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Jani Rytkönen

EFFECT OF HEAT DENATURATION OF BOVINE MILK BETA-LACTOGLOBULIN ON ITS EPITHELIAL TRANSPORT AND ALLERGENICITY

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Abstract

Beta-lactoglobulin (β-lg) is the main whey protein in bovine milk. It belongs to the lipocalin protein family, and it is one of the main milk allergens. Resistance to hydrolysis is a particular feature of β-lg making it possible that β-lg reaches the small intestine in its native form. Heat treatments during milk processing may change the native structure of bovine β-lg and change its intestinal transport properties. Heat induced conformational alterations may also expose new antigenic sites. However, there have been no previous studies on the effects of heat treatment on the transport of β-lg or on its sensitizing properties.

Cow's milk allergy is one of the most important food allergies affecting about 2.4% of infants. Milk proteins, including β-lg, in breast milk substitute formulas are often the earliest foreign antigens in the diet of newborns. According to the hygiene hypothesis, natural infections and vaccinations may modify the immunological balance and decrease the risk of allergy.

Isoelectric precipitations followed by anion exchange and gel filtration were used to purify bovine milk β-lg in its native form. Transport of native and heat-denatured β-lg was compared in two *in vitro* cell models, Caco-2 and M-cells. Sensitization properties of native and heat-denatured β-lg were studied with an animal model using Hooded-Lister rats. Effects of BCG vaccination in combination with the native β-lg were also studied. Effects of different sensitizations were assessed by antibody levels in serum and inflammation locally in the gastrointestinal tract.

Heat denaturation of β-lg made its transport slower in both enterocytes and M-cells. M-cells were more effective transporters of both native and heat-denatured β-lg than caco-2 cells. Animals generated higher levels of IgE when sensitized with native β-lg, but heat-denatured β-lg induced a more intense inflammatory cell reaction in the gastrointestinal tract. Vaccination with BCG decreased serum IgE concentration and modified the predominant site of the inflammatory cell response in intestine.

The results indicate that, heat denaturation of β-lg and BCG vaccination, change both the systemic and the mucosal response to bovine milk β-lg. The reasons for this remain speculative. The effect of BCG vaccination is consistent with the hygiene hypothesis. The observed alteration of transport properties could be one mechanism by which heat denaturation modifies the allergenic properties of this protein, but additional studies are necessary to assess whether other mechanisms, such as exposure of new antigenic determinants are also relevant.

Keywords: beta-lactoglobulin, biological transport, Caco-2 cells, M-cells, milk hypersensitivity, protein denaturation

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Abbreviations

AGP	α-1-acid glycoprotein
α-la	alpha-lactalbumin
APC	antigen-presenting cells
ApoD	apolipoprotein D
ATCC	American type cell collection
$_{\alpha 1}$m	α_1-microglobulin
BCG	bacillus Calmette Guerin
β-lg	beta-lactoglobulin
BSA	bovine serum albumin
C8γ	complement component 8γ
CBR	Coomassie brilliant blue
CD	celiac disease
CMA	cow's milk allergy
CMP	cow's milk proteins
DAPI	4'-6-Diamidino-2-phenylindole
DMEM	Dulbecco's modified eagle medium
DTH	delayed type hypersensitivity
ELISA	enzyme-linked immunosorbent assay
FA	food allergy
FRET	fluorescence resonance energy transfer
GI	gastrointestinal
GLY	glycodelin
HRP	horseradish peroxidase
HSA	human serum albumin
IEC	intestinal epithelial cell
IELs	intraepithelial lymphocytes
IFN-γ	interferon gamma
IgE	immunoglobulin E
IgG	immunoglobulin G
IgM	immunoglobulin M
IL	interleukin

IUIS	International Union of Immunological Societies
kDa	kilo Dalton
LP	lamina propria
LPLs	lamina propria lymphocytes
LPS	lipopolysaccharide
MW	molecular weight
NGAL	neutrophilic lipocalin
OBP	odorant binding protein
OVA	ovalbumin
PBS	phosphate buffered saline
PEF	pulsed electric field
PGDS	prostaglandin D synthase
PPs	Peyer´s patches
RBP	retinol binding protein
RMCPII	Rat mast cell protease II
TEER	transepithelial electrical resistance
Th	T helper
Th1	T helper 1
TJs	tight junctions
Tlc	tear lipocalin
TMB	tetramethyl benzidine
UHT	ultra high temperature
WPC	whey protein concentrate

List of original papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals:

I Heikura J, Suutari T, Rytkönen J, Nieminen M, Virtanen V & Valkonen K (2005) A new procedure to isolate native β-lactoglobulin from reindeer milk. Milchwissenshaft 60:388-391.

II Rytkönen J, Valkonen K, Virtanen V, Foxwell RA, Kyd JM, Cripps AW & Karttunen TJ (2006) Enterocyte and M-cell Transport of Native and Heat-Denatured Bovine β-Lactoglobulin: Significance of Heat Denaturation. Journal of Agricultural and Food Chemistry 54:1500-1507.

III Rytkönen J, Karttunen TJ, Karttunen R, Valkonen KH, Jenmalm MC, Alatossava T, Björkstén B & Kokkonen J (2002) Effect of heat denaturation on beta-lactoglobulin-induced gastrointestinal sensitization in rats: Denatured βLG induces a more intensive local immunologic response than native βLG. Pediatric Allergy and Immunology 13:269-277.

IV Rytkönen J, Karttunen TJ, Karttunen R, Valkonen KH, Björkstén B & Kokkonen J (2004) BCG vaccine modulates intestinal and systemic response to β-lactoglobulin, Pediatric Allergy and Immunology 15:408-414.

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1 Introduction

Bovine beta-lactoglobulin (β-lg) is the major protein of bovine whey, but it is not found in human milk. It belongs to the lipocalin protein family, and is one of the major milk allergens. At physiological pH it is a dimer consisting of two identical amino acid chains (162 amino acids) each having a molecular mass about 18 kDa. β-lg is an exceptionally stable protein in an acidic environment. At pH 2 it dissociates reversibly to its monomers but its structure remains native. Furthermore, native β-lg is almost totally resistant to pepsin degradation at low pH. Resistance to acid hydrolysis as well as to proteases allows some of the β-lg to remain intact after gastric digestion. This increases the probability that intact β-lg as well as its digested fragments will be absorbed as antigens. It has also been shown that bovine β-lg binds small ligands such as fatty acids and vitamins. Although the physical and chemical properties of bovine β-lg are well characterized, its biological functions *in vivo* are not yet known.

During dairy processing of liquid milk products, different heat treatments such as pasteurization change the native structure of bovine milk β-lg (Villamiel *et al.* 1997) and may alter its intestinal transport properties and allergenicity, as the digestibility of the protein changes (Reddy *et al.* 1988, Kitabatake & Kinekawa 1998). Heat related conformational changes may also expose new antigenic sites with different allergic properties (Davis & Williams 1998, Song *et al.* 2005). However, there are no previous studies comparing hypersensitivity reactions to native and heat-denatured bovine milk β-lg in the gastrointestinal tract or at a systemic level.

Transport is the first step in intestinal absorption of nutritional antigens, and the transport properties of proteins may affect their allergenicity. Transport of native β-lg has been studied *in vitro* with Caco-2 cells (Caillard & Tome 1995, Puyol *et al.* 1995), however there are no studies comparing intestinal transport of native and heat-denatured β-lg. It has been established that the dosage of orally given antigen is important in the genesis of tolerance, although several other factors, like the presence of additional antigens, adjuvant, age and duration of exposure, are also important. It is assumed that the induction of tolerance requires that whole antigen molecules are absorbed, suggesting that degradation rates during transport through enterocytes might modify the immunological response (Chehade & Mayer 2005).

Intestinal transport of nutrients is mainly via enterocytes (Snoeck *et al.* 2005). Another important epithelial cell phenotype is M-cells that occur over organized mucosal lymphoid follicles (follicle associated epithelium, FAE) in the intestinal tract. M-cells deliver foreign material by transepithelial transport from the lumen to organized mucosal lymphoid tissues and therefore are important in the regulation of immunological reactions to nutritional and other luminal antigens. Antigen processing in M-cells differs from that in enterocytes, where the majority of antigens are processed in lysosomes. Vesicular transport without degradation is the major pathway in M-cells, transferring unprocessed antigens into the lymphatic system (Niedergang & Kraehenbuhl 2000).

Exposure, dosage and intestinal transport properties of nutritional antigens are important factors modifying the immunological response. However, reactions to other environmental antigens may also change the response of the immunological system. The so-called “hygiene hypothesis” favors the idea that lack of microbial challenge leads to Th2 dominance and to a high risk of allergy, whereas encountering infections leads to Th1 dominance and to a lower risk of allergy (Liu & Murphy 2003). Bacillus Calmette-Guérin (BCG) vaccination is thought to modify the immune response possibly decreasing the risk of allergy to some antigens in both human and experimental animals (Koh *et al.* 2001). Whether reaction to β-lg is altered by BCG is a matter of controversy.

Hypersensitivity reactions against cow’s milk (CM) proteins affect about 2.4 % of infants thus, making cow's milk allergy (CMA) one of the most important food allergies (Saarinen *et al.* 1999). Breast milk is the primary source of nutrition in infants. After weaning, breast milk is commonly substituted with CM. Allergy to CM is a common food allergy in early childhood. CM proteins including β-lg in breast milk substitute formulas are often the earliest foreign antigens encountered, and therefore may be the key antigens in the development of more widespread food sensitization and respiratory allergies.

2 Review of the literature

2.1 Lipocalins

Lipocalins are generally small (160-180 amino acids), extracellular proteins sharing similar properties: they can bind small hydrophobic molecules, form complexes with other soluble macromolecules, and bind to specific cell-surface receptors. Proteins in the lipocalin protein family have been primarily classified as transport proteins, but they also have numerous other functions; including olfaction, pheromone transport, enzymatic synthesis of prostaglandins, mediation of cell homeostasis and the regulation of immune response. Lipocalins show low levels of sequence similarity, but all lipocalins have conserved sequence motifs, which form the basis of their classification. The so-called kernel lipocalins share three conserved sequence motifs, while outlier lipocalins share only one or two motifs (Flower 1996, Flower *et al.* 2000). In contrast to relatively low sequential identity, the crystal structures of lipocalins are highly conserved (Virtanen 2001). Lipocalins are found in vertebrates, invertebrates, bacteria, and plants (Akerstrom *et al.* 2000a). Ten different lipocalins that have been identified in humans are shown in Table 1.

Table 1. Lipocalins of human origin.

Lipocalin (abbreviation)	Reference
Retinol-binding protein (RBP)	Cowan *et al.* 1990, Zanotti & Berni 2004
Neutrophilic lipocalin (NGAL)	Kjeldsen *et al.* 2000, Goetz *et al.* 2002
Apolipoprotein D (ApoD)	Rassart *et al.* 2000
α_1-microglobulin (α_1m)	Akerstrom *et al.* 2000b, Larsson *et al.* 2004
Complement component 8γ (C8γ)	Schreck *et al.* 2000, Ortlund *et al.* 2002
Tear lipocalin (Tlc)	Redl 2000, Breustedt *et al.* 2005
Prostaglandin D synthase (PGDS)	Urade & Hayaishi 2000
Odorant binding protein (OBP)	Tegoni *et al.* 2000, Briand *et al.* 2002
α-1-acid glycoprotein (AGP)	Fournier *et al.* 2000
Glycodelin (GLY)	Koistinen *et al.* 1999, Halttunen *et al.* 2000

Many mammalian allergens have been found to be members of the lipocalin protein family. The lipocalin allergens from different species include can f1 and can f2 (dog), mus m1 (mouse), rat n1 (rat), equ c1 and equ c2 (horse) and bos d5 (bovine β-lg) (Virtanen *et al.* 1999, Rouvinen *et al.* 2001). Among the other lipocalins β-lg is the only food allergen, the rest of the known lipocalin allergens being respiratory allergens (Rouvinen *et al.* 2001). The reason for the allergenicity of lipocalins is not known, but it is thought that molecular mimicry at T-cell level between allergenic and endogenous lipocalins could be a mechanism (Virtanen *et al.* 1999). This might explain the allergenicity of β-lg as it has a high amino acid and structural similarity to human glycodelin (Koistinen *et al.* 1999). One other factor explaining the allergenicity could be that lipocalins are often present in secretions and are therefore easily spread to the environment (Rouvinen *et al.* 2001).

2.2 Beta-lactoglobulin

β-lg is a major protein of bovine whey, the average concentration in bovine milk being about 2-3 g/l (Kontopidis *et al.* 2004). In addition to bovine milk, β-lg can be found in the milk of many mammals, such as reindeer (Rytkonen *et al.* 2002), pig (Conti *et al.* 1986), horse (Conti *et al.* 1984), ewe (El-Zahar *et al.* 2004), cat (Halliday *et al.* 1991), and dog (Pervaiz & Brew 1986); but it is not present in human milk (Sawyer & Kontopidis 2000). β-lg belongs to the lipocalin protein family (Sawyer & Kontopidis 2000), and is one of the major milk allergens (Wal 2001). It has been shown that bovine β-lg binds small ligands such as fatty acids and vitamins, as expected for a member of lipocalin protein family (Kontopidis *et al.* 2002). Although the physical and chemical properties of bovine β-lg are well characterized, its biological functions *in vivo* are not yet known.

2.2.1 Secretion and structure

β-lg, as most of the milk proteins is produced in the secreting epithelial cells of mammary gland, under the control of prolactin hormone (Larson 1972). Messenger RNA coding β-lg is synthesized in the mammary gland, and it is translated into 180 amino acid long pre-β-lg (Yoshikawa *et al.* 1978). Pre-β-lg contains a highly conserved signal peptide of 18 amino acids. The mature protein itself contains 162 amino acids and has a molecular weight of 18400 D (Sawyer & Kontopidis 2000). The amino acid sequence of bovine β-lg A (www.pdb.org, code 1BEB):

```
1   LIVTQTMKGL DIQKVAGTWY SLAMAASDIS LLDAQSAPLR YVEELKPTP
51  EGDLEILLQK WENDECAQKK IIAEKTKIPA VFKIDALNEN VLVLDTDYK
101 KYLLFCMENS AEPEQSLVCQ CLVRTPEVDD EALEKFDKAL KALPMHIRLS
151 FNPTQLEEQC HI 162
```

At neutral pH β-lg is a dimer formed by two homologous monomers, while it dissociates into native monomers at acidic pH <3.5 or basic pH >7.5 (McKenzie & Sawyer 1967). Main variants of β-lg are A and B, and their amino acid sequence differ by only 2 amino acids in positions 64 (Asp-Gly) and 118 (Val-Ala)(Sawyer & Kontopidis 2000). This change in amino acid composition changes the isoelectric point of β-lg; variant A has a isoelectric point at pH 5.1 and variant B at pH 5.3 (Stastna & Slais 2005).

The three-dimensional structure of monomeric bovine β-lg is based on 9 strands of anti-parallel β-sheets (A-I), eight of them create a conical barrel or calyx with a binding site for hydrophobic ligands within the barrel. Like many lipocalins, bovine β-lg can bind a variety of different small hydrophobic ligands including retinol, and fatty acids (Sawyer *et al.* 1998). Strands A-D form one surface of the barrel while strands E-H form the other (Kontopidis *et al.* 2002). Bovine β-lg monomer has two disulfide bonds between cysteine residues (Cys^{66}-Cys^{160} and Cys^{106}-Cys^{119}) and one free thiol group (Cys^{121}) (Sakurai & Goto 2002). The so-called EF loop forms a lid-like structure, closing and opening the binding site inside the calyx. At low pH the lid closes and binding of ligands is inhibited or impossible, but at high pH lid opens, making the ligand binding site accessible. (Tanford & Nozaki 1959, Brownlow *et al.* 1997, Qin *et al.* 1998, Kontopidis *et al.* 2004). The 9th β-strand (I) flanks the 1st strand and is outside of the calyx and forms a part of the dimer forming interface. The AB loop also takes part in the formation of the dimer interface (Qin *et al.* 1998, Sakurai & Goto 2002).

2.2.2 Biological function

There have been several suggestions about the role of β-lg in milk, but a clear physiological function has not been found. β-lg belongs to the lipocalin protein family, of which many are transport proteins. On this basis a transport role has been suggested for β-lg. Taking into consideration the stability of β-lg in the acidic environment, such a transport role would be reasonable and also receptors for β-lg have been identified in bovine and rat cells (Papiz *et al.* 1986, Mansouri *et al.* 1997, Mansouri *et al.* 1998, Sawyer & Kontopidis 2000).

It is also possible that β-lg is involved in the uptake of fatty acids and retinol. It has been shown that β-lg enhances the activity of pregastric lipase (Perez *et al.* 1992) and increases uptake of fatty acids and retinol (Burczynski *et al.* 1990, Puyol *et al.* 1995). It has been suggested that bioactive peptides formed from β-lg are useful to the offspring (Pihlanto-Leppala *et al.* 1997, Pihlanto-Leppala 2000, Sawyer & Kontopidis 2000, Yamauchi *et al.* 2003), and that β-lg enhances passive immunity. β-lg binds to the gut wall and may displace some harmful organisms from the neonatal intestine (Ouwehand *et al.* 1997).

It has also been suggested that β-lg might be a convenient nutritional protein in milk "left over" from another function in the mother. This is supported by the fact that cow's genome includes a pseudogene (a non-expressed sequence of DNA that is very similar to a normal gene) that shows great homology to β-lg genes expressed in some other species (Passey & Mackinlay 1995, Sawyer & Kontopidis 2000). Although several functions for β-lg have been proposed, its function *in vivo* has not been confirmed.

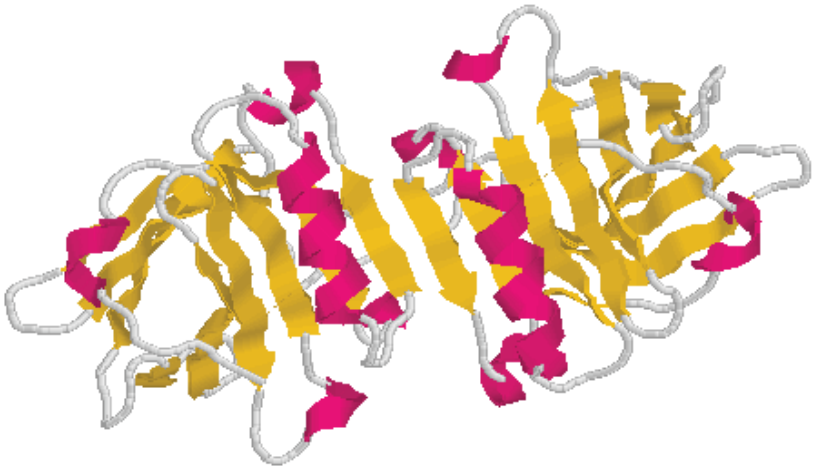

Fig. 1. Three dimensional structure of bovine β-lg (www.pdb.orb, code 1BEB), picture generated with Protein Explorer v. 2.45 beta (www.proteinexplorer.org).

2.2.3 Purification of β-lg

Numerous methods for the purification of β-lg from bovine milk have been developed. The first purification method for bovine milk β-lg was developed in 1934 (Palmer 1934). This method has been used as a basis for many other methods developed since. Fats and caseins are removed first; usually fats are removed by centrifugation of the raw milk and after that the caseins are separated by acid precipitations, the remaining liquid is called whey. β-lg can be isolated from whey in many different ways. Methods used for the isolation of β-lg from milk are summarized in Table 2. A purification method utilizing the exceptional stability of β-lg against proteases has also been developed. Pepsin can be used, since it hydrolyzes all the other proteins in whey, leaving β-lg intact (Kinekawa & Kitabatake 1996). For large scale purifications, the most suitable methods are precipitations followed by ion exchange (Outinen *et al.* 1996, de Jongh *et al.* 2001).

Table 2. Purification methods for β-lg from bovine milk.

Reference	Fat removal	Casein removal	Fractionation of whey proteins	Final purification
Palmer 1934		Acidic precipitation at pH 5.8	Na_2SO_4 precipitation, 30ºC	Dialysis Crystallization
Ashaffenburg & Drewry, 1957	Na_2SO_4 precipitation, 40ºC	Na_2SO_4 precipitation, 40ºC	Acidic precipitation at pH2 $(NH_4)_2SO_4$ precipitation at pH 6	Dialysis Crystallization
Armstrong *et al.* 1967	$(NH_4)_2SO_4$ precipitation, 20ºC	$(NH_4)_2SO_4$ precipitation, 20ºC	Acidic precipitation at pH2 $(NH_4)_2SO_4$ precipitation at pH 6	Dialysis Crystallization
Monaco *et al.* 1987	Centrifugation	$CaCl_2$ precipitation at pH 6.6	Dialysis Anion exchange	Gel filtration Crystallization
Felipe & Law 1997	Centrifugation	Acidic precipitation at pH 4.6 Filtration, Dialysis	Gel filtration	
Heddleson *et al.* 1997		Acidic precipitation at pH 4.6 Centrifugation	N-retinyl-celite affinity chromatography	
Chiancone & Gattoni 1991		Acidic precipitation at pH 4.6 Centrifugation, filtration	Bio affinity chromatography	
de Jongh *et al.* 2001	Centrifugation	Acidic precipitation at pH 4.4-4.5	Base precipitation at pH 7.2 Centrifugation Anion exchange chromatography	Gel filtration

2.2.4 Heat denaturation

Heating is an important process in the manufacture of most dairy products, e.g. yogurt, milk powder, and ultra high temperature (125-130°C) milk. It is used to modify functional properties and also to ensure the safety of the food products (Oldfield *et al.* 2005). During such processing of liquid milk products, different heat treatments such as pasteurization cause changes in the native structure of bovine β-lg (Villamiel *et al.* 1997), and may alter its sensitizing properties as digestibility of the protein changes (Reddy *et al.* 1988, Kitabatake & Kinekawa 1998). In addition, these conformational alterations can expose new antigenic sites (Davis & Williams 1998, Song *et al.* 2005).

Thermal denaturation of β-lg proceeds through different steps that are characterized by well defined temperature thresholds and by the temperature dependence of their rate constants. These steps lead to both reversible and irreversible modifications. Some of them are non-correlated and some are sequential (Iametti *et al.* 1996). Heat denaturation of β-lg starts at as low temperature as 30ºC. Dimeric β-lg dissociates easily to monomers in temperatures between 30-55ºC. This reaction is reversible (Sawyer 2003). At temperatures between 40-55ºC Tanford transition (N↔R transition) occurs (Tanford *et al.* 1959, Tanford & Taggart 1961). The main structural rearrangement occurring during the transition is a change in the conformation of the EF loop (amino acids 85-90). At low pH, the loop closes the entrance to the calyx, and at high pH it is open (Oliveira *et al.* 2001). After the transition the buried carboxyl group (Glu89) and Tyr42 are exposed, and free cysteine (Cys121) is available for intermolecular reactions (Qi *et al.* 1997, Qin *et al.* 1998, Oliveira *et al.* 2001). The Tanford transition occurs also when pH is about 7.5 (Tanford *et al.* 1959).

Partly irreversible heat denaturation of β-lg occurs in two phases, and is affected by the concentration of β-lg, salt and sugar, the pH and ionic strength of the liquid (Qi *et al.* 1995, Qi *et al.* 1997). The first phase of heat denaturation occurs at 58-60ºC, and affects mainly the α-helix structures. Reversible structural changes begin at the surface, area Lys8-Try19 (β-sheet) at 65ºC and progresses to the Thr125-Lys135 area (α-spiral). These structural changes become irreversible at temperatures beyond 80ºC (Casal *et al.* 1988). As the denaturation process progresses, the intra-chain disulphide bridges begin to break and the free sulphydryl groups can now react with each other and aggregation of β-lg can take place (Roefs & De Kruif 1994, Creamer *et al.* 2004).

2.2.5 Stability in the digestive tract

β-lg is an exceptional protein in regards to its stability in the acidic environment of the stomach. At pH 2, as found in the stomach it dissociates reversibly to its monomers, but its structure is still native (Sakai *et al.* 2000). Furthermore, native β-lg is almost entirely resistant to pepsin degradation at low pH (Reddy *et al.* 1988, Astwood *et al.* 1996, Takagi *et al.* 2003). Studies with simulated gastric fluids have shown that β-lg can remain intact in the environment of the stomach (Astwood *et al.* 1996, Fu *et al.* 2002). The exceptional resistance to degradation in the stomach makes it possible to detect intact β-lg in the small intestine (Mahe *et al.* 1991); however most of the β-lg is finally digested in the small intestine (Kitabatake & Kinekawa 1998).

Although it has been suggested that β-lg ingested by the mother could be secreted intact in breast milk in intact form, there is conflicting evidence about the prescence of bovine β-lg in human breast milk. Some studies have reported β-lg in the milk of mothers (Axelsson *et al.* 1986, Makinen-Kiljunen & Palosuo 1992, Sorva *et al.* 1994, Fukushima *et al.* 1997). However a study by Conti questions the validity of these findings as they found that cross reactivity with the C-terminal fragment of human β-casein exists, undermining immunological methods of β-lg detection. No bovine β-lg peptides with a molecular mass >10000 Da were detected in milk samples from healthy mothers on a diet rich in bovine milk and dairy products (Conti *et al.* 2000).

In contrast to extreme resistance of native β-lg to digestion, heat denaturation of β-lg at temperatures higher than 80ºC causes irreversible structural changes, and makes β-lg more easily digested. Studies with heat-denatured β-lg show that heat denaturation made β-lg more prone to digestion by pepsin at pH 2 (Reddy *et al.* 1988, Kitabatake & Kinekawa 1998). It is therefore probable that the majority of heat-denatured β-lg is hydrolyzed in the stomach, whilst *in vivo* studies show that native β-lg is found intact in the small intestine (Mahe *et al.* 1991).

2.3 Allergy

Current nomenclature for allergy for global use states that allergy is *a hypersensitivity reaction initiated by specific immunologic mechanisms.* Allergies can be divided to antibody-mediated and cell-mediated (Johansson *et al.* 2004).

Hypersensitivity reactions have been classically divided into four types as introduced by Gell and Coombs (Gell & Coombs 1963). These types are anaphylaxis (Type I), antibody-mediated cytotoxic reactions (Type II), immune complex-mediated reactions (Type III), and delayed type hypersensitivity (Type IV). This classification was proposed over 40 years ago, and it is still used. Of the four major types of hypersensitivity reactions, type I is best understood and referred as the classical allergic reaction. Type I reactions are immediate hypersensitivity or anaphylactic reactions. They are manifested with one or more of the following clinical reactions: urticaria, angiodema, cardio-respiratory (anaphylactic) shock, asthma, rhinitis or conjunctivitis (Descotes & Choquet-Kastylevsky 2001). Type II reactions are antibody mediated. Cytotoxic antibodies, mainly IgM and IgG cause the reactions. Cell damage caused by these cytotoxic antibodies can occur by two different mechanisms. First cells can be damaged by the direct action of macrophages, neutrophils and eosinophils that are linked to immunoglobulin-coated target cells. Second complement can be activated by antibody-mediated reactions and result in cell lysis (Descotes & Choquet-Kastylevsky 2001). Type III reaction cause tissue injury mediated by immune complexes. Microprecipitates are formed in small vessels and cause secondary damage to cells. These microprecipitates are formed when precipitating antibodies (mainly IgM) recognize antigens and form immune complexes. Type IV reactions are mediated by T-lymphocytes and usually manifest as skin eruptions (Descotes & Choquet-Kastylevsky 2001).

2.3.1 Induction of IgE production

Clinically, IgE mediated immediate or atopic allergy, is considered the most important and most prevalent form of allergy. Allergens contain both B-cell and T-cell epitopes; B-cell epitopes are regions on the surface of a protein recognized by soluble membrane bound antibody molecules, and T-cell epitopes are short peptides bound to major histocompatibility complexes (MHC) that can be recognized by T-cells (Toseland *et al.* 2005).

To elicit IgE antibody production, conformational epitopes on the surface of the allergen must first be recognized by B-cells, which also require Th2 type cells to induce them to transform to IgE secreting plasma cells. Th2 type cells recognize allergens as processed linear peptides. Therefore non-processed allergenic proteins cannot be recognized by T-cells, and allergens must first be processed by APC's and then presented on their surface in conjunction with MHC II class molecules (Geha *et al.* 2003).

Many factors have been suggested to be responsible for the induction of the Th2 type response. Genetic factors background of the host can promote the shift to Th2 type responses (Geha *et al.* 2003). GATA3 is a transcription factor, which has been shown to regulate the balance between Th1 and Th2 response by inducing production of IL-4. It promotes Th2 and inhibits the Th1 response (Usui *et al.* 2003). Also environmental factors: changes of the commensal flora, reduced exposure to natural infections, reduced family size are capable of promoting the shift (Romagnani 2000, Geha *et al.* 2003).

There are lots of suggestions as to why certain protein might be allergens (Bufe 1998, Huby *et al.* 2000, Bredehorst & David 2001). An ovoid shape (Rouvinen *et al.* 1999), repetitive motifs in the amino acid sequence (Pomes *et al.* 1998), heat stability (Taylor & Lehrer 1996), and resistance to gastrointestinal degradation (Astwood *et al.* 1996) have all been suggested as reasons for allergenicity. Enzymatic activity can also cause increased allergenicity. It is shown that some allergens are able to increase their own permeability in the bronchial epithelium (Herbert *et al.* 1995), and decrease the proliferation of Th1 cells (Shakib *et al.* 1998) by their proteolytic activity thus biasing the immune response towards the Th2 type.

2.3.2 Food allergy

European Academy of Allergy and Clinical Immunology (EAACI) defines food hypersensitivity as any adverse reaction resulting from ingestion of food. It can be a food allergy involving an immunologic reaction or it can be non-allergic food hypersensitivity (Bruijnzeel-Koomen *et al.* 1995, Johansson *et al.* 2004). The term *food allergy* is appropriate only when immunologic mechanisms have been demonstrated. If IgE takes part in the allergic reaction to food, the term *IgE-mediated food allergy* can be used (Johansson *et al.* 2004).

Food allergy is recognized as an universal problem in westernized countries (countries that have adapted to a western diet, hygiene standards and lifestyle). It appears to be on the increase, as are other atopic disorders (Sampson 2004). It is not surprising that a wide variety of gastrointestinal hypersensitivity disorders develop, since the gastrointestinal tract is the first to confront an enormous variety of food allergens (Sampson 1999). About 20% of people in industrialized countries report adverse reactions to food in a questionnaire (Young *et al.* 1994). The prevalence of confirmed allergy is, however much lower: 6-9% in children and 1-2% in adults (Sampson 2004).

2.3.2.1 Food allergens

Although the diversity of the human diet is enormous, only a small number of foods account for the majority of food allergies (Sampson 2004). A food allergen may be defined as a substance that reacts with IgE antibodies, induces allergic sensitization or induces allergic reaction. There are no structural features that predict that a certain antigen will be a potent food allergen (Aalberse 1997). Some of the most common food allergens are listed in Table 3.

Table 3. Some of the most common food allergens (http://www.allergen.org, official list of allergens IUIS, International union of immunological societies, allergen nomenclature subcommittee).

Food	Allergen	Trivial name
Cow´s milk	Bos d5	β-lg
	Bos d4	α-lactalbumin
	Bos d8	Caseins
Egg	Gal d1	Ovomucoid
	Gal d2	Ovalbumin
	Gal d3	Ovotranferrin
Fish	Gad c1	Parvalbumin
Potato	Sola t1	Patatin
Shrimp	Pen a1	Tropomyosin
Peanut	Ara 1	Vicilin
	Ara 2	Conglutin

2.3.3 Cow´s milk allergy and β-lg

Hypersensitivity reactions against cow's milk proteins (CMP) affect up to 2.4% of infants thus making cow's milk allergy (CMA) one of the most important food allergies (Saarinen *et al.* 1999). Breast milk is the primary source of nutrition in infants. After weaning, breast milk is commonly substituted with CM products. Allergy to CM is one of the most common types of food allergy in early childhood. A recent study showed that 45% of patients with allergic reactions to cow's milk in SPT had β-lg specific IgE antibodies (Natale *et al.* 2004). Infant formulas usually contain β-lg which is often the earliest foreign antigen for a child (Exl & Fritsche 2001), and therefore it may be the key antigen in the development of more widespread food sensitization. Antigenicity of β-lg was first discovered in the 1960´s. Studies have shown that the immunoreactivity of β-lg requires an intact tertiary structure (Kurisaki *et al.* 1982, Otani *et al.* 1985).

B-cell epitopes of β-lg have been studied with protein fragments generated from β-lg after tryptic proteolysis (Selo *et al.* 1999) or cyanogen bromide treatment (Selo *et al.* 1998), also synthetic peptides have been also used (Miller *et al.* 1999). The main epitopes detected in these fragments are: 21-40, 41-60,102-145, and 148-168. The functional capacity of the epitopes was not studied. Allergens have to be bivalent (i.e. contain two

epitopes) to induce a release of immune mediators in mast cells. A study using tryptic peptides and synthesized peptides identified one bivalent B-cell epitope at the C terminal end of β-lg, consisting of amino acids 149-162 (Fritsche *et al.* 2005).

Three human T-cell epitopes for bovine β-lg were identified in a study of five milk allergic patients (amino acids 30-47, 97-117, and 142-62) (Sakaguchi *et al.* 2002). They are in the β-sheet region of the β-lg molecule. The main epitope was amino acids 97-117; this sequence has been previously identified as a T-cell epitope (Inoue *et al.* 2001). The core sequence recognized most effectively was fragment 101-112. This epitope is buried within the β-lg molecule, but its edge is orientated toward the surface (Sakaguchi *et al.* 2002). Murine T-cell epitopes have been studied with mice models (Tsuji *et al.* 1993, Totsuka *et al.* 1997). The strongest T-cell responses in Balb/c mice were directed against peptide fragments containing amino acids 67-75, 71-79 and 80-88 from β-lg (Totsuka *et al.* 1997).

2.3.3.1 Significance of milk processing in the pathogenesis of CMA

Thermal treatments used in processed liquid milk products may change the allergenic properties of milk proteins. When proteins are denatured by heat, the original tertiary structure is often lost. Many of the native protein epitopes recognized by antibodies are also lost (Davis & Williams 1998, Song *et al.* 2005). In many case heat treatments reduce the allergenicity of a protein. It has been shown by ELISA inhibition studies that heat treatment reduces the IgE reactivity of β-lg, but does not totally eliminate it (Ehn *et al.* 2004). On the contrary, heat denaturation and unfolding of the protein can expose some new allergenic epitopes. It has been shown that heat denaturation of β-lg exposes at least some new epitopes, which are not accessible in the native protein (Davis & Williams 1998, Song *et al.* 2005).

Milk processing can also alter the digestibility of milk proteins as described in section 2.1.3. Heat denaturation of β-lg makes it more susceptible for degradation by hydrolyzing enzymes (Takagi *et al.* 2003). Whether this change in digestibility has any effect on the allergenicity of β-lg is not known. It has been shown that heat denaturation of whey have an effect in its capacity to induce a humoral reaction. Heat-denatured whey was did not induce antibody production; however native whey induced an IgG antibody reaction, especially against β-lg (Enomoto *et al.* 1993). Heat denaturation of whey proteins did not have any effect on its ability to induce oral tolerance; both native and heat-denatured whey proteins induced oral tolerance (Enomoto *et al.* 1993).

Homogenization of milk is also a part of normal milk processing. During homogenization, large lipid globules are broken to smaller globules. This process increases the surface area of the milk lipid globules and part of the milk lipid membrane is replaced by milk proteins. In raw milk most of the antigenic determinants are inside the casein micelles, while homogenization of milk increases the exposed antigenic determinants (Poulsen *et al.* 1987).

There has been conflicting information about the effects of homogenization on the allergenicity of milk. Some animal studies have shown that processed (pasteurized and homogenized) milk cause more hypersensitivity reactions than raw milk. It was shown that pasteurized and homogenized milk induced anaphylactic shock (Poulsen *et al.* 1987),

increased the production of milk specific immunoglobulin production, induced degranulation of mast-cells (Poulsen *et al.* 1990), and increased IgE production (Nielsen *et al.* 1989). Unhomogenized pasteurized or raw milk caused fewer symptoms. On the contrary, recent studies in humans show that subjects with self-reported hypersensitivity reactions to homogenized milk tolerated homogenized and unhomogenized milk equally well (Paajanen *et al.* 2003), and there were no differences in milk specific antibody production in milk-tolerant subjects consuming homogenized or unhomogenized milk products (Paajanen *et al.* 2005a). It was also concluded that processing of milk did not affect the severity of hypersensitivity reactions in milk intolerant subjects.

2.3.4 Gastrointestinal processing of proteins

Proteins are an essential part of the human diet. To maintain a positive nitrogen balance and to provide all the essential amino acids, a normal healthy adult requires about 0.83 g/kg/d of protein (Rand *et al.* 2003). Ingested dietary proteins are degraded first by gastric acidity and pepsin, and then by intestinal luminal digestive enzymes and brush border peptidases. Sequential digestion with pepsin and trypsin normally degrades most of the protein antigens. Although this system is very effective, a small fraction of the ingested proteins remain unaffected, and can be absorbed intact (Heyman 2001).

Protein antigens can be taken up from the intestinal lumen by enterocytes and M-cells. In enterocytes, transcytosis of proteins normally takes place via two main pathways (Snoeck *et al.* 2005). The degradative pathway is the major route, accounting for about 90% of the transported proteins. Antigens are transported in small vesicles and larger phagosomes and then digested in phagolysosomes. Total hydrolysis of proteins to amino acids is not achieved, but peptides with sizes varying from 2000 to 4000 D are generated. Enterocytes can act as non-professional APCs, but due to the polarized nature of the enterocyte, antigen presentation is only possible at the basolateral surface (Hershberg & Mayer 2000). Furthermore, it has been observed that enterocytes can release intestinal epithelial exosomes, which contain MHC class II/peptide complexes, at their basolateral surfaces, and they could act as a link between luminal antigens and the gut associated local immune system (Van Niel *et al.* 2003, Mallegol *et al.* 2005). The minor pathway through enterocytes- direct transcytosis without degradation- is responsible for about 10% of the total transport (Heyman & Desjeux 1992, Chehade & Mayer 2005). The recent discovery that CD23 mediated transport through intestinal epithelium can provide a “protected” transport pathway for food antigens might be one explanation for the transport of intact antigens through the mucosa (Bevilacqua *et al.* 2004). It has been shown that sensitized animals have an abnormally high expression of CD23, forming a direct transport pathway that rapidly conveys antigens in intact form from the intestinal lumen to the underlying tissues (Yu *et al.* 2003, Bevilacqua *et al.* 2004). It has been shown that intact protein is transported significantly more in the patients with atopic eczema, Crohn’s disease, and CMA (Heyman *et al.* 1988, Majamaa & Isolauri 1996, Malin *et al.* 1996). Paracellular leakage is not common through healthy intestinal mucosa (Heyman & Desjeux 1992).

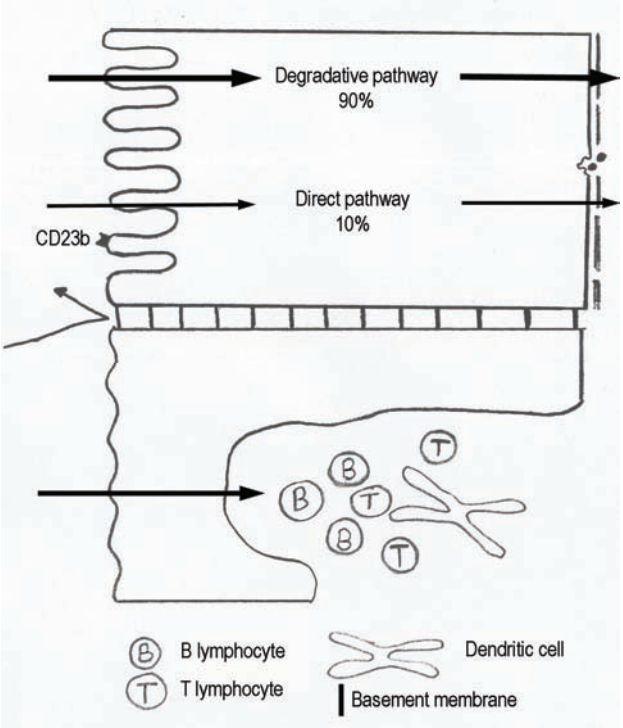

Fig. 2. Transport pathways through intestinal mucosa (Heyman *et al.*, 2001; Tu *et al.*, 2005).

Another route for proteins to pass through the intestinal wall is via M-cells. They take up particles using a process which is reminiscent of phagocytosis, and involves actin re-arrangement (Niedergang & Kraehenbuhl 2000). There is some controversy about the cellular compartments of M-cells. Lysosome-like structures are present in M-cells (Niedergang & Kraehenbuhl 2000), also it is shown that endosomal compartments of M-cells are acidified (Allan & Trier 1991) and they contain the endosomal protease cathepsin E (Finzi *et al.* 1993). Nevertheless it is generally assumed that antigens are transported through M-cells without degradation (Niedergang & Kraehenbuhl 2000). This might be due to fact that endocytotic vesicles have only a short way to travel before exocytosis occurs and proteins may bypass degradative enzymes (Niedergang & Kraehenbuhl 2000). Transport of particles is fast and antigens can be transported within 10-15 min from the intestinal lumen to the basolateral pocket of M-cells (Neutra *et al.* 1999). Most of the antigens transported across FAE by M-cells are probably captured by immature DCs in the subepithelial dome region, which can in turn present processed antigens to the immune system (Neutra *et al.* 2001).

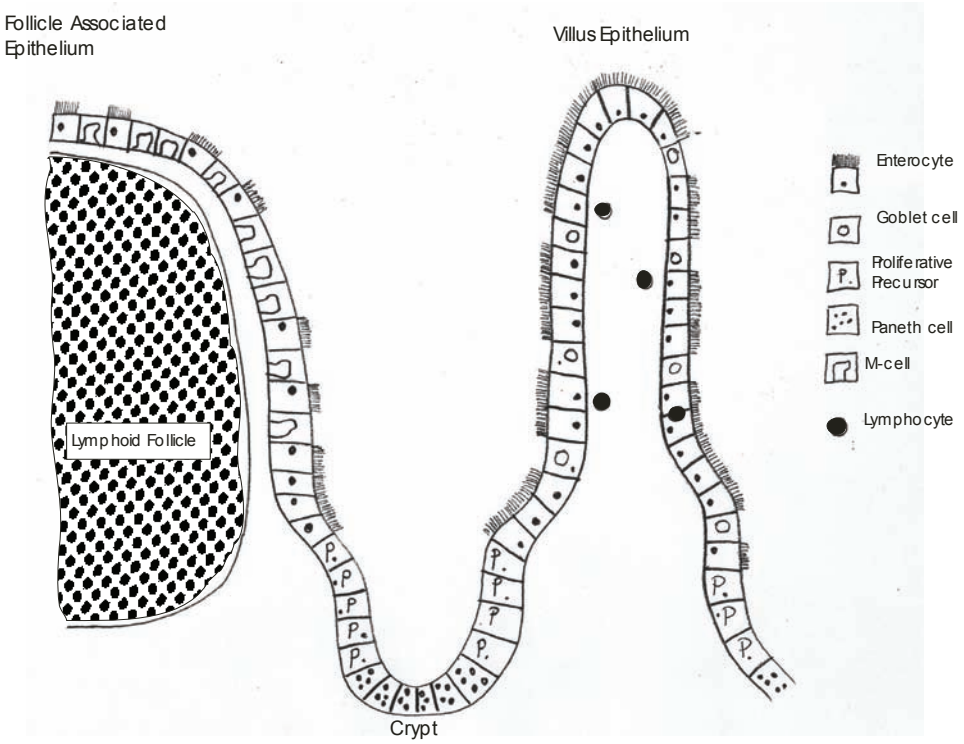

Fig. 3. Enterocytes and M-cell in intestine (modified from Neutra *et al.* 1996).

2.3.5 Microbes, vaccinations and the development of allergy

Epidemiological studies have suggested that the increase in the prevalence of the allergic disorders that has occurred in recent decades is attributable to a reduced microbial burden in Westernized countries during childhood. This is the basis of the so-called hygiene hypothesis (Strachan 1989, Romagnani 2004). Mechanisms to explain why reduced exposure of children to pathogenic and nonpathogenic microbes results in enhanced responses by Th2 cells remain controversial (Romagnani 2004). At first it was proposed that lack of age-related physiological shifting of antigen specific responses from the Th2 to the Th1 phenotype was responsible (Romagnani 1994, Martinez 2001). Later, reduced T regulatory cell activity was suggested as a mechanism (Yazdanbakhsh *et al.* 2002).

The composition of the gut microbial flora seems to be associated with the development of allergic diseases. High amounts of potential pathogens (clostridia) in the intestine are associated with clinical manifestations of allergy and IgE production; on the contrary bacteroides seem to down-regulate IgE responses (Sepp *et al.* 2005). Thus a balanced gut microbiota may protect children against allergic diseases (Sepp *et al.* 2005). Also it is suggested that probiotic bacteria (Lactobacillus GG) may down regulate hypersensitivity

reactions and intestinal inflammation in patients with food allergy (Majamaa & Isolauri 1997), this might be connected to the finding that Lactobacillus GG stabilizes the mucosal barrier and decreases the transport of proteins (Isolauri *et al.* 1993), thus probiotic bacteria might be useful in the treatment of food allergy (Majamaa & Isolauri 1997).

In addition to natural infections and intestinal microbes, various vaccinations have been suggested as having either protective or inductive effects on the development of allergy (Bach 2001, Bager *et al.* 2002). Population studies with tuberculosis vaccination (Bacillus Calmette-Guerin) have shown vaccination to be either inversely related (Gruber *et al.* 2001) or unrelated to asthma and allergies (Alm *et al.* 1997, Jentoft *et al.* 2002). Clinical studies, strongly support a negative correlation (Martinati & Boner 1997, Gruber *et al.* 2002).

2.3.6 Oral tolerance

The gastrointestinal tract is the largest immunological organ in the body. The lumen of the intestine is lined with a single layer of epithelial cells. Under this cell layer are great numbers of lymphocytes in the loose connective tissue, the lamina propria. The surface epithelium is exposed to a wide range of dietary proteins and microbes. Despite massive dietary antigenic exposure, only few individuals develop food allergies. This is because of the development of oral tolerance to dietary proteins (Chehade & Mayer 2005).

Oral tolerance means an active inhibition of immune responses to an antigen by means of prior exposure to that antigen through the oral route (Chase 1946). Many factors play a role in the induction of oral tolerance to a dietary antigen. The dose and nature of antigen are important, as also factors that are inherent to the host like; age, genetics, and intestinal flora. Oral tolerance is a crucial mechanism for the general well being of humans, as it prevents the development of excess food hypersensitivities (Chehade & Mayer 2005).

Table 4. Factors involved in oral tolerance (adapted from Strober et al. *1998, Chehade & Mayer 2005).*

Factor	Effects
Antigen Dose	High-dose results in lymphocyte anergy or deletion
	Low-dose results in activation of regulatory T cells
	Low-dose results in more profound DTH response
Form of the antigen	Soluble antigen is more tolerogenic than particulate
Normal mucosal microflora of the host	
Genetics of the host	
Age of the host	Neonates have stronger immunologic reactions

2.4 Animal models of food allergy

Lots of information about the basic immunological mechanisms involved in food allergy is available. In human studies it is not ethically appropriate to experimentally sensitize patients with food allergens. Therefore it is only possible to study naturally sensitized subjects. Also, structural analysis of the gastrointestinal tract and mechanistic studies, are limited in human studies.

Limitations in human studies make animal models important in analysis of the allergenicity of new food stuffs and for the analysis of the mechanisms of induction of allergic reaction and factors modifying induction (Andre *et al.* 1994, Astwood *et al.* 1996, Miller *et al.* 1998, Dearman *et al.* 2001). Lots of important information has been provided by animal experiments on the extent to which gut immune responses depend on the age, the dose or the frequency of antigen administration and the conditions needed to induce allergic reactions (Dahlgren *et al.* 1991). It has been shown that allergic reactions tend to become more frequent in consecutive generations, indicating a cumulative effect of factors transferring from the mother to the offspring (Herz *et al.* 2001, Valkonen *et al.* 2002).

2.4.1 Animal species

Most food allergy studies have been on rodents, such as mouse, guinea pig and rat. Some studies have used dog and pig models (del Val *et al.* 1999, Helm *et al.* 2002). Normally, so-called high IgE responder strains of mouse and rat are used in food allergy models. The most common rat strains used in allergy studies are Brown Norway and Hooded Lister. Brown Norway rats are considered to be an optimal model for oral sensitization studies, since they produce high IgE response to oral sensitization without any adjuvants (Knippels *et al.* 1999a, Knippels *et al.* 1999c). This strain is not however an optimal model for all food allergy studies, as also non-IgE mediated food allergies are important clinical problems in humans (Sampson 1999). The clearest delayed type response is shown in Hooded Lister and Wistar rat strains (Knippels *et al.* 1999b). The most commonly used mouse strains are the Balb/c and C3H/hej strains (Kato *et al.* 1999, Li *et al.* 1999). Inbred dogs with a strong IgE response have also been used for allergy studies (Ermel *et al.* 1997, del Val *et al.* 1999).

2.4.2 Immunization route

The optimal route for sensitization with food allergens is an important factor in animal allergy models. The oral route is the most comparable to the clinical situation, but conventional oral administration without adjuvant usually results in the development of tolerance instead of sensitization (Dearman *et al.* 2001, Dearman & Kimber 2001, Penninks & Knippels 2001). Also, differences in physiological degradation of the antigen have to be considered. Antigens might be partially or totally degraded in the gastrointestinal tract. This processing might change the animal's immunological reaction

to the antigen. Induction of oral tolerance is an important aspect from a therapeutic viewpoint, but it can make the evaluation of allergenicity of certain food antigen difficult. However, gavage feeding has been shown to induce an IgE response without adjuvants (Knippels *et al.* 1998b, Dearman *et al.* 2001). Carrageenan, saponin and cholera toxin have been used as adjuvants in oral sensitization studies. It is important to note that even though their main function is to induce increased permeability of the mucosa, they might simultaneously modify the immune response by promoting Th2 activation and IgE response (Dearman *et al.* 2001). More extensive IgE production is possible with intraperitoneal sensitization, and it is considered the most reliable way to compare allergenicity of different proteins (Dearman *et al.* 2001). It is important that test animals are bred and maintained with a specified antigen-free diet. This can be a difficult as some commercial rat feeds, whilst claiming to be free of contaminating proteins do contain some potentially allergenic impurities (Brix *et al.* 2005). In sensitization studies animals have to be raised on an antigen-free diet for two consecutive generations for reliable results (Knippels *et al.* 1998a, Pecquet *et al.* 2000a, Pecquet *et al.* 2000b, Frossard *et al.* 2001, Brix *et al.* 2005).

2.4.3 Milk allergy models

Several milk allergy models have been developed using both different animal species and milk antigens. Animal species including guinea pigs, rats and mice have been used (Kitagawa *et al.* 1995, Li *et al.* 1999, Miller *et al.* 1999, Frossard *et al.* 2004a, Frossard *et al.* 2004b). Animals have been sensitized with raw milk, pasteurized milk, homogenized milk or with purified milk proteins like; β-lg and caseins. Table 5 lists some milk products and proteins studied using animal models.

Table 5. Bovine milk products and proteins studied by using animal models (modified from Valkonen et al. 2002).

Milk product / protein	Reference
Untreated milk	Poulsen *et al.* 1987, Poulsen *et al.* 1990
Pasteurized milk	Poulsen *et al.* 1987
Homogenized milk	Poulsen *et al.* 1987
Fermented milk	Terpend *et al.* 1999
Milk proteins	Kitagawa *et al.* 1995
Protein hydrosylates	Ju *et al.* 1997, Pecquet *et al.* 2000a
Whey proteins	Enomoto *et al.* 1993, Ju *et al.* 1997, Pecquet *et al.* 1999
Caseins	Murosaki *et al.* 1998
β-lg	Adel-Patient *et al.* 2000, Frossard *et al.* 2001, von der Weid *et al.* 2001

2.4.3.1 Systemic response to β-lg

The most common way to analyze the systemic response to antigens is the measurement of total antibody levels or antigen specific levels from sera of the test animals. It is assumed that IgG_1 and IgE antibodies are related to a Th2 type response and IgG_{2a} class antibodies to a Th1 type response in rodents (Stevens *et al.* 1988). Many assays for β-lg specific IgE measurement have been developed (Fritsche & Borel 1994, Fritsche *et al.* 1997, Kato *et al.* 1999, Miller *et al.* 1999, Pecquet *et al.* 1999, Adel-Patient *et al.* 2000, Pecquet *et al.* 2000a, Pecquet *et al.* 2000b). β-lg specific IgG, IgG1, IgG2a and IgM levels have also been measured (Kato *et al.* 1999, Adel-Patient *et al.* 2000, Frossard *et al.* 2001). The passive cutaneous anaphylaxis (PCA) test has been used to measure IgE mediated reactions to β-lg (Ju *et al.* 1997, del Val *et al.* 1999). Delayed type hypersensitivity (DTH) reactions can be measured by injecting β-lg intradermally into the ear or footpad of an immunized animal, and measuring the increase of ear/footpad thickness by dial gauge microcaliper (Pecquet *et al.* 1999, Pecquet *et al.* 2000a, Pecquet *et al.* 2000b). Systemic anaphylactic reactions can be graded after a β-lg challenge by observing the behavior of the animals and scoring the symptoms according to established criteria (Frossard *et al.* 2001). Blastogenic response to an antigen is a measure of specific T-cell response (Kato *et al.* 1999, Pecquet *et al.* 2000a).

2.4.3.2 Local response to β-lg

Local reactions to β-lg can be assessed *in vitro* by stimulating various tissue types and measuring the secreted cytokines in the growth medium. Spleen cells, intestinal mucosal lymphocytes and Peyer´s patch cells were stimulated with β-lg and then IL-4, IL-10, IL-12 and TNF-a levels measured by Kato *et al.* (Kato *et al.* 1999). Stimulation of individual tissue types gives information about local Th1/Th2 balance in the organ of origin. These cells can be also be used in immunospot assays where β-lg specific antibody secreting cells are counted (Kato *et al.* 1999). Secreted β-lg specific antibodies in the lumen of the sensitized animals can be measured (Pecquet *et al.* 2000a).

Mast-cell activation is also a good marker of immunological activity. Activation of mast cells can be measured either by measuring the enzyme concentrations related to de-granulation of the mast cells or by measuring the levels of components released by mast-cells. Rat mast cell protease II (RMCPII) is a specific marker for gut mucosal mast cell degranulation (Ju *et al.* 1995, Fritsche *et al.* 1997), and rat chymase rchyII is another marker of mast-cells degranulation (Gee *et al.* 1997, Ju *et al.* 1997). The classic method is to stain the mast cells with Giemsa-stain and microscopically observe the cell structure for evidence of mast-cell degranulation. The local structural effects in tissues caused by sensitization to β-lg can be assessed by immunohistochemical staining of tissue samples followed by microscopical analysis.

Table 6. Immunological and other relevant assays for quantification of the sensitization in animal models of milk allergy (modified from Valkonen et al. 2002).

Response	Assay and reference
Systemic Response	
Serological response	Total serum IgE, IgG, IgA levels (Kato *et al.* 1999, Adel-Patient *et al.* 2000)
	Levels of β-lg specific serum antibody levels (Miller *et al.* 1999, Pecquet *et al.* 2000a, Pecquet *et al.* 2000b)
T-cell response	Activation of T-lymphocytes (Kato *et al.* 1999)
Skin tests	Passive cutaneous anaphylaxis (Ju *et al.* 1997, Li *et al.* 1999)
	DTH reactions (Pecquet *et al.* 1999, Pecquet *et al.* 2000a)
Systemic acute response	Systemic anaphylaxis, blood pressure, respiratory function (Frossard *et al.* 2001)
Growth	Weight (Bevilacqua *et al.* 2001)
Local response	
Local antibody production	Quantification of antibody secreting cells, immunospot (Kato *et al.* 1999)
	Intestinal fluid antibodies (Pecquet *et al.* 2000a)
Mast cell activation	Release of mast cell products (Li *et al.* 1999)
	RMCTP II and Chymase levels in blood (Ju *et al.* 1995, Fritsche *et al.* 1997, Ju *et al.* 1997)
	Functional tests for mast cell activation (Fritsche & Borel 1994)
Structure of intestinal mucosa	Mucosal structure (Bevilacqua *et al.* 2001)

2.5 Intestinal *in vitro* cell models for absorption studies

2.5.1 Caco-2 cells

The human intestinal Caco-2 cell line has been used over twenty years as a model of the intestinal barrier, enterocytes. Originally this cell line was obtained from a human colon adenocarcinoma. In culture process, this cell line undergoes spontaneous differentiation that leads to the formation of a monolayer of cells, expressing several morphological and functional characteristics of mature enterocytes (Pinto *et al.* 1983, Sambuy *et al.* 2005). Caco-2 cells are normally grown on permeable filter supports, which allow free access of ions and nutrients to both sides of the monolayer. Since these conditions lead to improved morphological and functional differentiation of the cells, they have been proposed as a good physiological model for intestinal transport studies (Hidalgo *et al.* 1989, Sambuy *et al.* 2005). After the cell monolayer has reached confluence transepithelial electrical resistance measurement can be used to monitor the integrity of the monolayer (Sambuy *et al.* 2005).

2.5.2 M-cells

The other absorptive cell type in the mucosa of the small intestine is the M-cell. M-cells can be distinguished from enterocytes by their unique morphological features, such as a diminished apical brush border and deep invaginations of the basolateral plasma membrane that contain lymphocytes and phagocytic leukocytes (Niedergang & Kraehenbuhl 2000). M-cells play an important role in the initiation of mucosal and systemic immune responses, due to their transport activity (Neutra *et al.* 1996). The origin of M-cells is a matter of controversy; they either develop from stem cells located in the crypts or are results of further differentiation of enterocytes (Niedergang & Kweon 2005). *In vitro* models of M-cells rely on the latter theory, they use enterocytes as a starting material for the transformation to M-cells (Kerneis *et al.* 1997, Gullberg *et al.* 2000). In these models Caco-2 cells are grown to confluence and either lymphocytes from mice Peyer's patches (Kerneis *et al.* 1997, Kerneis *et al.* 2000, Tyrer *et al.* 2002) or Raji B-lymphocytes (Gullberg *et al.* 2000) are added to the culture. Lymphocytes migrate through the pores of the filters. After four days of co-culture they can be found between the cells and in pockets formed by the invagination of the basolateral membrane (Niedergang & Kraehenbuhl 2000). The brush border also becomes disorganized and apical sucrase-isomaltase expression is lost. At the same time, the mucosal cells develop transcytotic activity for both inert particles and noninvasive bacteria (Kerneis *et al.* 1997, Tyrer *et al.* 2002). This shows that differentiated enterocytes acquire most of the morphological and functional features of M-cells (Niedergang & Kraehenbuhl 2000).

3 Aims of the study

β-lg is a lipocalin type protein and an important allergen in bovine milk. The structure of β-lg is altered by heat treatments. Heat denaturation modifies its sensitivity to proteolytic degradation, but there is only fragmentary information about the effects of denaturation on the allergenic properties of β-lg. Intestinal absorption characteristics of proteins are one factor affecting allergenicity, but no information on the effect of heat denaturation on *in vitro* transport properties of β-lg are available. Natural infections and vaccinations have also been suggested as important factors affecting the development of food allergies.

The main aims of this doctoral thesis were to evaluate the effect of heat denaturation of bovine milk β-lg on its transport properties and its allergenicity. Preliminary study of the immunomodulatory potential of BCG vaccination on β-lg hypersensitivity was also undertaken. The detailed aims are shown below (numerals I-IV referring to scientific articles belonging to this thesis):

1. To purify β-lg from bovine milk in its native form and characterize it. (I)
2. To evaluate the effect of heat denaturation of β-lg on its transport properties in two *in vitro* cell models. (II)
3. To evaluate the effect of heat denaturation of bovine β-lg on its hypersensitivity in an animal model. (III)
4. Using an established animal model, evaluate the impact of early BCG vaccination on the markers of systemic and gastrointestinal (GI) sensitization to β-lg. (IV)

4 Materials and Methods

Materials and methods are described in detail in the original articles (I-IV).

4.1 Beta-lactoglobulin

4.1.1 Purification (I, III, IV)

β-lg (I) was purified from bovine milk by a modified method (de Jongh *et al.* 2001). For immunizations of experimental animals (III, IV) β-lg (Sigma Chemical Co, St Louis, USA) was purified using anion exchange chromatography (Valkonen *et al.* 2000).

4.1.2 Labeling (II)

β-lg was labeled with BODIPY-FL dye according to the manufacturers instructions (Molecular Probes, Oregon, USA). The BODIPY-labeled β-lg was then separated from non-bound dye by PD-10 gel filtration column and kept at -20 ºC until used in transport experiments.

4.1.3 Heat denaturation (II,III)

Heat denaturation of β-lg was done prior to the transport experiments and immunizations. Heat denaturation was done at 90º for 30 min to achieve a complete and stable denaturation of β-lg. Denaturation was confirmed by using SDS-PAGE analysis.

4.1.4 Characterization (I, II)

For characterization of β-lg, reduced and non-reduced SDS-PAGE was carried out with 4% stacking gel and 15 % separation gel followed by CBR or fluorescent scanning (Valkonen *et al.* 2000). For determination of the isoelectric points isoelectric focusing was used. Gels were scanned with a Storm scanner for fluorescent label in blue mode with an excitation at 450 nm and emission at >520 nm. For Western blotting proteins were transferred to nitrocellulose membrane overnight (Valkonen *et al.* 2000). Both primary antiserum (anti bovine milk β-lg) and HRP-labeled secondary antibody were diluted 1:3000 in PBS-Tween. Detection kit with HRP was used according to manufacturer's instructions.

4.2 Cell culture (II)

4.2.1 Caco-2 cells

Caco-2 (ATCC HTB-37) cells were grown in DMEM supplemented with 10% v/v fetal calf serum, 1% v/v non-essential amino acids and 1% v/v antibiotics in 75 cm^2 tissue culture flasks. Cells were seeded on the inverted transwells and incubated overnight at 37ºC. Thereafter the transwells were placed back into the plates, and cells were grown for 21-28 days to differentiate them. Intact epithelial structure was confirmed by transepithelial electrical resistance measurements (TEER). Cell cultures with TEER value higher than 150 Ωcm^2 were used (Tyrer *et al.* 2002).

4.2.2 M-cells

Peyer's patch (PP) cells were isolated from the small intestines of BALB/c mice. Small intestines were collected and intestinal contents were removed. PP's were dissected and forced through nylon mesh and individual cells collected. Isolated PP cells were diluted in medium and apical medium from Caco-2 cell cultures was replaced with this solution. Cells were grown for 2 days to differentiate to M-cells (Tyrer *et al.* 2002).

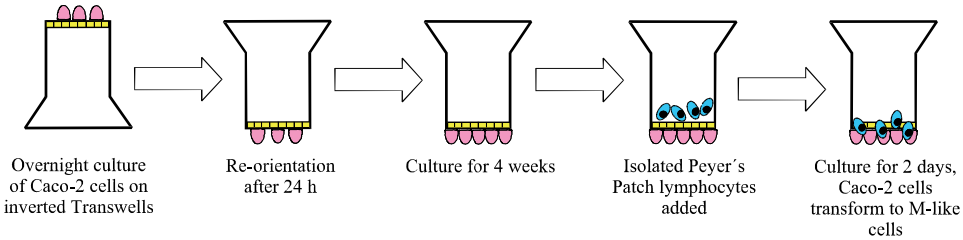

Fig. 4. M-cell culture protocol.

4.2.3 Transport experiments

Apical culture medium was removed from each transwell (Caco-2 cells and M-cells) and replaced with medium containing native or heat-denatured labeled β-lg. Transwells were incubated at 37 °C for 24 h. This was followed by collecting the apical and basolateral culture media for further analysis after this incubation period. Transwell membranes were also collected.

4.2.4 Fluorometric measurement

Fluorescence of the samples containing β-lg from labeling reaction and transport experiments were measured. The effect of fluorescence quenching was assessed by a measurement of the samples after trypsinization. The fluorescence values were measured by using a fluorescence plate reader (an excitation at 485 nm and an emission at 538 nm).

4.2.5 Fluorescence microscopy

Transwell membranes were fixed in a solution of neutral-buffered formalin, 10% v/v formalin. Non-specific binding was blocked with 2% w/v bovine serum albumin and anti ZO-1 antibody was added. Specimens were probed with anti-rabbit IgG alexa-546 conjugate, and counter-stained with DAPI. Cells were examined by epifluorescence microscopy with filters to rhodamine (Alexa 546), DAPI and fluorescein (BODIPY-FL). Digital photographs were taken and image analyses were performed by MetaMorph imaging software.

4.3 Animal model (III, IV)

4.3.1 Animals

Conventionally out-bred Hooded-Lister rats (Laboratory Animal Center, University of Oulu, Oulu, Finland) were used in animal experiments. The stock of rats was HsdOla:LH. The study was approved by the Ethical Committee for Animal Studies, University of Oulu.

4.3.2 Sensitization

The effect of heat denaturation of β-lg in animals was studied with immunizations by using native or heat-denatured bovine milk β-lg and pertussis vaccine containing whole inactivated bacteria (Public Health Institute, Helsinki, Finland). Accordingly, animals were fed native or heat-denatured milk products. Schema of immunization is shown in Figure 5. The effect of BCG vaccine on β-lg sensitization of animals was studied by immunizing animals additionally with subcutaneous injection of BCG vaccine. Animals were also immunized with native β-lg and fed with native milk products. All the animals were allowed water and food *ad libitum*. On day 131, the animals in all groups were sacrified and tissue samples were taken from the antrum of the stomach, duodenum, proximal jejunum (< 5 cm from the pylorus), and colon. Blood samples were obtained by a cardiac puncture. Sera were separated and stored at –20ºC.

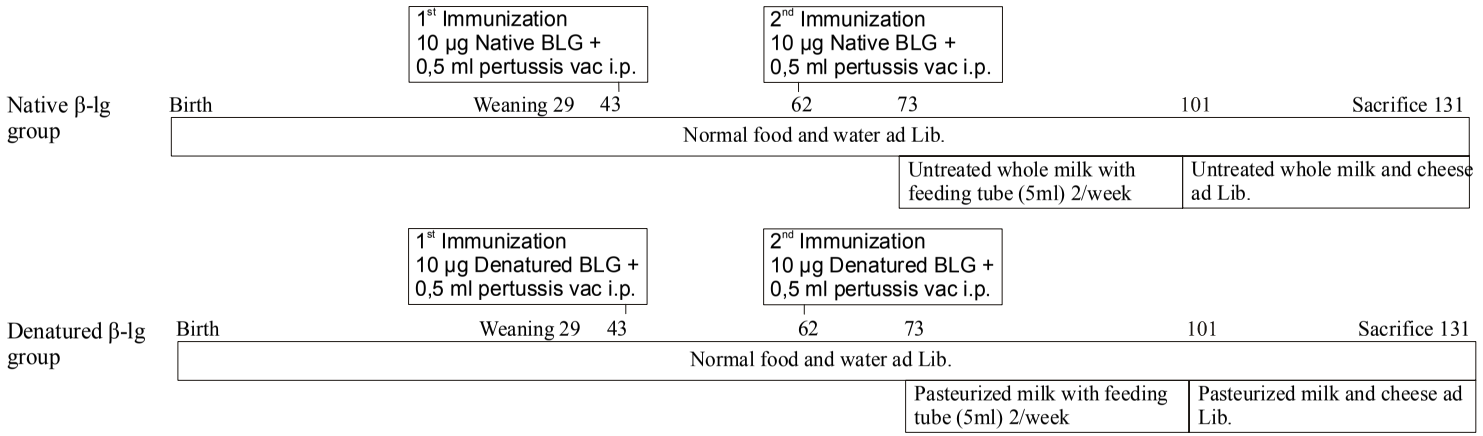

Fig. 5. Schema of the immunization procedure and diets of the experimental animals.

4.3.3 Histology

Specimens were fixed in neutral buffered formalin and emmebedded in paraffin. Sections cut at 5 µm were stained with hematoxylin and eosin. Coded sections were studied by one investigator blinded for the animal group data. Epithelial cells, intraepithelial lymphocytes, and eosinophils were counted (total 300 cells) in the specimens obtained from the duodenum and jejunum. The results were expressed as numbers of intra-epithelial lymphocytes or eosinophils per 100 epithelial cells. The densities of mononuclear inflammatory cells and eosinophils were counted separately in the glandular deep-lamina propria and foveolar (superficial) lamina propria of gastric antral mucosa, in the villous lamina propria of duodenal mucosa, and in the inter-cryptal glandular lamina propria of colon mucosa. Three random fields of lamina propria were counted from each specimen and the mean was calculated. The results of the cell counts are expressed as a number of cells/0.001 mm^2. In the duodenal samples, the number of lymphoid follicles in the whole sample area was counted and expressed as a number of follicles/mm^2.

4.3.4 Measurement of IgE, IgG and cytokine concentrations

4.3.4.1 IgE concentration

ELISA was developed for the measurement of rat serum IgE concentrations and validated by measuring samples with known concentrations of rat IgE and by confirming inter-assay reproducibility. Microtiter plates were coated with monoclonal mouse anti-rat IgE antibody and blocked with human serum albumin. All samples were analyzed as duplicates. Biotin-conjugated monoclonal mouse anti-rat IgE, and then horseradish peroxidase conjugated streptavidin were added. Thereafter substrate to HRP [3,3', 5,5'Tetramethyl-Benzidine (TMB)] was added. After 30 min, the reaction was terminated and the optical densities (OD) were read at 450 nm.

4.3.4.2 Specific IgG_1 and IgG_{2a} concentrations

ELISA for the measurement of β-lg/OVA specific IgG_1 and IgG_{2a} antibodies, microtiter plates were coated with OVA (Sigma) or β-lg. The coating antigen was OVA in the standard lanes and β-lg for the samples. Rat sera and blanks were added to duplicate wells. The reference serum, a rat serum known to contain antibodies to OVA was used. Monoclonal mouse anti-rat IgG_1 (clone MARG1-2; Biosource Int.; 3 mg/ml) and IgG_{2a} (clone MARG2a-1, Biosource Int.; 1 mg/ml) antibody followed by alkaline phosphatase (ALP)-conjugated rabbit anti-mouse IgG were added. A substrate to ALP [para-nitrophenyl phosphate (pNPP)] was added. After 30 min, the reaction was terminated. The ODs were read at 405 nm.

4.3.4.3 Interferon-γ and interleukin-4

For the evaluation of spontaneous secretion of interferon (IFN)-γ and interleukin (IL)-4, fresh specimens taken from the rat duodenum were placed immediately in cell culture medium and incubated at 37ºC for 5 h. The supernatants were frozen at -70ºC. Secreted IFN-γ and IL-4 were measured with a commercial kit (R&D systems, Oxon, UK).

4.4 Statistics (I-IV)

The data were analyzed with the SPSS 12.0.1 package (SPSS Inc., Chicago, USA). All statistical tests were two-sided, and p-values of 0.05 or less were considered significant. Non-parametric Mann-Whitney U test or Kruskal-Wallis tests were used for continuous variables with skewed distribution when different groups were compared, and for correlation analyses. Spearman rank correlation test was used. Fischer's exact test was used for dichotomous variables.

4.5 Ethical considerations

Animal experiments were approved by the Ethical Committee for Animal Studies, University of Oulu.

5 Results

5.1 Beta-lactoglobulin

5.1.1 Purification, characterization and labeling of β-lg (I, II)

β-lg purified from bovine milk by using the method developed in this study was characterized with several methods. SDS-PAGE analyses showed only a monomer of the β-lg with molecular mass of 18.000 D (Figures 2 and 4, article I), which was also recognized in Western blot analysis (data not shown). This indicates that β-lg was in native form as non-covalent aggregates were not present. Native PAGE analysis showed also that isolated β-lg was free from contaminating proteins (Figure 5, article I). Purified β-lg was also characterized by isoelectric focusing (Figure 6, article I). Isoelectric points were 5.1 for variant A and 5.3 for variant B, which are in agreement with previous results for native bovine milk β-lg (Stastna & Slais 2005).

Labeled β-lg was characterized by SDS-PAGE followed by fluorescent scanning and Western blot. Fluorescent scanning of SDS-PAGE gel showed that only a band with molecular mass of 18.000 D was present corresponding to the mass of a monomeric β-lg. A weak additional band with molecular mass of 36.000 D was detected with Western blot corresponding to the β-lg dimer.

5.1.2 Heat denaturation of β-lg (II, III)

Heat denaturation of β-lg was performed after the labeling of the protein. Non-reduced SDS-PAGE was used in analyzing both the native and heat-denatured β-lg. Results show clearly that heat denaturation causes aggregation of β-lg molecules (Figure 1, article II) as shown earlier (Creamer *et al.* 2004). Heat treatment caused also fluorescence quenching of the labeled β-lg. Native β-lg had a fluorescent intensity value of 1195 when concentration was adjusted to 1 mg/ml, heat denaturation diminished the value to 180, indicating fluorescence quenching.

Trypsin treatment raised the intensity of the fluorescence of the heat-denatured β-lg drastically (170/2400), while that of the native β-lg almost doubled (1250/2540) after trypsin treatment. This indicates that even the fluorescence of the native labeled β-lg was quenched significantly. These observations confirm that by using trypsinization, quenching could be greatly eliminated. However, after trypsinization, the fluorescent intensity of the heat-denatured β-lg was still about 7% less than that of the native β-lg (2400/2540). This decrease in fluorescent intensity suggests that a permanent loss of the fluorescence or some irreversible quenching have taken place during the heat denaturation.

5.2 Beta-lactoglobulin transport *in vitro (II)*

5.2.1 Integrity of cell monolayers (II)

Microscopic examination of immunostained cultured Caco-2 and M-cells showed that the human ZO-1 protein, which is a peripheral membrane protein of approximately 225 kDa, was located regularly at the cell junctions (Figure 6). This regular staining pattern indicated that the tight junctions between the cells were regularly arranged in both cell types. Additional evidence for an epithelial barrier function before transport studies was obtained by confirming that the transepithelial electrical resistance (TEER) value was greater than 150 Ω/cm^2. This indicates that Caco-2 cells had differentiated into enterocyte-like cells forming confluent cell monolayers, and were suitable for co-culture and transport experiments.

5.2.2 Transport route (II)

Fluorescence micrographs show that in living cells, both native and heat-denatured β-lg is located in the cytoplasm of the cells, while in dead/injured Caco-2 cells β-lg is located in the intercellular spaces around the tight junctions (Figure 2, article II). This indicates that the transport of native as well as heat-denatured β-lg was transcellular in both Caco-2 and M-cells.

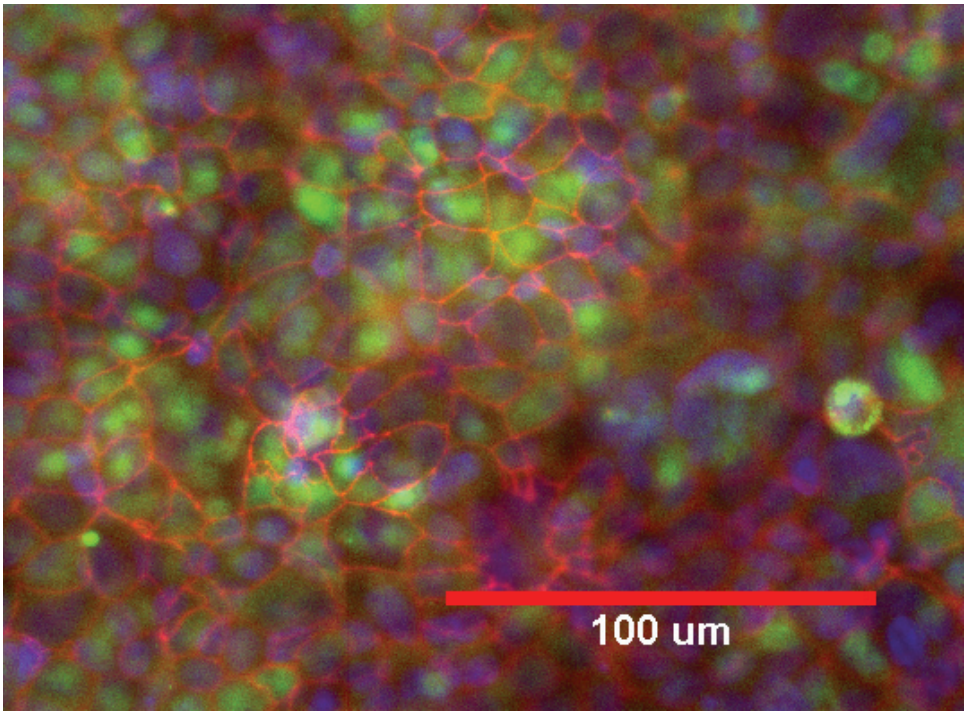

Fig. 6. Fluorescent micrograph of M-cells, showing tight junctions (red), nuclei (blue), and β-lg (green).

5.2.3 Amount of transported β-lg (II)

5.2.3.1 Qualitative analysis

Qualitative analyses of transported β-lg were completed with a reduced SDS-PAGE followed by fluorescent scanning and Western blotting. Fluorescent scanning and Western blotting indicated that native β-lg was transported through Caco-2 cells and through M-cells (Figure 3, article II). Transported heat-denatured β-lg could not be visualized with either fluorescent scanning or Western blotting (Figure 4, article II).

5.2.3.2 Quantitative analysis

Some permanent loss of fluorescent intensity occurred during the heat denaturation of native labeled β-lg. To eliminate this decrease in comparison of transport rates of native and denatured β-lg, we calculated the proportion of the transported β-lg to that of the

non-transported β-lg by using the fluorescent intensities measured after trypsin treatment (Table 1, article II).

Native β-lg was transported in higher amounts by both Caco-2 cells (p=0.003) and M-cells (p=0.000) than heat-denatured β-lg. In addition native β-lg was transported in larger quantities by M-cells than by Caco-2 cells (p=0.004) while no significant difference between Caco-2 and M-cells in transport of heat-denatured β-lg was observed (p=0.34).

5.2.3.3 Degradation of β-lg during transport

Degradation of β-lg during transport in Caco-2 and M-cells was assessed qualitatively by a reduced SDS-PAGE followed by fluorescent scanning and Western blotting. These analyses indicate that heat-denatured β-lg is degraded in both cell types during the transport since it was not detected by either fluorescence scanning or Western blotting (Figure 4, article II). In contrast, native β-lg was not totally degraded since it was detected by both fluorescence scanning and Western blotting (Figure 3, article II).

Second assessment of degradation of transported β-lg was based on the release of the quenched fluorescence, which can be used to monitor degradation of labeled proteins (Jones *et al.* 1997). Trypsin treatment did not increase the fluorescent intensity of the transported native or heat-denatured β-lg (Table 1, article II). This suggests that most of the native and heat-denatured β-lg degraded during transport in Caco-2 and M-cells, while some native β-lg was still intact as shown by visible bands in fluorescent scan and Western blot (Figure 3, article II).

5.3 Immunological response in animals

5.3.1 Local response (III, IV)

5.3.1.1 Morphology of the gastrointestinal tract

The intensity of the mucosal inflammatory cell reaction was analyzed in the different parts of the gastrointestinal tract. Results of these analyses are shown in the Table 7. In comparison with native bovine milk β-lg, immunization with heat-denatured β-lg caused a significant rise in the amount of the mononuclear inflammatory cells (p=0.01/p=0.001) and eosinophils (p=0.001/p=0.006) in antral mucosa when compared to rats immunized with native β-lg or with un-immunized controls. In addition the densities of duodenal intraepithelial lymphocytes (p=0.01) and lamina propria eosinophils (p=0.009) were significantly higher in the controls than in rats immunized with native β-lg. The number of lymphoid follicles in the jejunum was significantly higher in the controls compared to the native β-lg group (p=0.037).

In the analysis of the effect of BCG vaccination, eosinophilic counts in the gastric lamina propria were significantly higher in the BCG/β-lg (p=0.002) and control (p=0.006)

groups when compared with native β-lg group, while in the duodenum the controls showed more eosinophils than the BCG/β-lg group (p=0.002). Rats immunized only with β-lg had significantly less IELs in the duodenum than the rats immunized with BCG/β-lg (p=0.015) or controls (p=0.01), a similar difference between β-lg and BCG/β-lg groups were seen in the jejunum as well (p=0.027). Jejunal intraepithelial eosinophil counts were similarly lower in the β-lg group when compared with BCG/β-lg group (p=0.045). Lamina propria mononuclear inflammatory cell densities were mostly similar, but in the colon, the BCG/β-lg group showed highest density differing significantly from the control group (p=0.02). Showing a similar trend in comparison with the β-lg group (p=0.09), thus showing evidence for a chronic inflammatory reaction.

Table 7. Quantification of mucosal inflammation in untreated controls and in rats immunized with heat-denatured, native or native β-lg accompanied with BCG vaccination. Median values are shown.

Anatomic site	Nat β-lg	Den β-lg	BCG/β-lg	Controls
Antrum				
Mononuclear inflammatory cells	341¤	700¤	427	331¤
Eosinophils	128*	400*	277*	320*
Duodenum				
Mononuclear inflammatory cells	7830	6400	8152	7750
Eosinophils	578+	500	318+	578+
Intraepithelial lymphocytes	7.9#	12.5	14.6#	16.2#
Intraepithelial eosinophils	0	0.8	0.4	1.0
Jejunum				
Mononuclear inflammatory cells	7950	8200	8354	7699
Eosinophils	895	700	925	867
Intraepithelial lymphocytes	15.5@	15.4	23.3@	17.9
Intraepithelial eosinophils	1.0”	0.8	1.7”	1.2
Colon				
Mononuclear inflammatory cells	462	400	658%	373%
Eosinophils	71	70	178	124

¤ p=0.001; β-lg vs. den β-lg, p=0.001, den β-lg vs. controls
* p=0.002; BCG/β-lg vs. β-lg, p=0.006; β-lg vs. controls, p=0.01; β-lg vs. den β-lg, p=0.001; den β-lg vs. controls
+ p=0.002; BCG/β-lg vs. controls, p=0.009; β-lg vs. controls
# p=0.01 β-lg vs. controls, p=0.015 BCG/β-lg vs. β-lg
@ p=0.027; BCG/β-lg vs. β-lg
“ p=0.045; BCG/β-lg vs. β-lg
% p=0.02 BCG/β-lg vs. controls

5.3.1.2 Cytokine production

Spontaneous cytokine secretion of duodenal specimens taken from experimental animals after immunizations and long term exposure was measured to get insight of Th1/Th2 balance in the different animal groups. Secretion of IFN-γ and IL-4 from duodenal samples was low during the five hour incubation period, and there was no significant difference in

the number of animals with detectable secretion between the immunized groups. However, median secretion of IFN-γ was higher in rats immunized with heat-denatured β-lg as compared with controls (p=0.038).

5.3.2 Systemic response (III, IV)

5.3.2.1 IgE levels

The rats immunized with native bovine milk β-lg showed a higher total IgE concentration in serum than the rats immunized with heat-denatured β-lg (Table 8; p=0.031). Total IgE concentrations were lower in the BCG/β-lg than in the native β-lg group (p=0.046).

5.3.2.2 β-lg specific IgG_1 and IgG_{2a} levels

The levels of β-lg specific IgG_1 and IgG_{2a} were low in all groups of animals and, in some cases, below the measurement range of the assay, making the conclusion about specific IgG response unsatisfactory. IgG_1 levels were higher in the control group than in the group immunized with native bovine milk β-lg (Table 8; p=0.037). IgG_{2a} concentrations did not show any significant differences, but there was clearly a tendency for β-lg group to show lower values than controls (p=0.055).

5.3.2.3 IL-4 and IFN-γ levels

The rats immunized with heat-denatured β-lg showed higher levels of spontaneous secretion of IFN-γ than control group (Table 8; p=0.038). IL-4 levels did not show any differences between groups.

Table 8. Measurement of total serum immunoglobulin IgE, β-lg-specific IgG1 and IgG2a from serum, and spontaneous secretion of IFN-γ or IL-4 from duodenal mucosa in controls and in animals immunized with heat-denatured, native or native β-lg accompanied with BCG vaccination. Median values are shown.

Variable	Nat β-lg	Den β-lg	BCG/β-lg	Controls
IgE (ng/ml)	500*	180*	150*	200
IgG_1 (OD)	0.021+	0.03	0.04	0.03+
IgG_{2a} (OD)	0.06	0.06	0.07	0.09
IL-4 (pg/ml)	0	7.7	-	9.3
IFN-γ (pg/ml)	0	28.1^	-	0^
IL-4 (Pos %)	36%	55%	50%	70%
IFN-γ (Pos %)	36%	55%	25%	10%

* p=0.031; β-lg vs. Den β-lg, p=0.046; BCG/β-lg vs β-lg. + p=0.037; nat vs. controls. ^ p=0.038; den vs. controls

6 Discussion

Our main aim was to investigate the effects of heat denaturation on bovine milk β-lg, focusing on the heat induced modifications and their significance in the pathogenesis of hypersensitivity. This was studied using an animal model and by analyzing effects on *in vitro* transport properties of the native and heat-denatured β-lg. The results of animal experiments favor the idea that heat denaturation of β-lg modify its allergenicity. The results of transport experiments suggest that heat denaturation modifies the intestinal transport properties of β-lg, and provide one potential mechanism by which the effect of heat denaturation on the allergenicity could be mediated. Furthermore, the results indicate that BCG vaccination modifies the host response to native β-lg.

6.1 Purification and labeling β-lg

We used the mild isolation method for β-lg purification. It was developed from a method originally described by De Jongh (de Jongh *et al.* 2001), designed for semi large-scale isolations. We modified the method for our purposes at in analytical scale; one liter of fresh milk was used in each batch. This method uses milder isolation conditions than most of the other purification methods, and therefore it should not be denaturing for native bovine β-lg. Extreme pHs and high ionic strengths applied in several previous methods (Table 2) were not used. Previous methods may cause irreversible denaturation of isolated β-lg. Avoiding extreme conditions enabled us to isolate β-lg in as native form as possible.

There is no simple way to determine the degree of denaturation of β-lg. Many spectroscopic methods have been used to study the folding and refolding of β-lg. These can be also used to determine how close to the native state an isolate is (Ngarize *et al.* 2004). SDS-PAGE analysis is normally used to check the purity of isolated proteins, but it can also be used for evaluation of the quality of the protein. We confirmed, by SDS-PAGE analysis, that β-lg purified with our method did not contain any impurities or covalent aggregates of β-lg (Figure 2, article I). Only the monomers of β-lg were detected indicating that no covalently bound aggregates were formed during the purification process. These analyses confirmed that β-lg was isolated in native form and without any impurities. This isolated

native β-lg can be used in applications requiring strictly native form of β-lg without impurities.

We used fluorescently labeled β-lg in our transport studies. A fluorescent label was chosen to avoid the occupational safety problems of radioactive labels. The label used, BOPIPY-FL, has excitation/emission wavelengths (503nm/512nm) comparable to fluorescein (494nm/518nm). This similarity allowed us to use the same filters, as are used for fluorescein.

The used fluorescent dye conjugate, a succimidyl ester, reacts with lysine residues and the amino terminal α-amino group of a protein, forming covalent bonds. In β-lg, there are a total of 15 lysine residues that can react with the active label. Such chemical modification could possibly alter the biological properties of β-lg, by changing the three dimensional structure or other characteristics. It has also been suggested that labels may modify the solubility of proteins (Brinkley 1992). The possible effects of labeling on biological functions have not been studied with β-lg, but for some other proteins, such as surfactant protein B, assays have not indicated any significant BODIPY- related physiological changes (Diemel *et al.* 2001).

When using fluorescence labels, the effect of fluorescence quenching caused by fluorescence energy transfer (FRET) is an important factor to be taken into consideration. In our study, fluorescence quenching caused the fluorescent intensity of the labeled β-lg to drop significantly when it was heat-denatured (30min, 90ºC). Prior to the heat denaturation step, the fluorescent intensity of labeled native β-lg was about 2-fold lower when compared to that after the trypsin treatment and for denatured β-lg, the increase was more than ten fold. This is in agreement with earlier observations showing that fluorescence quenching of labeled proteins can be reduced with trypsinization of the labeled protein (Schade *et al.* 1996, Jones *et al.* 1997). The use of trypsinization treatment allowed us to compare the transport rates of heat-denatured and native β-lg, which would have been impossible without this treatment. Also this reduction of quenching made it possible to estimate the degree of degradation of the transported β-lg. It was noted that 7% of fluorescence was lost in the heat denaturation step when compared to that of native β-lg. To take this decrease of fluorescence into consideration and estimate the relative amount of transported β-lg, we calculated the ratio of non-transported vs. transported β-lg in each experiment and used this as a measure of the transported β-lg.

6.2 Effect of heat denaturation on β-lg transport

In transport studies of Caco-2 and M-cells we found that native β-lg was transported more effectively than heat-denatured β-lg. Furthermore, we found that M-cells transported both native and heat-denatured β-lg more readily than Caco-2 cells. There are no previous cell model studies of either differences in β-lg transport between Caco-2 and M-cells or differences in the transport of native and heat-denatured β-lg.

As M-cells are located among enterocytes in living animals, comparison of the transport properties of these cell types was not possible until development of the M-cell model by Kerneis in 1997 (Kerneis *et al.* 1997), and it has proven to be useful in transport studies (Kerneis *et al.* 2000, Liang *et al.* 2001, Tyrer *et al.* 2002). The finding that M-cells are more

effective transporters of β-lg than enterocytes is in agreement with earlier studies using the Kerneis model, showing that M-cells are more effective transporters of inert particles and microbes than enterocytes (Liang *et al.* 2001, Tyrer *et al.* 2002).

The mechanism behind more effective transport of native β-lg remains speculative. The finding might be partly explained by the size differences between the two forms of β-lg. Native β-lg has a diameter of 1.75 nm but aggregates of heat-denatured β-lg can reach diameter of 50 nm in diameter (Sawyer & Kontopidis 2000, Carrotta *et al.* 2001). The larger size of heat-denatured β-lg aggregate, could explain the lower transport rate of β-lg, at least in M-cells. It has been shown that transport of particles through M-cells is size dependent, being more effective for small particles (Liang *et al.* 2001, Tyrer *et al.* 2002).

Heat denaturation also changes the three dimensional structure of β-lg and this might be an other reason for a less effective transport of heat-denatured β-lg. Receptors for β-lg have been found in rat and bovine cells (Mansouri *et al.* 1997, Mansouri *et al.* 1998) but the presence of such receptors in Caco-2 cells and their importance in intestinal absorption is not known. Structural changes in β-lg (ligand) might change the binding affinity or totally block binding to the putative receptor and thus cause less effective uptake of β-lg in the Caco-2 cells.

An additional aim of our research was to study the degradation of β-lg during the transport. Heat-denatured β-lg was shown to be degraded during transport, as SDS-PAGE analysis did not detect non-degraded heat-denatured β-lg after transportation (Figure 4, article II). We gathered some additional, indirect evidence of degradation by measuring the reduction of the fluorescence quenching of transported β-lg. If transported β-lg is not degraded, trypsinization should increase its fluorescent intensity. Trypsinization did not cause significant reduction of fluorescence quenching in either transported native or heat-denatured β-lg (Table 1, Article II), suggesting that the majority of transported β-lg was degraded with no difference in degradation between heat-denatured and native β-lg in either of the cell types. On the other hand, our SDS-PAGE gel analysis results indicated that a small portion of the native β-lg was transported intact through the cell monolayers, but heat-denatured β-lg was more probably degraded. The reasons for the divergence between the two assays are not clear, but could be related to the insensitivity of the fluorometric assessment to minor increases in intensity caused by fluorescence release. The finding that heat-denatured β-lg is more degraded during transport is in concordance with earlier studies where it has been shown that heat denaturation of β-lg at higher temperatures than 80ºC (Reddy *et al.* 1988, Kitabatake & Kinekawa 1998) makes it more susceptible to degradation.

We showed that native and heat-denatured β-lg were transported through the cell layer via the transcellular pathway and not between the cells (paracellular route). The transport route was similar in both cell types (Figure 2, article II). This is in agreement with earlier studies showing that transport of β-lg, like other nutritients, is mostly transcellular and paracellular leakage is very unlikely (Heyman & Desjeux 1992, Caillard & Tome 1995).

Our study is the first one, in which the *in vitro* M-cell model has been adapted to study transport efficiency and digestion during transport of a nutritional allergen. One problem when using the *in vitro* M-cell model might be the presence of macrophages and dendrite cells derived from the original isolate of cells from mice Peyer's patches (Makala *et al.* 2003). These cells can take part in the processing of antigen during the *in vitro* transport. This obstacle could be bypassed by using the co-culture model developed by Gullberg *et*

al., which uses human B-cell lymphoma raji cells instead of isolated mouse Peyer's patch cells (Gullberg *et al.* 2000). The raji cell line does not contain any cells that might contribute to the transport or digestion process. M-cells differentiated from Caco-2 cells have some of the functional features of M-cells, but the genes induced may reflect a more general FAE phenotype, not entirely the same as natural M-cells (Lo *et al.* 2004). However, it has been proven that in contrast to conventional Caco-2 cells, an *in vitro* generated M-cell monolayer is capable of transporting antigen in a form in which it could be processed and presented to T lymphocytes, indicating that *in vitro* M-cell model has some of the physiological properties of true M-cells (Liang *et al.* 2001).

Quantitative and qualitative differences in the epithelial transport of native and heat-denatured bovine milk β-lg may be factors that modify immunological host responses. It has been shown that several factors are important in the genesis of oral tolerance including genetic background, the host microbial flora, the use of additional adjuvants and the age of the host (Strobel & Mowat 1998, Chehade & Mayer 2005). Oral tolerance is associated with the presence of an immunologically relevant antigen, therefore, the amount of antigen absorbed in intact form is likely to influence the induction of oral tolerance (Strobel & Mowat 1998). The degradation observed during transport and the quantitatively less effective transport of heat-denatured β-lg could be important factors in the development of oral tolerance, or on the contrary the hypersensitivity reactions caused by this antigen. More detailed analysis of the transported β-lg and its properties is necessary before it can be concluded that the changes in the intestinal transport are an important immunological consequence of heat denaturation of β-lg.

6.3 Immunizations with β-lg

An important factor in animal models of immunization is the purity of the antigen. We used β-lg purified by anion exchange. The purity of β-lg was confirmed by SDS-PAGE analysis to make sure that no proteins other than β-lg were present. The purity of antigen in immunizations is an important factor since it has been shown that commercial β-lg preparations from various sources can contain endotoxins and lipopolysaccharides (LPS) and they might have a significant effect on the outcome of the sensitization studies with β-lg (Brix *et al.* 2003, Brix *et al.* 2004).

The structure of the antigen is important when studying a modified antigen like heat-denatured β-lg. In our study the denaturation temperature of β-lg was crucial. Heat denaturation of β-lg is not easy to perform reproducibly, as heat denaturation of β-lg is a very complex phenomenon (Fessas *et al.* 2001). The temperature reached is the most important factor. If temperature is too low the denaturation can be reversible. At higher temperatures changes in the three dimensional structures are more stable, but they may still be partly reversible. To avoid renaturation of β-lg after the heat denaturation we used a high denaturing temperature and long denaturing time i.e. 90ºC for 30 min. The denaturation was also performed just minutes prior to immunization. These procedures assured that β-lg was in the heat-denatured state when immunizations were done. Stabilizing agents, such as thioredoxine or iodoacetamide (Negroni *et al.* 1998, del Val *et al.* 1999) could have been used to stabilize the heat-denatured β-lg, but we avoided this because it could change the

structure of β-lg in an uncontrolled way and have additional effects on the pattern of immune reactions. Furthermore, such stabilizing agents are not present in commercial milk products.

6.4 Heat denaturation of β-lg modifies mucosal and systemic responses

One of the aims of this thesis was to develop an animal model to study the hypersensitivity reactions caused by bovine milk β-lg. This was achieved with by the immunizations of animals with native or heat-denatured β-lg. Animals were also fed with the corresponding milk preparations i.e. raw or processed commercial fat-free milk. Our results showed that heat denaturation of β-lg changed its sensitization properties. The main findings were that native β-lg causes a more intense rise of serum IgE concentration compared to that of heat-denatured β-lg. On the other hand heat-denatured β-lg causes a greater increase of mucosal inflammatory cells in the GI tract. The latter effect was most pronounced in the proximal part of the GI-tract. Based on increased serum IgE levels, the reaction to native β-lg has features of a Th2 balanced response while the reaction induced by heat-denatured β-lg has more Th1 features.

Mechanisms of different reaction patterns for native and heat-denatured β-lg remain speculative. Heat denaturation of β-lg makes it more susceptible to degradation in the stomach; this might be one explanation for the higher levels of IgE in sera of animals immunized with native β-lg, since easily degradable proteins are unlikely to persist in the gastrointestinal tract for a sufficient time to provoke an immune response (Huby *et al.* 2000). Heat treated whey protein concentrate (WPI) has been shown to induce oral tolerance, but not a humoral response as measured by antibody production. On the contrary, oral sensitization with native WPI has been shown to induce oral tolerance together with a measurable production of antibodies, mainly IgG class antibodies specific to β-lg (Enomoto *et al.* 1993). We did not evaluate the oral tolerance in our model, but in agreement with this study, we demonstrated that native β-lg induced more antibody production than heat-denatured β-lg.

In our model heat-denatured β-lg caused more inflammation in the GI tract, resembling a delayed type hypersensitivity reaction instead of a Type I allergic reaction as measured by total IgE levels. It is assumed that low dose immunization induces a more profound DTH reaction than high dose immunization (Strober *et al.* 1998). Smaller amounts of heat-denatured versus native β-lg were transported by enterocytes and M-cells in our *in vitro* studies. If this corresponds to the *in vivo* transport properties of β-lg, this might be one reason for a more pronounced DTH reaction caused by heat-denatured β-lg. Along with the DTH type reaction, increased IFN-γ secretion was induced in animals treated with heat-denatured β-lg. The same trend has been observed in a human study of patients with DTH cow's milk allergy, their IFN-γ production was also up-regulated (Veres *et al.* 2003, Paajanen *et al.* 2005b).

6.5 BCG vaccination modifies the effects of sensitization to native β-lg

Mycobacterium species are known to be potent inducers of the Th1 response (Mosmann & Sad 1996). BCG vaccine contains *Mycobacterium bovis,* and thus it may be capable of inducing a Th1 response and simultaneous suppression of the Th2 response. It has been suggested that vaccination with BCG can modify immune responses, possibly decreasing the risk of allergy in both human and experimental animals (Koh *et al.* 2001). Whether BCG alters the response to β-lg is a matter of controversy. Our results showed that BCG vaccination modulates the responses to native β-lg in the animal model, but the pattern of different components of the response was not consistent in terms of Th1/Th2 features. BCG vaccination decreased the amount of IgE in the sera when compared to the native β-lg group. This suggests that BCG vaccine modulated the response by suppressing the Th2 response, which is in agreement with the results of a previous study (Koh *et al.* 2001). On the other hand, BCG induced eosinophilic infiltration in the gastric mucosa, which is indication of Th2 activity. Thus it seems that the systemic and local effects of BCG vaccination may be different. In our animal model, there was a (non-significant) observation of a BCG vaccination-related shift in the inflammation site to more distal parts of the GI tract, when compared to inflammation caused by native β-lg only. This shift of inflammation site, together with the discrepancy in the Th1/Th2 pattern at local and systemic levels emphasizes the importance of extensive analysis of the response, including both systemic and local effects. Morphologic analysis of different parts of the GI mucosa seems to be of value in demonstrating possible divergence in reaction patterns at different anatomical locations.

6.6 Clinical significance of the heat induced changes of β-lg

β-lg is one of the first milk allergens identified and it has been studied more extensively than other milk allergens. It is easy to isolate from milk, which makes β-lg a very popular model protein for all kinds of proteomic studies. In addition to β-lg, many other possible allergens have been identified in bovine milk. The other main allergens of bovine milk are caseins (α_{s1}, α_{s2}, β and κ), BSA, lactoferrin, and IgG heavy chain (Wal 2001). In recent years a change in prevalence of sensitization to different proteins has been observed, the importance of whey proteins as milk allergens has decreased, but on the other hand the importance of the caseins has increased (Natale *et al.* 2004). This change in the pattern of allergenicity of different milk proteins could be due both to changes in processes applied to milk or to improvement in the analytical methods detecting and identifying IgE reactive CMP`s. Recent study showed that 90% of CMP allergic subjects had antibodies to α_{s2}-casein and 45% to β-lg (Natale *et al.* 2004), indicating that β-lg is still one of the main allergens causing cow's milk allergy.

The effects of dairy processing on β-lg and thus the allergic reactions it causes have not attracted much interest until now. Some studies have been carried out with pasteurized and homogenized milk (Poulsen *et al.* 1987, Paajanen *et al.* 2003, Paajanen *et al.* 2005a), but there have been no systematic studies of the effects of pasteurization on β-lg. Our studies are the first to analyze the effects of heat denaturation on the transport of β-lg and the

hypersensitivity reactions it causes. Heat treatments of β-lg have been studied by the dairy industry, as β-lg plays an important role in the fouling of pasteurization equipment; causing practical and economical problems (Visser & Jeurnink 1997).

Although our results suggest that a simple step in milk processing can be an important factor in the pathogenesis of milk allergy; they do not show that routine pasteurization would have a similar effect. For consistent and irreversible denaturation we used a high temperature (90ºC) for along time (30min). In routine pasteurization, the temperature is 72ºC for, only 15 s, in the UHT (ultra high temperature) process, milk is heated to 125-130ºC for 0.5-2 seconds (Council Directive 92/46/EEC). Due to these differences in heat treatments, structural changes induced by pasteurization are probably less advanced and partially reversible (Villamiel *et al.* 1997, Chen *et al.* 2005). Therefore our findings cannot be directly applied to routinely processed milk.

Even more importantly, the allergological significance of heat denaturation of β-lg in human subjects cannot be determined based on our observations in animals and in *in vivo* cell experiments. Animals demonstrate inter-species differences in their immunological responses and propensity to develop allergic reactions. Hooded-Lister rats are prone to allergic reactions and responses seen in them are probably not directly comparable to humans. Furthermore, immunization by peritoneal injection does not correspond to natural sensitization to food antigens via the oral route. Human clinical studies would be necessary before any conclusions about the significance of heat denaturation of β-lg are made.

6.7 Future aspects

More investigations are needed to study the mechanisms underlying the differences in response to heat-denatured and native bovine milk β-lg. Protein degradation may be one of key factors in the observed difference in allergenicity. Digestion of the heat-denatured β-lg in the gut might expose new sequential antigenic epitopes not found in native β-lg. These epitopes might be the main factor in the induction of more a pronounced inflammatory reaction in the proximal intestine (the gastric mucosa). The amount transported through the mucosa is important when studying initiation of allergic reactions. According to our results, native β-lg is absorbed more effectively and probably in a more intact form than heat-denatured β-lg. These might be important factors in the induction of the classical IgE type reaction in which conformational epitopes are known to be important.

It has been shown that rapid transepithelial transport occurs after sensitization with a particular antigen (Berin *et al.* 1997). In Balb/c mice, IgE antibodies bound on CD23 in epithelial cells are the mediators of this rapid transport of antigens (Bevilacqua *et al.* 2004). Recently it has been shown that human intestinal cells also contain CD23b, which is an isoform of CD23. CD23 is also expressed in Caco-2, as used in our study (Tu *et al.* 2005). A modification to our method could be used to study the effect of specific IgE on transepithelial transport, and degradation of an allergen in a controlled cell culture environment. Alternatively, the effect of the sensitization with β-lg on the intestinal transport could be studied using intestinal segments of animals sensitized to β-lg.

As degradation of the antigen may be important in determining the type of hypersensitivity reaction, the degradation status of β-lg after transport is an important

factor to consider. Mass spectrometric analysis of the transported material can give detailed information about differences in β-lg peptides formed during the transport of native and heat-denatured protein. These peptides could be analyzed for the presence of known linear allergenic epitopes.

Further characterization of the role of β-lg and the fragments formed during transport on the pathogenesis of IgE mediated allergy reactions could be studied with the immediate allergy model based on cultured mast cells and basophiles. These cells express FcεRI receptors on their surface (Wilson *et al.* 2002), which can be primed with IgE class antibodies from sensitized animals. The ability of transported β-lg and its fragments to elicit release of immune mediators from these two cell types could be measured. This would give further information of the sensitizing capacity *in vitro*. Similarly, sera from CMA patients could be used to study differences in reactivity of the peptide epitopes formed during the transport of native and heat-denatured β-lg.

Recent clinical studies have shown that non-IgE mediated food allergy is more common that it had been thought. This indicates that it is important to study the effects of sensitization not only on antibody levels but also locally in the intestinal mucosa. Evaluation of the whole GI-tract is apparently important as the so-called gut allergy can manifest as abnormalities in any part of the GI tract (Kokkonen *et al.* 2004, Macdonald & Monteleone 2005). Our animal studies showed that local inflammatory reactions in the mucosa were widespread starting from the stomach and continuing to the colon. This emphasizes the importance of evaluating the whole GI tract when studying the local reactions.

As clinically important allergies are becoming increasingly common in Westernized countries it is important to evaluate all the possible reasons for this trend. The increase has been linked to the hygiene hypothesis and the increased variety of food stuffs available for consumption. Although our results suggest that industrial processing of food might have a role, the evaluation of this possibility requires further study. In addition to clinical studies, epidemiological studies evaluating the relationship between the emergence of processed foods, including milk products and the incidence of food allergies could provide some information of the allergological importance of food processing.

If our findings hold true in the clinical setting, further development of food processing techniques would be required. A possible alternative to heat processing might be a cold pasteurization of milk products. In cold pasteurization non-thermal preservation techniques like pulsed electronic field are used (Vega-Mercado *et al.* 1997, Bendicho *et al.* 2002). The effect of this treatment on β-lg has been studied and the results indicated that electronic pulses partially modify the native structure of β-lg (Perez & Pilosof 2004).

7 Conclusions

This study evaluated the effects of heat denaturation of bovine milk β-lg. Our results showed clearly that even a simple step in the milk processing such as heat denaturation of β-lg can cause a significant difference in transport properties and the hypersensitivity reactions it causes. Our observations also showed that adjuvants used in immunization experiments have a significant effect on the outcome. Based on the results presented in this thesis the main conclusions are:

1. β-lg can be purified in its native form and without any impurities from bovine milk by use of our method. The method could be used in future applications requiring strictly native and pure β-lg.
2. Heat denaturation of β-lg affects its transport properties in *in vitro* cell models, reducing the transport rate in both the enterocyte and M-cell model.
3. Heat denaturation makes β-lg more prone to degradation; most of the heat-denatured β-lg is degraded as it is transported through the enterocyte and M-cell monolayers.
4. Heat denaturation of β-lg changes its sensitizing properties in animal model.
   – Native β-lg induces a stronger systemic response than heat-denatured β-lg.
   – Heat-denatured β-lg induces a stronger local reaction than native β-lg.
5. Immunization with BCG vaccine has an effect on immune reaction caused by β-lg.
   – It suppresses the Th2 response and modifies the site of local hypersensitivity reaction.

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