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Applicability of epithelial models in protein permeability/transport studies and food allergy

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Measurement of protein transport across the intestinal barrier might be a relevant approach in allergenicity risk assessment. Traditionally, studies on protein transport, were performed using stable cell lines cultured as a monolayer. One of the major advantages of these models is their relatively low price and easy handling. However, monolayers lack a physiologically relevant environment (presence of other cell-types and a mucus layer), which may have an effect on transport characteristics and thus correct prediction of protein allergenicity. This paper summarizes the most widely used epithelial models and discusses their benefits and limitations for measuring protein transport and allergic sensitization to food.

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Introduction

Incorporation of new proteins into food crops and the introduction of new protein sources onto the food market (e.g. rapeseed) can lead to the introduction of new food allergens, and consequently increasing the risk for the susceptible food-allergic population. For that reason, allergenicity assessment of these new proteins is needed.

In the past 10 years, we have made huge efforts to establish the features that characterize an allergen [1]. However, despite these efforts, we still do not know exactly what makes a protein an allergen. Allergies are complex diseases in which two phases can be distinguished: (1) the *sensitization phase*, in which a protein is exposed to the mucosal immune system, is recognized as an allergen and induces the production of immunoglobulin E (IgE). (2) The *symptomatic phase*, in which IgE is bound to the surface of effector cells via specific receptors (FcεRI) and binding of two IgE molecules with the allergen, which induces the release of inflammatory mediators responsible for allergic symptoms. For both phases, proteins need to come into contact with the immune system and therefore needs to be transported over the protective epithelial layers of our body. Unfortunately, the role of allergen transport and transport route (respiratory, skin or oral) in food

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allergy is relatively unaddressed in the literature. For that reason, it is necessary to expand our knowledge in this field to enhance our understanding on the sensitization mechanism.

Emerging evidence suggests that the skin may be a highly relevant inductive site for allergic sensitization to food proteins. Conversely, some routes of exposure have been proposed to be inherently tolerogenic (e.g. oral and sublingual exposure). Transport via the gut may lead to different immunological responses as well. Transport of soluble milk allergens via epithelial cells led to anaphylaxis, while transport of the aggregated form of the same milk allergen after heating led to sensitization in a cow's milk mouse model [2]. These findings suggest that the transport of soluble protein via villous epithelial cells was the main pathway for anaphylactic responses, while transport of the aggregated forms via Peyer's Patches (PP) was needed for sensitization. Furthermore, defects in the integrity of the epithelial barrier have also been reported in food allergy. Clinical studies in children with cow's milk allergy demonstrated that intestinal permeability increased after, but not before the allergen challenge [2,3]. A recent study based on small intestinal biopsy specimens exposed to food allergens *in vitro*, showed decreased expression of tight junction (TJ) proteins (i.e. occludin, claudin-1, and ZO-1) in tissues obtained from food allergic patients compared to healthy subjects [3]. Both studies suggest, that in sensitized individuals intestinal permeability and the passage of allergens is enhanced. For that reason, evaluation of protein transport across the intestinal barrier and its effect on epithelial permeability might be a relevant parameter in allergenicity risk assessment [4]. The applicability of different epithelial cell models to study these aspects is discussed in the present review.

The intestinal epithelium

The intestinal epithelium is the largest interface between the host and the environment. It regulates fluxes of ions and nutrients and limits host contact with luminal antigens [5]. Anatomically, the intestinal mucosa is divided into three layers: (i) the first, which is closest to the intestinal lumen, consists of a single layer of epithelial cells attached to a basement membrane; (ii) the second layer, the lamina propria, consists of subepithelial connective tissue, immune cells, and lymph nodes; (iii) the third layer is known as the muscularis mucosae and is composed of smooth muscle fibres [6].

The first layer of epithelial cells forms a biochemical and physical barrier which separates microbiota in the lumen from the underlying mucosa (Fig. 1a) [7]. The lymphoid tissue in the mucosa is organized into *inductive sites* (Peyer's patches and mesenteric lymph nodes) and *effector sites* (normal intestinal mucosa), which are responsible for the induction phase of an immune response such as sensitization [8,9].

At the bottom of the crypts of the first layer, a pool of pluripotent stem cells can differentiate into five epithelial cell types: absorptive columnar cells (enterocytes), goblet cells, endocrine cells, Paneth cells, and M (microfold) cells (Fig. 1a) [10]. Goblet cells and Paneth cells secrete, respectively, mucus and antimicrobial proteins (defensins, cathelicidins, and histatins) to protect the epithelial surface from intruding bacteria. M cells and enterocytes mediate transport of luminal antigens and living bacteria across the epithelial monolayer to the underlying lymphoid cells, such as antigen-presenting cells (e.g. dendritic cells and intestinal macrophages) [11]. The permeability and polarity of the first epithelial layer are maintained by the apical junctional complex, which is composed of TJs, adherent junctions, and the subjacent desmosomes (Fig. 1b). Permeability depends mainly on the TJs, which are composed of transmembrane proteins such as occludin, claudin, junctional adhesion molecule A, and tricellulin (Fig. 1b) [5,12].

Transport through the epithelium

Although most dietary proteins are degraded by digestive enzymes and absorbed as amino acids and di/tripeptides, some can resist the gastric environment (pH 1–2 and pepsin hydrolysis). Large immunogenic peptides and intact proteins are capable of reaching the lumen of the small intestine and triggering immune cells in the mucosa [5]. Thus, resistance to gastrointestinal digestion might contribute to allergenicity. However, there are also examples of pepsin-sensitive allergens whose resulting fragments still show IgE-binding activity [13]. It can be envisioned that the combination of gastrointestinal digestion and protein transport is an important factor for allergenicity.

Once the dietary proteins and peptides reach the small intestine, they can be transported across the epithelial intestinal barrier to the underlying basolateral side and distributed throughout the body. **Transport of proteins** across the intestinal mucosa depends on size (influenced by aggregation), polarity, and shape. Proteins can be transported via the *paracellular route* or via *transcellular routes* (Fig. 1c). *Paracellular transport* is the transfer of compounds through the intercellular space and is regulated by the integrity of the TJs [14]. Normally, small hydrophilic compounds (up to 600 Da) are transported by this route, although small proteins (less than 3.5 kDa) can also pass.

Transcellular transport comprises the absorption of compounds via *passive diffusion*, *vesicle endocytosis*, and *carrier-mediated transport* (Fig. 1c). The main route of transcellular protein transport is endocytosis, which is known to occur in different cell types. The transcellular transport of large particles has traditionally been ascribed to M cells overlying Peyer's patches, while soluble particles are transported via the epithelial cells [15]. It has even been suggested that transport via M cells will induce a local or systemic immune response towards the antigen, while soluble antigens transported via enterocytes will lead to suppression of the immune

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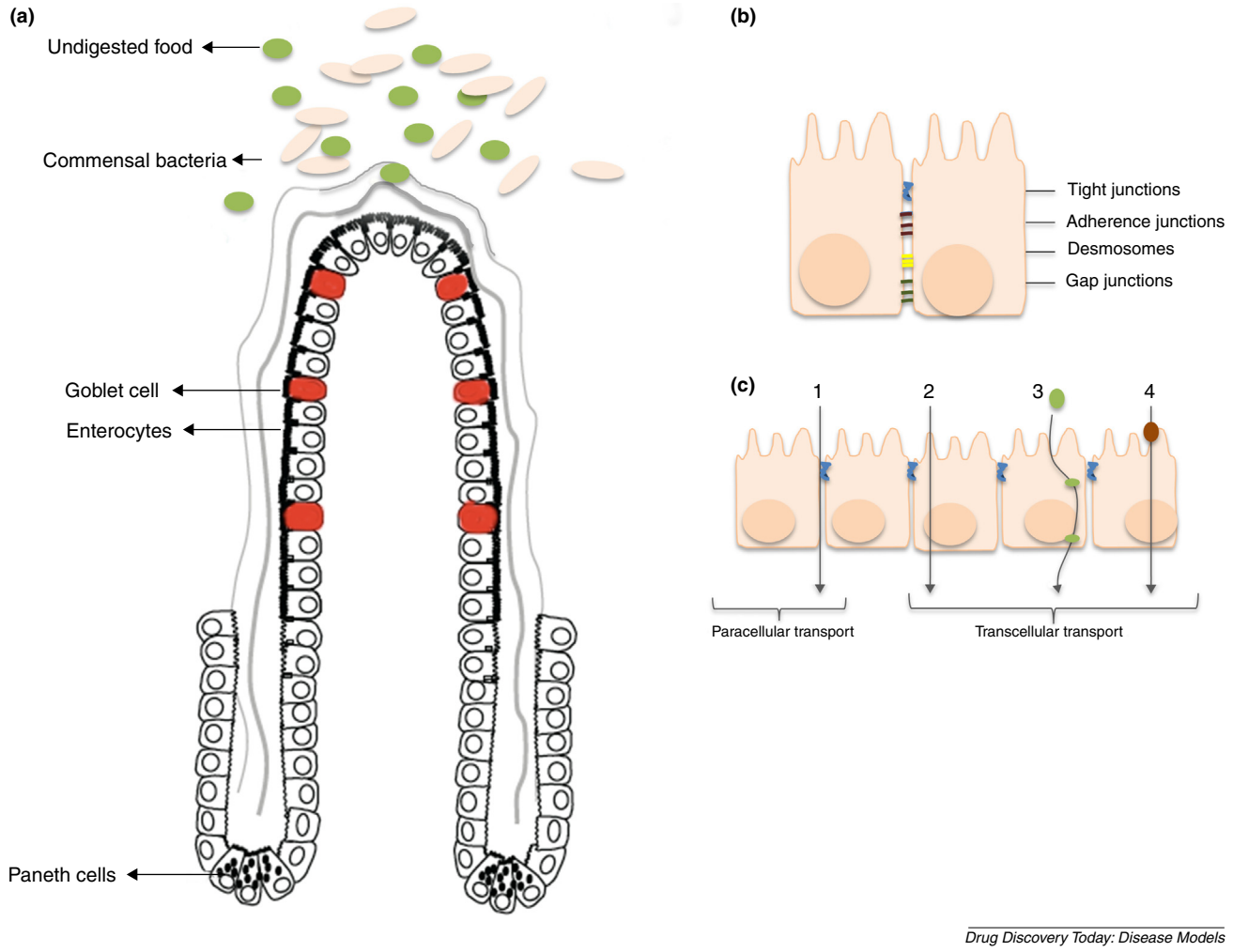

Figure 1. Components of intestinal mucosa. **(a)** Simplified scheme of intestinal cells and structures in the small intestine. **(b)** Schematic drawing of polarized epithelial cells with different types of intercellular contacts. **(c)** Different types of transport through the intestinal epithelium: 1, paracellular transport; 2, passive diffusion; 3, vesicle-mediated transcytosis; 4, carrier-mediated uptake.

system and induction of tolerance to the antigen [16]. The passage of aggregated antigens through M cells in combination of soluble antigens through IEC is thought to be critical in the onset of milk allergy [Roth Walter].

Alternatively, intestinal DCs and macrophages have the capacity to sample directly in the intestinal lumen by extending dendrites between epithelial cells. Antibodies such as IgA, IgG, and IgE can also be involved in enterocytic protein transport in the form of *carrier-mediated transport* [5]. IgE binds to the antigen and the CD23 receptor, which transports the IgE-antigen complex across the cell without lysosomal degradation [17].

Intestinal epithelium models used in protein permeability/transport studies

An epithelial model used to study protein transport should preferentially include all the components of the intestinal mucosa (e.g. mucus layer and epithelial and M cells). However, in practice this is not always feasible, with the result that various models have been developed. These models differ in complexity and applicability (Tables 1 and 2). It is therefore important to define the study objective (e.g. transport via M cells and/or enterocytes, allergenic activity, mucus effect, permeability) in order to identify the best intestinal model [18]. Irrespective of the cell model chosen, the main readout parameters are the effects on epithelial barrier function, absorption, and trans-epithelial transport of the test compound. The reliability of such studies depends on the uniformity and integrity of the confluent and polarized cell monolayer.

Tumor cell line models

Cell lines used to study the transport and absorption of proteins include **Caco-2**, **HT-29**, **T84**, and **IPEC-J2** (Table 1). In all cases, cells are grown in a Transwell system to form a monolayer (Fig. 2).

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Table 1. Comparison of the different cell lines used to study protein transport

	Caco cell line	HT29	IPEC-J2 Cell line
Growth	Grows in culture as an adherent monolayer of epithelial cells	Grows in culture as an adherent monolayer of epithelial cells	Grows in culture as an adherent monolayer of epithelial cells
Differentiation	Takes 14–21 days after confluence under standard culture conditions	Takes 21–28 days after confluence under standard culture conditions	Takes 4–9 days after confluence under standard culture conditions
Cell morphology	Polarized cells with tight junctions and brush border at the apical side	Polarized cells with tight junctions, without ciliar border at the apical side	Microvilli on their apical side and tight junctions sealing neighboring cells together
Mucus production	In co-culture with HT29-MTX	Yes	Yes
Electrical parameters	High electrical resistance	Moderate electrical resistance	High electrical resistance
Digestive enzymes	Expresses typical digestive enzymes, membrane peptidases and disaccharidases of the small intestine (lactase, aminopeptidase N, sucrase-isomaltase and dipeptidylpeptidase IV)	Expresses sucrose-isomaltase, aminopeptidase N, dipeptidylpeptidase-IV and alkaline phosphatase, but lactase is absent.	
Active transport	Amino acids, sugars, vitamins, hormones	Amino acids, sugars, vitamins, hormones	Low active transport
Receptors	Vitamin B12, vitamin D3, EGFR (epidermal growth factor receptor), sugar transporters (GLUT1, GLUT2, GLUT3, GLUT5, SGLT1)	Receptors usually expresses in intestinal epithelium	Receptors usually expresses in intestinal epithelium
Cytokine production	IL-6, IL-8, TNFx, TGF-x1, thymic stromal lymphopoietin (TSLP), IL-15	IL-6, IL-8, TNFx, TGF-B, TSLP, IL-15, IL3, GM-CSF, VGF	IL-1α, -1β, -6, -7, -8, -12A, -12B, -18
Applicability	Studies about protein/drugs transportation	Studies about proteins/drugs transportation	Studies about proteins/drugs transportation
Interaction with immune system	Co-culture with different immune cells	No described	No described
Expenses	Low	Low	Low

Monolayers of human colon carcinoma cell lines, the so-called **Caco-2**, have been extensively used over the last 20 years to predict the permeability of the intestinal mucosa to proteins [19]. The polarized monolayer of these well-differentiated columnar absorptive cells expresses a brush border on their apical surface with typical small intestinal enzymes and transporters. The cells differentiate into a polarized apical and basolateral membrane mimicking the luminal and microvilli side (apical) of the intestine and intercellular TJs. Caco-2 exhibit features of enterocytes of the small intestine. During differentiation, cells progressively express digestive enzymes [20]. Conversely, the electrical properties and ionic conductivity and permeability of the differentiated Caco-2 cells resemble those of the colonic crypt cells [19].

The Caco-2 model has many limitations, such as trans-epithelial electrical resistance (TEER (used to address the integrity of the monolayer)), which is higher in Caco-2 monolayers (up to 500 ohm/cm^2) than in human intestine (12–69 ohm/cm^2) owing to over-expression of TJs [21]. Moreover, no mucus layer is produced on the apical side of Caco-2, thus limiting studies of the protein–mucosa interaction. Despite these limitations, the Caco-2 cell line has proved to be the best model to date to study intestinal absorption and toxicity.

HT-29 cells stem from a human colon adenocarcinoma cell line that contains both absorptive and mucus-secreting cells [19]. Under normal growth conditions, HT-29 cells grow as a multilayer of non-polarized, high glucose-consuming, and undifferentiated cells. When glucose is removed from the growth medium and replaced with a different carbon source, the cells differentiate after three to four weeks in culture, leading to the appearance of both absorptive cells with characteristics similar to those of the differentiated Caco-2 cells and to goblet-like mucin cells. These differentiated cells express brush border-associated hydrolases that are typical for the small intestine. The cells have brush border microvilli even though enzyme activity is much lower than for normal intestinal epithelial cells, and they do not express lactase.

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Table 2. Comparison of different types of culture

	Caco cell line	Organoids	Primary culture
High-throughput	High	Low	Low
Expenses	Low	High	High
Redout	Protein/drugs transportation	Protein and drugs metabolism	Transport and metabolism
Advantage	Easy manage	Maintain the integrity of the mucosa, with all the specialized cells represented	Multi-cell system. All intestinal regions can be used Less labor intensive than Ussing chamber technology
Disadvantages	No mucus, no specialized cell, no 3D structure	The apical side is not accessible	Biological variations Limited viability
Applicability on food allergy	Yes	Yes, specially, interaction with specialized cells	Yes, transport and interaction with specialized cells

The **T84 cell line** is an epithelial model used to study protein transport. The cell line was obtained from a pulmonary metastasis of a human colon carcinoma. When the cells are grown on microporous filter supports coated with collagen, they form a cell monolayer with a highly polarized morphology, few microvilli, and a very high TEER, thus indicating the presence of well-differentiated TJs. Chloride secretion in T84 cells is regulated, as is typical of colonic crypt cells [22,23].

The porcine intestinal enterocyte cell line (**IPEC-J2**) is a non-transformed, permanent intestinal cell line that was originally isolated from the jejunal epithelium of a neonatal unsuckled piglet. The cells have already proven to be a valuable tool in the characterization of epithelial cell interactions with enteric bacteria and viruses providing insight into initial host–pathogen and host–non-pathogen (e.g. commensal or probiotic) interactions [24]. The strength of the IPEC-J2 cell line as an *in vitro* model originates from its morphological and functional similarities with intestinal epithelial cells *in vivo*. No brush border enzyme activity has been described in IPEC-J2 cells. High TEER values and low active transport rates are obtained when IPEC-J2 cells are cultured in fetal bovine serum.

The Caco-2 model is mostly used to study protein/allergen transport. For example Roth Walter *et al.* studied the transport of native and aggregated β-lactoglobuline in a Caco-2 model and compared this with an *in vivo* mouse study [2]. The transport of Ber e 1 and Ses i 1 was studied by Moreno *et al.* [25]. The other epithelial cell models were also used to study allergens. HT-29 was used to study endocytosis of Ara h2 [26], T84 to study effect of Gly m 5 on TJ proteins [27] and IPEC J2 to study Gly m 1 uptake [28].

Co-culture of the Caco-2 cell line and HT29-MTX cells

Co-cultures of Caco-2 cells with HT29-MTX (HT29 cells treated with methotrexate) have been developed in an attempt to overcome the lack of mucus in Caco-2 cultures [21]. Caco-2 and HT29-MTX are derived from intestinal absorptive and goblet cells, respectively. The human intestinal cell line Caco-2 differentiates into enterocytes, while HT-29MTX cells produce mucins, heavily glycosylated proteins that form a surface protecting layer on epithelial cells (mucus). The difficulty of this model is to culture the right Caco-2/HT-29 MTX ratio.

Triple co-culture of Caco-2, HT29-MTX, and **RajiB lymphoma** was recently applied to complete the intestinal mucosa model [29]. RajiB cells can differentiate into a M-cell phenotype by co-culturing with Caco-2 enterocytes. This relevant model is complex owing to the presence of three non-adherent cell lines in RajiB, and the conversion of M-cell has to be closely monitored [30]. Both co-culture models were, to the best of our knowledge, not used for protein transport studies. Rytkonen *et al.* used a co-culture of Caco-2 cells with PP from mice to study the transport of heated and native β-lactoglobulin [31]. However the co-culture of human cells with mouse cells seems a bit odd.

Intestinal organoids

Collectively, the intestinal organoid system (Fig. 2) enables culture and expansion of intestinal stem cells *ex vivo*. Importantly, the resulting epithelial structures faithfully recapitulate the homeostasis and architecture of the functional intestinal epithelium. To date, intestinal organoids have been shown to have multiple applications, including analysis of endogenous stem cell characteristics and gene function, as well as disease modelling [32]. With the recent development of efficient gene-editing tools, it is now possible to rapidly engineer cultured intestinal organoids to generate highly physiological models of human gastrointestinal disease for use as research tools [33]. Despite the requirement for more expensive technology than tumor cell lines, the genetic profile of intestinal organoids seems closer to that of the intestinal cell epithelium. Therefore, this system could be an alternative for the study of protein transport and

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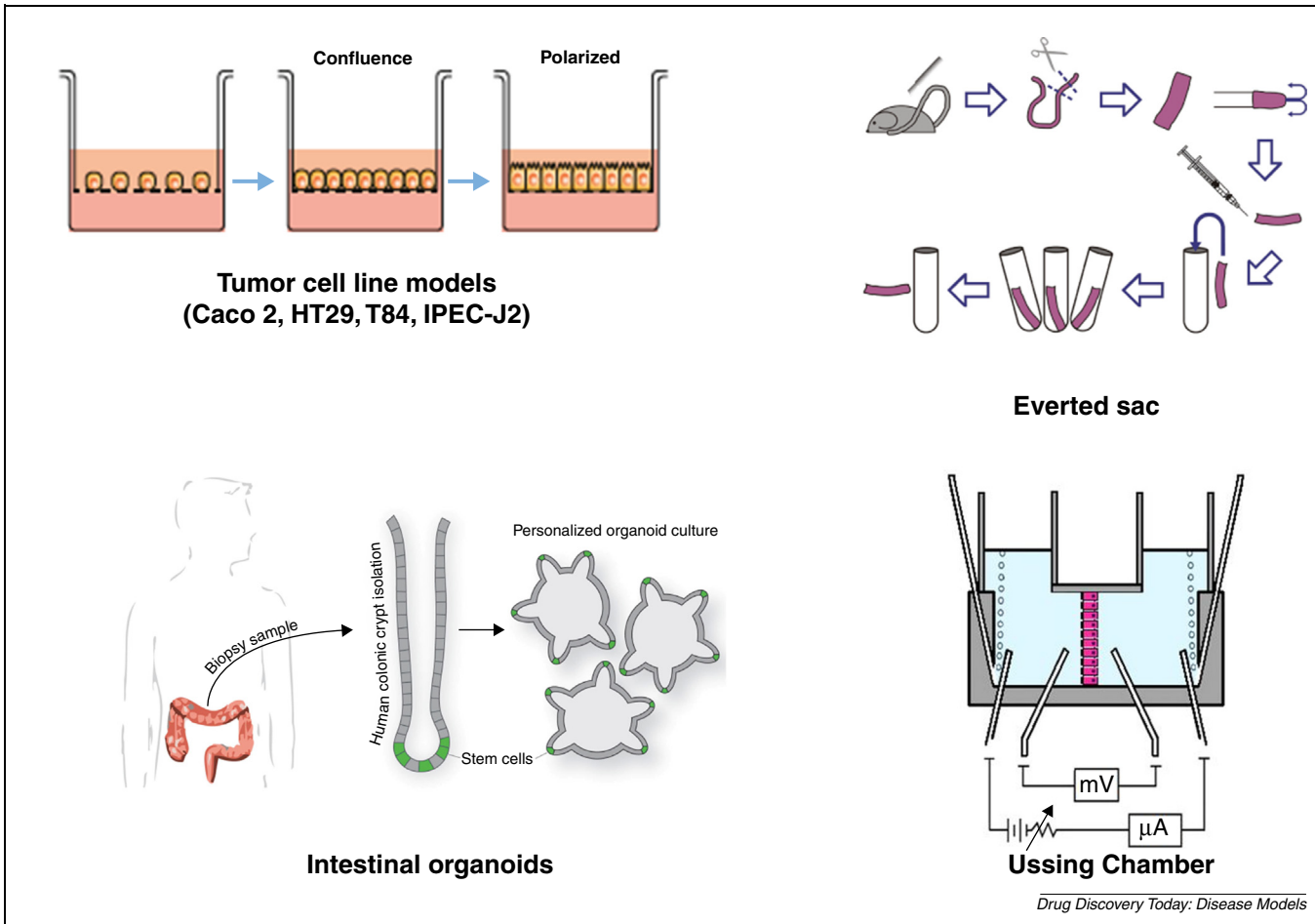

Figure 2. Different types of epithelial models used to study protein transportation. **(a) Tumor cell line models**, epithelial cells are seeded on plastic structures (Transwell) separating the bsasolateral and apical sides and allowing the polarization of the cells. **(b) Intestinal epithelial organoids**, three-dimensional structures of cultured intestinal cells from isolated intestinal crypts including stem cells, allowing to remake a mini-intestine including all cell types. **(c) Everted sac**, intestinal segments are cut and inverted so that the apical side of the mucosa is on the outside. **(d) Ussing Chamber**, an intestine segment placed on an apparatus for measuring epithelial membrane properties in both side and the efficiency of transportation.

associated diseases. However, the inwards orientation of the epithelial cells (directed to the lumen of the organoids), makes the apical side relatively inaccessible for direct experimental stimulation. This will impede protein transport studies. Furthermore, availability of human tissue is often a bottleneck and hinders their possibilities as a standard approach. To the best of our knowledge no literature was found on the use of organoids in allergen transport or permeability studies.

Ex vivo *models*

The major drawbacks of the single cell models described above can be overcome by using intestinal tissues. In these models, the asymmetrical distribution of proteins and lipids in the two plasma membrane domains facing the intestinal lumen, the internal milieu, and the presence of highly organized structures (TJs) joining adjacent cells and separating the two membrane domains, enable selective processes of absorption, transport, and secretion to take place across the intestinal mucosa [34]. The maintenance of these characteristics *ex vivo* is particularly important for the study of absorption, metabolism, and toxicity.

Traditionally, *ex vivo* intestinal models for intestinal protein transport studies have been based on animal tissue. The techniques used include the **everted sac technique** [35] and **the Ussing chamber** [36] (Fig. 2). The everted sac is a segment of animal intestine that is everted and used to assess protein transport. In the case of the **Ussing chamber**, a fresh intestinal segment is mounted into a complex apparatus for measuring protein transport and epithelial membrane properties. Both techniques provide an accurate measurement of intestinal permeability. The most relevant advantage for food allergy is the possibility of studying the effect of sensitization on intestinal protein absorption, using intestinal tissue from sensitized animals [37,38].

However, both techniques have several limitations. First, tissue viability is rapidly lost (2 h); second, the tissue can be damaged during isolation, which may lead to overestimation

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of protein transport. On the contrary, the presence of the muscle layer in the everted sac method may lead to underestimation of protein transport [35]. Third, interspecies differences in anatomy, physiology, metabolism, diet, and microbiota complicate extrapolation of data to humans [4,39]. Pigs share more physiological and immunological similarities with humans than rodents, and the use of minipigs is becoming increasingly common in nutritional research [40].

The recently developed **InTESTine**™ method is a medium-throughput alternative to the Ussing chamber and is based on intestinal tissue from pigs that is incubated on a rocker platform in a high oxygen incubator [41]. The viability of tissue could be retained for 2 h, paracellular absorption transport resembles that of human intestinal tissue in the Ussing chamber [42], and the transport of macromolecular proteins is studied using radioactively labelled proteins [14]. This method seems to be a good alternative that should be evaluated in future studies on food allergy.

Food allergy studies based on epithelial models

It can be envisioned that sensitization to food allergens begins with the transport of these allergens across the intestinal epithelium. Transport of luminal antigens occurs typically via M cells but intestinal epithelial cells also have the capacity to transport luminal antigens across the intestinal wall, but with a different capacity than M cells do [43]. Furthermore, the ability of allergens (intact or fragmented) to cross the epithelial barrier could be based on the increased permeability of TJs or on their immunogenic activity [2,31].

Most studies with food allergens using epithelial models focus on the effect on TJs. For instance, Price *et al.* showed that peanut allergens were able to alter the intestinal barrier permeability and TJ localisation in a Caco-2 model. The allergens passed through the epithelial monolayer by the paracellular pathway [41]. Zhao *et al.* used a T84 porcine model and reported that incubation with β conglycinin from soy (Gly m 5) induced the downregulation of TJ proteins (claudin-3, occludin, and ZO-1) [27]. The study from Cavic *et al.* [44] demonstrated that Act c 1 (actinidin), which is a kiwifruit allergen, exhibits persistent proteolytic activity during digestion. Exposure of T84 cells to this allergen, resulted in impairment of the epithelial barrier, which was related to the degradation of occludin by the proteolytic action of actinin. Furthermore, the alteration of this single TJ protein led to nonselective paracellular transport of allergens. Not all allergens affect epithelial permeability, for example Moreno *et al.* [25] showed that the transcellular transport of purified 2s albumins Ber e 1 (Brazil nut) and Ses i 1 (Sesame seed) within Caco-2 monolayers, did not affect permeability as observed with no change to allergen absorption rate and TEER. The same is true for wheat allergen ω 5 and lipid transfer protein (LTP) [45]. This paper also shows that digestion of the wheat allergen ω 5 protein enhanced their transcellular transport capacity. Besides digestion also other intrinsic properties and processing steps might influence protein transport. Roth-Walter *et al.* showed, *in vitro* and *in vivo*, that pasteurization of the soluble milk protein β-lactoglobulin (which causes aggregation) shifted transport from transcytosis through enterocytes to transport via Peyer's patches. The *in vivo* study also showed that aggregated β-lactoglobulin induced IgE formation (Th2-associated antibody) and Th2 cytokine production (IL-5, IL-13, IFN-γ, IL-10) with respect to the soluble β-lactoglobulin. The findings of this study suggested that the transport of soluble protein via villous epithelial cells was the main pathway for anaphylactic responses, while transport of aggregates via PP induced sensitization [2]. So it can be hypothesized that parameters such as transport route (M-cells or epithelial cells) and/or transported protein size (intact or fragmented) could help us to predict the allergenic potential of proteins, but more tests are needed to confirm this.

Both increased the permeability of the epithelial T84 monolayer, and thus affected the apical-to-basal movement of proteins, such as horseradish peroxidase, through both the transcellular and paracellular pathway [46]. Moreover, there is evidence that mediators, released from mast cells (e.g. tryptase and tumor necrosis factor alpha) contribute to increased epithelial paracellular permeability [47]. The latter will take place in already sensitized individuals.

In summary, we can make the following assumptions: (1) allergens may affect TJs (e.g. protease activity), (2) digestion and processing influence protein transport, (3) allergens must cross the gastric barrier in an immune reactive form, (4) size and solubility determines transport route and immunological response, and (5) immunological status (release compounds mast cells and Th2 cytokines) may increase paracellular transport (sensitized persons). However, further studies with food allergens in the models described above are required in order to clarify the precise scenario for proteins necessary to induce food allergy.

Conclusions

Unfortunately, there is lack of data on the transport capabilities of many food allergens and their route of exposure to the mucosal immune system. It is highly likely that gut permeability and allergen transport play a role in the development of food allergy or tolerance. However, more data is needed on the permeability and absorption of food allergens to draw any conclusions regarding the influence of intestinal permeability on the allergenic potential of proteins. Furthermore, the combination of processing and digestion on permeability should be explored, since they may have a huge effect on the transport capacities of allergens and thus the immunological response thereafter. Understanding the role of protein transport and gut permeability, will help us to develop better

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in vitro models to measure these important parameters and to predict allergenicity of new proteins, in the future.

Conflict of interest

The authors declare that they have no conflicts of interest.

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