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Symposium on 'Dietary influences on mucosal immunity'

How dietary antigens access the mucosal immune system

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The intestinal epithelium is a selective barrier where incompletely-digested food antigens are transmitted to the immune system. Food antigens are often the starting point of intestinal diseases such as food allergy or coeliac disease. The intestinal epithelial cells (IEC) take up and process food antigens mainly by fluid-phase transcytosis involving two functional pathways, one minor direct pathway without degradation and another major lysosomal degradative pathway. Among the peptidic metabolites generated during transepithelial transport of luminal antigens, some have a molecular mass compatible with a binding to restriction (major histocompatibility complex; MHC) molecules; the latter can be up regulated on enterocytes, especially in inflammatory conditions. Indeed, interferon-γ not only increases the paracellular absorption of antigens, but also their transcytosis across epithelial cells. It has been reported that enterocytes may even directly present peptidic epitopes to underlying T-cells. As a new potential way of transmitting peptidic information to the local or systemic immune system, the secretion by IEC of antigen-presenting vesicles called exosomes and bearing MHC–peptide complexes has recently been proposed. Many other factors such as nutritional or environmental factors can also influence the properties of the epithelial barrier and the outcome of the immune response to lumen antigens.

Intestinal absorption: Food antigens: Food allergy: Epithelial barrier: Mucosal immunity

The initiation of an immune response to ingested food antigens requires an interaction between these antigens and the gut-associated lymphoid cells, and the mechanisms and pathways by which dietary antigens access the mucosal immune system are of major importance in the outcome of the response. Although normal immune response to ingested food antigens is mainly oral tolerance, dietary antigens are sometimes the starting point of intestinal diseases such as food allergy or coeliac disease, where specific antigens are responsible for an abnormal stimulation of the mucosal immune system.

Most ingested proteins are digested within the gut lumen by secreted enzymes or peptidases inserted in the enterocyte microvillus membrane. However, a small quantity of intact antigen does escape this enzymic breakdown and is available for intestinal absorption. The resistance to enzymic hydrolysis (Mahé *et al*. 1991) is highly variable and often conditions the allergenicity (Astwood *et al*. 1998). For example, cow's milk proteins are degraded by sequential digestion with pepsin and trypsin, but β-lactoglobulin, the major allergen in milk, is more resistant to hydrolytic enzymes than α-lactalbumin or caseins (Marcon-Genty *et al*. 1989). Indeed, after milk ingestion in healthy volunteers, about half the β-lactoglobulin is recovered in an intact form at the jejunal level and is available for intestinal absorption (Mahé *et al*. 1991). The two pathways for intestinal absorption of lumen antigens (paracellular *v*. transcellular) as well as the influence of local factors (intestinal maturation, infection, inflammation) seem to play an important role in the activation or suppression of local immune cells.

Intestinal epithelial cells

To be presented to the immune system, exogenous antigens are directly taken up by professional antigen-presenting cells such as dendritic cells or B-cells, and processed before being expressed at the plasma membrane as major

Abbreviations: HRP, horseradish (*Armoracia rusticana*) peroxidase; Ig, immunoglobulin; MHC, major histocompatibility complex.

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histocompatibility complex (MHC) class II–peptide complexes and presented to naive or memory $CD4^+$ T-cells.

After oral administration, however, food antigens do not interact directly with professional antigen-presenting cells and T-cells distributed within the lamina propria, but need to cross the intestinal barrier before being recognized. The pathway and mechanism of transepithelial antigen transport appear to be a critical regulatory step in the outcome of the immune response (allergy *v*. oral tolerance). There is evidence that tolerance after feeding is dependent on gut processing of fed antigens, and that 'gut-processed' antigens can transfer tolerance (Bruce & Ferguson, 1986; Furrie *et al*. 1995). The precise role of the intestinal epithelium in this phenomenon is not clearly understood. The intestinal epithelium is composed of absorptive cells joined at their apical poles by junctional complexes preventing the passage of macromolecules between cells. In addition, mucus and entrapped secretory immunoglobulin (Ig) A also inhibit the absorption of lumen dietary antigens. Nevertheless, the enterocytes do have the capacity to transport a small proportion of antigenic material from the intestinal lumen to the underlying tissues by transcytosis. The evidence that enterocytes are involved in intestinal transport of food antigens was provided initially in rats by Walker's group in the 1970s (Cornell *et al*. 1971), and this concept was extensively confirmed later, including in human intestine where it was reported that β-lactoglobulin was able to enter epithelial cells (Wheeler *et al*. 1993) of the duodenum, and to cross the intestinal mucosa (Saidi *et al*. 1995).

In order to understand how food proteins are endocytosed by epithelial cells and how they are processed during their transepithelial transport, *in vitro* studies have been developed, using Ussing chambers, to analyse transmucosal transport of proteins across intestinal fragments from experimental animals or endoscopic biopsies from human intestine (Heyman & Desjeux, 1996).

Using radiolabelled material, it was shown that >90% of the endocytosed protein is degraded during transepithelial transport, while 10% is transported intact to the serosal compartment (Heyman *et al*. 1982). Both the direct and degradative pathways are transcellular, at least under normal conditions, as shown by the inhibitory effect of metabolic inhibitors and cytoskeleton-disrupting drugs on the transepithelial transport. Paracellular diffusion of antigens through the tight junctions joining epithelial cells is negligible under physiological conditions, since the integrity of tight junctions is maintained even at sites of desquamation (Madara, 1990).

Food antigens can be absorbed in the stomach (Curtis & Gall, 1992), small intestine (Ducroc *et al*. 1983) and colon (Heyman *et al*. 1989*a*). Transcytotic activity increases from the proximal to the distal part of the digestive tract, with the degradative pathway predominating, particularly in the distal intestine (M Heyman, unpublished results). This observation might be due to the stimulating role of the intestinal microflora on antigen transport. Indeed, absorption of the model protein horseradish (*Armoracia rusticana*) peroxidase (HRP) is four times lower in germ-free mice than in conventional mice (Heyman *et al*. 1986).

Intestinal epithelial cells and antigen presentation

Intestinal epithelial cells have been described as non-professional antigen-presenting cells (Bland & Warren, 1986; Kaiserlian *et al*. 1989; Hershberg *et al*. 1997), at least *in vitro*. The studies on epithelial antigen processing and presentation were stimulated by the discovery of MHC molecule expression by absorptive enterocytes (Mayer & Shlien, 1987; Brandtzaeg *et al*. 1992). Thus, one might postulate that MHC class II molecules (human leucocyte-associated antigen-DR), which are moderately expressed in basal conditions *in vivo*, and highly up regulated in inflammatory conditions, may interfere with the nature and/or the quantity of antigen-derived peptides formed during transepithelial transport. Indeed, a recent study has shown that the processing of HRP by the HT29-19A intestinal cell line led to the production of 10% intact protein, 40% peptides and 50% amino acids (Terpend *et al*. 1998) during apical to basolateral transport, indicating that half the protein endocytosed may still retain the potential to react with immune cells (Fig. 1). It is possible that binding to restriction molecules protected peptides and proteins from a total degradation. Interestingly, the peptides formed during the transepithelial transport had a molecular mass

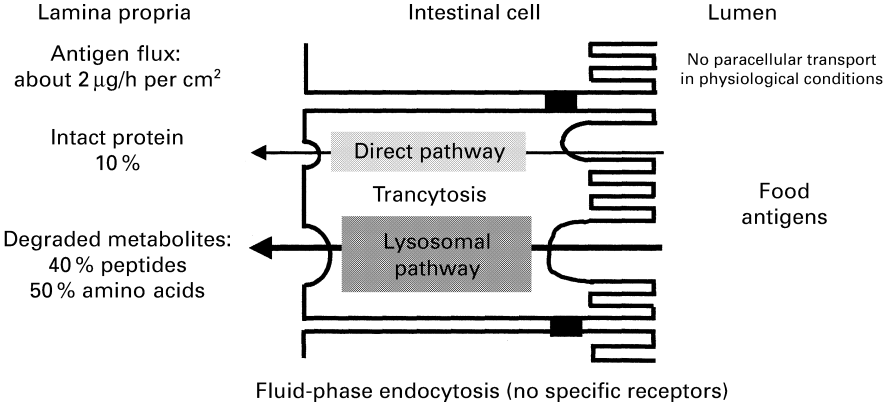

Fig. 1. Diagrammatic representation of the absorption of food antigens by intestinal epithelial cells as exemplified by the processing of horseradish (*Armoracia rusticana*) peroxidase by the HT29-19A intestinal cell line during apical to basolateral transport. (Data from Terpend *et al.* 1998.)

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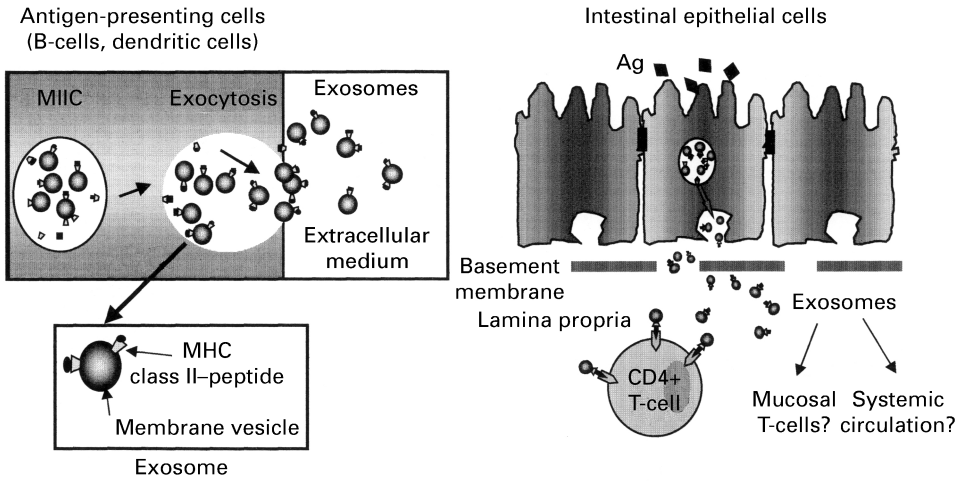

Fig. 2. Diagrammatic representation of the process by which intestinal epithelial cells can present peptides to $CD4^+$ T-cells. MHC, major histocompatibility complex; MIIC, MHC class II–enriched compartment; Ag, antigen. (From Van Niel *et al.* 2001.)

(approximately 1100 Da) compatible with a binding to restriction molecules.

Recently, Hershberg re-evaluated the antigen-presenting capacity of intestinal cell lines and showed that it is highly dependent on the extent of intestinal inflammation (Hershberg *et al*. 1997), and that it is polarized to the basolateral membrane (Hershberg *et al*. 1998). One of the remaining questions is to understand how epithelial cells can present peptides to $CD4^+$ T-cells, since most of the intra-epithelial lymphocytes are $CD8^+$ T-cells, and $CD4^+$ T-cells are separated from the enterocytes by a basement membrane, limiting their direct interactions with epithelial cells. Recent studies have identified that intestinal epithelial cells secrete exosome-like vesicles which express MHC class II–peptide complexes, at least in inflammatory conditions (Van Niel *et al.* 2001; Fig. 2). These exosomes may act as messengers to transmit peptidic information to non-adjacent T-cells. Indeed, exosomes are small membrane vesicles secreted by various cell types. Professional antigen-presenting cells such as dendritic cells or B lymphocytes secrete MHC class I- and class II-carrying exosomes that stimulate T-cell proliferation *in vitro* (Denzer *et al*. 2000). The role of epithelial-derived exosomes in the induction of oral tolerance or hypersensitivity is currently a matter of investigation. On the other hand, it is noteworthy that either differential induction of immediate-type hypersensitivity or T-cell proliferation may occur, depending on changes in peptide structure or MHC haplotype, at least when conventional antigen-presenting cells are concerned (Soloway *et al*. 1991). This possibility might explain why an association between human leucocyte-associated antigen haplotypes and susceptibility to various diseases such as coeliac disease has often been reported.

Factors influencing antigen absorption

Intestinal absorption of food antigens is highly dependent on developmental or environmental factors, including maturity of the intestinal mucosa, sites of absorption, presence of inflammation or infection, and intestinal microflora.

Intestinal maturation

During the neonatal period, gut closure to macromolecules varies greatly according to the animal species. In man, although the intestinal permeability seems to be increased in premature infants (Axelsson *et al*. 1989), gut closure occurs rapidly, and growth factors in colostrum activate the maturation of the intestinal mucosa so that a normal permeability is rapidly observed after birth (Heyman *et al*. 1988). Not only the age of the host but also the stage of maturation of epithelial cells along the crypt–villus axis influence antigen uptake. The intestinal mucosa comprises mature villus cells and immature crypt cells located in the proliferative compartment. *In vitro* studies in mice (Fig. 3) have indicated that immature crypt cells endocytose more proteins than mature villus cells (Heyman *et al*. 1989*b*). These results may be important, since in many pathological situations the increased crypt:villus may explain an enhanced permeability to macromolecules.

Sites of absorption

The location of antigen sampling is not confined to the absorptive epithelium, but also takes place in the epithelium overlying Peyer's patches (Ducroc *et al*. 1983). Peyer's patches are secondary lymphoid organs recognized as the inductive site where the priming of naive T- and B-cells for mucosal immune responses to particulate antigens such as viruses or bacteria occurs, whereas the adjacent absorptive epithelium is rather an effector site with memory T-cells and IgA-secreting plasma cells (Fig. 4). They may also participate, at least partly, in the acquisition of oral tolerance for soluble antigens (Gonnella *et al*. 1998). In Peyer's patches M-cells are specialized in the rapid transport of particulate antigens, mainly bacterial or viral antigens, and due to their weak lysosomal system and their close proximity to antigen-presenting cells, they can transmit native antigens to underlying immune cells. Soluble antigens are transported by the absorptive epithelial cells; they are mostly degraded during their transport, but peptides or protein fragments can reach

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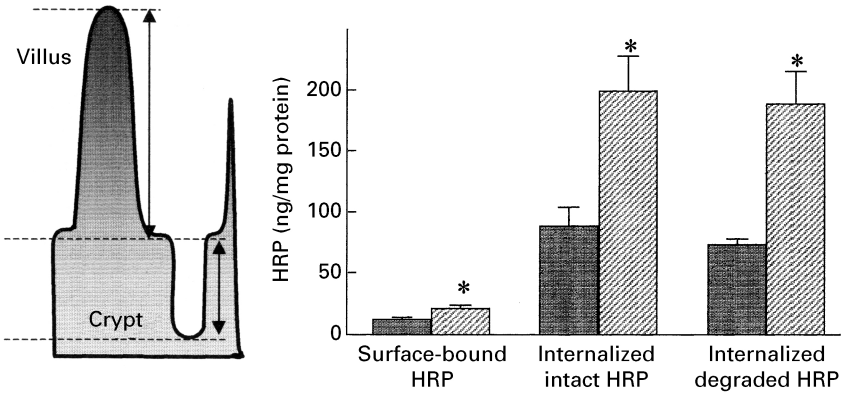

Fig. 3. Results of an *in vitro* study showing that mouse immature crypt cells (▩) endocytose more horseradish (*Armoracia rusticana*) peroxidase (HRP) than mature villus cells (▭). Values are means with their standard errors represented by vertical bars for ten measurements. Mean values were significantly different from those for mature villus cells: $*P < 0{\cdot}05$. (From Heyman *et al.* 1989*b*.)

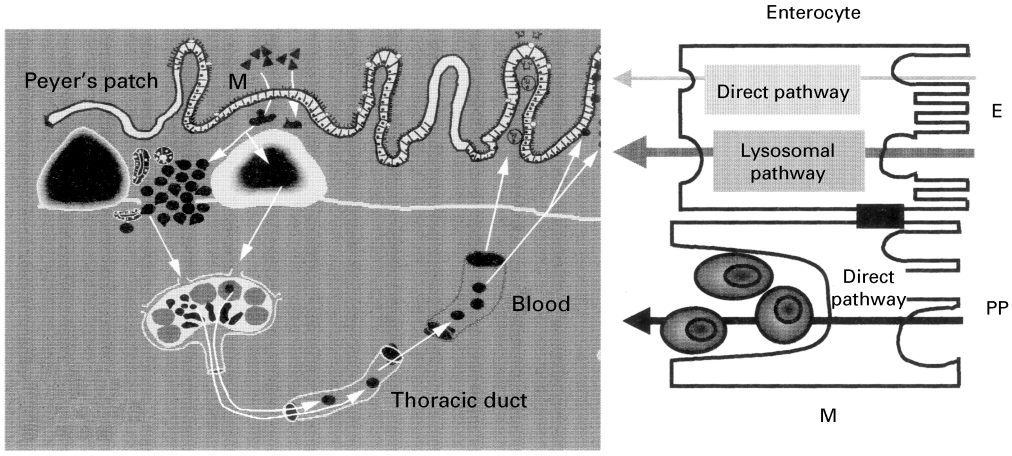

Fig. 4. Diagrammatic representation of the sites of antigen absorption showing that antigen sampling is not confined to the absorptive epithelium (E), but also takes place in the epithelium overlying Peyer's patches (PP). (○), T blast; (●), immunoglobulin A blast; •, secretory immunoglobulin A; ◂, antigens, M, M-cells.

the lamina propria and interact with the mucosal immune cells.

Intestinal inflammation or infection

Most probably, the way the antigen is seen by the mucosal immune system is important in order to elicit either an immunogenic or a tolerogenic response. Inflammation and infection are situations in which antigen-presenting cells are more likely to induce an activation rather than a suppression of T-cells. This situation is probably due to the fact that pro-inflammatory cytokines not only up regulate antigen-presenting molecules and co-stimulatory molecules on immune cells, but also activate the transport of antigenic material across the gut. Interferon-γ is one of the pro-inflammatory cytokines, together with tumour necrosis factor α, which modifies the transport pathways of antigenic macromolecules. Interferon-γ increases paracellular transport of HRP, first by disrupting tight junctions between epithelial cells (Madara & Stafford, 1989), but also by increasing the rate of transcellular transport (transcytosis) across the epithelial layer. Interferon-γ increases total protein fluxes without modifying, importantly, the intracellular processing of antigens (Terpend *et al.* 1998; Fig. 5). Other cytokines such as tumour necrosis factor α have been shown to increase the permeability of HT29-19A cell monolayers and to damage tight junctions, as shown by freeze-fracture etching (Rodriguez *et al.* 1995). This alteration in the tight junctions between epithelial cells leads to a paracellular leakage and to an increase in the antigenic load transmitted to the mucosal immune system.

The quantity of antigen absorbed, together with the effect of enteric infections and inflammation, are thought to influence the outcome of antigen presentation to underlying T-cells and to direct the immune response toward tolerance or allergy. During viral or bacterial infections of the digestive tract, intestinal permeability to food antigens generally increases, due to the epithelial damage induced by infectious agents and to the inflammatory reaction. In the context of such an inflammatory environment, the local antigen-presenting cells (mainly dendritic cells) are switched from a tolerogenic state to an immunogenic state

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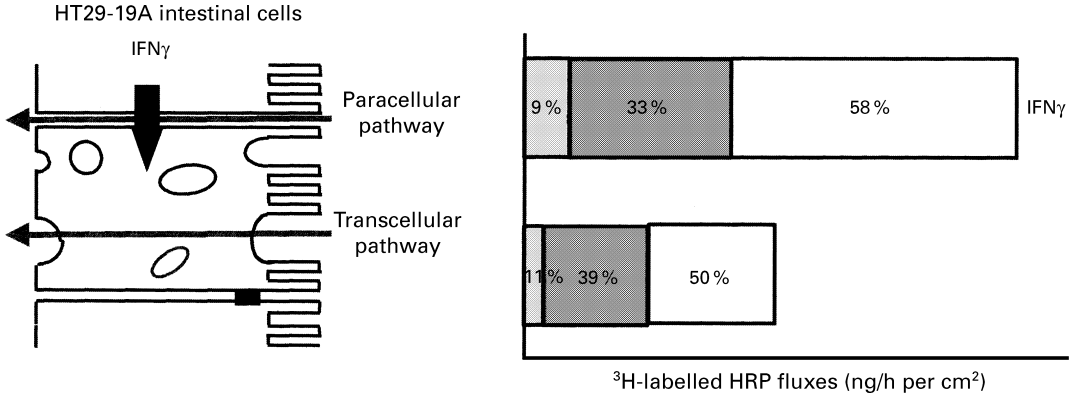

Fig. 5. The effect of interferon-γ (IFNγ) on the transport pathways of antigenic macromolecules. IFNγ increases paracellular transport of horseradish (*Armoracia rusticana*) peroxidase (HRP) by disrupting tight junctions between endothelial cells and by increasing the rate of transcellular transport across the endothelial layer, without modifying the intracellular processing of antigens. (▤), Intact HRP; (▬), peptides; (▭), amino acids. (Data from Terpend *et al.* 1998.)

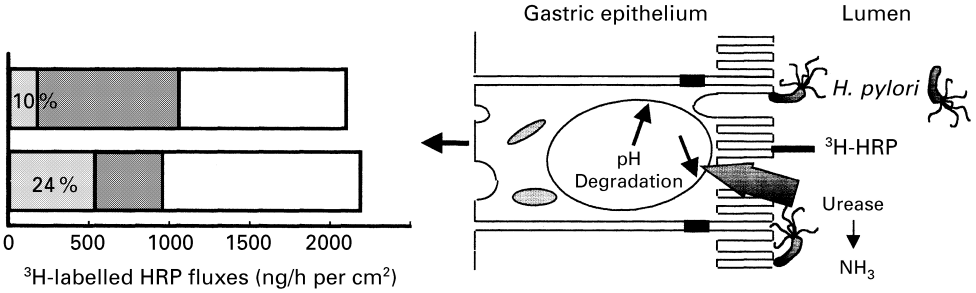

Fig. 6. The effect of *Helicobacter pylori* on the processing of horseradish (*Armoracia rusticana*) peroxidase (HRP) by the gastric epithelium. *H. pylori* secretes high amounts of an enzyme involved in ammonia production. Ammonia increases lysosomal pH and thus inhibits acid proteases in enterocytes, resulting in an increase in intact HRP (▤) transport. (▬), Peptides; (▭), amino acids; ^{3}H-HRP, ^{3}H-labelled HRP. (From Matysiak-Budnik *et al.* 1998.)

(Williamson *et al.* 1999). Few studies have reported a clear link between increased uptake of food antigens and the development of food allergy (or the breakdown of oral tolerance). Infections, by disrupting the epithelial barrier and increasing antigen absorption, may lead to intestinal dysfunction and persistant diarrhoea. Rotavirus infection, which is the most frequent cause of diarrhoea in childhood, has been shown to disturb antigen handling by the gut (Heyman *et al.* 1987). Bacterial enteric infections also lead to intestinal lesions, such as the effacement of microvilli or damage to the intercellular junctions by cytotoxins (Heyman *et al.* 1989*a*). These lesions may interfere with the barrier function of the epithelium or with the endocytic capacity of enterocytes (Heyman *et al.* 1989*a*; Philpott *et al.* 1998). It was reported recently that the gastric pathogen *Helicobacter pylori* induces altered protein processing by the digestive epithelium, leading to increased transepithelial transport of intact proteins. In fact, this increase in intact protein transport is due to an inhibition of the lysosomal degradation of the protein during intestinal transport. *H. pylori* secrete high amounts of urease, an enzyme involved in the production of NH_3. This lysosomotrophic weak base is responsible for the increase in the lysosomal pH and thus inhibits acid proteases (cathepsins) in enterocytes (Matysiak-Budnik *et al.* 1998; Fig. 6). This result may be linked to the observation that persistent gastric inflammation is often observed, even after eradication of the bacteria, suggesting that sensitization to bystander food antigens may have occurred. This hypothesis is strengthened by the observation that *H. pylori* infection is often associated with the development of allergic disorders (Corrado *et al.* 1998; Figura *et al.* 1999).

Food allergy

Abnormalities in intestinal permeability are associated with an inflamed gut and, conversely, allergic inflammation is involved in the epithelial dysfunction and altered antigen handling. It is now well recognized that a constitutive abnormality in intestinal antigen handling is not the primary cause for the development of food allergy, but rather a secondary phenomenon. This phenomenon has been illustrated by studies on macromolecular absorption in jejunal biopsies from infants with cow's milk allergy, during the active symptomatic phase of the disease and after treatment with a cow's milk-protein-free diet. During active cow's milk allergy, there was an eightfold increase in the absorption of the bystander protein HRP, an increase in Cl^- secretion and an alteration of the epithelial integrity (Heyman *et al.* 1988). After several months on a cow's milk-free diet, i.e. during

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the symptom-free period, the protein absorption, Cl^- secretion and paracellular permeability had returned to normal values, indicating that the increased intestinal permeability to antigens was not the primary cause of the disease.

Subsequent experiments analysing intestinal biopsies from children with cow's milk allergy clearly confirmed the role of cow's milk antigens in the alteration of antigen handling by the gut (Saidi *et al*. 1995). In these studies bovine β-lactoglobulin (the sensitizing antigen) and human α-lactalbumin (a self antigen) were placed on the luminal surface of the jejunal biopsies from infants with active cow's milk allergy or from treated (symptom-free) infants. In infants with active allergy, β-lactoglobulin (but not human-α-lactalbumin) induced an increase in Cl^- secretion, and an increase in ionic conductance, an index of epithelial integrity, whereas no such effects were observed in treated allergic infants. Moreover, the absorption of the sensitizing antigen, β-lactoglobulin, was faster and higher in active cow's milk-allergic infants than in treated infants. These results suggested that in the sensitized human intestinal mucosa β-lactoglobulin triggers the release of mediators, probably mast cell mediators, in turn altering intestinal function.

Cow's milk allergy displays an heterogeneous expression, and can be associated with a subnormal small intestinal mucosa with increased IgE plasma cells, or with a partially or totally flat intestinal mucosa in cow's milk-sensitive enteropathy (Host, 1997). In children with cow's-milk allergy oral provocation with milk antigens induces the release of the pro-inflammatory cytokine tumour necrosis factor α, and of eosinophil cationic protein (recovered in faeces; Majamaa *et al*. 1996; Kapel *et al*. 1999). In parallel, it has been reported that mononuclear cells from infants with active cow's milk allergy and intestinal symptoms release more tumour necrosis factor α after stimulation than those of children having recovered from the disease (Heyman *et al*. 1994).

Recently, a major breakthrough in the understanding of the role of the intestinal epithelium in the absorption of food antigens in sensitized individuals has been reported. It was shown that CD23, the low-affinity receptor for IgE, had an important role to play in the absorption of allergen by intestinal epithelial cells (Yang *et al*. 2000). The authors showed that in HRP-sensitized rat jejunum CD23 was up regulated at the surface of intestinal epithelial cells. In these animals the increased absorption of HRP was inhibited by anti-CD23 antibodies. In addition, IgE was shown to mediate the CD23-dependent HRP absorption, indicating that in allergic animals the sensitizing antigens are specifically transported as IgE–antigen complexes through a CD23-mediated transport. This receptor-mediated transcytosis may decrease the degradation of allergens during their transepithelial transport, and therefore may contribute to strengthen the allergic reaction.

Intestinal microflora

Another important factor in the development of oral tolerance and in antigen absorption is the intestinal microflora, not only the commensal microflora, but also probiotics such as lactic acid bacteria or bifidobacteria present in fermented milks and shown to present beneficial effects to the host. Intestinal microflora is mandatory to the establishment and maintenance of oral tolerance. Indeed, Moreau and co-workers (Moreau & Corthier, 1988; Gaboriau-Routhiau & Moreau, 1996) showed that the recovery of oral tolerance after a transient breakdown mediated by bacterial toxin, was possible in conventional mice but not in germ-free mice, indicating the importance of the gut flora in the establishment of oral tolerance. The beneficial effect of probiotics in the stabilisation of the epithelial barrier has been demonstrated in various animal models. In neonatal rats fed cow's milk the permeability to HRP increased, but milk supplemented with *Lactobacillus casei* strain GG restored a normal permeability, suggesting a protective effect of the lactic acid bacteria (Isolauri *et al*. 1993). In another recent study feeding guinea-pigs with a milk fermented with *Bifidobacterium breve* and *Streptococcus thermophilus* was shown to reinforce the intestinal barrier to HRP and β-lactoglobulin (Terpend *et al*. 1999). In this context, it is interesting to note that non-pathogenic enteric bacteria, interacting directly with a model human epithelium, have recently been shown to attenuate the synthesis of pro-inflammatory effector molecules (nuclear factor kappa β) elicited by diverse pro-inflammatory stimuli (Neish *et al*. 2000). Thus, the use of non-pathogenic enteric organisms such as probiotics are now being explored as therapeutic agents in inflammatory bowel disease (Gupta *et al*. 2000).

Altogether these results suggest that both the bacteria of the resident intestinal microflora and probiotic bacteria are important in maintaining intestinal homeostasis.

Conclusion

Dietary antigens incompletely degraded by digestive enzymes are delivered in an antigenic form into the intestinal lumen. A small proportion is endocytosed and processed by intestinal epithelial cells, and a small proportion reaches the serosal compartment in an intact form, while the major proportion is transferred as amino acids and peptidic epitopes which may be free or linked to exosome-like vesicles. It is likely that intact proteins can interact with professional antigen-presenting cells such as dendritic cells in the lamina propria, or in the case of a sensitized host, with IgE bound to the mast cells, whereas peptides may be directly presented to T-cells. Normally, these interactions lead to the development of oral tolerance. The importance of mucosal permeability in the development of food allergy or sensitization processes is difficult to rank, because, on the one hand, oral tolerance has been shown to be linked to the ingestion of large amount of food antigens, leading to clonal anergy, but on the other hand, food allergy occurs frequently after a gastroenteritis, a condition associated with an increased intestinal permeability to food antigens. Thus, it seems that to develop an abnormal immune response to food antigens, not only the amount of antigen absorbed is important, but also the way the antigen is encountered by the gut-associated lymphoid tissue. It seems that an infectious environment is capable of playing an adjuvant role, probably by allowing dendritic cells to

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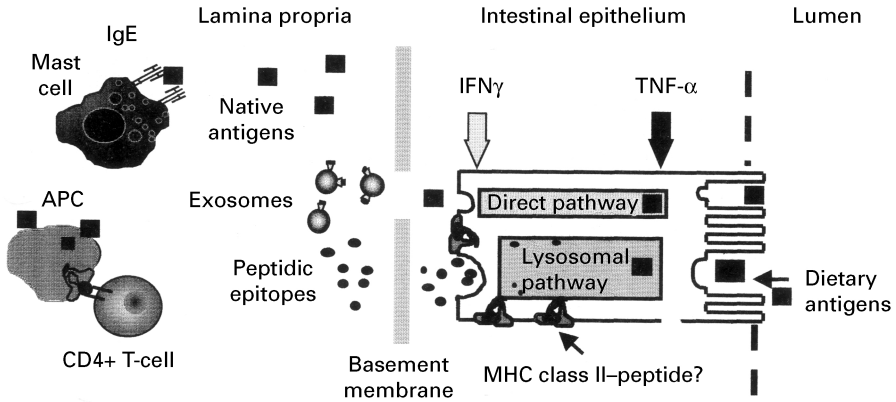

Fig. 7. Diagrammatic representation of the outcome of antigen presentation, showing that it differs depending on the inflammatory environment. APC, antigen-presenting cell; IgE, immunoglobulin E; IFNγ, interferon-γ; TNF-α, tumour necrosis factor α. MHC, major histocompatibility complex.

express co-stimulatory molecules, and elicit an immunogenic response rather than the normal tolerogenic response (Fig. 7).

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