

REVIEW

Protein transport across the small intestine in food allergy

Marit Reitsma^{1,2}, Joost Westerhout¹, Harry J. Wichers², Heleen M. Wortelboer¹ and Kitty C. M. Verhoeckx¹

¹ TNO, Zeist, The Netherlands

² Food and Biobased Research, Wageningen University and Research Centre, The Netherlands

In view of the imminent deficiency of protein sources for human consumption in the near future, new protein sources need to be identified. However, safety issues such as the risk of allergenicity are often a bottleneck, due to the absence of predictive, validated and accepted methods for risk assessment. The current strategy to assess the allergenic potential of proteins focuses mainly on homology, stability and cross-reactivity, although other factors such as intestinal transport might be of added value too. In this review, we present an overview of the knowledge of protein transport across the intestinal wall and the methods currently being used to measure this. A literature study reveals that protein transport in sensitised persons occurs para-cellularly with the involvement of mast cells, and trans-cellularly via enterocytes, while in non-sensitised persons micro-fold cells and enterocytes are considered most important. However, there is a lack of comparable systematic studies on transport of allergenic proteins. Knowledge of the multiple protein transport pathways and which model system can be useful to study these processes may be of added value in the risk assessment of food allergenicity.

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1 Introduction

In the near future, a shortage of protein sources for human consumption is foreseen and, therefore, alternative and sustainable protein sources (e.g. insects and algae) are now being explored for the production of food and feed. However, safety issues, such as the risk of allergenicity of novel proteins are often a bottleneck in bringing these products to the food market due to the absence of predictive, validated and accepted methods for risk assessment. New proteins or genetically modified foods are currently assessed for their allergenic potential using an allergenicity assessment strategy advised by the Food and Agriculture Organization and the World Health Organization. This strategy is based on a weight of evidence approach that recognises that no single endpoint can be used to predict human allergenic potential [1, 2] and focuses on characterising the protein/gene source, amino acid sequence homology to known allergens, in vitro cross-reactivity with

known allergens and protein stability in a static pepsin digestion model [3]. However, these methods are mostly subjective and no guidance on procedures and interpretation of the outcome is available as yet. For example, several reviews and studies have indicated that in vitro digestion using the simulated gastric fluid method, protein digestion by pepsin in acidic conditions, is not always a good predictor of allergenicity [4, 5]. A comparative study by Fu et al. found no correlation between simulated gastric and intestinal fluid stability of allergenic and non-allergenic protein and between major and minor allergens [6]. Furthermore, it was shown (own experience) in a dynamic digestion model (TNO's intestinal model for the gastrointestinal tract) that even less stable proteins might reach the intestinal tract in an intact, at least immunogenic, form.

We hypothesised that transport of food proteins and peptides across the gastrointestinal barrier is needed to induce sensitisation or to elicit an allergic reaction, making it an important parameter in allergy research, next to digestion of these proteins. Therefore, we collected information on how proteins are transported in both sensitised persons and non-sensitised persons. This information can be used to estimate whether intestinal passage of protein or immune-reactive protein fragments can be incorporated into a new method for risk assessment of allergenicity of a protein. For this reason

Correspondence: Dr. Kitty C. M. Verhoeckx, TNO, Utrechtseweg 48, 3704 HE Zeist, The Netherlands
E-mail: kitty.verhoeckx@tno.nl

Abbreviations: DC, dendritic cell; HRP, horseradish peroxidase; M cell, micro-fold cell

a comprehensive literature study was performed using the open-access databases NCBI-PubMed, Scopus and Web of Science to review the current knowledge on the mechanisms of protein transport across the small intestine and which methods are currently being used to study the intestinal absorbance of proteins.

2 Transport of allergens across the intestinal tract

The gastrointestinal barrier has different functions to fulfil, e.g. absorb nutrients and exclude “unwanted” compounds, such as bacteria and allergens. The intestinal tract is composed of different layers, glycocalyx, luminal mucosa, which are composed of a monolayer of epithelial cells, lamina propria, which is connective tissue scaffold containing the blood and lymphatic vessels, and the muscularis mucosa (muscle layer) [7]. The lamina propria is highly folded to form villi, increasing the intestinal surface. This surface is covered by a heterogeneous population of epithelial cells, including absorptive intestinal epithelial cells (enterocytes), enteroendocrine cells (L-cells), mucin-secreting goblet cells and microfold cells (M cells) [8].

The secretive and absorptive cells have a highly folded apical membrane, forming uniform micro-villi, express brush border enzymes and membrane-embedded transporter proteins. The epithelial cells are joined at their apical side by tight junctions and other integral membrane proteins (e.g. claudins and occludins) that prevent the passage of macromolecular compounds larger than 600 Da [9, 10]. In addition, secretory Ig A (IgA) and mucus (secreted by goblet cells) also restrict the absorption of dietary antigens [11]. Mucus, a mixture consisting mainly of water, glycoproteins (e.g. mucin), lipids and proteins, forms a protective layer of 50–450 μm on top of the intestinal epithelial cell layer [12]. Although no reports on the relation between protein absorption and intestinal mucus were found, thickness of the mucus layer can influence the absorption rate of drug compounds [12], making it acceptable to reason that also food protein absorption can be limited or facilitated by the mucus.

Intestinal micro-biota is pivotal to health homeostasis. Although a clear relation between gut micro-biota and allergy has been established many times, the mechanism behind this is not clear yet. As summarised by Gigante et al., a difference in gut micro-biota can be seen between atopic (prone to develop allergies) and healthy persons. Furthermore, the gut micro-biota plays a crucial role in oral tolerance induction as germ-free mice do not develop tolerance [13]. Moreover, lack of intestinal bacteria in germ-free mice leads to a decreased intestinal surface, decreased intestinal cell renewal, and a thinner mucus layer [14]. However, no evidence was found on the role of micro-biota in protein transport, but due to the date present, it can be envisioned that micro-biota will play a relevant role.

After the consumption of food, the majority of food proteins are degraded by the action of gastric, pancreatic and small intestinal brush border proteases (e.g. pepsin, trypsin and chymotrypsin), resulting in a mixture of free amino acids, di- and tri-peptides, intact proteins and other products of proteolysis. Shimizu et al. estimated that 90% of intact proteins, absorbed by enterocytic cells, is degraded within these cells by intra-cellular enzymes [15]. Still, 10% of the non-degraded proteins are able to cross the intestinal barrier intact. The fact that intact proteins cross the intestinal barrier was also shown by Husby et al., who measured intact ovalbumin in human blood after egg consumption using HPLC in combination with ELISA [16]. Already in 1913, van Alstyne reported the transport of intact oligopeptide across the intestine. In this study, blood from dogs fed with milk caused anaphylaxis in milk protein sensitised guinea pigs [17]. Furthermore, Smith et al. described in a review the amount of absorbed intact proteins, e.g. horseradish peroxidase (HRP), α - and β -lactalbumin and BSA from undetectable to 12% of ingested proteins, depending on the protein used, the use of ex vivo or in vivo studies, age of the animal and region of the intestine used [18].

2.1 Para-cellular and trans-cellular transport routes of proteins

Transport of proteins across the intestinal tract depends on size, polarity, shape, aggregation status and 3D structure of the protein and may occur either via the para-cellular route or via trans-cellular routes (Fig. 1). Para-cellular transport refers to the transfer of compounds through the inter-cellular space between the cells and is regulated by the integrity of tight junctions [19, 20]. Para-cellular transport is only considered to occur for small hydrophilic compounds (up to 600 Da [9, 10]), but Pauletti et al. and Rubas et al. described that the maximal radius of a protein, which could pass the intestinal barrier via the para-cellular mechanism was estimated at 15 Å ($\pm$ 3.5 kDa) [19, 21]. Proteins transported via the para-cellular route are not exposed to lysosomes in the enterocyte and are therefore not degraded [15]. In healthy adults, however, para-cellular protein/antigen transport is not considered of much importance [22–25]. This is in contrast to already sensitised persons, where the integrity of the tight junctions is decreased due to the presence of mast cells, enhancing the amount of non-degraded protein entering the human body [26] as will be more extensively explained in Section 2.4.

The trans-cellular transport route comprises the absorption of compounds via passive diffusion, carrier-mediated transport, or endocytosis (Fig. 1). As proteins are mostly hydrophilic macro-molecules, passive diffusion across the lipid bi-layer of the cell membrane is considered minimal and will therefore not be discussed in this review. Carrier-mediated transport routes are present in the human intestine for amino-acids [27] and di- and tri-peptides [22], but literature on protein carrier-mediated transport was not found. The

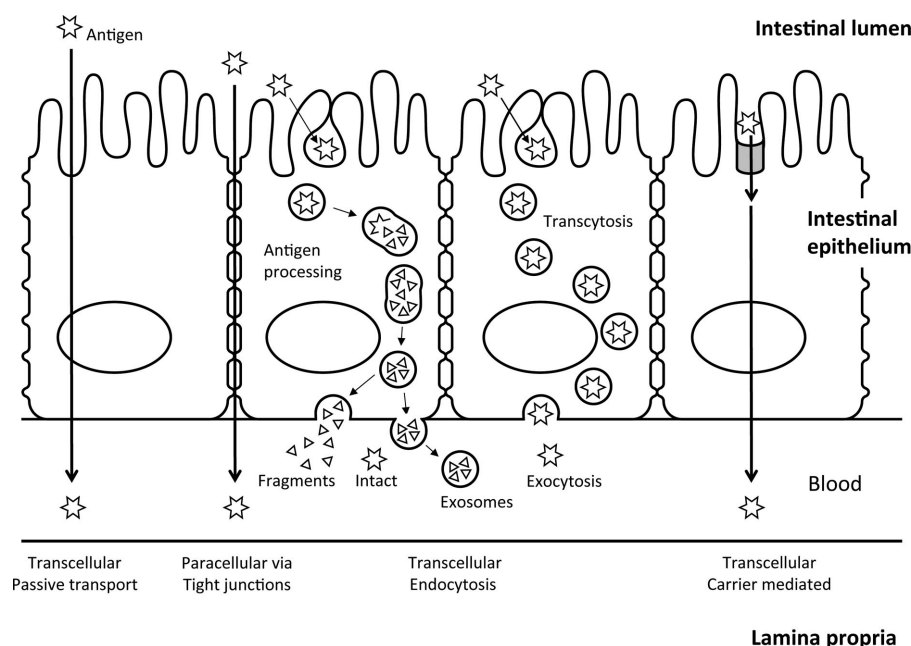


Figure 1. Different passage routes across the intestines.

main route of trans-cellular protein transport is endocytosis, which is known to occur in different cell types and will be described in the following sections.

2.2 Enterocytes

Enterocytes or absorptive intestinal cells are epithelial cells and are the most abundant cells in the intestinal barrier. Due to the formation of tight junctions between two adjacent enterocytes, they are involved in para-cellular transport, but additionally they are also involved in protein and peptide absorption via the trans-cellular route. According to So et al. [28], soluble particles such as proteins can be endocytosed by enterocytes, after which they are transported in small vesicles or larger phagosomes and are either digested in lysosomes or left intact (trans-cytosis) [29], but the fragments as well as the intact proteins may still be allergenic, as was shown by Terpend et al. In this study, HT29–9A cells (an enterocytic cell line) were incubated with HRP; 1% HRP was endocytosed by the cells, of which 90% was subsequently hydrolysed intra-cellularly. However, the authors showed that 40% of the hydrolysed protein had a molecular mass of at least 1100 Da, which is still large enough to bind MHC-II molecules [30]. In another study, it was estimated that 2% of intact proteins can reach the intestinal lymph and portal circulation under physiological conditions [31].

Belut et al. described another trans-cellular pathway of antigen uptake by enterocytic cells, which is facilitated by IgE and only occurs in sensitised persons, when luminal antigen-specific IgE is present [32]. This IgE forms a complex with the antigen and binds to the CD23 receptor, which is over-expressed on the apical membrane of enterocytes in

sensitised persons. The CD23 receptor transports the IgE-antigen complex trans-cellularly without lysosomal degradation across the intestinal membrane [9, 33]. The importance of CD23 in this transport system was shown by Bevilacqua et al. who exposed the intestine of HRP sensitised and non-sensitised mice to HRP. The HRP sensitised mice showed increased transport of intact HRP, which was eliminated after the addition of anti-CD23 antibodies, whereas the transport of partly degraded HRP was not changed [33]. The involvement of IgE was further demonstrated by Yu et al. who passively immunised naive mice by injecting immune serum from a mouse that had been actively sensitised to HRP. IgE depletion of this serum eliminated the induced increase in intestinal trans-epithelial antigen transport [34].

O'Brien et al. showed that after antigen uptake with or without subsequent lysosomal degradation, antigens with a size of at least 18–20 amino acids long can be presented on enterocytic MHC-II molecules [35], or secreted into exosomes. Thereafter, the antigen can be presented to T cells by MHC-II molecules on the enterocyte, but this rarely happens [36]. It is more likely that antigen-containing exosomes or tolerosomes will present the antigens on their MHC-II molecules to T cells [9, 36, 37], possibly reaching them via pores in the intestinal basement membrane or via the blood circulation. Inflammatory conditions in the intestine may increase exosome secretion from intestinal epithelial cells as was shown in vitro when intestinal epithelial cells increased secretion of exosomes after the addition of the pro-inflammatory cytokine IFN- γ [36]. Exosomes are thought to induce tolerance toward the antigen it contains. However, not all studies confirm this effect [38]. It has been suggested that intestinal epithelial derived MHC-II containing exosomes are tolerogenic in the absence of co-stimulatory molecules, but can activate T cells in presence of co-stimulatory molecules [25].

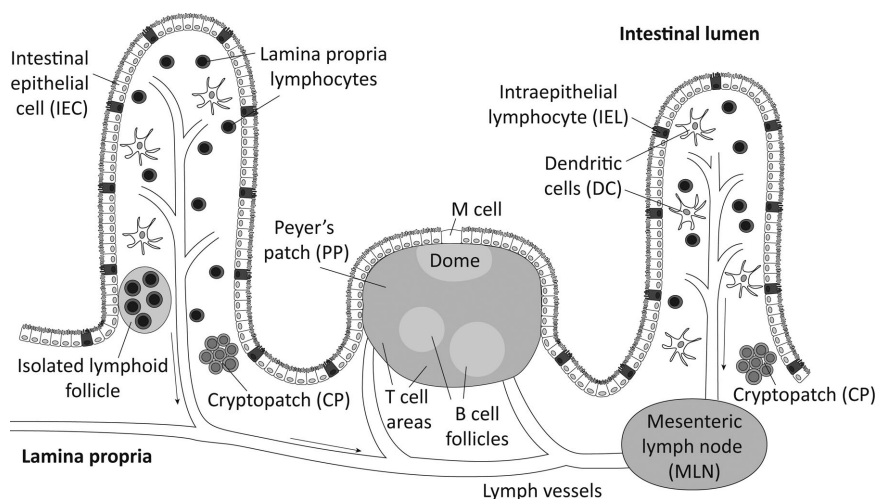


Figure 2. The intestine at a cellular level, based on Spahn and Kucharzik [41].

Besides IgE, also IgA and IgG are involved in enterocytic protein transport. Polymeric IgA and IgG reach the intestinal lumen trans-cellularly via, respectively, the polymeric IgA receptor and the neonatal Fc receptor on enterocytes [39, 40]. However, in contrast to IgE, binding of luminal antigens to IgA prevents their uptake, and binding of IgA to antigens that have already crossed the intestinal tract leads to secretion back into the intestinal lumen. Therefore, antigen-specific IgA is considered to be protective against sensitisation and allergic reactions. Transport of IgG–antigen complexes across the intestines in the neonate seems to be tolerogenic, but in adults this role is less clear [40].

2.3 M cells

M cells are specialised epithelial cells that are part of the Peyer's patches in the intestinal tract (Fig. 2), but they can also be found in the gut independently of these patches [42]. Since M cells contain fewer lysosomes, have a thinner glycocalyx layer compared to enterocytes and do not present membrane-associated enzymes, the chance of compounds to be degraded during trans-cellular transport is low. M cells have been shown to transport proteins, bacteria, viruses and other particles of up to 1 μm [43], either via phagocytosis [44], endocytosis [45] or pinocytosis [43]. For example, HRP has been shown to be absorbed by rabbit and piglet tonsil and intestinal Peyer's patch cells [45, 46]. Another study showed that in vivo exposure of murine intestine to IgA and IgG against mouse mammary tumour virus revealed binding and internalisation of both Igs by M cells [47].

The mechanism by which M cells transport proteins and/or antigens has been suggested to occur via a basal pocket, such as invagination in M cells creating "M cell pockets" where B and T cells, macrophages and dendritic cells (DC) appear to be present [48, 49]. The exact function of these pockets is unknown but it is thought that these

pockets may shorten the intra-cellular distance for antigens to travel before being displayed to antigen-presenting cells, which then migrate to antigen-specific lymphocytes in underlying lymphoid follicles inducing T-cell proliferation. This process results in the development of IgA-producing B cells, some of which move into the vasculature and then back to the mucosal surfaces, efficiently seeding specific mucosal immunity [43, 50, 51].

Considering antigen transport, most research has been focused on the uptake of antigens by either M cells or enterocytes. The current perception is that particulate or aggregated antigens are taken up by M cells, inducing a local or systemic immune response towards that antigen and inducing the production of IgA. Soluble antigens, on the other hand, are thought to be predominantly absorbed by epithelial cells, leading to suppression of the immune system, induction of tolerance, towards this antigen [11, 52]. A comparison between soluble and aggregated milk proteins (α -lactalbumin and β -lactoglobulin) revealed both in vitro and in vivo an uptake of aggregated milk proteins by Peyer's patches, containing mostly M cells, inducing a stronger immune reaction than the soluble milk proteins that were predominantly transported into intestinal epithelial cells. However severity of orally induced anaphylactic reactions were greatly impaired by aggregation of the milk proteins, implying that aggregated antigens can induce sensitisation, while allergic reactions following this sensitisation are mainly caused by soluble antigens [53]. However, M cells have been proven to transport not only insoluble, but also soluble peptides and the onset of tolerance versus sensitisation also seems to be influenced by particle size [54, 55].

2.4 Mast cells

Shea-Donohue et al. estimated that about 2% of resident intestinal cells are mast cells and during immune responses this amount increases [56]. Mast cells are involved in

eliciting symptoms during allergic reactions, but they are also indirectly involved in the transport of allergens.

In this respect, food hypersensitivity reactions are known to occur in two phases. The first phase is mast cell independent and antigen-specific with antigen uptake occurring via trans-cytosis by intestinal epithelial cells, which is increased by sensitisation to the allergen. The next phase is mast cell-dependent, increased after sensitisation, and during this phase intestinal permeability to non-specific bystander proteins is also increased [26]. Using colonocytes this increased intestinal permeability was shown to be triggered by IgE-induced tryptase release by mast cells, which activates the protease-activated receptor 2 on colonic epithelial cells, causing redistribution of several tight junction proteins and inducing increased para-cellular permeability to macromolecules [57]. As small intestinal enterocytes also contain this protease-activated receptor [58], we expect a similar interaction between activated mast cell components and enterocytes in the small intestine.

2.5 DCs

DCs constitute a major cellular component of the intestinal lamina propria, and play a pivotal role in the balance between tolerance and sensitisation in the intestine. DCs are antigen-presenting cells and are able to intercalate between intestinal epithelial cells, by virtue of extended dendrites without disrupting the epithelial barrier by opening tight junctions and forming new tight junctional complexes with adjacent epithelial cells, a process that is up-regulated in inflammatory conditions [59]. In this way DCs are able to take up soluble compounds from the lumen, although protein up-take by dendrites is controversial [60] and no literature data of direct protein or peptide sampling by DCs from the intestinal lumen was found.

In addition, Chambers et al. showed that intestinal (Peyer's patch) DCs obtained from sensitised and subsequently challenged mice can induce allergen-specific IgE in naive mice in the absence of an allergen challenge [61]. However, in general, intestinal DCs are thought to be more tolerogenic compared to DCs from other organs (e.g. the spleen) as they activate relatively more regulatory T cells [62]. Both the level of DC maturation and the type of DC could be of influence in this "tolerogenic" role; immature DCs are more likely to induce tolerance [36] and plasmacytoid DCs have been shown to decrease sensitisation [63].

2.6 Macro-phages

Besides DCs, macro-phages have been shown capable of transporting antigens across the intestinal tract using pseudopodia [64]. After antigen uptake, the antigen-containing macro-phages enter the systemic circulation, leading to systemic reactions towards this antigen. Whether or not Peyer's

patch macro-phages transport compounds out of Peyer's patches depends on the compound size; (latex) particles with a diameter $<5\ \mu\text{m}$ can be transported via macro-phages, while particles with a diameter of $5\text{--}10\ \mu\text{m}$ mostly remain inside the Peyer's patches, eliciting local mucosal effects [18,65].

Similar to intestinal DCs, intestinal lamina propria macro-phages display a non-inflammatory phenotype compared to macro-phages from other tissues, e.g. blood monocytes, from which they stem. Unlike other macro-phages, intestinal macro-phages do not express receptors for, among others, LPS, IgG and IL-2, and express low levels of all major pro-inflammatory cytokines, even after phagocytosis, an action which they perform avidly [66]. On top of this non-inflammatory phenotype of intestinal macro-phages, these cells have also been described as critical actors in intestinal tolerance induction to food protein antigens [67].

2.7 Goblet cells

The last epithelial cell type that must be discussed is the goblet cell, whose main function is the production of mucin. Recently McDole et al. showed in vivo, in healthy persons, that goblet cells transported 10 kDa peptides from the intestinal lumen to underlying lamina propria DCs. However, this transport is probably size limited as it was shown that large particles ($0.02\text{--}1\ \mu\text{m}$) cannot enter via this pathway [68].

From such literature data it can be concluded that protein transport in non-sensitised persons occurs via the M cells and enterocytes, while in sensitised persons the para-cellular route with involvement of mast cells, and trans-cellular route via enterocytes are considered the most important.

3 Measurement of allergen transport across the intestinal tract

Food proteins need to cross the gastrointestinal tract in order to induce sensitisation or elicit an allergic reaction. Therefore, we assume that the assessment of allergen or protein transport across the intestinal barrier might be a relevant, additional parameter in allergenicity risk assessment. The different techniques to assess intestinal protein transport will be discussed in the following sections. An overview of reviewed transport studies and allergens used is given in Table 1.

3.1 In vitro assays

Several in vitro methods have been used to study intestinal protein absorption, and an overview is presented in Table 1. Various cell lines (e.g. Caco-2 and HT-29) can be used to study the epithelial transport of proteins in vitro. These cell lines are from different origins and species and all have their advantages and disadvantages. The Caco-2 cells, a cell line of human colonic origin, exhibit many properties of small

Table 1. In vitro absorption studies using different techniques, proteins and analysis methods. For each study the transport of intact protein is presented

Technique	Species	Protein	Food-related	Intact protein	Analysis	Reference
Caco-2, everted sac	Human, Wistar rat	γ -Conglutin	Lupin	Yes	Immunoblotting	[69]
Caco-2, HT-29	Human	FITC-tetanus toxoid, FITC-ovalbumin	No	Assumed	Laser scanning confocal microscopy for fluorescence intensities	[28]
HT-29	Human	FITC-tetanus toxoid	No	Assumed	Fluorescence microscopy	[70]
Caco-2	Human	Ber e 1, Ses i 1	Brazil nut, Sesame seed	Yes	HPLC, ELISA	[98]
Caco-2	Human	β -Lactoglobulin, albumin	Milk	Assumed	^{125} I-radioactive labelling	[72]
HT29-19A	Human	Horseradish peroxidase	No	Yes	HPLC, ^{3} H-labelling	[30]
Caco-2	Human	β -Lactoglobulin, α -lactalbumin	Milk	Yes	HPLC, ^{14} C-labelling	[73]
Ussing chamber	Rabbit	β -Lactoglobulin	Milk	Yes	ELISA, HPLC, SDS page, ^{14} C-labelling	[74]
Ussing chamber	Rabbit	Horseradish peroxidase	No	Yes	Enzymatic activity, ^{3} H-radioactive labelling	[75]
Ussing chamber	Rabbit	BSA	No	Yes	Immunoblotting, ELISA, ^{125} I-radioactive labelling	[76]
Ussing chamber	Human	Horseradish peroxidase	No	Yes	Enzymatic activity, ^{3} H-radioactive labelling	[77]
Ussing chamber	Pig	Horseradish peroxidase	No	Yes	Enzymatic activity	[78]
Ussing chamber	Wistar rat pups	Horseradish peroxidase	No	Yes	^{125} I-radioactive labelling combined with HPLC gel filtration	[79]
Ussing chamber	Human	Horseradish peroxidase	No	Yes	Enzymatic activity, ^{125} I-radioactive labelling	[77]
Introduction of material into the gut lumen (ileal loop)	BALB/c mouse	Ara h1, Ara h2, Ara h3	Peanut	Unknown	Transmission electron microscopy, fluorescence microscopy, detection of protein bodies	[54]

FITC, fluorescein isothiocyanate.

intestinal epithelium as they form a polarised monolayer of well-differentiated columnar absorptive cells expressing a brush border on their apical surface with typical small intestinal enzymes and transporters. The cells form very dense tight junctions, whose tightness resembles more the colonic than small intestinal tissue [8]. HT-29 cells stem from a human colon adenocarcinoma cell line that contains both absorptive and mucus secretive cells [30]. Compared to the Caco-2 cell line or the human intestine, the HT-29 expresses few carriers and is mostly used to study the effect of mucin on transport [80]. One of the major advantages of using cell lines to study the intestinal transport of proteins is the relatively high throughput at which different proteins can be studied. On the other hand, due to the lack of a physiologically relevant

environment (e.g. interactions with other cell-types, intestinal fluid composition and mucus presence) [81], prediction of human oral exposure to allergens on the basis of in vitro studies with cell lines might be prone to errors. Other in vitro methods for measuring intestinal transport of proteins include the Ussing chamber technique and the everted sac technique [75–79, 82]. Both techniques have a higher resemblance to the in vivo situation as they require small sections of ex vivo intestinal tissue rather than cell mono-layers. In both the Ussing chamber technique and everted sac technique, a section of the gut is used. In the everted sac method, the intestinal section is everted and both ends are tied after filling the sac with buffer. The ‘sausage’ is placed into buffer with the compound of interest. With the Ussing chamber

Table 2. In vivo absorption studies using different techniques, proteins, and analysis methods. For each study the transport of intact protein is presented

Technique	Species	Protein	Food-related	Intact protein	Analysis	Reference
Oral exposure	Pig	IgG, β -lactoglobulin, albumin, trypsin	Colostrum	Yes	Immunoblotting	[87]
Oral exposure	Human	α -Lactalbumin	Milk	Assumed	^{125} I-radioactive labelling	[88]
Oral exposure	Human	β -Lactoglobulin	Milk	Assumed	RIA	[89]
Oral exposure	Human	Ovalbumin	Egg	Yes	Gel permeation chromatography, ELISA	[16]
Oral exposure	Brown Norway rat	β -Lactoglobulin	Milk	Assumed	ELISA	[90]
Oral exposure	BALB/c mouse	HSA	No	No	^{125} I-radioactive labelling	[91]
Oral exposure	ddY mouse	Ovalbumin	Egg	Yes	ELISA, Immunoblotting	[71]
Oral exposure, everted sac	Guinea pig, hamster, rabbit	Albumin, γ -globulin, β_2 - γ -globulin	Egg/milk	Assumed	Fluorescence microscopy	[83]
Oral exposure	BALB/c mouse, CD rat rabbit	IgA	No	Assumed	Colloidal gold-conjugation electron microscopy	[47]
Oral exposure	ddY mouse	14–16 kDa rice allergen	Rice	Yes	ELISA, Immunoblotting	[92]

technique, the intestinal segment is mounted in an Ussing chamber where one side will be exposed to buffer with the compound of interest (apical or lumen side) and the other side to buffer without the compound of interest (basolateral or mucosal side).

Heyman et al. and Majamaa et al. both used the Ussing technique with jejunal biopsies from children to study the transport of HRP [77, 82]. However, due to the limited availability of healthy human tissue, intestinal tissue from rabbit [75], piglets [78] or rats [79] is often used.

No literature could be found on intestinal protein absorption using the everted sac technique with human tissue. However, the everted sac technique has been applied for investigating the absorption of human γ -globulin and ovalbumin across the ileum of rabbits and guinea pigs [83], as well as γ -conglutinin absorption in rats [69]. A disadvantage of everted sac method against the Ussing chamber technique is that with everted sac the muscle layer is still present and this may lead to the underestimation of the protein transport value. A major disadvantage of using ex vivo human tissue is its relatively limited viability and low throughput. Normally, ex vivo tissue is used within 2–3 h, because intestinal oedema and disruption of the villi will occur when tissue is incubated in vitro [84]. The disadvantage of using animal tissue may be the inter-species differences in anatomy, physiology, metabolism, diet and micro-biota, which complicates the extrapolation of data to humans [80, 85]. But an advantage of using animal ex vivo tissue is the possibility to investigate the effect of sensitisation on the intestinal absorption of proteins by immunising the tissue in vivo prior to the in vitro studies [29]. Pigs share more physiological and immunological similarities to humans than rodents, and the use of (mini)pigs is becoming increasingly common in nutritional research [86]. Therefore, we recently developed InTESTine™, a medium

throughput alternative for the Ussing chamber technique using intestinal waste tissue from pigs, incubated on a rocker platform in a high oxygen incubator. In this system, viability of tissue could be retained for 2 h and the para-cellular absorption transport resembles that of human intestinal tissue in Ussing chambers (Wortelboer et al., unpublished results) and transport of macro-molecular proteins was studied using radioactive labelled proteins (Verhoeckx et al., unpublished results).

3.2 In vivo assays

The most physiologically relevant way to study intestinal protein absorption is by oral exposure to the protein in vivo, as all types of intestinal cells, blood circulation and mucus are present. Preferably, to elucidate the protein absorption in human, in vivo experiments are performed in humans, as this is the target species. However, the majority of experimental studies on protein absorption described in literature have been performed in animals (Table 2). Consequently, the interpretation of the findings could be difficult due to inter-species differences. Also, the possibility of separating variables that influence protein absorption is limited in in vivo studies [81].

The number of in vivo studies of intestinal protein absorption in humans is limited, though we were able to find some examples. Paganelli and Levinsky measured β -lactoglobulin by RIA in the serum of healthy human subjects, indicating that β -lactoglobulin was present in the blood after 0.5–3 h of milk consumption [89]. Husby and colleagues investigated the absorption of ovalbumin and β -lactoglobulin into the blood of healthy adults by HPLC combined with ELISA. The study revealed that intact ovalbumin was absorbed by seven of eight individuals, whereas β -lactoglobulin was not

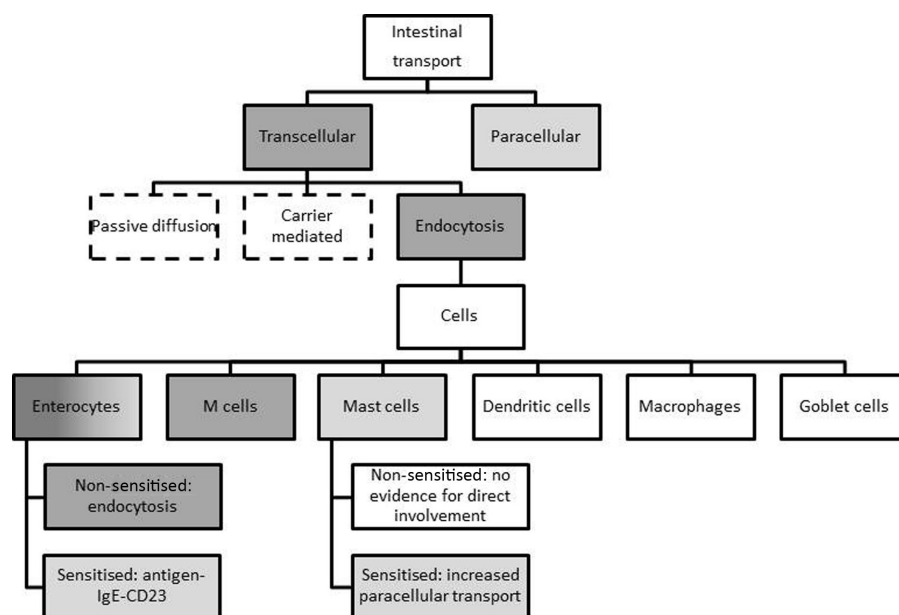


Figure 3. Different mechanisms and cells involved in intestinal protein transport. Boxes indicate they have no (dashed lines), or a possible (no filling) role in intestinal protein transport in sensitised (light grey) or non-sensitised (dark grey) persons.

detected in any of the subjects [93]. Jakobsson et al. measured α -lactalbumin from human milk in the serum of breast fed infants by RIA [88]. In the same project, these researchers discovered that the absorption of α -lactalbumin decreased as age increased, indicating that the intestinal wall becomes less permeable, also mentioned as closure of the gut. A comparable observation was made by Robertson et al., who measured the absorption of cows' milk protein β -lactoglobulin into the blood of preterm and term neonates by RIA [94]. This observation appears to be applicable to all species, although the rate at which the decrease in protein absorption occurs is different between species [95].

It is clear that in *in vivo* protein transport studies many different animal species are used, e.g. rats, mice, guinea pigs, dogs, pigs, calves, cows, steers and sheep [22, 96]. In the absence of a so-called golden standard in predicting or identifying food allergens by use of intestinal transport studies, the use of larger animals, particularly pigs, might be better comparable to the human situation [96].

3.3 Protein analysis

A crucial step in protein transport studies is the analytical method to be used to detect the intact protein. In protein transport studies, proteins are often labelled radioactively [30, 72, 75, 76, 79, 91, 97] or fluorescently [28, 70, 83] for relatively easy detection. However, the detection of fluorescent or radioactive labelled proteins in plasma does not necessarily indicate that the protein is absorbed intact [91, 97]. The same is true for using transmission electron microscopy [46, 47, 61, 83], fluorescence microscopy [28, 61, 70, 83], light microscopy [46, 54], ELISA [16, 71, 76, 90, 92, 97, 98] and/or a RIA [88, 89]. A positive result in an ELISA or RIA could be caused by only a specific part of the protein, so even if

the protein is degraded intra-cellularly it may still be recognised by the antibody used in these assays. Immunoblotting [69, 71, 87, 92] and high-performance liquid (gel permeation) chromatography [16, 30, 79, 99] are good alternatives, since these techniques separate the proteins by their molecular weight, thus proving the presence of intact protein. Another appropriate method, but unfortunately not always possible, is measuring the functional activity of the protein. For example, Heyman et al. used an HRP enzyme activity assay to measure intact HRP, since only intact HRP shows enzyme activity [100]. A more quantitative method, however not applied in the screened literature, is the use of LC coupled to MS. However, the use of these techniques requires special skills and equipment, fragments and intact proteins cannot always be measured simultaneously and the concentration of transported proteins and fragments is often at the lower LOD. Furthermore, the MS spectra of intact proteins are very complex and are difficult to recognise in biological samples containing more than one protein.

4 Conclusion and discussion

New proteins or genetically modified foods are currently assessed for their allergenic potential using an allergenicity assessment strategy advised by the Food and Agriculture Organization and World Health Organization. This strategy is based on a weight of evidence approach that recognises that no single endpoint can be used to predict human allergenic potential [1, 2].

The goal of this review was to study how protein and immune-reactive protein fragments are transported across the intestinal wall, and whether it can be advised to develop a new model for allergenicity risk assessment based on this protein transport.

Proteins are transported across the intestinal barrier via many different routes and these routes appear to depend on the health status of the individual. The different routes and cells involved are summarised in Fig. 3. The data indicate that in non-sensitised persons, absorption of proteins via M cells and trans-cellular transport via enterocytes are considered very important, whereas in sensitised persons, proteins can also be transported via the para-cellular transport route with involvement of mast cells, and trans-cellular via IgE-mediated transport via the CD23 receptor on enterocytes. The current perception is that particulate or aggregated antigens are taken up by M cells, inducing a local or systemic immune response towards that antigen and inducing the production of IgA. Soluble antigens on the other hand are thought to be predominantly absorbed by epithelial cells, leading to suppression of the immune system, induction of tolerance, towards this antigen [11, 52]. For this reason, transport route (M-cells or epithelial cells) and/or transported protein size (intact or fragmented) could be possible parameters to predict the allergenic potential of proteins. However, at the moment more research is needed since no comparative study on proteins with different allergenicity was found in the literature and the studies performed on individual allergens cannot be compared due to inter-laboratory differences, differences in transport methods and the analytical methods used.

Enough evidence was found that intact protein can cross the intestinal wall, which indicates that there is a potential risk of immunological sensitisation. This urges the need to develop a good protocol to determine the route of transport. In our opinion, the use of ex vivo intestinal segments or in vivo experiments is crucial next to a reliable instrumental analytical method (e.g. LC-MS) to analyse intact and fragmented proteins preferably simultaneously.

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