interstitium (Collan and Kalima, 1974). Pre-lymphatics, which frequently end at the junctions between endothelial cells of lymph capillaries or lacteals, are present to allow the movement of fluid, and chylomicra, with little impedance unlike the passage through the amorphous mucopolysaccharide gel itself which would offer resistance to flow. In addition large opening in lacteals themselves (75-600nm), also known as clefts, may allow the direct entry of particles once they have diffused through the interstitium (Figure 6.2). The lymphatics also have a fragmented basement membrane (Figure 6.3) offering little resistance to the passage of solutes, fluids and large particles into lymphatic vessels.

Another possibility is the entry of particles directly through postcapillary venules despite the absence of large gaps in their walls. Endothelial cells are abundantly populated with endocytic vesicles (650 per μm^3) and have been shown to phagocytose particles (Leak, 1976). As early as 1924, Aschoff proposed that endothelial cells were part of the RES system suggesting that such cells were capable of phagocytic events. More recently Aschoff’s hypothesis has been shown to be correct from the observations that endothelial cells and Kupffer cells originate/differentiate from a common stem cell (Weiss and Greep, 1977) and from the even more striking experimental evidence from Kreuter and co-workers (1995) of the phagocytic uptake of polysorbate coated poly(butyl cyanoacrylate) nanoparticles by the blood-brain vessel endothelial cells, once thought to be an even more insurmountable obstacle than GIT. The authors further demonstrated the penetration of nanoparticles to deeper cerebral tissues (Purkinje cells) and a dose-dependant analgesia with surface bound enkephalin nanoparticles. This
may indirectly explain the difficulty with which ligand-coupled nanospheres could be located and quantified in mesentery nodal tissue, (the chemical quantitation of which was subsequently coupled with villous tissues) since nanospheres may have directly entered the bloodstream itself via venous endothelial cells rather than negotiating a path through the lymphatic system.

Support for this hypothesis is also provided by the extensive studies conducted by LeFevre and co-workers (reviewed in LeFevre and Joel, 1984) who reported that "few particles seem to penetrate beyond the mesenteric nodes", presumably phagocytosed by the numerous macrophages that populate such regions and forms a secondary line of defence against particles that have managed to breach the intestinal epithelium. Their findings demonstrated that carbon microparticles were confined exclusively to the mesenteric lymph nodes macrophages, none being detected in the livers, spleens or lungs of mice exposed to carbon for two months (Joel et al., 1970). The appreciable quantities of both invasin (13 ± 5%) and TL-coupled (18 ± 4%) nanospheres in the bloodstream would not have been possible if nanospheres had utilised the lymphatic pathway. However this does not explain why nanospheres are then able to resist sequestration by the RES system, namely the hepatic Kupffer cells and splenic macrophages of the red pulp. One possibility is the negatively charged surface or the presence of ligand remnants prevents opsonisation of nanospheres, thus preventing complement mediated phagocytosis.
Figure 6.2. Diagrammatic representation of a cross-sectioned intestinal villus and the enclosed lacteal. The rat unlike, humans, have lacteals that terminate with several branches. "Pre-lymphatics" occur in the interstitial gel surrounding the lacteal and are thought to allow the movement and clearance of fluid, particles etc.
Figure 6.3 Three-dimensional representation of a villous lacteal. The major pathway for the translocation of fluids and particulates from the interstitium into the lacteal lumen is the intracellular clefts [large arrows]. The uptake of particulate components appears to be also via endocytic vesicles of endothelial cells of the lacteals [black arrows] and prelymphatic vessels are though to terminate at the junctions of endothelial cells, thus delivering particles/fluid at the site of absorption.
In addition, the possible adsorption of host components onto nanospheres during their translocation through various bodily tissues may render them unrecognisable, in immunological terms, by the RES, a method that is frequently exploited by bacteria (Finlay, 1990).

Yet another possibility is that the sheer numbers of nanospheres reaching these organs overwhelms \(3.6 \times 10^{10}\) for invasin; \(2 \times 10^{12}\) for TL) their phagocytic capacity if large numbers of particles occupy available RES receptors or exhaust opsonising factors. Given the relatively small deposition of ligand-coupled nanospheres in the liver and spleen (invasin = 0%; TL = 3.8 ± 2.5%), which manifests 85-95% of the total intravascular phagocytic activity (Saba, 1970), the fact that mesenteric lymph node macrophages do not readily migrate to the bloodstream and the small numbers of total white blood cells in the rat blood \(10^4/mL\), or \(1.3 \times 10^5\) for 200g rat [Baker et al., 1979]), it is unlikely that circulating nanospheres are contained within plasma macrophages. Such blockade of the RES with placebo substances such as carbon, latex particles and dextran sulphate was once thought of as a method to maintain higher levels of circulating colloidal carriers (Illum et al., 1986) and to effect passive targeting to desired organs. Subsequent injections of radio-iodinated latex particles resulted in only 30% of the injected dose accumulating in the liver and spleen, compared with 90% for unblocked animals. In a similar approach Profitt et al (1983) were able to increase by 50% the tumour targeting ability of \(^{111}\)Indium-labelled liposomes in mice bearing EMT6 tumours by suppressing the RES with empty liposomes. Further haematological studies are needed with ligand-coupled nanospheres to ascertain the possibility that saturation of the RES system has occurred since impairment of RES function can have serious consequences, particularly in patients with cancer or septicaemia as mononuclear phagocytes are active in preventing the spread of metastases or blood-borne bacterial/viral infections (Poste, 1983). Indeed any oral colloidal drug delivery system accumulating in the RES would need to be readily biodegradable so that RES overload does not occur with repeated dosing. In this regard it seems unlikely that materials with long biodegradable times, such as polymethacrylate or certain
blends of poly-co-lactide-glycolide polymers, can be envisaged as long-term circulating depot delivery systems continually releasing their drug payload, as proposed by a number of workers.

The eventual excretion of systemically absorbed nanospheres was not examined in the studies but is an issue of crucial importance, particularly from the recent findings that chronic administration of poly(butyl) cyanoacrylate nanoparticles, once presumed and proposed as a leading oral biodegradable carrier, can lead to hepatic inflammatory reactions (Fernandez-Urrusuno et al., 1995). Rypacek et al (1985) have suggested that non-degradable polymers which are too large to pass through the glomerular membrane can still be cleared from the systemic circulation either by being exuded by the intestinal mucosa or excreted with bile to the intestinal lumen. However they observed that the total amount of polymer (molecular weight average = 70,000) excreted with bile during the perfusion of an isolated liver amounted to no more than 0.015% of the polymer introduced into the perfusion circuit.

6.3 Practical considerations and future perspectives on the use of oral colloidal delivery systems

The plethora of in vivo animal studies performed within the last 5 years, easily outweighing the number studies conducted since the early experiments of Herbst in 1844, alone demonstrates the general acceptance of the potential of the mammalian intestine to absorb and translocate nanoparticulates. This flurry of activity may partly be due to the use of the word "M-cell" by the World Health Organisation in its recent grant application form, but is more likely as a result of the increasing awareness of the need to explore alternative patient-friendly oral delivery systems for the uncomplicated delivery of peptide and protein therapeutic agents.
The field of intestinal particulate uptake can be arbitrarily split into four levels. The first level is the demonstration that particulate uptake is a physiological process employed by eukaryotic animals as a means of immune surveillance of potentially pathogenic particulates in the intestinal lumen. Thus the presence of M-cells in amphibian animals, the selective adherence of *E. coli* to rabbit M-cells 4h after oral inoculation compared to normal enterocytes (Inman and Cantey, 1983), and the unique features of the M-cell are a reflection of the physiological role played by the GALT in translocating particulates. More recently studies by Hodges and co-workers (1995) have shown the ability of non-lymphoid intestinal tissue in translocating greater numbers of particles than PP's, in agreement with the studies described here. The ability to modulate epithelial particulate uptake with the use of ligands, and alterations in the hyrophobicity of particulate surface (Eldridge et al., 1990; LeFevre and Joel, 1984) comprises the second level, and provides the strongest evidence to date of the existence of the phenomena. We are now approaching the third level in this paradigm, and probably the most critical step, in unequivocally demonstrating that therapeutic levels of gut-labile molecules can be delivered by oral nanoparticulate systems in laboratory animals with reproducibility and consistency. However the small number of studies conducted in this area so far have revealed conflicting results. While Steiner and co-workers (1994) have claimed the high, but variable, levels of systemic delivery of calcitonin in rats and dogs (25-50% of the subcutaneous dose) with intestinally applied microspheres, the mechanism of which still remains a mystery, Lowe and Temple (1994) have concluded from that calcitonin and insulin encapsulated in isobutylcyanoacrylate nanocapsules "gave no significant overall enhancement of peptide absorption" compared to the free drug in solution" leading to the "conclusion that the nanocapsules released the peptides into the intestinal lumen, with small amounts then being absorbed but the rest largely degraded."

It appears, therefore, that the true test for such systems (fourth level), either for oral immunisation or peptide delivery will ultimately require investigations in human volunteers for a number of reasons (curiously this was one of the main intended objectives of the radioidinated work as described in chapter 2). Firstly
it should be emphasized that, considering the extensive 200-300 m² mucosal surface area of the human intestine- larger than most laboratories- and the copious amount of lymphoid tissue in the oropharyngeal cavity, what might appear to be a low level particle translocation in laboratory animals could represent a substantial number of absorbing particles in humans. Secondly, for oral immunisation, there are differences between the GALT of man and laboratory animals even at a fundamental level. The PP themselves are distributed differently. In humans PP are macroscopically invisible and are distributed in small clusters throughout the intestine, being particularly concentrated in the terminal ileum. In rats and mice, however, PP are found in approximately 10-26 clusters distributed evenly along the length of the ileum. Further, there are differences in the numbers and size of PP depending on the age of humans (Comnes, 1965) unlike in rats where they remain relatively unchanged in macroscopic appearance throughout life. In humans PP develop in utero and increase in size and number with exposure to the gut flora, but then decrease with age. At the microscopic level the synthesis of IgA in human tissues is not restricted to the mucosae to the extent seen in rats. Unlike rats which synthesise mostly dimeric IgA, humans synthesize both monomeric and dimeric forms of two subclasses of IgA, the monomeric form not apparently being involved in the protection of mucosal surfaces because of its inability to combine with secretory component (Spencer et al., 1986). Furthermore there are important differences in the isotypes of immunoglobulin that are expressed by B-cells in PP's. Thirdly, for peptide delivery, specific problems associated with each peptide/protein drugs remain. For example insulin, a potent drug with a narrow therapeutic window, precise dosing is critical and is further dependent and complicated on the amount of meal intake, the latter which might interfere with absorption of nanoparticles. For human growth hormone there are additional problems with the timing of administration. As succinctly described by Sim Fass, president of Biotechnology General (New York) "Human growth hormone is released naturally in a pulsatile manner, with one large spike of the hormone during the night.....if we could use an oral system to administer that spike, less hGH would be needed, perhaps with greater effect" (in Edgington, 1991).
There however remains a paucity of studies even to assess the potential of nanoparticle translocation in humans. In an attempt to produce alternative gamma scintigraphic media, Ercan et al (1993), employing 2.5μm latex microspheres labelled with Tc⁹⁹, enthused about the 98% retention of particles in the GIT of human subjects, oblivious to the efforts of others to promote particle translocation across the mammalian intestine !. This incidental observation is in agreement with studies of Jani et al (1989, 1990, 1992) performed in this laboratory demonstrating the inability of particles above 1μm to translocate the rat intestine efficiently (systemic absorption = 0.78% after 10 days) and the complete absence of 3μm sized particles in the systemic circulation.

Throughout our studies we could not demonstrate any significant translocation of microspheres through the PP’s or by the lymphatic pathway (as assessed by the quantification of mesentery nodal tissue and histological photomicrographs). This could be due to the rapid clearance of ligand-coupled nanospheres from such regions but is probably related to the low numbers of such cells in the GIT and the possible saturation of this pathway with limited volumetric capacity (Ermak et al., 1993). Further, direct entry into mesenteric blood vessels cannot be ruled out. However since the ligands (TL and Invasin) have been shown to be internalised by normal enterocytes, the vast numbers of the latter are, we believe, are responsible for this redirection of nanosphere uptake, particularly since the recent demonstration that TL binds both M-cells, goblet cells and villous enterocytes with equal affinities (Giannasca et al., 1994). Several investigators have championed the selective targeting of nanoparticles or coupled antigens to M-cells (Giannasca et al., 1994; Jepson et al., 1995), mainly by using sutured-closed intestinal loops encompassing PP’s, with the use of fucose-binding lectins such as Ulex europeus I but these have been shown to be toxic with repeated dosing (Grant, 1992). Further how this situation is to be reflected in humans, where there are to our knowledge no reports of differential (non-toxic) lectin binding studies with M-cells, remains to be determined. Critical issues pertaining to M-cells such as the entanglement of particles with the thick
mucus en route, the limited capacity of the M-cell, high esterase activity of PP’s (Owen and Bhalla, 1983) and variability between individuals, could undercut the practicality of such an approach. Lectin such as peanut agglutinin, which is mitogenic for normal and malignant epithelial cells, may complicate matters in individuals with predisposed, yet unidentified, colonic polyps or pre-malignant tissue (Ryder et al., 1994).

It is understandable why directing antigens to M-cells is the focus of much attention at the present time since the efficacy of many drugs is often limited by their potential to reach their therapeutical site of action. In contrast site-specific delivery of bacterial/viral epitopes would not only increase the amount of immunogens reaching the PP’s but reduce the large quantities of soluble immunogens that must be administered orally to stimulate a mucosal response, mainly because the majority of the antigens never traverses the epithelia before being degraded or cleared by the gut. Further, PP’s are thought to be the inductive sites for a generalised mucosal response (ie not restricted to the GIT mucosa) due to their preponderance of B-cells pre-programmed to differentiate and secrete IgA, and to the more recent discovery that activation of T-cells (Th₂ response), that secrete cytokines such as interleukin-5 and -6 which direct antigen-specific surface IgA-positive B cells to become IgA producing plasma cells, are induced in PP continually being supplied to distant mucosal effector sites for regulation of IgA responses (McGhee and Kiyono, 1993). In addition, cytopathic pathogens such as Salmonella that appear to utilise M-cells as portals of entry, may be blocked at their site of entry. However, at the time of writing, a number of reports in the literature have appeared which seem to indicate the over-importance attached to PP in immune regulation and for oral immunisation. Nanno et al (1994), utilising a new spontaneous recessive mutation that induces a generalised lack of lymph nodes and PP in mice, demonstrated that the development and maturation of intestinal intraepithelial T-lymphocytes is still possible, indicating that the lamina propria is able to present luminal antigens to T-cells ie. passage of the latter through PP is not required. More recently van Loon and Patriarca (1995) have even suggested at that the concurrent
administration of oral rehydration solution (ORS) with certain oral vaccines (mainly for diarrhoeal illness) may obviate the need to encapsulate such vaccines. They have theorised that due to the substantial water absorption in the small intestine, the use of ORS could result in the net flow of vaccine towards the mucosa, thus facilitating delivery of antigens from the lumen to M-cells and other GALT, and due to the alkalinity of ORS may also preserve the integrity of vaccines on exposure to gastric acid.

Lectin- or glycosylated mediated delivery of coupled drugs or nanoparticles to more generalised areas of the GIT such as colonic adenocarcinoma, that express both additional/novel endogenous lectins and glycans, may be a more feasible approach than trying to pin-point M-cells. Further, the enhanced endocytic capacity of tumour cells may allow high concentration of anti-tumour drugs encapsulated in nanoparticles to be internalised, resulting in an increased anti-tumour effect. The lack of antitumour specificity displayed by cytotoxic agents for gastrointestinal cancers, or indeed any cancers, results primarily from the low level of biochemical differentiation between the host and tumour cells, together with the heterogenous nature of tumour cell populations, thus limiting the effectiveness of current therapeutic approaches. Although much progress has been made in reducing the severity of cytotoxic-induced side effects by multiple administration schedules and timing of administration to coincide with various phases of cancer cell differentiation, there is a limit to their effectiveness. By virtue of their high-specificity of glycoconjugate binding to altered glycan expression of oncogenic transformed cells, lectins have been predicted to be extremely specific homing molecules which may be conjugated to tumouricidal drugs. The recent demonstration of the specific binding of the terminal N-acetylglucosamine binding lectin, *Griffonia simplicifolia* I-A4, to the Tn antigen, but not to a closely related tumour-associated epitope (sialylated Tn), that are both expressed exclusively in human colorectal cancers (including epithelia derived from patients or their first-degree relatives with an inherited tendency to develop colonic cancer), illustrates the remarkable specificity of the interaction that may be exploited for the site specific delivery of anti-tumour agents loaded
nanoparticles (Chen, 1994).

Finally a brief mention on the production and types of polymeric materials that have been proposed as encapsulation media is warranted particularly since poly (lactide-co-glycolide) polyesters are the only FDA-approved biodegradable polymers in common use for encapsulation and still occupy a pre- eminent place among bioerodible drug delivery devices. Polymeric materials should ideally degrade to natural metabolites to avoid potential toxicity particularly in the case of those products that resemble metabolites which may interfere with various biochemical pathways eg. N-acetylglucosamine disrupts glucogenesis by inhibiting glucose incorporation leading to hepatic carcinomas. This is further complicated by the scarce pharmacokinetic data available for monomeric constituents of polymeric materials. There are likely to be additional stability problems with each specific drug to be encapsulated as revealed recently by the aggregation of small molecular weight peptides in poly(lactide-co-glycolide) microspheres (Lu and Park, 1995). Such aggregation, protein denaturation or chemical degradation during formulation, storage, or by undesirable microenviroments within nanoparticles may alter the release kinetics of such drugs with narrow therapeutic indices, leading to unpredictable and hazardous release profiles. Crucially, a balance between systemic appearance of nanoparticles and their biodegradability has to be achieved since they may be degraded *en route* during intestinal translocation, captured by the RES leading possibly to "dose-dumping" in unintended organs, or slowly erode over a matter of days. Use of ligands to aid epithelial translocation could resolve a part of this equation. Recent observations that poly(alkylcyano acrylate) nanoparticles were more cytotoxic to murine hepatic cells than the degradation products and that induced hepatic inflammatory reactions were fully reversible on cessation introduces additional complexity (Fernandez-Urrusuno., 1995) concerning the biodegradability of polymeric materials.
6.4 Prospectus

It is now over a quarter of century since Bangham and Horne (1965) reported their observations on phospholipid smectic mesophases which led to the recognition of vesicles, but it is only relatively recently that a limited number of liposome encapsulated drugs have entered clinical trials and employed in clinical practice eg amphotericin B. Concepts and technologies that were at the cutting edge of basic research 15-20 years ago have now matured into clinically useful and commercial viable therapies. Perhaps the leading example of this is the widespread success of transdermal delivery systems from low molecular weight drugs such as nicotine patches, oestradiol (for hormone replacement therapy) and nitroglycerin for angina. These successes are due probably to the emergence of drug delivery research as a truly interdisciplinary field, combining elements of both physical science and biology. From this perspective it is hoped that studies described in this thesis has made a modest attempt to couple the interaction of physical systems with physiological processes.

In a similar fashion, it will be few years before a product based on oral colloidal drug delivery systems might be expected to enter the market place. Even up to 5 years ago the subject of intestinal particulate translocation was largely at the observational step but as a result of the explosion of research into M-cells, oral peptide delivery etc it is now at the edge of the determinant level. Recombinant DNA technology has allowed the production of large amounts of many different peptides and proteins, often specifically for potential development into pharmaceuticals. It is now common place to express large proteins in mammalian cells to produce correctly glycosylated proteins in the 10,000 litre scale. However as described by John Patton, the former Genetech’s drug delivery principal scientist “The recombinant wave has given the idea that the sky is the limit, but cloning a protein is child’s play compared to effective delivery - light years of difference in the scale of difficulty” (in Edgington, 1991). Although a variety of non-oral devices are on the market such as intranasal delivery of desmopressin, a peptide for diabetes insipidus, and other products in
development as suppositories etc, trying to promote this outside the western world - such as in the Hindu Kush - could evoke a rather unpleasant response.

Among these it would appear that the most likely product to reach the market stage are compounds intended for oral immunisation, given the intense research in the field and the heavyweight backing of the Children’s Vaccine Initiative of the National Institutes of Health as well as the World Health Organisation’s goal to develop a single-dose oral delivery of major paediatric vaccines. Further, the attractive attributes of oral vaccines such as the simplicity of administration, which does not require sterile syringes with needles and trained personnel, less stringent requirements for preparation of orally delivered antigens than for injectable ones, and fewer problems with storage of dry, lyophilised oral vaccines than with liquid injectables, give further impetus to this subject.

The pre-clinical evaluation of an orally administered HIV-1 vaccine, consisting of branched peptide immunogens entrapped in controlled release microparticles, shown to induce both high levels of serum IgG and neutralizing antibodies (O’Hagan et al., 1995), and the multitude of Investigational New Drug Applications submitted to the FDA, alone indicates the remarkable acceleration in the field of intestinal particulate translocation. It further supports the concept of intestinal particulate uptake despite the lack of agreement on the size and extent of uptake. Our data, based on the quantitative analysis of the material comprising the latex nanospheres, extracted from tissues convinces us that the route has a viability that requires further study. If procedures such as the attachment of lectins and invasin-type ligands to the surface of colloidal carriers routinely and reproducibly increases uptake without deleterious effects on the epithelia, coupled with continuing advances in the understanding of phagocytic events in non-professional phagocytes and cytoskeletal function that has occurred in the last decade, then the possibility of delivering realistic levels of proteins and peptides orally grows.
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