Oral sensitization to food proteins and immune mediated effects; a Brown Norway rat food allergy model

Orale sensibilisatie voor voedingseiwitten en immunologisch gemedieerde effecten; een model voor voedselallergie onderzoek in de Brown Norway rat

(met een samenvatting in het Nederlands)

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Voor mijn ouders
Voor Mariëlle

Contents

CHAPTER 1

General Introduction

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contaminated with for instance polybrominated biphenyls (PBB's) or polychlorinated biphenyls (PCB's) [1]. In addition, food allergic reactions may occur in susceptible individuals. There is a lack of tools for research in pathophysiological mechanisms involved in food allergy. Likewise, no good tools are available for the prediction of the allergenic potential of new proteins to be introduced in food. The aim of the work presented in this thesis was the development of enteral animal models for food allergy research and research on the allergenicity of food proteins.

Adverse reactions to foods

Adverse reactions to foods (Fig. 1) can be distinguished between toxic, nontoxic reactions, and aversion. In food aversion, the food is not tolerated for psychological reasons. Toxic reactions will occur in any exposed individual provided that the dose is high enough. Nontoxic adverse reactions to foods, often also referred to as food hypersensitivity, may be defined as a qualitatively and/or quantitatively extremely different reaction to food, which is not so much caused by the food itself, but rather by a specific trait of the person who takes the food. These reactions can be divided into reactions due to food allergy, food intolerance, and food aversion. Food allergy may be defined as a food hypersensitivity in which the reactions are primarily immunologically mediated, while non-immunologically mediated mechanisms play the major role in food intolerance. Food intolerance may be divided into enzymatic, pharmacologic, and undefined food intolerance. Enzymatic food intolerance is caused by a metabolic disorder of individuals, e.g. lactase deficiency. Pharmacologic food intolerance occurs in individuals who are abnormally reactive to substances in the food, like vasoactive amines. In case the mechanism is unknown, the term undefined food intolerance is used.

Based on the immunological mechanisms underlying allergic reactions, 4 different types of allergy can be distinguished as described by Gell and Cooms: type I, the immediate type or immunoglobulin E (IgE) mediated hypersensitivity; type II, the antibody-dependent cytotoxic hypersensitivity; type III, the immune-complex mediated hypersensitivity; type IV, the cell-mediated (delayed type) hypersensitivity. Only IgE-mediated (type I) allergic reactions are for certain known to play a major and primary role in food allergy [2,3]. IgE-mediated (food) allergy often occurs as a part of the so called atopic syndrome. People with atopy are considered to have a hereditary trait (the atopic constitution) associated with a greater risk of development of IgE-mediated allergies. However, up to 10% of the children of healthy, non-atopic parents was also calculated to develop atopic diseases [4]. Although, genetic factors play a major role in the development of allergic diseases, other factors, like the introduction of new allergens and air pollution, are also thought to be responsible for the recent increase in the prevalence of allergic diseases [5,6,7,8].

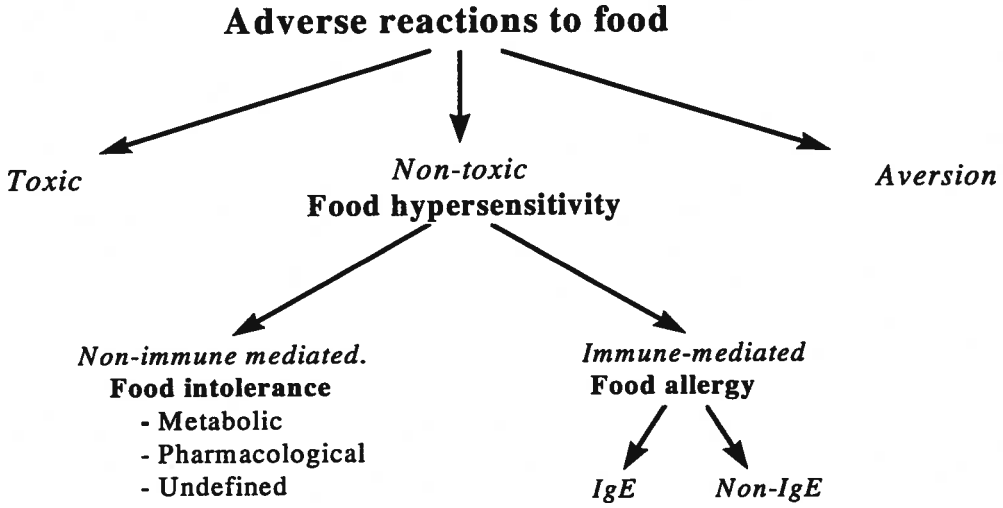

Figure 1. Schematic presentation of adverse reactions to food.

Prevalence of IgE mediated food allergy

Epidemiological studies on the prevalence of food allergy are limited and the majority of the studies has been performed in the paediatric population. The prevalence of food allergy in children is estimated to be about 1.5-5% of the general population, corresponding to about 8-10% of the paediatric population [8,9,10], and around 1% in adults [11,12,13]. Food allergy in children usually appears to be a transient phenomenon and the allergic symptoms tend to subside with age [14,15]. Over 75% of food allergic children has "outgrown" their respective reactivities within 5 to 9 years after the onset of clinical symptoms [16,17]. However, some food allergies, like allergic reactions to peanuts, are more persistent and often do not diminish or disappear while growing up [13,18]. The decreased incidence of food allergy with age suggests that immaturity of the immune system may be an important factor in the pathophysiology of the disease

epidemiological studies on food allergy prevalence indicated that a food hypersensitivity could only be confirmed in 8-20% of children suspected of having food hypersensitivity according to the parents [11,24,25,26].

Diagnostic Tests for IgE mediated food allergy

A correct diagnosis of food allergy is often difficult, although several procedures are available for the diagnosis of food allergy. Since food allergy is usually associated with atopy, a family history gives a good indication of the existence of an atopic constitution. To demonstrate sensitization to the offending food, skin prick tests (SPT), radio-allergo-sorbent tests (RAST) and enzyme-linked immunosorbent assays (ELISA) are often performed. The sensitivity and the specificity of the tests are controversial since the diagnostic concordance of skin-prick tests in suspected food allergic symptoms is only around 60-70% [13,27]. This is mainly due to the allergen source used in the diagnostic tests. As a result of the limited knowledge on food allergens, the various food extract used for diagnosis are not well defined and standardized and the results obtained with these different extracts may vary substantially. Freshly prepared extracts usually give better results than commercially available extracts, especially in case of fruit or vegetable derived allergens [28]. Other tests, like the basophil histamine release assay in the diagnosis of food allergy remains controversial, since the concordance is estimated to be around 50% [29]. However, in the diagnosis of food allergy, a combination of SPT and RAST is mostly used. In addition, an elimination diet can be used for diagnosis, in which the patient avoids possible offending foods for several weeks. Following this "exclusion" phase, patients that improve subsequently test excluded foods one by one, to see which provoke a reaction.

The double-blind, placebo-controlled food challenge (DBPCFC) has been labelled as the golden standard for the diagnosis of food allergy [2,30]. Reproducible reactions to the test food, but not to the placebo, constitutes a positive result. However, this method is not applicable for patients with suspected anaphylactic sensitization to the offending food. To obtain a correct food allergy, diagnosis the results of multiple tests have to agree with each other. After the diagnosis of food allergy is established, an elimination diet in which the offending food is avoided is mostly recommended. Since food allergy tends to decrease with age, repeated testing is suggested to confirm the persistence of food allergy for specific allergens.

Clinical manifestations of IgE mediated food allergy

The type I or immediate type hypersensitivity is characterized by the production of food

allergen-specific IgE and the activation of mast cells or basophils. Food-allergen specific IgE antibodies bind to the high affinity IgE receptors (FcεRI) present on mast cells throughout the body tissues and basophils in the circulation. Upon renewed contact with the food-allergen, the allergen binds to the Fab region of cell-associated IgE and subsequently cross-links the membrane-bound IgE molecules. Cross-linking of several IgE molecules will result in an intra-cellular signal causing degranulation of the mast cells and basophils. The release of chemical mediators such as histamines, leukotrienes, prostaglandins, platelet-activating factor, and newly formed cytokines cause the allergic symptoms (Fig. 2). These mediators induce a variety of food allergy associated clinical symptoms involving the gastrointestinal tract, the skin, the respiratory tract, and the circulatory system, as listed in Table 1 [31-34]. The symptoms may occur within minutes to days after ingestion of the offending food [35] and may sometimes result in an anaphylactic shock and sometimes death [36,37].

Table 1. Clinical aspects of food allergy

Oral cavity	Oral allergy syndrome (itching and swelling of lips, mouth or throat)
Gastrointestinal tract	Vomiting, cramps, diarrhea, abdominal pain, angioedema
Skin	Urticaria, atopic dermatitis (eczema), angioedema
Respiratory tract	Asthma, rhinitis, bronchospasm, wheezing, angioedema
Cardio-vascular	Decrease in blood pressure, anaphylaxis

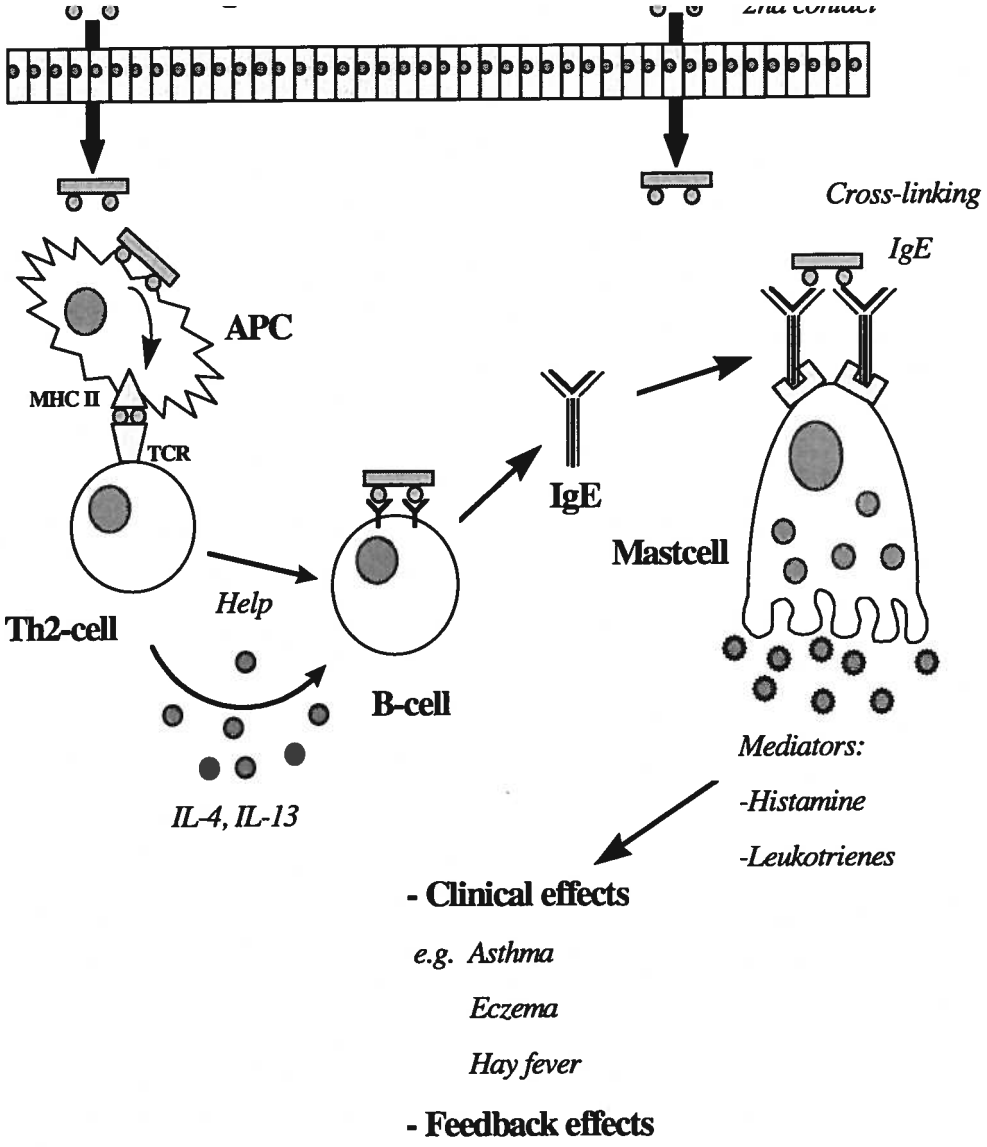

Figure 2. Schematic presentation of the mechanisms in type I, IgE mediated, hypersensitivity reactions.

Mechanism of IgE-mediated allergy

The recognition of food allergens and the subsequent production of IgE antibodies is a complex process, involving different cell types and molecular interactions. Specific immunity towards antigens in general is exerted by B cells and T cells, resulting in long-lasting protection. B cells recognize intact protein antigens via their membrane-inserted immunoglobulins, T cells recognize antigenic peptides via their T cell receptor (TCR). T cells only recognize the processed antigens (8-25 amino acids) in association with major histocompatibility complex (MHC) molecules on the surface of antigen presenting cells (APC) [38]. Besides recognition and binding of the antigen, a second signal is required for the activation of naive B and T lymphocytes [39]. Recognition of antigen without the second signal leads to inactivation, resulting in apoptosis or rendering into functionally unresponsive lymphocytes (anergy) [40,41].

T cell activation and their cytokine profiles

Two T cell subsets can be distinguished based on the expression of the accessory molecules CD4 and CD8. CD8+ T cells recognize peptides in association with MHC class I, present on all nucleated cells, and become activated by virus-infected or malignant cells and will eliminate them by cytotoxicity. In addition, they can act as suppressor cells that regulate the activity of CD4+ T cells. CD4+ T cells recognize peptides in association with MHC class II, present on APC and B cells. CD4+ T cells regulate the activity of cytotoxic T cells and B cells, and other cells from the innate immune system [38]. Peptides are recognized by the TCR associated with a CD3 complex, consisting of a γ-, δ-, ε-, ζ-, and η-chain, which regulates the signal transduction into the cytoplasm [42]. The TCR consists of two transmembrane polypeptide chains, which comprises a α-chain and a β-chain or a γ and δ-chain, with a variable (V) and a constant (C) part. Each T cell bears receptors of a single specificity and a functional TCR is constructed upon DNA rearrangements leading to a selection of different V, J (joining), and D (diversity) and C segments which leads to the enormous TCR polymorphism [43]. Besides binding of the TCR/CD3 complex in association with either CD4 or CD8 to peptides in the groove of the MHC molecule present on the APC, several other interactions between the T cell and APC are necessary to activate the T cell. A second costimulatory signal for T cell activation is delivered by CD28 or CTLA-4. Binding of CD28 or CTLA-4 to their ligands present on the APC, B7-1or B7-2, results in T cell activation and subsequent interleukin (IL)-2 production [44,45]. Besides IL-2, other cytokines may be produced as well by stimulated T cells.

Two different subsets of effector CD4+ T helper (Th) cells were described in the mouse and human based on different cytokine production profiles. T helper-1 (Th1)

enhance eosinophil maturation [46-48]. Later, a third subset, the Th0 cell was described which produces a cytokine profile of both Th1 and Th2 cytokines [48,49]. This subset is either the common precursor for the Th1 and Th2 cells or constitutes a third effector population. Th1 and Th2 cells inhibit each others actions and their balance determines which effector mechanism is activated. This balance is influenced by various factors, including cytokines produced by other cells, the nature and dose of the antigen, concurrent infections, the use of adjuvantia, and the genetic make-up of the exposed individuals [48,50-52].

B cell activation

Humoral immunity is mediated by B cells. The membrane-inserted IgM and IgD molecules function as receptors for naive protein antigens. Binding of the antigen to these Ig molecules results in signal 1, and requirement for signal 2 depends on the nature of the antigen recognized. Most antigens, such as allergens, are thymus-dependent antigens indicating that B cells need costimulation from activated Th cells to produce specific antibodies [44,53,54]. Antigen binding leads to internalization, proteolitical cleavage into epitopes, and presentation of the epitopes on the surface of the B cell in MHC class II molecules [44]. Specific recognition of such epitopes by an effector Th cell induces the expression of CD 40-ligand (CD40L) on the T cell. Subsequent interaction with its receptor CD40 on the B cell, the so called cognate T-B cell cooperation, delivers signal 2 and can lead to activation of the B cell [55,56]. Activated B cells express B7 molecules, can function as APC, and become receptive for cytokines that augment proliferation and differentiation [57,58]. Via membrane-bound and soluble signals, the T cell allows the B cell to mature into either long lived memory cells, while most differentiate into plasma cells that initially secrete large amounts of IgM. In the course of a response they can change the constant part (isotype) of the Ig molecules by gene-segment rearrangement leading to secretion of IgG or other isotypes, which activate distinct effector mechanisms after binding to the antigen [59].

T cells in IgE-mediated allergy

Several studies have shown that CD4+ Th2 cells play an important role in the pathophysiology of allergic diseases. T cell clones from atopic donors, specific for environmental allergens, were shown to have a Th2 phenotype with high production of IL-4 and IL-5 and little or no IFN-γ, whereas T cell clones from non-atopic donors upon stimulation with antigen produced IFN-γ and no or little IL-4 [60-63]. These data suggest different functional subsets of CD4+ T cells in atopic and normal individuals.

The Th2 cell-derived cytokine IL-4 has been shown to induce B cell switch to IgE [64], a phenomenon wich has also been reported for IL-13 [65]. As a consequence, atopic individuals have elevated levels of IgE. In contrast, IgE synthesis is inhibited by IFN-γ, a Th1 cytokine [64]. Moreover, most CD8+ T cells produce IFN-γ [66] and have been suggested to suppress IgE responses. However, a subset of CD8+ T cells (Tc2) cells are known to produce a similar cytokine profile as Th2 cells, although their role in IgE-mediated reactions is not yet clear.

CD8+ T cells were found to be active early in the induction phase of the immune response, suggesting an ideal position to skew the immune response into a Th1 response [67]. Moreover, IL-12, which is obligatory in the generation of Th1 cells, plays an important role in developing human cytotoxic CD8+ T cells [68,69]. Recently it has been demonstrated that γδ T cells produced type 1 or type 2 cytokines [70]. This is of critical importance as γδ T cells produce these cytokine with rapid kinetics and upon first encounter with the antigen, and thus may be one of the sources for the cytokine that influence CD4+ and CD8+ polarization [71,72].

As described above, our knowledge on the pathophysiological mechanisms involved in the development of food allergy as well as the development of immune mediated effects upon challenge has greatly increased over the past decades. Nevertheless, many questions have remained unanswered. Because tools for research into these issues are rather lacking, new models suitable for mechanistic studies will be of great value.

Food allergens

In theory, every food (glyco)protein can potentially be a food allergen. Most food allergens are glycoproteins with a molecular weight between 10 and 60 kD [73,74]. Factors that determine the allergenicity of food proteins are poorly known, but an important factor may be the digestibility of the protein in the gastro-intestinal tract since it is known that food allergens are relatively stable to acid- and heat-treatment, and relatively resistant to digestive breakdown [74-76]. However, even small molecules are known to cause sensitization either directly or via the hapten-carrier mechanism [77]. It is also known that carbohydrate structures on proteins in part determine or influence the allergenicity of proteins [78,79]. In particular with respect to B cell epitopes, since carbohydrate structures may for an important part determine the secondary and tertiary structure of proteins and as such may strongly determine the conformational B cell epitopes. The allergenicity not only differs between proteins from different food products, but also between proteins from one product. For instance, cow's milk contains proteins that only play a minor role in allergic reactions, while other milk proteins demonstrate strong allergenic properties. Proteins for which many patients are sensitized are often referred to as "major allergens". The most frequently observed food allergies

Several (bio)technological techniques can be applied to reduce the antigenicity of food proteins to produce for instance hypoallergenic infant formulas. Biotechnological techniques are also available to synthesize for instance new proteins or new biological varieties for applications in food. For such biotechnologically derived protein products (novel foods), allergenicity may also pose a major concern. For safety reasons, it is of importance to evaluate the residual antigenicity of modified protein products, to screen on possible cross-reactivity to prevent reactions in previously sensitized individuals, and to test for sensitizing properties of new and/or modified protein products. Although well validated models to determine the allergenic potential of new dietary proteins are not available yet, several methods may currently be applied to generate some relevant information with respect to the antigenicity and allergenicity of proteins.

In vivo antigenicity assays

To determine the antigenicity of proteins, several well validated assays are operational. These assays are based on parenteral application of the test proteins to laboratory animals, in which the guinea pig is the most regular test species. Yet, these assays are also operational for other species, like the rat. Within these antigenicity assays, several well validated *in vitro* and *in vivo* tests and analyses can be applied to qualitatively and quantitatively determine specific immune responses and immune mediated effects as a measure for the antigenicity of the test protein. In general, these parenteral antigenicity assays demonstrate high sensitivity.

However, although the information from antigenicity assays may be of major relevance, it must always be recognised that such assays only provide information on the antigenicity of proteins. In general, any protein that may be recognized as an antigen (foreign protein) will induce a humoral and cellular immune response upon injection and will most likely give a positive testing result in such assays. Whether a protein has a high or low potency of inducing food allergic reactions in (susceptible) humans can not be concluded or predicted based only on the results of these parenteral antigenicity assays. Natural barriers such as the gastrointestinal acid denaturation and digestion and the mucosal/epithelial layers, which are all known to prevent, reduce, or in any other way influence the contact between food antigens and the local and systemic immune system [82,83], are not modelled or taken into account in such assays. Therefore, it seems not justified to restrict or withhold the application of new proteins based only on a positive testing result in a parenteral antigenicity assay. More discriminating approaches, that include an evaluation of the influence of other factors than the antigenicity only, are therefore of importance.

Physico-chemical and immunochemical analyses

In addition to *in vivo* antigenicity assays, several (combinations of) physico-chemical and immunochemical analyses are used routinely to determine antigens. In general, immunochemical methods also show high sensitivity in demonstrating the presence of antigens or in detecting antigen-specific antibodies. However, these *in vitro* analyses are not directly suitable to study the allergenicity of new proteins, since antibodies or sera obtained from already sensitized subjects are needed. Yet, immunochemical analyses can be very useful in the control on hypoantigenicity of modified protein products or to study possible immunological cross reactivity. In addition, these analyses can be used to determine possible sensitization using sera collected from patients (diagnostic procedures) or test animals (*in vivo* antigenicity assays).

Mast cell and basophil degranulation tests

Determination of allergenic proteins or fragments that are able to cause activation of mast cells and basophils is possible using *in vitro* mast cell or basophil degranulation tests. For these assays, mast cells or basophils are isolated and subsequently loaded with antigen-specific cytophylic antibodies. The sensitized cells are subsequently incubated with the antigen or test product and possible activation and degranulation of the cells can be determined by means of for instance the measurement of histamine release. Well validated *in vivo* counterparts for the detection of mast cell activation is the Passive Cutaneous Anaphylaxis (PCA) test or the Active Systemic Anaphylaxis (ASA) test.

As is the case for immunochemical analysis, mast cell and basophil degranulation tests are not directly suitable to determine the allergenicity of new food proteins because sera from already sensitized subjects are needed. However, the assays are useful to control on hypoallergenicity of modified protein products or to study possible immunological and allergological cross reactivity.

Human studies

In the evaluation of the potential allergenicity of food products, patient assays such as skin prick tests or challenge procedures may also be used. For instance, these assays are applicable in the evaluation of the residual allergenicity of hypoallergenic products, in the evaluation of cross-allergenicity, or in the evaluation of the possible allergenicity of food products derived from biotechnologically derived crops in which a gene from a known allergenic source species has been introduced. However, the use of patients in such assays for non-diagnostic purposes requires careful ethical consideration. Since

The role of the gastrointestinal tract physiology in food allergy and in the evaluation of the allergenicity of food proteins

Many elements of the gastrointestinal tract physiology influence the ultimate allergenicity of food proteins. These include the pH, digestive enzymes, bile, peristalsis, transit time, bacterial fermentation, and the intestinal barrier function, permeability, and absorption [82,84,85]. It should be recognized that primary, secondary, and tertiary structures of (glyco)proteins are affected to different degrees by digestion, indicating that B and T cell epitopes will be affected by digestion to different degrees. In addition, it should be recognized that digestion of food proteins is part of the normal sequence of events following consumption of food and that food allergic patients may well have become sensitized to digested allergens. Indeed, *in vitro* enzymatic digestion of food allergens does not necessarily diminish patient IgE binding [86], yet may even increase the IgE binding [87]. Based on human clinical observations, an important role of digestion with respect to the allergenicity of food proteins is often suggested, yet this role is still poorly investigated and documented. However, some attempts have been made to correlate the susceptibility to enzymatic breakdown of cow's milk proteins and the intestinal permeability of these proteins with their allergenic properties [88-90]. Evidence for an important role of digestion with respect to food protein allergenicity also comes from animals studies. Prefeeding of an endopeptidase inhibitor (aprotinin) to mice results in an inhibition of oral tolerance induction by protein feeding [91], while feeding of protein antigens to mice is known to induce substantial systemic tolerance for specific antibody and cell mediated immune responses under normal circumstances [92-97].

Because acid-denaturation and digestibility of a food constituent in the gastro-intestinal tract are likely to be important factors partly determining the allergenic potential of food proteins, knowledge on the acid-stability and digestibility of food proteins as well as on the residual antigenicity and allergenicity of absorbed fragments may be of major relevance in the evaluation of the allergenicity of (new) food proteins. Several food allergens or allergenic determinants were indeed reported to be relatively resistant to acid-denaturation and proteolytic digestion [86,87,98-103]. Unfortunately, still only limited information is available on differences in susceptibility to acid-denaturation and gastro-intestinal digestion between strongly allergenic food proteins and proteins that possess weak or virtually no allergenic potential. Therefore, evaluation of acid-stability and digestibility of food proteins will in most cases not yet provide conclusive information regarding their allergenic potential upon ingestion.

The intestinal barrier function, permeability, and absorption are hardly or not taken into account in the evaluation of the allergenicity of food proteins. Particularly in patients suffering from gastro-intestinal pathology, local damage may cause an increased

macromolecular absorption resulting in an increased systemic food allergen load. However, although knowledge on the passage of specific protein antigens and their fragments may provide some additional information in the evaluation of the potential allergenicity of protein products, the macromolecular exclusion by the epithelial barrier is a rather ancient concept [104-107]. This has become particularly evident from studies on the presence of food allergens and other food proteins in human breast milk [108-114]. From these studies, it became clear that macromolecular passage from the intestinal lumen into the circulation should be regarded a normal phenomenon also in healthy individuals. Therefore, an impaired macromolecular exclusion by the epithelial barrier is not considered of primary importance in the development of food allergy.

Animal models in food allergy research and research on allergenicity of food proteins

Because of the restrictions of all models or approaches described in the previous sections and the limited possibilities for human research, animal models suitable for food allergy research or research on the allergenicity of food proteins would be of real value. Several attempts to develop animal models for food allergy research have been conducted in the past. Although some of the attempts to develop enteral sensitization and/or challenge protocols for laboratory animals were rather successful or at least promising, these efforts hardly resulted in structured approaches aimed at the development of well validated enteral allergenicity models.

For food allergy research, 3 rodent species have frequently been used: the mouse, the guinea pig, and the rat, although occasionally other animals were used [115,116]. Many studies have been conducted using parenteral sensitization and enteral challenges [117-122]. In addition, effects of challenges have also frequently been investigated in *in vitro* studies with intestinal tissue or with for instance ligated gut [123-127]. Although effects upon oral challenge in these models of IgE mediated hypersensitivity were successfully investigated, the natural route of feeding during the sensitization period was not taken into account. The ideal model would include the possibility for oral sensitization.

In mice, immune priming or sensitization may occur after enteral protein administration if adjuvants are used [128,129] or if enteral exposure is performed at early stages of life [92,128,130,131]. However, under normal conditions, oral protein feeding of mice both through gavage as well as via the drinking water or diet most easily results in tolerance induction [92-97]. Particularly repeated exposure was demonstrated to result in systemic tolerance rather than priming of humoral and cellular responses [92,95]. The easy induction of an immunological tolerance upon enteral protein exposure of mice indicates that the mouse is not a most suitable species for studying oral sensitization. However, this preferential response makes the mouse most useful in oral

of value in studies on differences in sensitizing properties of [illegible]
and formulas based on modified protein products (hypoallergenic formulas) [134-137]. However, a significant difference in immunophysiology in guinea pigs when compared to other species, the limited knowledge on the guinea pig immune system, and the lack of tools for studying the guinea pig immune system are major draw backs for the use of this species in food allergy research. In addition, although the guinea pig proved very sensitive in studying oral sensitization to proteins and a reduced sensitizing potential of hypoallergenic products could be demonstrated with this species, it remains questionable and to be evaluated whether a guinea pig assay will demonstrate any specificity in investigating differences in allergenic sensitizing potential of food proteins. For instance, it should be emphasized that most studies on oral sensitization demonstrated anaphylactic sensitization. No or only very limited information is available whether the guinea pig is able to discriminate between anaphylactic and non-anaphylactic sensitization upon oral protein antigen exposure. Such a dichotomy in immune response is known to exist in other species like the mouse, rat, and man.

For the rat, oral sensitization to food proteins administered through the diet or by intra-gastric dosing, often in combination with an adjuvant to facilitate the immune response, was also reported [138-141]. Although tolerance induction may also occur in rats [142], it was not observed to be the general response upon oral antigen feeding. Since the rat is commonly used in routine toxicity testing, knowledge on the oral sensitizing properties of food proteins in the rat would enable the evaluation of such properties in a perspective to the total of information on the potential effects of a product on the health state. Moreover, a second advantage of the rat as a species for research on effects of substances on or interactions with the immune system is the rather broad knowledge on the rat immune system and the availability of many tools for studies in this field. Finally, recent studies demonstrated that upon intra peritoneal injection of food antigens, specificities of induced antibodies were similar to those in man [143], but comparable studies based on oral sensitization of rats have not been reported yet.

Scope of this thesis

The primary aim of the work presented in this thesis was the development of an enteral rat model for food allergy research and research on the allergenicity of food proteins.

First, we developed an intra-gastric dosing protocol, without the use of an adjuvant, for inducing specific humoral (IgG and IgE) and cellular immune responses in the Brown Norway rat (BN rat; high IgE responder strain) using the well defined chicken egg white allergen ovalbumin as a model antigen (Chapter 2). In Chapter 3, we have demonstrated the influence of exposure to soy-proteins via the diet of parental generations of BN rats on the presence of soy-protein specific antibodies in their offspring bred and raised on a soy-protein free diet for several generations. In addition,

using an oral sensitization protocol, we examined the effect of dietary pre-exposure to soy-protein on oral sensitization with soy-protein in the BN rat. In Chapter 4, studies are described on the influence of genetically-based strain-specific characteristics of the immune system on the outcome of oral sensitization studies using BN, Hooded Lister (HL), Piebald Virol Glaxo (PVG), and Wistar rats. In Chapter 5, we described studies in which we exposed BN rats to different food-proteins and the specificities of induced antibodies in the enterally sensitized rats were compared with the specificities of antibodies in sera from food allergic patients to determine whether a comparable pattern of proteins is recognized by the rat and human immune system. Finally, in Chapter 6, studies on possible systemic and local immune mediated effects upon oral challenge of sensitized rats are described. Effects on the respiratory system, blood pressure, and permeability of the gastro-intestinal tract were examined *in vivo*. The main results obtained in the studies described in this thesis are summarized and discussed in the final Chapter (Chapter 7: Summarizing Discussion).

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CHAPTER 2

Oral sensitization to food proteins: a Brown Norway rat model

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Background: Although several *in vivo* antigenicity assays using parenteral immunization are operational, no adequate enteral sensitization models are available to study food allergy and allergenicity of food proteins. *Objective*: This paper describes the development of an enteral model for food allergy research in the Brown Norway (BN) rat. *Methods*: The animals were exposed to ovalbumin either *ad libitum* via the drinking water (0.002 to 20 mg/ml) continuously for 6 weeks or by gavage (1 mg/ml per rat). Gavage dosing was performed either daily, twice a week, once a week or once every two weeks during a period of 6 weeks. No adjuvants were used during the sensitization studies. *Results*: After intra-gastric administration of ovalbumin once or twice a week or once every two weeks, no or only a very low frequency of ovalbumin-specific antibody responses were detected. Daily intra-gastric dosing with ovalbumin resulted in antigen-specific IgG as well as IgE responses in almost all animals tested. Upon *ad libitum* exposure, ovalbumin-specific IgG but no ovalbumin-specific IgE was detected. The cellular response was examined by determination of delayed-type hypersensitivity (DTH) reactions in the animals dosed by daily gavage and in the *ad libitum* exposed rats. Both sensitization protocols sensitized for DTH. The response was most pronounced in *ad libitum* exposed rats at day 28 of exposure. *Conclusion*: These studies show that the BN rat may provide a suitable animal model for inducing specific IgG and IgE responses as well as specific T cell mediated hypersensitivity (DTH) to ovalbumin upon exposure via the enteral route without the use of adjuvants.

Introduction

Humans rather frequently suffer from more or less severe allergic reactions after consumption of dietary proteins [1,2]. Type I or Immunoglobulin E (IgE)-mediated allergic reactions are known to play a major and primary role in food allergy [3]. No methods to predict whether a protein has a strong or weak potency of inducing food allergic reactions in susceptible humans are available at present.

Several attempts have been made to develop animal models for food allergy research, mainly in mouse, guinea pig, and rat. Many of these studies have been conducted using parenteral sensitization and enteral challenges [4,5,6,7,8]. Although effects upon oral challenge in these models of IgE mediated hypersensitivity were successfully investigated, the natural route of feeding during the sensitization period was not taken into account. In mice, immune priming or sensitization may occur after enteral protein administration if adjuvants are used [9,10] or if enteral exposure is performed at early stages of life [9,11,12,13]. However, under normal conditions oral protein feeding of mice most easily results in tolerance induction [12,14,15,16,17,18]. This preferential response in mice indicates that the mouse is not a most suitable species for studying oral sensitization. For the guinea pig, several studies with oral sensitization to food proteins

have been described [19,20,21,22,23,24]. However, a significant difference in immunophysiology in guinea pigs when compared to other species and the limited knowledge on the guinea pig immune system and tools for studying the immune system are major draw backs for the use of this species in food allergy. For the rat, oral sensitization to food proteins administered through the diet or by intra-gastric dosing was also reported [25,26,27]. Although, sensitization to orally administered food proteins can be induced in rats, tolerance induction may also occur [28]. Nevertheless, since the rat is commonly used in routine toxicity testing, knowledge on the oral sensitizing properties of food proteins in the rat would enable the evaluation of such properties in a perspective to the total of information on the potential effects of a product on the health state.

The development of IgE mediated allergies, including food allergy, is more common in atopic humans who have a genetic predisposition to react with an elevated production of IgE antibodies to generally harmless substances. The Brown Norway (BN) rat is a high-immunoglobulin (particularly IgE) responder strain [29] and thus, to a certain degree, resembles atopic humans in their (genetic) predisposition to react with an overproduction of IgE to antigens. This preferential response indicates that the BN rat may be a suitable strain for food allergy research. Recently, Atkinson *et al.* [27] reported a Brown Norway (BN) rat model of food allergy using oral administration of ovalbumin in combination with intraperitoneal administration of carrageenan as an adjuvant to promote IgE production. We have reasoned that hyperstimulation of the IgE response in this way may influence the control mechanisms that occur under conditions of "physiological" antigen exposure. Although such predispositional conditions may likely play a role in the development of food allergy in humans, we preferred the development of an enteral animal model without the use of an adjuvant. We hereby report an intra-gastric feeding protocol, without the use of an adjuvant, for inducing specific humoral (IgG and IgE) and cellular immune responses in the BN rat using the well defined chicken egg white allergen ovalbumin as a model antigen.

Material and Methods

Animals and maintenance

Young male Brown Norway (BN) rats were obtained from Charles River (Sulzfeld, Germany). The rats were housed in an animal room maintained at 23 ± 3^{0}C, with a light/dark cycle of 12 h, and a relative humidity of 30-70% during the experiment and for at least 10 days prior to study initiation. The animals were housed in stainless-steel wire cages in groups of four and had free access to food and tap-water. The rats were bred and raised on a commercially available ovalbumin-free rodent diet (SDS Special Diet Service type RM3(E) FG SQC, Witham, UK). Pre-study blood samples were

Fluka Chemie, Buchs, Switzerland, purity: 70 %). Several oral dosing protocols were applied. The animals were exposed to OVA either *ad libitum* via the drinking water (0.002, 0.02, 0.2, 2 or 20 mg/ml) continuously during 6 weeks or by gavage, using a 18-gauge stainless steel animal feeding needle (1 mg OVA/ml tap-water; 1 ml/animal). Gavage dosing was performed daily, twice a week, once a week, or once every two weeks during 6 weeks. The OVA containing drinking water was refreshed twice a day to avoid turning sour. After the induction period, the animals were not exposed to OVA for one week before sacrifice, except for the animals used to perform delayed type hypersensitivity tests. Control animals received normal drinking water and either or not a daily gastric intubation with 1 ml of tap-water. Blood samples were obtained from the orbital plexus under light CO_2 anaesthesia at weekly intervals or by exsanguination from the abdominal aorta at sacrifice. After coagulation for 1 h at room temperature, the blood samples were centrifuged (Heraeus Minifuge T, Osterode, Germany) for 20 min. at 2000g and 4°C to obtain sera. The sera were stored at -20°C until analyses for anti-OVA specific IgG titers by Enzyme Linked Immunosorbent Assay (ELISA) and anti-OVA specific IgE by ELISA and passive cutaneous anaphylaxis (PCA)-test.

Positive control animals were injected intraperitoneally (i.p.) with 0.5 ml of a 0.2 mg/ml OVA solution in sterile saline on days 0, 2, 4, 7, 9, and 11. To potentiate the immune response, 0.2 ml of a 25 mg/ml $AL(OH)_3$ adjuvant suspension in sterile saline mixed with the 0.5 ml of OVA was injected on day 0. The animals were bled on day 28 by exsanguination from the abdominal aorta. Sera were prepared and stored as described before. Sera from positive control animals were pooled and used as positive control samples in the OVA-specific IgG and IgE ELISA's and in the PCA-test.

Assays for anti-OVA antibodies

Initially, only sera obtained from blood samples collected at day 28 and 42 were analysed for OVA-specific antibodies. At second instance (see results section), sera obtained from the *ad libitum* exposed animals were analysed for all time points (weeks 1-7). Serum IgG and IgE-specific for OVA were determined by ELISA. For the detection of OVA-specific IgG, 96-wells microtiter plates (Flat-bottomed, Maxisorp, NUNC, Roskilde, Denmark) were coated overnight at 4°C with 100 µl/well of a 5 µg/ml solution of OVA (Serva, Feinbiochemica, Heidelberg/New York, purity: >98%) in carbonate buffer, pH 9.6. The plates were washed three times with tap-water containing 0.4% Tween 20 (Merck, Hohenbrunn, Germany). This was followed by the addition of 100 µl/well phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA; Sigma Chemicals Co., St. Louis, USA) and 0.02% Tween 20 (PBS/BSA-Tween 20). After 1 h incubation at 37°C, the plates were washed and serial dilutions of rat serum in PBS/BSA-Tween 20 were added to the wells and incubated for 1 h at 37°C. After washing, 100 µl/well peroxidase conjugated goat anti-rat IgG (H+L) (Zymed, San Francisco, USA, diluted 1:500) in PBS/BSA-Tween 20 was added. After incubation for

1 h at 37°C, the plates were washed again and an enzyme substrate solution of 3,3',5,5'-tetramethylbenzidine (TMB; Sigma Chemicals Co., St. Louis, USA, 100 µl/well; 6 mg/ml DMSO) was added. The plates were developed at room temperature for 5 to 15 min. Finally, 100 µl/well of 2N H_2SO_4 was added. Optical densities were read spectrophotometrically at 450 nm with an ELISA plate reader (Microplate Reader, Biorad Laboratories, Richmond, USA). A presera pool was used as negative control. The pooled preserum was measured at a 1:4 dilution. The average extinction in negative control wells, to which three times the standard deviation was added, provided the reference value taken to determine the titer in the test sera. Each test serum was titrated starting at a 1:4 dilution and the reciprocal of the furthest serum dilution giving an extinction higher than the reference value was read as the titer. All analyses were performed in duplicate. The serum pool derived from the i.p. immunized animals was used as a positive control sample. Positive and negative control samples were incorporated for each 96-wells plate.

For the detection of OVA-specific IgE, 96-wells microtiter plates were coated overnight at 4°C with 100 µl/well mouse anti-rat IgE (MARE-1, Zymed, San Francisco, USA) at a concentration of 1.5 µg/ml in carbonate buffer, pH 9.6. The plates were washed and 100 µl/well of PBS/BSA-Tween 20 was added. After incubation for 1 h at 37°C, the plates were washed and diluted rat serum samples were added and incubated for 2 h at 37°C. The plates were washed and subsequently 100 µl/well of an 1 µg/ml solution of an OVA-digoxigenin (DIG) conjugate was added. The DIG was obtained from Boehringer (Mannheim, Germany) and coupling to OVA was performed according to the manufacturer's instructions. The labelled OVA was separated on a sephadex G-25 column (Boehringer, Mannheim, Germany) and labelling efficiency was determined spectrophotometrically at 280 nm. Incubation with OVA-DIG was performed for 1 h at 37°C. After washing, 100 µl/well peroxidase conjugated sheep anti-DIG Fab fragments (Boehringer, Mannheim, Germany) diluted 1:3000 in PBS/BSA-Tween 20 was added. After incubation for 1 h at 37°C, the plates were washed again and an enzyme substrate solution of TMB was added. Plate development, measurement and titer elaboration were as described for the OVA-specific IgG ELISA.

Passive Cutaneous Anaphylaxis

PCA was tested essentially as described previously by Ovary *et al.* [30]. Naive (untreated) BN rats were shaven on the back and flanks and injected intradermally with 0.1 ml of the test sera in serial dilutions, followed 64 h later with an intravenous injection of 1 ml of a 1:1 mixture of a solution of OVA (Fluka Chemie, Buchs, Switzerland, purity: 70 %, 5 mg/ml sterile saline) and a solution of Evans blue (2% in sterile saline). After 20-30 min., the animals were examined for positive responses. The diameter of dye extravasation at the site of the serum injection was measured. The reaginic titer was read as the reciprocal of the furthest dilution giving a coloured spot

were tested for DTH reactions. At day 28 or 42 (separate groups of rats) of dosing, the DTH response was assessed by an ear swelling test. Rats received a subcutaneous injection of 25 µl OVA (Serva, Feinbiochemica, Heidelberg/New york, purity: >98%, 25 mg/ml) in PBS in one ear and 25 µl PBS in the contralateral ear. Increasements in ear thickness were measured 24 h after challenge using an electronic micrometer (d=0.01 mm). OVA-specific DTH reactions were calculated by subtracting the ear thickness of the PBS-challenged ear from that of the OVA-challenged ear. DTH reactions were compared between naive (control) and sensitized animals. DTH responses of naive and sensitized animals were analysed for statistical significance of differences by two-tailed Student's *t*-test. Data were considered significantly different if $p<0.05$.

Results

Negative controls

Pre-study blood samples were always tested for ovalbumin (OVA) specific antibodies. No anti-OVA antibodies of the IgG or IgE class were detected in the presera of the animals. These sera were pooled and used as negative control in the ELISA's and PCA tests.

Positive controls

OVA-specific IgG and IgE were demonstrated in the pooled day 28 serum from the positive control animals (n=8, ^{2}log IgG titer of 21 and ^{2}log IgE titer of 15 in the OVA-specific ELISA's; data not shown). This serum pool was subsequently used as positive control in the ELISA's and PCA tests.

Administration of OVA by gavage

Rats exposed to OVA by gavage once a week (n=4) or once every two weeks (n=4) did not develop OVA-specific antibody responses. Upon intra-gastric dosing with OVA twice a week, 1 out of 4 animals developed an OVA-specific IgG (^{2}log titer 12 both at day 28 and 42) and an OVA-specific IgE response (^{2}log titer 3 at day 28 and ^{2}log titer 8 at day 42) (data not shown). The administration of OVA by daily gavage induced an OVA-specific IgG and IgE response in almost all animals (Fig. 1). After 28 days of exposure, both OVA-specific IgG (^{2}log titer 12.9 ± 2.8 (mean ± SD), range ^{2}log titer: 7-15) and OVA-specific IgE (^{2}log titer 6.7 ± 2.9, range 2 to 9) were detectable in all 7 tested animals. The same animals were used for measurement of delayed type hypersensitivity (DTH) reactions at day 28 (see section Delayed Type Hypersensitivity below). In a different group of animals exposed to OVA for 42 days, OVA-specific IgG was detectable in all tested animals (n=8, ^{2}log titer 12.8 ± 3.2, range 7 to 16) whereas OVA-specific IgE was detectable in 7 out of 8 animals (^{2}log titer in responders 6.5 ± 2.8,

range 2 to 9). The same animals were used for measurement of DTH reactions at day 42 (see below).

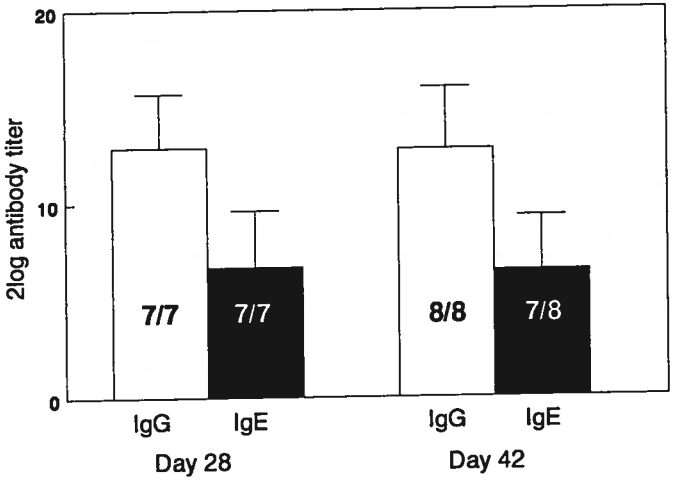
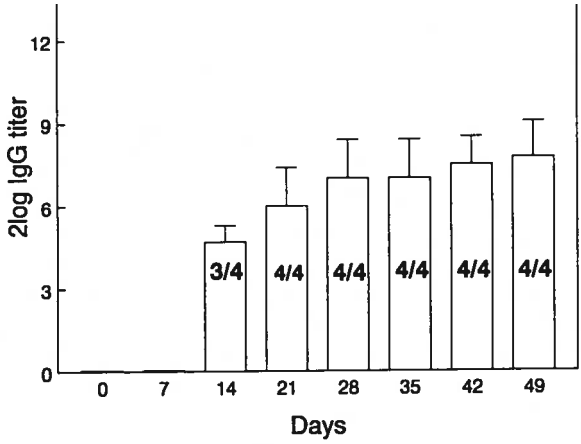


Figure 1. Ovalbumin (OVA)-specific IgG and IgE titers upon daily intra-gastric dosing of young BN rats with OVA (1 mg/rat) for 28 or 42 days. The data are presented as mean ^{2}log Ig titer ± SD of 7 (day 28) or 8 (day 42) rats per group. The number of animals developing an IgG or IgE response at the respective time-points are indicated in the bars.

***Ad libitum* exposure to OVA via the drinking water**

Antibody determinations by ELISA demonstrated that animals (n=4) exposed to 0.002, 0.02 or 0.2 mg/ml OVA via the drinking water did not develop OVA-specific antibodies whereas exposure to 2 or 20 mg/ml OVA via the drinking water resulted in OVA-specific IgG responses (^{2}log titer 7 ± 1.4, range 6 to 9 for 2 mg OVA/ml and ^{2}log titer 7.8 ± 1.3, range 6 to 9 for 20 mg OVA/ml, all data from sera collected at sacrifice). OVA-specific IgE could not be demonstrated in the day 28 and 42 samples from *ad libitum* exposed animals. Therefore, all serum samples obtained from the 20 mg/ml exposed animals (at weekly intervals) were analysed for OVA-specific antibodies. No OVA-specific IgE was detectable at any time-point investigated. OVA-specific IgG (Fig. 2) was first detectable at day 14 in 3 out of 4 animals (^{2}log titer 4.7 ± 0.6, range 4 to 5). At day 21, OVA-specific IgG was detectable in all animals (^{2}log titer 6 ± 1.4, range 4 to 7). Maximum titers were detected at day 28 (^{2}log titer 7 ± 1.4, range 5 to 8). No significant changes in OVA-specific IgG titers occurred from day 28 until termination of the induction period (day 42). In addition, OVA-specific IgG titers at day 49, one week after termination of the induction period, did not differ significantly from those seen at day 42. The average ^{2}log titer was 7.8 ± 1.3, with a range of 6 to 9 at termination of the study.



BN rats upon *ad libitum* exposure to OVA (20 mg/ml) via drinking water for 42 days. After the induction period, the animals were not exposed to OVA for one week. IgG titers were determined in blood samples obtained at weekly intervals. The data are presented as mean ^{2}log IgG titer ± SD of 4 rats per group. The number of animals developing an IgG response at the respective time-points are indicated in the bars. OVA-specific IgE could not be detected in animals exposed via drinking water.

Passive Cutaneous Anaphylaxis test (PCA)

In order to confirm the OVA-specific IgE ELISA-results, several representative sera were also tested in a PCA test (Table 1). Sera that were positive in the OVA-specific IgE ELISA in general also showed positive PCA results, although the ELISA tended to be more sensitive. In some cases, sera that were positive in the IgE ELISA did not induce PCA responses. Competitive reactions due to high IgG titers may have played a role in this phenomenon. Sera negative in the ELISA all were also negative in the PCA test.

Table 1. Comparison between ELISA and PCA results of representative sera (^{2}log IgG and IgE/reaginic antibody titers in sera from ovalbumin sensitized rats).

Serum sample	IgG[1]	IgE[1]	PCA[2]
A	12	-	-
B	17	9	2
C	-	-	-
D	14	11	3
E	-	-	-
F	-	-	-
G	17	11	4
H	14	5	1
I	17	12	-
J	17	13	6
K	15	12	4

1: results of triplicate analysis, 2: results of duplicate PCA tests.

Delayed Type Hypersensitivity (DTH)

Possible priming for DTH responses in animals exposed to OVA via drinking water or by daily gavage was investigated by performance of ear swelling tests at day 28 and 42. Upon *ad libitum* exposure, strong DTH responses were measured at day 28 (Fig. 3a). In animals exposed to OVA via the drinking water for 42 days, significant DTH responses were also determined, but the responses were weaker than those observed at day 28. In intra-gastrically dosed animals no significant DTH responses were measured at day 28 (Fig. 3b). After 42 days of intra-gastric dosing, a DTH responsiveness had developed which was comparable to the day 42 DTH responsiveness in animals exposed to OVA through the drinking water.

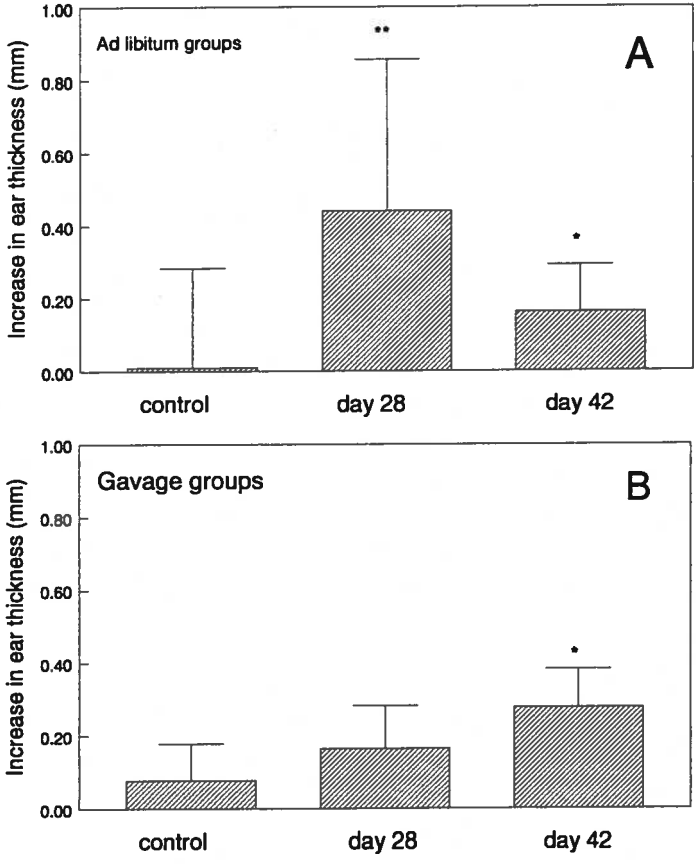


Figure 3. DTH responses in animals *ad libitum* exposed to OVA via the drinking water (20 mg/ml) continuously for 28 or 42 days (A) or animals exposed to OVA (1 mg/rat) by daily gavage for 28 or 42 days (B). Control animals received tap-water *ad libitum* with or without a daily gavage with tap-water (1 ml/rat) for 28 or 42 days. The DTH responsiveness was determined by ear swelling test, OVA (6.25 µg in 25 µl PBS) was injected in one ear and 25 µl PBS in the contralateral ear. The data are presented as mean (differences in thickness between the OVA injected and contralateral ear) ± SD of 8 rats per group. Statistics: two-tailed Student's *t*-test comparison between sensitized and control animals. $^{*}p<0.05$, $^{**}p<0.01$.

In the present paper, studies on various oral dosing protocols to sensitize Brown Norway (BN) rats to ovalbumin (OVA) without the use of an adjuvant are presented. The most significant results are summarized in table 2.

Table 2. Overview on the examined parameters for humoral- and cellular immune responses at day 28 and 42 in BN rats exposed to ovalbumin either *ad libitum* via the drinking water or by daily gavage.

	Ad libitum Day 28	Gavage Day 28	Ad libitum Day 42	Gavage Day 42
IgG response	++	++	++	++
IgE response	-	++	-	++
PCA	-	++	-	++
DTH	++	-	+	+

++: strongly positive, +: positive, -: negative.

From these results it appears that remarkable differences in immune responses to food antigens may occur depending on the dosing protocol applied. Administration of OVA *ad libitum* via the drinking water (2 or 20 mg/ml) resulted in OVA-specific IgG production but no specific IgE, whereas daily intra-gastric dosing of 1 mg OVA resulted in OVA-specific IgG as well as OVA-specific IgE responses. Upon intra-gastric dosing with 1 mg OVA once every two weeks or once or twice a week, no or only a very low frequency of antibody induction was noted. Cellular immune responses were studied by performing DTH tests. The DTH responses were most pronounced in *ad libitum* exposed animals at day 28. At day 42, both sensitization regimes primed for comparable DTH responses, but the responses were weaker than the day 28 DTH responsiveness in *ad libitum* exposed rats.

The strong DTH responsiveness in *ad libitum* exposed rats associated with the absence of an IgE response suggests a dichotomy in T cell function as is also observed in mice and humans. In the latter species, CD4+ T helper-1 (Th-1) cells, producing interleukin-2 (IL-2) and interferon-gamma (IFN-γ), promote cellular immune responses and, to a certain extend, IgG production [31,32,33]. Activated CD4+ T helper-2 (Th-2) cells, producing IL-4, IL-5, IL-6, IL-9, IL-10 and IL-13, strongly support humoral immune responses, particularly IgE responses [32,33]. Similarly, for the rat there is also evidence, although not as pronounced as for mice, for a functional dichotomy in Th cell functions [34,35], with IL-4 and IFN-γ having the same cross-regulatory function as in the mouse [36]. In the *ad libitum* exposed animals in the present studies, specific IgG

but no specific IgE was detected. This suggests a Th-1-like response, which is in accordance with the pronounced DTH response seen in these animals. In the gavage dosed animals, a Th2-like response seems to dominate with pronounced specific IgE and IgG responses and a weaker DTH responsiveness. However, this suggested dichotomy is not reflected in differences in DTH responsiveness upon prolonged exposure, since there was no difference in DTH reaction between *ad libitum* and gavage dosed animals at day 42.

Various factors may play a role in the development of the distinct immune responses upon *ad libitum* and gavage administration of food proteins. Estimation of the OVA intake by the *ad libitum* exposed rats indicates an OVA intake of 0.2 and 2 g/kg body weight (BW)/day for 2 and 20 mg/ml exposed rats respectively, versus approximately 5 mg/kg BW/day for the gavage dosed rats, based on a default liquid consumption figure of 100 g/kg BW/day. However, *ad libitum* exposure to 0.002, 0.02 and 0.2 mg/ml OVA corresponding to an estimated OVA intake of 0.2, 2 and 20 mg/kg BW/day respectively, (assuming a liquid intake of 100 ml/kg BW), comparable to the 5 mg/kg BW/day for gavage dosing, did not induce antibody production. Therefore, a difference in OVA intake is not likely to account for the observed differences in immune responses. A factor that may have played a role in the differences in immune responses upon gavage and *ad libitum* exposure to OVA is a difference in the OVA intake over the day. With gavage dosing, the animals received a daily bulk dose of OVA whereas upon *ad libitum* exposure, a more gradual intake of OVA over a prolonged period will have occurred. This difference may (in part) account for the observed differences between the intra-gastrically and *ad libitum* exposed animals. However, several other factors may have played a role in the revealed differences [25,26,37]. One of the factors which influences the potential immunological response to ingested protein is gastro-intestinal digestion as was shown by Hanson *et al.* [38]. Prefeeding of an endopeptidase inhibitor to mice reversed the outcome of intra-gastric protein feeding to mice from the induction of systemic non-responsiveness to priming for later protein-specific antibody responses. Moreover, studies by Catto-Smith *et al.* [39] demonstrated that intra-gastric administration of proteins may interfere with normal gastric functions such as the gastric emptying rate and as such may affect the digestive breakdown. An altered digestive breakdown of food proteins may result in a different spectrum of digestive fragments. Such differences may also (in part) account for the observed differences upon intra-gastric dosing and *ad libitum* exposure to OVA. At present no data are available to definitively explain the observed differences between the several intra-gastric dosing protocols. However, a difference in antigen load may well account for the differences seen since only daily intra-gastric dosing resulted in specific IgG and IgE responses whereas upon less frequent intra-gastric administration (i.e. lower antigen load) no or only a very low frequency of antibody induction was noted.

Our studies show that the BN rat can be sensitized by the enteral route without the

with intraperitoneal administration of carrageenan as an adjuvant induced specific IgE resulting in levels of reaginic antibodies comparable to the levels induced upon daily intra-gastric dosing of 1 mg OVA, without the use of adjuvant, in our studies. It remains to be elucidated whether the observed differences are due to the applied dose levels of OVA or to the use of the adjuvant. In the studies reported by Atkinson *et al.* [27] intra-gastric administration of 10 mg OVA twice a week resulted in responder percentages of about 60% to 100%. We performed several successive studies in which we sensitized BN rats by daily gavage with 1 mg OVA. The percentage of IgE responders in our studies in general exceeded 80%. However, occasionally, no OVA-specific IgE responses were induced upon daily gavage dosing with OVA in our studies. One of the major factors that may negatively affect the results of oral sensitization studies is unscheduled dietary pre-exposure of the test animal or their parental generations to the antigen under investigation. For guinea pigs [23] and rats [Knippels *et al.* J Allergy Clin Immunol, in press], it was demonstrated that dietary exposure of animals to the proteins under investigation affects the results of oral sensitization studies with the offspring and that at least 2 generations of animals have to be bred on a specified antigen free diet to avoid any influences in this respect. As far as we could reveal, the animals used in our studies met this condition, since pre-study blood samples evaluated on a routine basis prior to the initiation of our studies were always negative for OVA-specific IgG and IgE antibodies. Although we have not been able to determine the cause why sensitization did not occur in some of our experiments, the same phenomenon was reported from oral sensitization studies with rats performed by Jarrett *et al.* [26].

The studies reported here show that the BN rat provides a suitable animal model for inducing specific IgG and IgE responses as well as specific T-cell mediated hypersensitivity (DTH) upon daily intra-gastric dosing of 1 mg OVA without the use of an adjuvant. Currently, studies are in progress to investigate differences in oral sensitizing potentials of various food proteins using the model reported in this paper and to qualitatively and (semi)quantitatively compare the results with human clinical data. In addition, studies are in progress to characterize the rat model in more detail with respect to immune-mediated effects upon challenge and to study mechanisms involved in sensitization.

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CHAPTER 3

Continued expression of anti soy-protein antibodies in rats bred on a soy-protein free diet for one generation; the importance of dietary control in oral sensitization research

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Background: One of the major factors that may have negatively affected the results of many oral sensitization studies in animals has been unscheduled dietary pre-exposure of the test animal or their parental generations to the antigen under investigation. *Objective:* The influence of dietary pre-exposure to soy-protein on oral sensitization studies with soy-protein in Brown Norway (BN) rats was investigated. *Methods*: BN rats bred on a soy-protein containing diet for several generations (Routine Bred [RB] animals), were placed on a soy-protein free diet during and for at least 6 months before breeding (F0). Four generations of offspring were bred on a soy-protein free diet (F1, F2, F3 and F4). RB and F4 animals were exposed to soy-protein either *ad libitum* through drinking water or parenterally with an adjuvant. *Results:* In the F0 and F1 animals soy-protein specific IgG antibodies were still detectable whereas no soy-protein specific IgG was detectable in the other generations tested. In RB animals no significant increase in soy-protein specific IgG titers occurred after exposure to soy-protein. Enteral exposure of the F4 animals to soy-protein resulted in sensitization to soy-protein with increased soy-protein specific IgG titers. *Conclusions:* These studies demonstrate that there is a continued expression of anti soy-protein antibodies in rats bred and raised on a soy-protein free diet for one generation. Not only must the test animals be bred and raised on a specified antigen-free diet but their parental generations must also be bred in the same manner to avoid any problems in oral sensitization studies.

Introduction

Humans rather frequently have more or less severe allergic reactions after consumption of dietary proteins. Type I or IgE-mediated allergic reactions are known to play the major and primary role in food allergy [1]. Several (bio)technological techniques can be applied to reduce the antigenicity of food proteins or to synthesize new proteins or protein products for applications in food. For safety reasons, it is of importance to evaluate the residual antigenicity of modified protein products and to test for sensitizing properties of new or modified protein products. Although several *in vivo* antigenicity assays that use parenteral immunization are operational, it must be recognized that such assays only provide information on the antigenicity of proteins. Unfortunately, no well validated oral animal models to study food allergy and allergenicity of food proteins are available yet.

Several attempts have been made to develop animal models for food allergy research, mainly in mouse, guinea pig, and rat [2,3,4,5,6,7,8,9,10]. Although some of the attempts to develop enteral sensitization protocols were rather successful or at least promising, these efforts hardly resulted in structured approaches aimed at the development of well validated enteral allergenicity models. One of the major factors that may have negatively affected the results of many oral sensitization studies may have been unscheduled

dietary pre-exposure of the test animals or their parental generations to the antigen under investigation. This is of special importance since it is known that oral exposure to food antigens may easily induce an immunological tolerance in mice and rats [11,12,13,14,15,16].

To address this issue, we have investigated the influence of exposure to soy-proteins via the diet of parental generations of Brown Norway rats (BN; high IgE responder strain) on the presence of soy-protein specific antibodies in their offspring bred and raised on a soy-protein free diet for several generations. In addition, using an oral sensitization protocol (without the use of an adjuvant) to sensitize BN rats to food proteins as described in a previous paper [Knippels *et al.* Clin Exp Allergy, in press], we examined the effect of dietary pre-exposure to soy-protein on oral sensitization studies with soy-protein in the BN rat.

Materials and Methods

Animals and maintenance

Young, Brown Norway (BN) rats were obtained from Charles River (Sulzfeld, Germany). These animals, bred on a standard diet (containing soy-protein), were either used in oral sensitization studies immediately following a 10 day acclimatization period or were used to breed on a special soy-protein free rat diet prepared by TNO Nutrition and Food Research Institute. The rats were housed in an animal room maintained at 23 ± 3^{0}C, with a light/dark cycle of 12 h, and a relative humidity of 30-70% during the experiment and for at least 10 days before study initiation. The animals were housed in stainless-steel wire cages in groups of four and had free access to food and tap-water or soy-protein containing drinking water. All animal studies were approved by an independent ethical committee.

Diets

The standard diet was the open-formula stock diet for rats, mice and hamsters, which has been used at TNO Nutrition and Food Research Institute as the basal diet in many routine toxicity studies. The soy-protein free rat diet was prepared at the institute. The ingredient composition of both diets is given in Table I. The growth, behaviour and clinical signs of rats bred and raised on the soy-protein free diet were compared to those of rats bred on standard diet. No differences were found confirming nutritional adequacy of the soy-protein free diet (data not shown).

Table I. Composition of the standard diet and the soy-protein free diet

Standard diet		Soy-protein free diet	
Ingredient	Conc. (%)	Ingredient	Conc. (%)
Defatted soy 45% crude protein	11.0	Corn Gluten meal 60% protein	32.0
Fish meal 66% crude protein	7.0	Cornstarch	52,6
Meat meal	4.0	Cellulose	5.0
Wheat (whole ground)	38.5	$CaHPO_4$	1.5
Maize (whole ground)	26.0	Cornoil	3.1
Lucerne	3.0	Choline bitartrate	0.2
Soy oil	3.0	DL-Methionine	0.3
Whey powder, delactosed	2.0	L-Lysine	0.7
Yeast, unextracted, dry	3.0	L-Tryptophan	0.13
Premix[1,2]	2.5	AIN Mineral mix[3]	3.5
		AIN Vitamin mix[4]	1.0

1: Vitamine mixture (g or IU/kg diet): retinol (6400 IU), cholecalciferol (2100 IU), dl-α-tocopheryl acetate (0.045 g), menadione sodium bisulphite (0.003 g), thiamine (0.0025 g), riboflavin (0.003 g), pyridoxine (0.01 g), cyanocobalamin ($3.5x10^{-5}$ g), folic acid ($5x10^{-4}$ g), biotin ($1.5x10^{-5}$ g), nicotinic acid (0.0125 g), ca-D-pantothenate (0.0075 g).
2: Salt with trace elements (g/kg diet): iron (0.025), cobalt ($7x10^{-4}$), manganese (0.041), copper (0.008), zinc (0.012), iodine (0.0015), sodium chloride (1.8), calcium (2.9).
3: Mineral mixture (g/kg mix): NaCL (110), $K_3C_6H_5O_7 \cdot H_20$ (394), K_2SO_4 (52), MgO (28), $MnCO_3$ (3.5) $FeC_6H_5O_7 5H_2O$ (24), $5ZnO\ 2CO_3\ 4H_2O$ (1.6), $CuCO_3\ Cu(OH)_2\ H_2O$ (0.3), KIO_3 (0.08), $Na_2SeO_3\ 5\ H_2O$ (0.01), $CrK(SO_4)_2 \cdot 12H_2O$ (0.55), NaF (0.063), $CoCl_2 \cdot 6H_2O$ (0.127) and finely powdered sucrose to make 1 kg.
4: Vitamine mixture (g or IU/kg mix):retinyl palmitate/acetate (400.000 IU), cholecalciferol (248.400 IU), dl-α-tocopheryl acetate (5000 IU), menadione sodium bisulphite (0.4 g), thiamine-HCl (2 g), riboflavin (1.5 g), pyridoxine-HCl (0.7 g), cyanocobalamin (0.005 g), folic acid (0.2 g), D-biotin (0.06 g), nicotinic acid (9.0 g), D-Calcium pantothenate (4 g), inositol (10 g) and finely powdered sucrose to make 1 kg.

Breeding protocol

Animals, obtained from Charles River, were bred on a soy-protein containing diet (RB; routine bred) for several generations. These animals were either used in oral sensitization studies immediately after a 10 day acclimatization period or were fed a soy-protein free diet for at least 6 months (F0 animals). After at least 6 months on a soy-protein free diet, the F0 animals were bred on a soy-protein free diet. Blood samples were taken from the RB animals at arrival and at regular time intervals from the F0 animals. By using the F0 animals as the parental generation, four generations of animals (F1, F2, F3, and F4) were bred and raised on a soy-protein free diet. Blood samples were taken from animals of every generation at regular time intervals.

Sensitization protocol

Oral sensitization studies were performed with rats fed the routine (soy-protein containing) diet (RB) and with the fourth generation of offspring bred and raised on a soy-protein free diet (F4). Animals, 4-6 weeks old, were orally exposed to a soy-based infant formula (SBIF; Nutrilon, batch 00623, Nutricia, Zoetermeer, The Netherlands) or unheated soy-protein flower (USF; TNO ILOB, Wageningen, The Netherlands). The animals were exposed to soy-protein *ad libitum* through drinking water (20 or 40 mg protein/ml) continuously during 6 weeks. The soy-protein containing drinking water was refreshed twice a day to avoid turning sour. Blood was obtained from the orbital plexus under light CO_2 anaesthesia at weekly intervals from the RB animals exposed to soy-protein and at day 0, 28 and 42 from F4 animals in the enteral sensitization studies with soy-protein. After coagulation for 1 h at room temperature, the samples were centrifuged (Heraeus Minifuge T, Osterode, Germany) for 20 minutes at 2000g and 4^{0}C to obtain sera. The sera were stored at -20^{0}C until analyses for anti soy-protein specific IgG titers by ELISA were performed.

Positive control sera were obtained by intraperitoneal (i.p.) injection of animals with 0.5 ml of a 0.2 mg protein/ml SBIF or USF solution in sterile saline on days 0, 2, 4, 7, 9, and 11. To potentiate the immune response, 0.2 ml of a 25 mg/ml alum adjuvant suspension in sterile saline mixed with the 0.5 ml of SBIF or USF was injected on day 0. The animals were bled on day 28 by exsanguination from the abdominal aorta. Sera were prepared and stored as described above. These sera were pooled and used as positive control samples in the soy-protein specific IgG ELISA.

Assay for anti soy-protein specific IgG antibodies

Serum antibodies specific for soy-protein were measured by ELISA essentially as previously described [Knippels *et al.* Clin Exp Allergy, in press] with the exception of the coating which was performed with 100 µl/well of a 5 µg/ml solution of SBIF or USF in carbonate buffer, pH 9.6. For the detection of soy-protein specific IgG antibodies in the sensitization study using RB animals and in sera from animals bred and raised on a soy-protein free diet for several generations (F1, F2, F3 and F4 animals), a pooled serum from the third generation of offspring (F3) was used as a negative control, whereas a pooled preserum was used as a negative control in the sensitization study with F4 animals. The pooled negative control serum (either preserum or F3 serum) was measured at a 1:4 dilution. The average extinction in negative control wells, to which three times the standard deviation was added, provided the reference value taken to determine the titer in the test sera. Each test serum was titrated starting at a 1:4 dilution and the reciprocal of the furthest serum dilution giving an extinction higher than the reference value was read as the titer. All analyses were performed in duplicate. The serum pool derived from the i.p. immunized animals was used as a positive control sample. Positive and negative control samples were incorporated for each 96-wells plate.

Soy-protein specific antibodies in animals bred and raised on a standard or soy-protein free diet

Animals bred and raised on a soy-protein containing diet for several generations (RB) had soy-specific IgG antibodies at arrival (^{2}log titer 5 ± 0.7 [mean ± SD]) as determined by ELISA (Fig. 1). These animals were placed on a soy-protein free diet during and for at least 6 months before breeding (F0). After 6 months on a soy-protein free diet, soy-protein specific IgG was still detectable in these F0 animals (Fig. 1; ^{2}log titer 3.5 ± 1.2). The first generation of offspring (F1), which were bred and raised on a soy-protein free diet for at least one year, had lower soy-protein specific IgG titers (^{2}log titer 2 ± 0.5) compared with the F0 generation, but background extinction values as determined in sera from the third generation of offspring (F3) were not reached until the second generation of offspring (Fig. 1; F2: ^{2}log titer 0). Extinction values determined in sera from animals from the fourth generation of offspring bred on a soy-protein free diet did not differ significantly from those detected in F3 animals (Fig. 1).

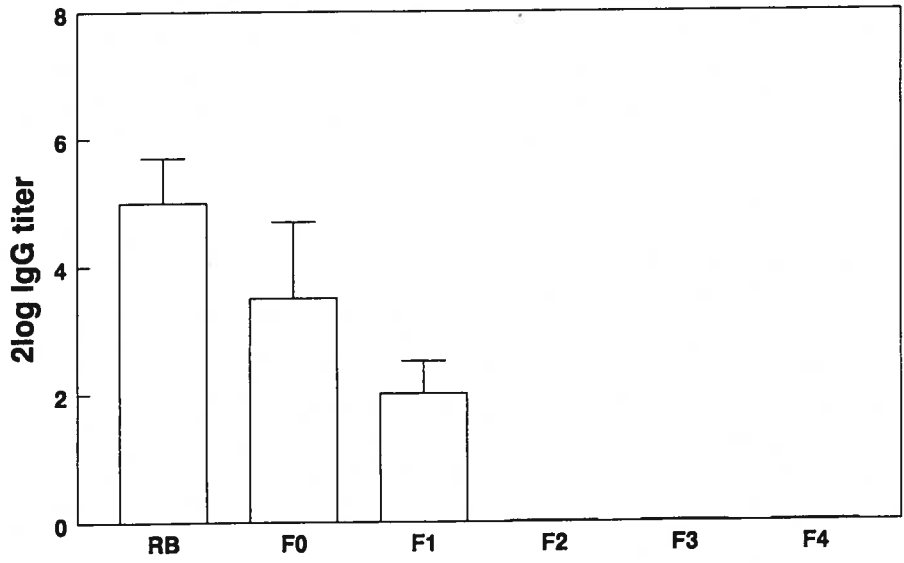

Figure 1. Soy-protein specific IgG antibody titers in BN rats bred and raised on a soy-protein containing diet for several generations (RB) and after being fed soy-protein free diet for at least 6 months (F0). After at least 6 months on soy-protein free diet, F0 animals were used to start breeding. Four generations of offspring were bred and raised on a soy-protein free diet (F1, F2, F3, and F4). Blood samples were taken from the RB animals at arrival and at regular time intervals from the F0, F2, F3 and F4 animals. Data are presented as ^{2}log IgG titer ± SD of groups of at least 4 rats from every generation of offspring measured by ELISA with pooled serum from F3 animals as negative control.

Exposure to soy-protein in animals fed a soy-protein containing diet

Young, male BN rats (n=4) bred and raised on a standard diet and exposed *ad libitum* to SBIF through drinking water (20 mg protein/ml) continuously for 42 days already had soy-protein specific IgG antibodies at study initiation as measured by ELISA with pooled F3 serum as a negative control (Fig. 2A; ^{2}log titer 5.5 ± 0.6, range: 5-6). Soy-protein specific IgG titers did not significantly change in RB animals upon either enteral (Day 42, termination of the treatment period: ^{2}log titer 4.8 ± 1.3, range 3 to 6) or parenteral (Day 28: ^{2}log titer 6) exposure to soy-protein (Fig. 2A; $p>0.05$ as determined by two-tailed Student's *t*-test comparison of antibody titers at days 7 to 42 and pre-study antibody titers).

Exposure to soy-protein in animals bred and raised on a soy-protein free diet for four generations

Young, male, BN rats, bred on a soy-protein free diet for four generations (n=4), were exposed *ad libitum* to SBIF (20 or 40 mg protein/ml) or unheated soy flower (USF: 20 or 40 mg protein/ml) through drinking water for 42 days. Soy-protein specific IgG was measured at days 28 and 42 by using pooled preserum as a negative control (Fig. 2B). After exposure to SBIF (20 mg/ml), soy-protein specific IgG was detectable in all animals at day 28 (^{2}log titer 12.3 ± 3.3, range 8 to 16) and day 42 (^{2}log titer 13.3 ± 2.8, range 10 to 16). Exposure to 40 mg/ml SBIF through drinking water also induced soy-protein specific IgG in all animals at day 28 (^{2}log titer 13.8 ± 1.0, range 13 to 15) and day 42 (^{2}log titer 14 ± 1.4, range 12 to 15). Both exposure to 20 mg/ml or 40 mg/ml USF resulted in soy-protein specific IgG in all animals at day 28 (20 mg/ml: ^{2}log titer 16 ± 1.4, range 14 to 16; 40 mg/ml: ^{2}log titer 17 ± 0) and day 42 (20 mg/ml: ^{2}log titer 15.5 ± 1.3, range 14 to 17; 40 mg/ml: ^{2}log titer 15.5 ± 0.8, range 15 to 16). Moreover, soy-protein specific IgG titers measured at day 28 in the pooled sera from animals injected with SBIF or USF (^{2}log titer 18.3 and ^{2}log titer 19.5 respectively) were higher when compared to the titers detected at day 28 in animals orally sensitized to either SBIF or USF. In comparison with the pre-study antibody titers, all F4 animals orally exposed to soy-proteins developed a statistical significant soy-protein specific antibody response ($p<0.05$ as determined by two-tailed Student's *t*-test).

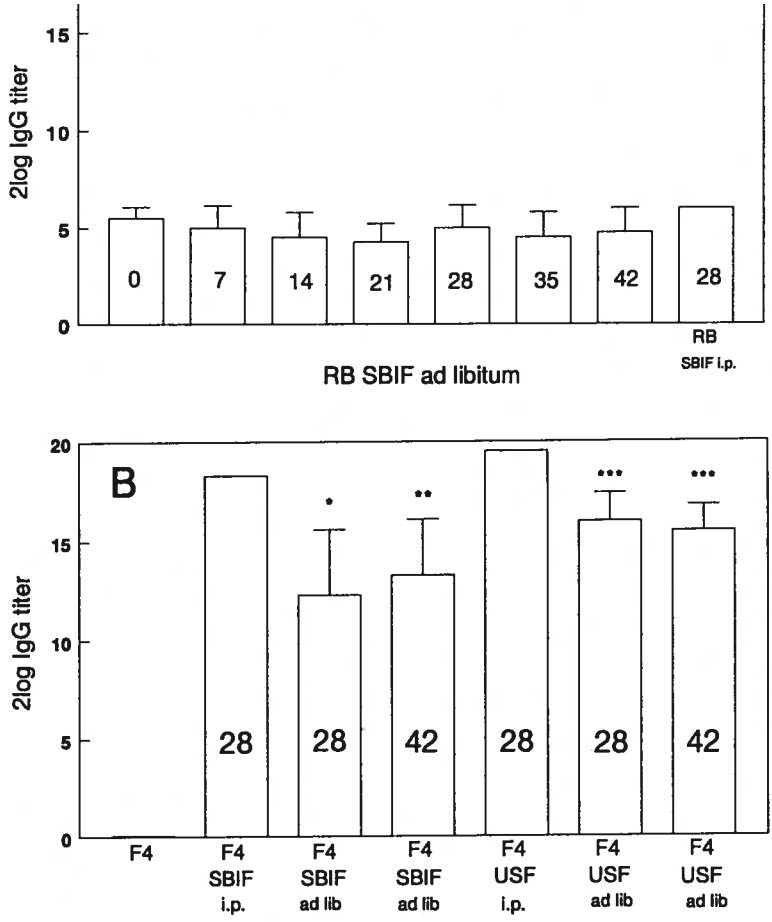

Figure 2. A, Soy-protein specific IgG titers in young BN rats fed soy-protein containing diet for several generations (RB) and exposed *ad libitum* to SBIF (20 mg protein/ml) through drinking water continuously for 42 days and in young RB animals parenterally injected with soy-based infant formula (RB soy i.p.) (Fig 2a). Blood samples were obtained at weekly intervals in the orally exposed RB animals and at day 28 in parenterally exposed RB animals. Data are presented as mean ^{2}log IgG titer ± SD of 4 rats per group as determined by ELISA in blood samples with a pooled serum from the third generation of offspring bred on a soy-protein free diet as negative control. **B**, Soy-protein specific IgG titers in BN rats bred and raised on soy-protein free diet for four generations (F4) and young F4 animals parenterally injected with either SBIF (F4 SBIF ip) or unheated soy flower (F4 USF ip) or exposed orally by administration of 20 mg protein/ml SBIF (F4 SBIF ad libitum) or 20 mg protein/ml USF (F4 USF ad libitum) *ad libitum* through drinking water continuously during 42 days. Soy-protein specific IgG titers were determined by ELISA in blood samples obtained at day 0, 28 and 42 by using a pooled preserum as a negative control. Data are presented as mean ^{2}log IgG titers ± SD of 4 rats per group. Respective time-points for specific antibody determination are indicated in the bars. No SD's are given for the i.p. sensitized animals because sera of individual animals were pooled. Statistical analysis was performed by two tailed Student's *t*-test comparison of pre-study antibody titers and antibody titers on oral exposure to soy-proteins.*$p<0.05$, **$p<0.01$, ***$p<0.0001$.

Discussion

One of the major factors that may have negatively affected the results of many oral sensitization studies in animals has been unscheduled dietary pre-exposure of the test animals or their parental generations to the antigen under investigation.

In this study, Brown Norway (BN) rats routinely bred on a soy-protein containing diet for several generations were fed a soy-protein free diet during and for at least 6 months before breeding. Several generations of offspring were bred on the soy-protein free diet. Soy-protein specific IgG antibodies were detectable in the routine bred animals on arrival in our laboratory. These antibodies were still detectable in serum samples obtained from these animals after a soy-protein free feeding period of 6-12 months. Moreover, anti soy-protein antibodies were even detectable in serum samples collected from F1 generation of offspring rats at ages of 6-12 months while these animals were still being fed a soy-protein free diet. In the second (F2), third (F3) and fourth (F4) generations of offspring bred on a soy-protein free diet no soy-protein specific IgG was detected anymore. These results show that the first generation of offspring, although not exposed to soy-protein via the diet, still expresses soy-specific antibodies.

After careful evaluation and studies on transfer of F3 or F4 animals to animal rooms in which soy-rich rodent diet had recently been present (all animals remained anti-soy protein IgG free; data not shown), we had to exclude the facility contamination with soy-protein as a possible cause for the expression of anti soy-protein IgG in the F1 animals. Experiments in rats have demonstrated that transfer of maternal immunity to offspring may occur transplacentally or through the milk [17]. However, after weaning, these antibodies were shown to fall to low levels by 5-6 weeks and to become undetectable by 7-8 weeks after birth [18]. Maternal derived soy-protein specific IgG transferred through the milk or transplacentally seems not to be a plausible explanation for the observed soy-protein specific IgG antibodies in the one year old F1 animals. As such, the observed soy-specific IgG antibodies in the offspring are probably not maternally derived. Another explanation could be priming of the neonatal immune system of the F1 animals by soy-proteins or their peptides. In the past few years it has become evident that, in humans in many cases, priming of the neonatal T cell system is initiated *in utero* [19,20]. Transplacentally transferred allergens, or maternally processed peptides from allergens, perhaps in conjunction with maternally derived IgG antibodies, may provide the initial triggers for sensitization [21]. Although in our studies, the F1 animals and their parental generation were not exposed to soy-protein or peptides during and for at least 6 months before breeding, soy-proteins may have been captured by the maternal animals during their exposure to soy-protein before breeding. Transplacental transfer of the captured soy-proteins or their peptides may result in sensitization of the offspring to soy-protein and subsequent antibody production which could explain the observed soy-specific antibodies in the F1 animals. Besides the possibility of antigen capture,

with soy-protein, anti soy-protein IgG positive routine bred animals (RB) and negative F4 animals were exposed to soy-protein *ad libitum* through drinking water for 42 days. In the RB animals, soy-protein specific IgG antibodies were already present at the start of the experiment and the levels did not increase during oral exposure. Both the induction of significant levels of serum antibody by protein feeding [24,25,26,27] and the phenomenon of active but self-limiting specific immune response despite continued exposure has also been described for other animals [27]. Moreover, intra peritoneal administration of soy-protein together with an adjuvant also did not result in significantly increased levels of soy-protein specific IgG in the RB animals. Exposure of F4 animals to soy-protein resulted in oral sensitization to soy-protein. Soy-protein specific IgG titers measured in the sera of these animals were approximately 2.5 to 3 times higher than those determined in the study with the RB animals ($p<0.05$ as determined by two-tailed Student's *t*-test), indicating that these animals became fully responsive to oral exposure with soy-protein. Moreover, on intra peritoneal administration, soy-protein specific IgG titers measured in the sera of these animals were approximately 4 times higher.

Our studies demonstrate that dietary exposure of BN rats to soy-protein affects the results of oral sensitization studies with soy-protein. After breeding the animals on a soy-protein free diet for at least two generations, soy-protein specific IgG antibodies were not detectable and oral sensitization to soy-protein could be demonstrated in anti soy-protein IgG negative animals. The same phenomenon has been described for oral sensitization studies with cow's milk-proteins in guinea pigs [9]. After breeding the animals on a milk-protein free diet, the F2 and later generations became fully responsive to oral exposure to cow's milk whereas impaired responsiveness was demonstrated in parental animals and the first generation of offspring bred on a cow's milk-protein free diet. Such studies in addition to ours indicate that besides the age of the animals, the dose of antigen, the presence of adjuvant, and the frequency of administration, which all may influence the immune response on oral antigen exposure [7,28,29,30], special caution should be paid concerning the diet when oral sensitization studies are performed with animals. Not only the test animals but also their parental generations must be bred and raised on an specified antigen-free diet in order to avoid any problems in oral sensitization studies.

At present, we have no indication whether (and if so, to what extend) the observations described for rats in this study and for guinea pigs in the study by Pahud *et al.* [9] are extrapolative to the human situation. However, these animal data suggest that despite the fact that the parental generation is not exposed to certain dietary antigens during a prolonged period, a non-hereditary transfer may occur to the first generation of offspring. This transfer may result in continued specific antibody expression against dietary proteins although the offspring never directly encountered these antigens through their diet. No specific antibodies were detectable in the F2 and later generations which

became fully responsive to oral exposure to the antigens. This observed phenomenon may have important implications, for instance with respect to the introduction of novel foods. Because theoretically, protective antibodies to newly introduced proteins may be absent, the chance of a subject getting orally sensitized to these proteins may be changed. Furthermore, the observed phenomenon may provide new insights into the development of the adoptive immune responses in young animals and infants and may theoretically open new prophylactic opportunities in disease control. Further studies in this respect would therefore be of value.

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CHAPTER 4

Humoral and cellular immune responses in different rat strains upon oral exposure to ovalbumin

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Background: Although several *in vivo* antigenicity assays using parenteral immunization are operational, no adequate enteral sensitization models are available to study food allergy and allergenicity of food proteins. *Objective*: In a previous paper, we described an oral sensitization protocol to sensitize Brown Norway rats (BN) to food proteins. In the present paper, the influence of genetically-based strain-specific characteristics of the immune system on the outcome of oral sensitization studies was investigated. *Methods and Results*: BN, Hooded Lister (HL), Piebald Virol Glaxo (PVG) and Wistar rats were daily administered 1 mg of ovalbumin (OVA) by gavage dosing for 42 days without the use of an adjuvants. OVA-specific IgG antibody responses were detected in all rats of the different strains except for the Wistar rats of which only 75% of the animals developed an OVA-specific IgG response. The highest OVA-specific IgG responses were detected in the BN rats followed by Wistar, HL and PVG rats. OVA-specific IgE responses were only detectable in the BN rats. The cellular immune response was examined by determination of delayed-type hypersensitivity (DTH) reactions in the animals one week after the 42 days induction period. The response was most pronounced in the HL and Wistar rats. PVG and BN rats showed comparable DTH responses but the responses were significantly weaker than those observed in HL and Wistar rats. *Conclusions*: It was concluded that the genetic make-up of different rat strains influences the outcome of oral sensitization studies. In addition, using the described oral sensitization protocol, the BN rat seems to be the most suitable strain for inducing oral sensitization.

Introduction

Humans rather frequently suffer from more or less severe allergic reactions after consumption of dietary proteins. Type I or Immunoglobulin E (IgE)-mediated allergic reactions are known to play a major and primary role in food allergy [1]. Although, several attempts have been made to develop animal models for food allergy research, mainly in mouse, guinea pig, and rat [2,3,4,5,6,7,8,9,10,11,12] these efforts hardly resulted in structured approaches aimed at the development of well validated enteral allergenicity models to study food allergy and the allergenicity of food proteins.

In a previous paper, we described an oral sensitization protocol, without the use of an adjuvant, to sensitize Brown Norway rats (BN) to food proteins [Knippels *et al.* Clin Exp Allergy, in press]. In the present study, we used this protocol to sensitize rats of different strains (BN, Hooded Lister (HL), Piebald Virol Glaxo (PVG), Wistar) to ovalbumin, a well defined chicken egg white allergen. Genetically-based strain-specific characteristics of the immune system may be of importance since the development of IgE mediated allergies in humans, including food allergy, is more common in atopic humans who have a genetic predisposition to react with an elevated production of IgE

antibodies to generally harmless substances. However, it must be recognized that IgE mediated allergies are observed in non-atopic humans as well. The BN and HL rats are high-immunoglobulin (particularly IgE) responder strains [13] and thus, to a certain degree, may resemble atopic humans in their (genetic) predisposition to react with an overproduction of IgE to antigens. The PVG strain was chosen because of its high susceptibility for stress, a factor which has a major influence on the function of the immune system. In addition, the Wistar was chosen since this strain is widely used in all kinds of experiments, including protocol toxicity studies. Using these four rat strains, the influence of strain differences on oral sensitization with ovalbumin was investigated by studying specific humoral (IgG and IgE) and cellular immune responses.

Materials and Methods

Animals and maintenance

Young male Brown Norway (BN) and Wistar rats were obtained from Charles River (Kent, UK) whereas Hooded Lister (HL) and Piebald Virol Glaxo (PVG) rats were purchased from Charles River Wiga GmbH (Sulzfeld, Germany). The rats were housed in an animal room maintained at 23 ± 3^{0}C, with a light/dark cycle of 12 h, and a relative humidity of 30-70% during the experiment and for at least 10 days prior to study initiation. The animals were housed in stainless-steel wire cages in groups of three or four and had free access to food and tap-water. The rats were bred and raised on a commercially available ovalbumin-free rodent diet (SDS Special Diet Service, type RM3(E) FG SQC, Witham, UK). Pre-study blood samples were always tested for ovalbumin specific antibodies. All animals studies were approved by an independent ethical committee.

Experimental design

Animals (n=6 to 8), 4-6 weeks old at study initiation, were exposed to ovalbumin (OVA, Fluka Chemie, Buchs, Switzerland, purity: 70 %) by daily gavage dosing during 6 weeks, using a 18-gauge stainless steel animal feeding needle (1 mg OVA/ml tap-water; 1 ml/animal). After the induction period, the animals were not exposed to OVA for one week. Blood samples were obtained from the orbital plexus under light CO_2 anaesthesia at weekly intervals. After coagulation for 1 h at room temperature, the blood samples were centrifuged (Heraeus Minifuge T, Osterode, Germany) for 20 min. at 2000g and 4^{0}C to obtain sera. The sera were stored at -20^{0}C until analyses for anti-OVA specific IgG and anti-OVA specific IgE titers by Enzyme Linked Immuno Sorbent Assay (ELISA). At day 49, the cellular immune response to OVA was investigated by delayed type hypersensitivity (DTH) testing.

Positive control animals (n=3 per strain) were injected intraperitoneally (ip) with 0.5

stored as described before. Sera from positive control animals were pooled and used as positive control samples in the OVA-specific IgG and IgE ELISA's.

Negative control animals (n=2 per strain) were not exposed to OVA. Presera were taken at day 0 and the animals were bled at day 49 by exsanguination from the abdominal aorta. Sera were prepared and stored as described before. Sera from negative control animals were pooled and used as negative control samples in the OVA-specific IgG and IgE ELISA's.

Assays for anti-OVA antibodies

Rat sera were tested for OVA-specific IgG and IgE antibodies by ELISA. For the detection of OVA-specific IgG, 96-wells microtiter plates (Flat-bottomed, Maxisorp, NUNC, Roskilde, Denmark) were coated overnight at 4°C with 100 µl/well of a 5 µg/ml solution of OVA (Serva, Feinbiochemica, Heidelberg/New York, purity: >98%) in carbonate buffer, pH 9.6. The plates were washed three times with tap-water containing 2% Tween 20 (Merck, Hohenbrunn, Germany). This was followed by the addition of 100 µl/well phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA; Sigma Chemicals Co., St. Louis, USA) and 0.02% Tween 20 (PBS/BSA-Tween 20). After 1 hr incubation at 37°C, the plates were washed and serial dilutions of rat serum in PBS/BSA-Tween 20 were added to the wells and incubated for 1 hr at 37°C. After washing, 100 µl/well peroxidase conjugated goat anti-rat IgG (H+L) (Zymed, San Francisco, USA, diluted 1:500) in PBS/BSA-Tween 20 was added. After incubation for 1 hr at 37°C, the plates were washed again and an enzyme substrate solution of 3,3',5,5'-tetramethylbenzidine (TMB; Sigma Chemicals Co., St. Louis, USA, 100 µl/well; 6 mg/ml DMSO) was added. The plates were developed at room temperature for 5 to 15 min Finally, 100 µl/well of 2N H_2SO_4 was added. Optical densities were read spectrophotometrically at 450 nm with an ELISA plate reader (Microplate Reader, Biorad Laboratories, Richmond, USA). A presera pool was used as negative control. The pooled preserum was measured at a 1:4 dilution. The average extinction in negative control wells, to which three times the standard deviation was added, provided the reference value taken to determine the titer in the test sera. Each test serum was titrated starting at a 1:4 dilution and the reciprocal of the furthest serum dilution giving an extinction higher than the reference value was read as the titer. All analyses were performed in duplicate. The serum pool derived from the ip immunized animals was used as a positive control sample. Positive and negative control samples were incorporated for each 96-wells plate.

For the detection of OVA-specific IgE, 96-wells microtiter plates were coated overnight at 4°C with 100 µl/well mouse anti-rat IgE (MARE-1, Zymed, San Francisco, USA) at a concentration of 1.5 µg/ml in carbonate buffer, pH 9.6. The plates were washed and 100 µl/well of PBS/BSA-Tween 20 was added. After incubation for 1 hr at 37°C, the plates were washed and diluted rat test serum samples were added and

incubated for 2 hr at 37°C. The plates were washed and subsequently, 100 µl/well of an 1 µg/ml solution of an OVA-digoxigenin (DIG) conjugate was added. The DIG was obtained from Boehringer (Mannheim, Germany) and coupling to OVA was performed according to the manufacturers instructions. The labelled OVA was separated on a sephadex G-25 column (Boehringer, Mannheim, Germany) and labelling efficiency was determined spectrophotometrically at 280 nm. Incubation with OVA-DIG was performed for 1 hr at 37°C. After washing, 100 µl/well peroxidase conjugated sheep anti-DIG Fab fragments (Boehringer, Mannheim, Germany) diluted 1:3000 in PBS/BSA-Tween 20 was added. After incubation for 1 h at 37°C, the plates were washed again and an enzyme substrate solution of TMB was added. Plate development, measurement and titer elaboration were as described for the OVA-specific IgG ELISA.

Measurement of Delayed Type Hypersensitivity

Rats of all four strains, exposed to OVA by daily gavage, were tested for DTH reactions. At day 49, one week after the sensitization period, the DTH response was assessed by an ear swelling test. Rats received a subcutaneous injection of 25 µl OVA (Serva, Feinbiochemica, Heidelberg/New york, purity: >98%, 0.25 mg/ml) in PBS in one ear and 25 µl PBS in the contra lateral ear. Differences in ear thickness were determined 24 hr after challenge using an electronic micrometer (d=0.01 mm). OVA-specific DTH reactions were calculated by subtracting the ear thickness of the PBS-challenged ear from that of the OVA-challenged ear. DTH reactions were compared between the four different rat strains. The DTH responses of sensitized rats from the different strains were analysed for statistical significance of differences by two-tailed Student's *t*-test. Data were considered significantly different when $p<0.05$.

Results

Negative and positive controls

Pre-study blood samples of the OVA exposed and negative control animals and endpoint sera of the negative control animals were always tested for ovalbumin (OVA) specific antibodies. No anti-OVA antibodies of the IgG or IgE class were detected in the tested sera. These sera were pooled and used as negative control in the ELISA's with the test samples from the sensitization study. Sera obtained at day 28 from positive control animals were positive for anti-OVA specific IgG and IgE and were pooled per strain and used as positive control in the ELISA's with the test samples from the sensitization study.

Strain-dependent antibody responses upon oral sensitization with OVA

Upon daily intra-gastric dosing with OVA, OVA-specific IgG responses were detectable

range 3-11). Moreover, only 6 out of 8 animals responded in the latter strain whereas all animals responded in the other 3 strains. With prolonged exposure, OVA-specific IgG titers decreased as evident from the titers determined at day 42 in the PVG (Fig. 1a: ^{2}log titer at day 42 3.5 ± 1.5, range 2-7) and HL rats (Fig. 1c: ^{2}log titer at day 42 4.8 ± 2.2, range 3-9). Moreover, one week after termination of the induction period, the number of animals expressing IgG antibodies had decreased from 8 out of 8 responding animals to 5 out of 8 in these 2 strains whereas the number of responders and titers did not change in the Wistar and BN rats upon prolonged exposure until day 49. OVA-specific IgE responses were only detectable in the BN rats (Fig. 2). The OVA-specific IgE responses were detectable from day 7 onwards (1 out of 6 animals responding) with maximum responses detectable at day 42 (^{2}log titer 6.3 ± 2.9, range 4 to 11) with 100% responders.

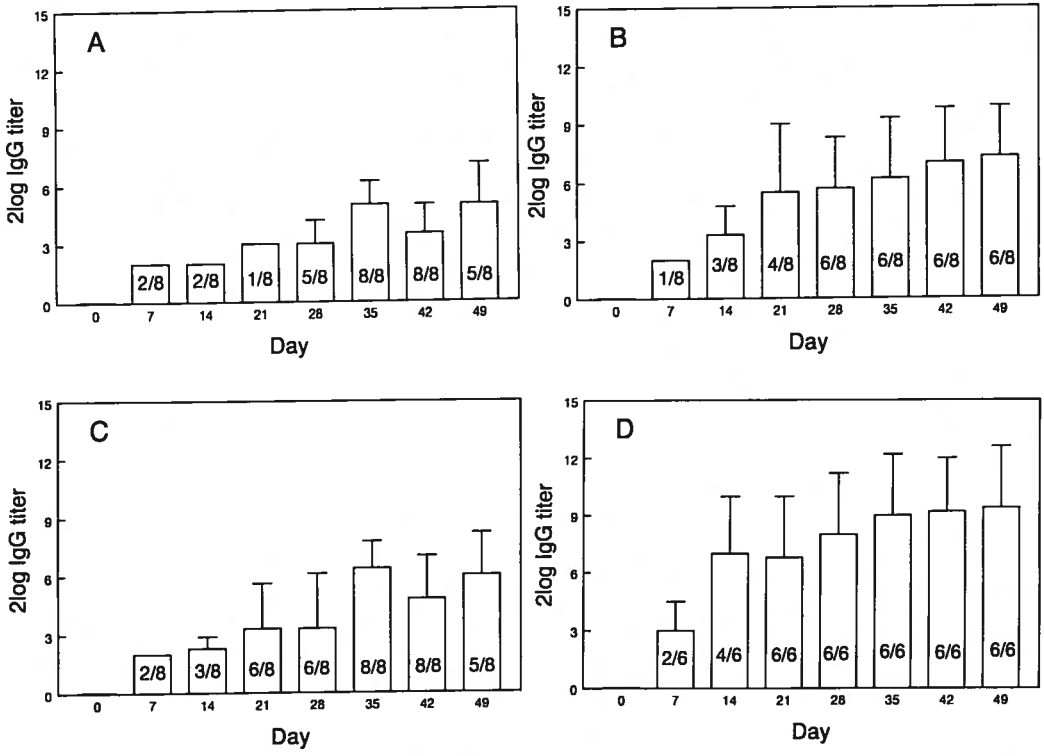

Figure 1. Time dependency of ovalbumin (OVA)-specific IgG response in young PVG (Fig. 1a), Wistar (Fig. 1b), HL (Fig. 1c), and BN rats (Fig. 1d) upon daily intra-gastric dosing with 1 mg OVA/rat/day during 42 days. After the induction period, the animals were not exposed to OVA for one week. IgG titers were determined in blood samples obtained at weekly intervals. The data are presented as mean ^{2}log IgG titer ± SD of 6 to 8 rats per group. The number of animals developing an IgG response at the respective time-points are indicated in the bars.

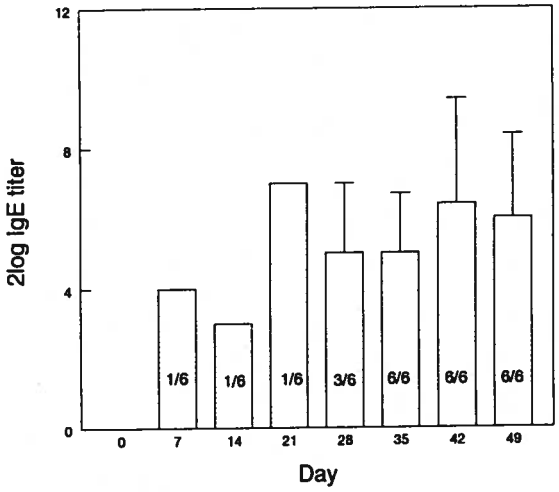

Figure 2. Ovalbumin (OVA)-specific IgE upon daily intra-gastric dosing of young BN rats with OVA (1 mg/rat/day) during 42 days. After the induction period, the animals were not exposed to OVA for one week. IgE titers were determined in blood samples obtained at weekly intervals. The data are presented as mean ^{2}log IgE titer ± SD of 6 rats per group. The number of animals developing an IgE response at the respective time-points are indicated in the bars. OVA-specific IgE could not be detected in exposed animals of the three other strains tested (PVG, Wistar, HL).

Strain-dependent cellular immune responses upon oral sensitization with OVA

Cellular immune responses were studied by performing delayed type hypersensitivity (DTH) tests at day 49, one week after the induction period. Possible priming for DTH responses in animals of all four strains exposed to OVA by daily gavage was investigated by performance of ear swelling tests. PVG and BN rats showed comparable DTH responses but the responses were significantly weaker than those observed in HL and Wistar rats (Fig. 3).

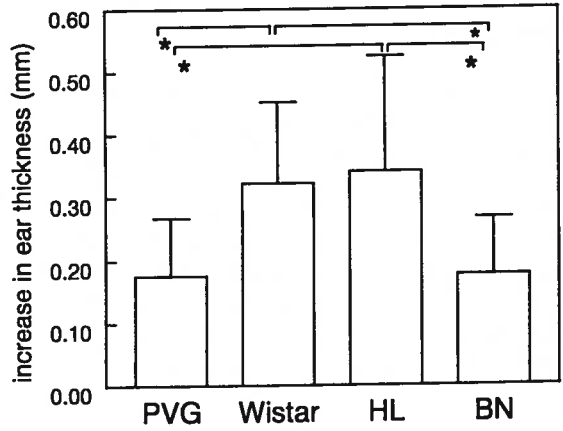

Figure 3. DTH responses in PVG, Wistar, HL, and BN rats exposed to OVA (1 mg/rat/day) by daily gavage during 42 days. The DTH responsiveness was determined by ear swelling test at day 49, one week after termination of the induction period. OVA (6.25 µg in 25 µl PBS) was injected in one ear and 25 µl PBS in the contra lateral ear. The data are presented as mean (difference in thickness between the OVA injected and contra lateral ear) ± SD of 6 to 8 rats per group. Statistics: two-tailed Student's *t*-test comparison between responses of animals of the different strains. *$p<0.05$

In a previous paper we described an oral sensitization protocol, without the use of an adjuvant, to sensitize Brown Norway (BN) rats to ovalbumin (OVA) [Knippels *et al.* Clin Exp Allergy, in press]. In the present study, we used the developed oral sensitization protocol to sensitize different rat strains to investigate the influence of strain differences on oral sensitization. BN, Hooded Lister (HL), Piebald Virol Glaxo (PVG) and Wistar rats were exposed to OVA by daily gavage dosing, without the use of an adjuvant, during 42 days. From the results it appears that remarkable differences occur depending on the rat strain used. Daily intra-gastric dosing with OVA resulted in OVA-specific IgG responses in all animals of the BN, HL and PVG strains but only in 75% of the Wistar rats. Moreover, the levels of OVA-specific IgG antibodies induced were highest in the BN rats followed by Wistar, HL and PVG rats. The latter two species showed a decrease in the levels of OVA-specific IgG antibodies and number of positive animals upon prolonged exposure, whereas no changes occurred in the BN and Wistar rats. The absence of OVA-specific IgG in 25% of the Wistar rats could be due to the induction of tolerance since it is known that repeated low dose protein feeding can induce tolerance resulting in an active suppression mediated by regulatory T cells [14,15]. In addition, OVA-specific IgE titers were determined in the rat sera. While all BN rats developed an OVA-specific IgE response, no OVA-specific IgE could be detected in sera of rats from the other 3 strains tested. Besides the humoral response, cellular immune responses were also studied by performing Delayed Type Hypersensitivity (DTH) tests one week after the induction period. The DTH responses were most pronounced in the HL and Wistar rats whereas PVG and BN rats showed comparable DTH responses which were significantly lower than those observed in the HL and Wistar rats.

Studies using experimental rat models to study mercuric chloride induced autoimmunity [16], collagen-induced arthritis [17], experimental allergic encephalomyelitis [18,19] and (immuno)toxicity of chemicals [20,21,22] all reported differences in susceptibility between rat strains resulting in apparently contrasting effects. In addition, the genetic make-up is known to have a marked influence on immune responsiveness in inbred strains of laboratory animals [23,24,25]. Therefore the observed differences in responsiveness of the different rat strains upon oral sensitization with OVA was not unexpected. In humans, the T-cell system in atopics and normal individuals responds in a qualitative different fashion to environmental allergens as reviewed by Holt [26]. T-cell cloning studies have revealed that atopics have a preference for establishment of T-memory for allergens that (by analogy with the murine system) is of the T helper-2 (Th-2) subset, being dominated by CD4+ T-cells secreting IL-4 and IL-5. Corresponding T-cell responses in normal individuals tend to be of T helper-1 (INF-γ secreting) phenotype. Similarly, for the rat, there is also evidence, although not as pronounced as for humans and mice, for a functional dichotomy in Th cell functions [27,28], with IL-4 and IFN-γ having the same cross-regulatory function

as in the mouse [29]. In the Wistar and HL rats, specific IgG but no specific IgE was detected upon daily gavage dosing with OVA. This suggests a Th-1-like response, which is in accordance with the pronounced DTH response seen in these animals. In the BN rats, a high-immunoglobulin responder strain [13] which to a certain degree resembles atopic humans in their (genetic) predisposition to react with an overproduction of IgE to antigens, a Th-2-like response seems to dominate with pronounced specific IgE and IgG responses and a weaker DTH responsiveness. The weak DTH response seen in the PVG rats combined with their less pronounced specific IgG response could be due to stress, caused by the daily gavage dosing, since PVG rats are known to make a more vigorous steroid response to stress which results in an immunosuppressive effect [18].

The studies reported here show the influence of the genetic make-up of different rat strains on the outcome of oral sensitization. The BN rat seems to be the most suitable rat strain for inducing specific IgG and IgE responses upon daily intra-gastric dosing of 1 mg OVA without the use of an adjuvant. Consequently, the differences in sensitivity between the rat strains may give perspective in using strains representative for specific human populations. Currently, studies are in progress to investigate differences in oral sensitizing potentials of various food proteins using the BN rat model and to compare the results with human clinical data. In addition, studies are in progress to characterize the BN rat model in more detail with respect to immune-mediated effects upon challenge and to study mechanisms involved in sensitization.

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CHAPTER 5

Comparison of antibody responses to hen's egg and cow's milk-proteins in orally sensitized rats and food allergic patients

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Background: Although several *in vivo* antigenicity assays using parenteral immunization are operational, no adequate enteral sensitization models are available to study food allergy and allergenicity of food proteins. *Objective*: To further validate a developed enteral Brown Norway (BN) rat sensitization model, specific protein recognition was studied to determine whether a comparable pattern of proteins is recognized by the rat immune system and the human immune system. *Methods*: The animals were exposed to either ovalbumin as a positive reference control, a hen's egg white-protein extract, or a cow's milk preparation by daily gavage dosing (0.5, 1, 2.5, 5, 10 or 15 mg protein/rat/day) for 9 weeks. No adjuvants were used during the sensitization studies. Using immunoblotting, specificities of antibodies against hen's egg white-proteins or cow's milk-proteins in sera from orally sensitized rats and food allergic patients were studied and compared. *Results*: The IgG and IgE antibodies to hen's egg white-proteins and cow's milk-proteins present in sera from orally sensitized rats and food allergic patients show a comparable pattern of protein recognition. *Conclusions*: Upon daily intra-gastric exposure to food allergens, the specificities of the induced antibody responses in the BN rat resemble those found in food allergic patients. These studies further support that the BN rat may provide a suitable animal model for food allergy research and research on allergenicity of food proteins.

Introduction

Humans rather frequently suffer from more or less severe allergic reactions after consumption of dietary proteins. Type I or Immunoglobulin E (IgE)-mediated reactions are known to play a major and primary role in food allergy [1]. Several (bio)technological techniques can be applied to reduce the antigenicity of food proteins or to synthesize new proteins or protein products for application in food. However, it is of importance to evaluate the (residual) antigenicity of new or modified protein products. Unfortunately, no well validated oral animal models to study food allergy and the allergenicity of food proteins are available yet.

We have previously reported an oral sensitization protocol to sensitize Brown Norway (BN) rats to food proteins without the use of an adjuvant [Knippels *et al.* Clin Exp Allergy, in press]. In later studies, we confirmed that, using this sensitization protocol, the BN rat was the most suitable strain for inducing specific-IgE responses when compared to Wistar, Hooded Lister, and PVG rats, which all were found not to produce measurable levels of antigen specific-IgE in the applied enteral induction protocol [Knippels *et al.* submitted]. Results from other studies [2,3] also indicated that the BN rat is a most suitable strain for oral sensitization studies. To further characterize our model, specific protein recognition was studied to determine whether a comparable spectrum of proteins is recognized by the rat and human immune system. We exposed

BN rats to a total hen's egg white-protein extract and cow's milk and the specificities of induced antibody responses were compared with the specificities of antibodies in sera from egg and milk allergic patients.

Since it is known that the dose of antigen influences the outcome of oral sensitization studies [4,5] we used different doses of the antigens to sensitize the animals by daily gavage dosing. To investigate whether the test animals were sensitized to the different antigen sources and to determine which dose of antigen induced the best response, antigen-specific antibodies were determined by Enzyme Linked Immunosorbent Assays and passive cutaneous anaphylaxis-tests. The sera with the highest levels of specific antibodies were subsequently used in immunoblotting to study specific protein recognition and to compare the binding patterns, with those of antibodies in sera from allergic patients.

Materials and Methods

Animals and maintenance

Young male Brown Norway (BN) rats were obtained from Charles River (Sulzfeld, Germany). The rats were housed in an animal room maintained at 23 ± 3^{0}C, with a light/dark cycle of 12 h, and a relative humidity of 30-70% during the experiment and for at least 10 days prior to study initiation. The animals were housed in stainless-steel wire cages in groups of four and had free access to food and tap-water. The rats were bred and raised on a commercially available hen's egg-protein and cow's milk-protein free rodent diet (SDS Special Diet Service, LAD1 (E) SQC, Witham, England). Pre-study blood samples were always tested for hen's egg-protein and cow's milk-protein specific antibodies to ensure the use of immunologically naive animals with respect to the antigens under investigation. All animal studies were approved by an independent ethical committee.

Materials

In these studies, three different antigen sources were used. A total hen's egg white (HEW) protein extract was prepared essentially according to the method of Bernhisel-Broadbent *et al.* [6]. Hen's eggs (free-range egg) were obtained from Albert Heijn, Zaandam, The Netherlands. A sample of 30 g of fresh egg white was added to 300 ml of sterile saline and the mixture was rotated overnight at 4^{0}C. The mixture was divided over several tubes and subsequently centrifuged for 10 min. at 2500g and 4^{0}C. The tubes were decanted and the supernatants were microcentrifuged for 15 min. at 17.000g and 4^{0}C and subsequently filter-sterilized through 0.2 µm filters (Costar Scientific Corporation, Cambridge, USA). The protein content of the extract was determined with a BCA protein assay (Pierce, Rockford, USA) according to the manufacturer's

In the studies presented in this paper, sera were used of 38 young patients, aged 7 months up to 8 years, with a previously proven IgE-mediated hen's egg white (20 patients) or cow's milk (18 patients) allergy as determined by a positive family history for atopic disease, a positive radioallergosorbent test (mean RAST class was 1.8 ± 1.17), and positive skin prick test (SPT≥2+; data not shown).

Experimental design

Animals, 4-6 weeks old at study initiation, were exposed to either OVA, HEW-proteins or CM-proteins by gavage dosing. Gavage dosing was performed daily during 9 weeks, using a 18-gauge stainless steel animal feeding needle. Groups of 6 BN rats were dosed orally with 6 different concentrations of one of the antigen sources (0.5, 1, 2.5, 5, 10 and 15 mg protein in 1 ml of tap-water per animal per day). Blood samples were obtained from the orbital plexus under light CO_2 anaesthesia at weekly intervals or by exsanguination from the abdominal aorta at sacrifice. After coagulation for 1 h at room temperature, the blood samples were centrifuged (Heraeus Minifuge T, Osterode, Germany) for 20 min. at 2000g and 4^{0}C to obtain sera. The sera were stored at -20^{0}C until analyses for specific protein recognition by IgG and IgE antibodies towards proteins in the HEW-protein extract and CM using SDS-PAGE and immunoblotting. In addition, anti-OVA specific IgG and IgE titers and anti-HEW protein and anti-CM protein specific IgG titers were determined by Enzyme Linked Immunosorbent Assay (ELISA). The method used to determine the antigen-specific IgE titers (digoxigenin-mediated assay, see Knippels *et al.* Clin Exp Allergy, in press) is not suitable to quantitatively determine specific IgE levels for mixtures of proteins. Therefore, anti-HEW-protein and anti-CM protein IgE titers were determined using passive cutaneous anaphylaxis (PCA)-tests.

Positive control animals (n=4 per antigen source) were obtained by intraperitoneally (i.p.) injection with 0.5 ml of a 0.2 mg protein/ml OVA, HEW-protein extract or CM solution in sterile saline on days 0, 2, 4, 7, 9, and 11. To potentiate the immune response, 0.2 ml of a 25 mg/ml $AL(OH)_3$ adjuvant suspension in sterile saline mixed with 0.5 ml of the OVA, HEW-protein extract or CM solution was injected on day 0. The animals were bled on day 28 by exsanguination from the abdominal aorta. Sera were prepared and stored as described before. Sera from positive control animals were pooled and used as positive control samples in the OVA, HEW-protein, CM-protein specific ELISA's and in the PCA-tests.

To investigate whether the test animals were sensitized to the different antigen sources and to determine which dose of antigen induced the best response, antigen-specific antibodies were determined by ELISA's and PCA-tests. The sera with the highest levels of specific antibodies were subsequently used in sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting to study specific protein recognition and to compare the binding pattern, with specific

protein recognition of antibodies in sera of allergic patients.

ELISA's for specific antibodies

Serum antibodies specific for OVA, HEW-proteins or CM-proteins were measured by ELISA. Antigen-specific IgG ELISA's were performed essentially as described previously [Knippels *et al.* Clin Exp Allergy, in press] with the exception of the coating which was performed with 100 µl/well of a 5 µg/ml solution of OVA or CM-proteins or with a 75 µg/ml solution of HEW-proteins in carbonate buffer, pH 9.6. OVA-specific IgE ELISA's were performed as described previously [Knippels *et al.* Clin Exp Allergy, in press]. To determine the antibody titer of the test sera, a pre-study serum pool was used as negative control. The pooled pre-study serum was measured at a 1:4 dilution. The average extinction in negative control wells, to which three times the standard deviation was added, provided the reference value taken to determine the titer in the test sera. Each test serum was titrated starting at a 1:4 dilution and the reciprocal of the furthest serum dilution giving an extinction higher than the reference value was read as the titer. All analyses were performed in duplicate. The serum pool derived from the i.p. immunized animals was used as a positive control sample. Positive and negative control samples were incorporated for each 96-wells plate.

Passive Cutaneous Anaphylaxis-tests

PCA was tested essentially as described previously [Knippels *et al.* Clin Exp Allergy, in press]. Naive (untreated) BN rats were shaven on the back and flanks and injected intradermally with 0.1 ml of the test sera in serial dilutions, followed 64 h later with an intravenous injection of 1 ml of a 1:1 mixture of a solution of OVA, HEW-proteins or CM-proteins (5 mg protein/ml in sterile saline) and a solution of Evans blue (2% in sterile saline). After 20-30 min., the animals were examined for positive responses. The diameter of dye extravasation at the site of the serum injection was measured. The reaginic titer was read as the reciprocal of the furthest dilution giving a colored spot of at least 5 mm in diameter. Positive and negative control sera as used in the ELISA's were assayed simultaneously with the test sera on each animal used for the PCA tests.

SDS-PAGE electrophoresis and immunoblotting

SDS-PAGE was performed essentially according to Leammli *et al.* [7] using 12% Tris-HCl polyacrylamide gels. Prior to laoding, protein samples were diluted (skimmed CM- proteins 1:80, HEW-proteins 1:40) in 63 mM Tris-HCl, 2% (w/v) SDS, 20% (v/v) glycerol, 0.01% (w/v) bromophenol blue, 1% (w/v) dithiothreitol, pH 6.8, and boiled for 10 min. A rainbow marker (Amersham International plc, UK) with molecular weights of 200, 97, 69, 46, 30, 21 and 14 kD was used as reference. Electrophoresis was performed for 15 min at 80 V followed by 1 h at 160 V. If not used for immunoblotting, gels were stained with Coomassie briljant blue R-250.

as described by [illegible] [5]. This membrane dry milk (Protifar; Nutricia, Zoetermeer, The Netherlands) or with 3% (w/v) BSA in 50 mM Tris-HCl, pH 7.4, 150 mM NaCl when electrophoresis was performed with HEW-proteins or CM-proteins, respectively. After 1.5 h blocking at room temperature, human or rat sera, diluted 1:5 in washing solution (0.1% Tween 20, 1% non-fat dry milk or BSA) were added and incubated overnight at room temperature. After washing, the membranes were incubated for 3 h at room temperature with peroxidase conjugated antibodies (rabbit anti-human IgG, rabbit anti-human IgE, goat anti-rat IgG or mouse anti-rat IgE, Zymed, San Francisco, USA), diluted 1:500 in washing solution. Subsequently, the blots were developed for peroxides activity using chloronaftol/DAB staining during approximately 5-20 min.

Results

ELISA's and PCA-tests with rat sera

Pre-study blood samples were all negative for OVA, HEW-protein or CM-protein specific antibodies. These sera were pooled and used as negative control in the ELISA's and PCA tests. Specific IgG and IgE antibodies were demonstrated in the pooled day 28 sera from the positive control animals (^{2}log IgG titer ≥ 11 in the OVA, HEW-protein and CM-protein specific IgG ELISA's and ^{2}log IgE titer ≥ 11 in the OVA-specific IgE ELISA's; data not shown). These serum pools were subsequently used as positive controls in the ELISA's and PCA tests.

All enteral treatment regime, induced specific antibody responses in the rats. In OVA exposed rats, most pronounced antibody responses were observed in the animals exposed to 1 mg OVA/rat/day. Maximum OVA-specific IgG titers in these animals were demonstrated at day 56 (Fig. 1a: ^{2}log titer 11.2 ± 5.2 (mean $\pm$ SD); range: 5 to 20) and maximum OVA-specific IgE responses at day 42 (Fig. 1b: ^{2}log titer 6.8 ± 3.03; range: 4 to 11). Higher doses of OVA induced less pronounced specific antibody responses and only 4 out of 6 animals responded upon daily gavage dosing with 10 mg OVA/rat (Fig 1). The optimal antibody responses in HEW-protein exposed rats were detected in animals exposed to 10 mg HEW-proteins by daily gavage dosing with maximum HEW-protein specific IgG responses detectable at day 49 with 100% responders (Fig. 2: ^{2}log titer 10.33 ± 4.97; range: 5 to 16). Rats exposed to lower doses of HEW-protein by daily gavage dosing developed lower levels of specific antibody responses as is shown in fig. 2 for the animals exposed to 2.5 mg HEW-protein/rat/day. The optimal antibody responses in CM-protein exposed rats were detected in animals exposed to 10 mg CM-proteins by daily gavage dosing with maximum CM-protein specific IgG responses detectable at day 56 with 100% responders (Fig.3: ^{2}log titer 4.8 ± 0.84; range: 4 to 6). Since we were not able to measure CM-protein and HEW-protein specific IgE responses by ELISA, reaginic antibody responses against CM-proteins and HEW-proteins were

determined by PCA-tests. Undiluted day 63 sera of 20 HEW-protein sensitized animals and all 34 CM-protein sensitized animals were analysed in PCA-test. Only 2 HEW-protein sensitized animals and 2 CM-protein sensitized animals showed a positive PCA reaction (data not shown).

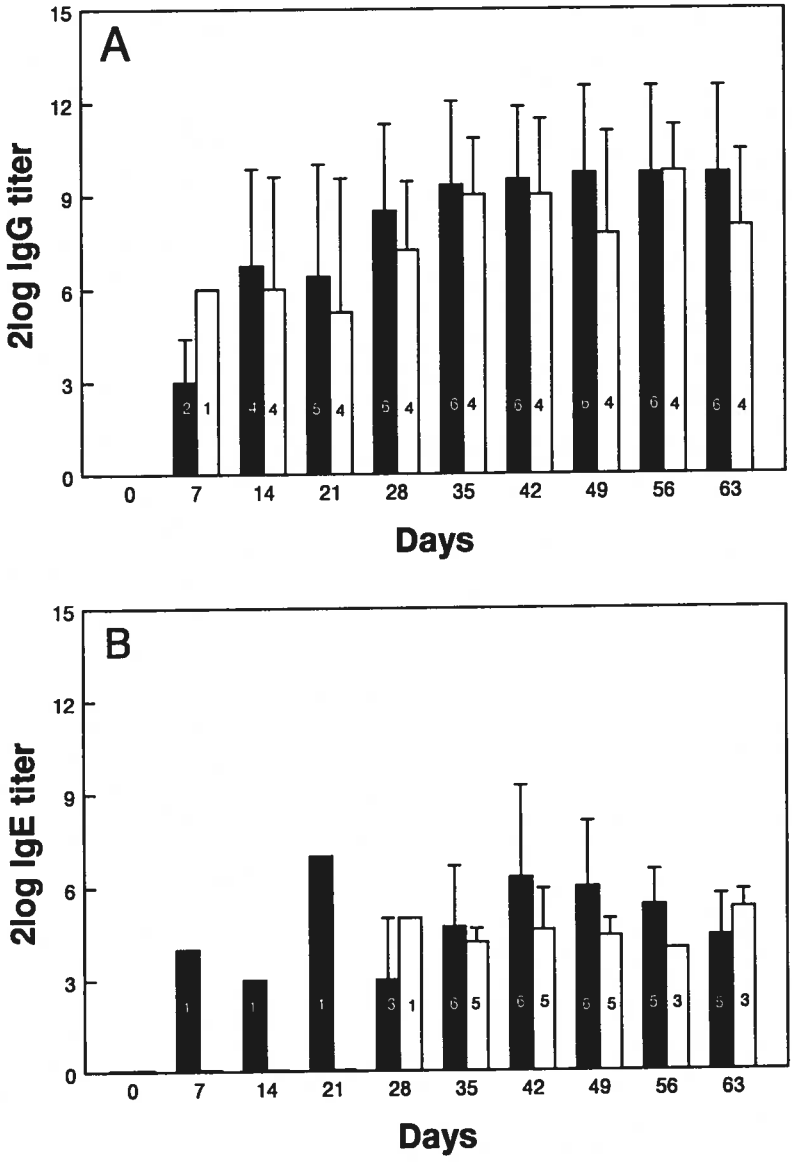
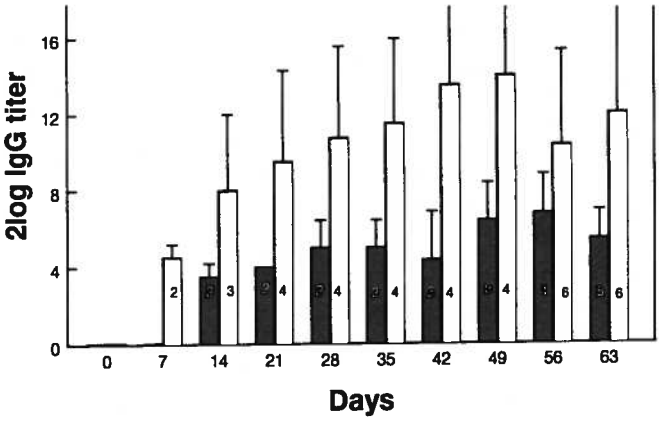


Figure 1. Time dependency of OVA-specific IgG (a) and IgE (b) response in young BN rats upon daily intra-gastric dosing with 1 mg OVA/rat/day (gray bars) or 10 mg OVA/rat/day (bright



responses in young BN rats upon daily intra-gastric dosing with 2.5 mg HEW-protein/rat/day (gray bars) or 10 mg HEW-protein/rat/day (bright bars) for 63 days. Immunoglobulin titers were determined in blood samples obtained at weekly intervals. The data are presented as mean ^{2}log IgG titer ± SD of 6 rats per group. The number of responders at the respective time-points are indicated in the bars.

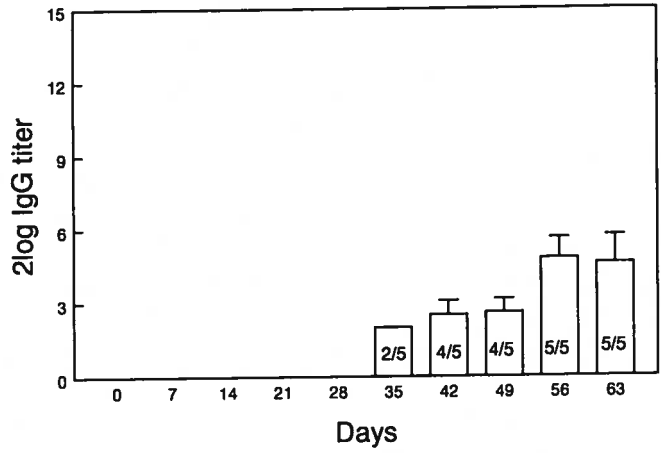


Figure 3. Time dependency of CM-protein specific IgG responses in young BN rats upon daily intra-gastric dosing with 10 mg CM-protein/rat/day for 63 days. IgG titers were determined in blood samples obtained at weekly intervals. The data are presented as mean ^{2}log IgG titer ± SD of 5 rats per group. The number of responders at the respective time-points are indicated in the bars.

SDS-PAGE and immunoblotting

The total Coomassie briljant blue staining of the proteins from the HEW-protein extract and CM after SDS-PAGE is presented in figure 4.

HEW-protein and CM-protein specific immunoglobulin binding was examined using immunoblotting with both sera from orally sensitized rats and food allergic patients. Figure 5 shows the binding of IgG and IgE antibodies to HEW-proteins on immunoblots after incubation with sera of egg allergic patients (Fig. 5a: specific IgG binding, Fig. 5c: specific IgE binding) or HEW-protein sensitized rats (Fig. 5b: specific IgG binding, Fig. 5d: specific IgE binding). The specific protein binding by IgG antibodies from HEW-

protein sensitized rats shows a comparable pattern as the protein binding by IgG antibodies in sera of egg allergic patients. Both sensitized rats and allergic patients mainly had IgG antibodies to ovotransferrin (77.7 kD), ovalbumin (46 kD), ovomucoid (28 kD), and lysozyme (14.3 kD), although antibodies against other proteins are also observed. Although ovalbumin and ovomucoid differ substantially in molecular weight, they migrate at very similar rates in SDS-PAGE, and are difficult to distinguish by this method [6,9]. The resemblance in the IgE blots is even more striking. All patients tested produced specific IgE antibodies against ovotransferrin, ovomucoid, and ovalbumin, whereas 4 patients demonstrated specific IgE antibodies against lysozyme. The same pattern is observed in specific IgE antibodies in HEW-protein sensitized rats. All animals demonstrated specific IgE antibodies against ovotransferrin, ovomucoid, and ovalbumin, whereas only 5 of the animals tested had produced detectable specific IgE antibodies against lysozyme. Figure 6 shows the binding of IgG and IgE to CM-proteins on immunoblots after incubation with sera of cow's milk allergic patients (Fig. 6a: specific IgG binding, Fig. 6c: specific IgE binding) or cow's milk sensitized rats (Fig. 6b: specific IgG binding, Fig. 6d: specific IgE binding). The specific protein binding by IgG antibodies from cow's milk sensitized rats resembles that by IgG antibodies in sera from cow's milk allergic patients. Both produce IgG antibodies to the main protein, β-lactoglobulin (18 kD), and to a lesser extend to several caseins (around 30 kD). All cow's milk allergic patients mainly had IgE antibodies against β-lactoglobulin and some very weak reactions against the caseins are observed in a few patients. Although the response in the rat IgE blot is very weak, the observed IgE antibodies also seem to be mainly directed against β-lactoglobulin. As demonstrated in Figure 4, both CM and the HEW-protein extract contain a large number of different proteins. The majority of proteins present in CM and the HEW-protein extract had not induced detectable IgE antibody responses in the BN rats, which is in accordance with the observations from sera from the allergic patients.

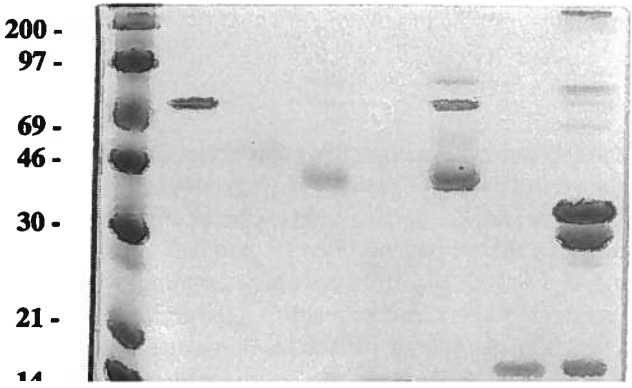


Figure 4. SDS-PAGE of proteins from cow's milk, proteins from hen's egg white-protein extract, and purified proteins (Sigma Chemicals Co., St Louis, USA). *Lane 1*, molecular weight marker, *lane 2*, ovotransferrin, *lane 3*, ovomucoid, *lane 4*, ovalbumin, *lane 5*, lysozyme, *lane 6*, hen's egg white extract, *lane 7*, β-lactoglobulin, *lane 8*, cow's

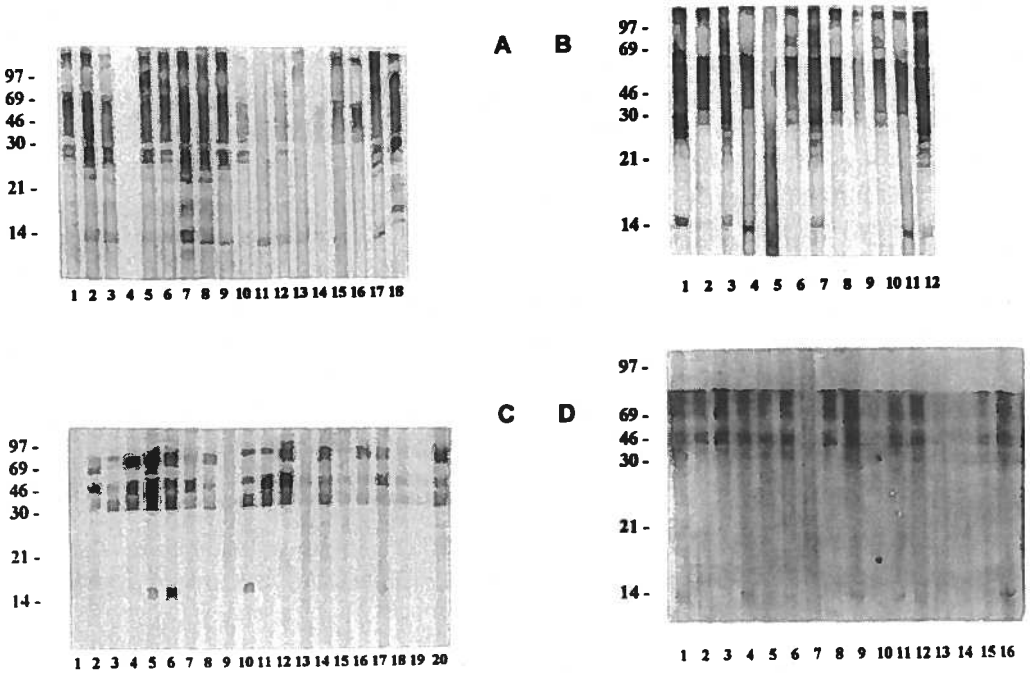


Figure 5. Immunoblot analysis of IgG and IgE antibodies against hen's egg white-proteins present in sera from hen's egg allergic patients (blot a and c for IgG and IgE, respectively) and sensitized BN rats (blot b and d, respectively). Molecular weights are indicated on the left in kD. **Blot A**: *Lane 1 to 3, 5 to 13 and 15 to 18*, sera from egg allergic patients; *lane 14*, serum of a non-allergic subject; *lane 4*, blanc. **Blot B**: *Lane 1 to 4, and 6 to 12*, sera from rats orally sensitized with hen's egg white-proteins; *lane 5*, serum from a rat orally sensitized with cow's milk-proteins. **Blot C**: *Lane 1*, blanc; *lane 2 to 8, and 10 to 20*, sera from egg allergic patients; *lane 9*, serum from a non-allergic subject. **Blot D**: *Lane 1 to 6, 8, 9, and 11 to 16*, sera from rats orally sensitized with hen's egg white-proteins; *lane 7*, blanc; *lane 10*, serum from a rat orally sensitized with cow's milk-proteins.

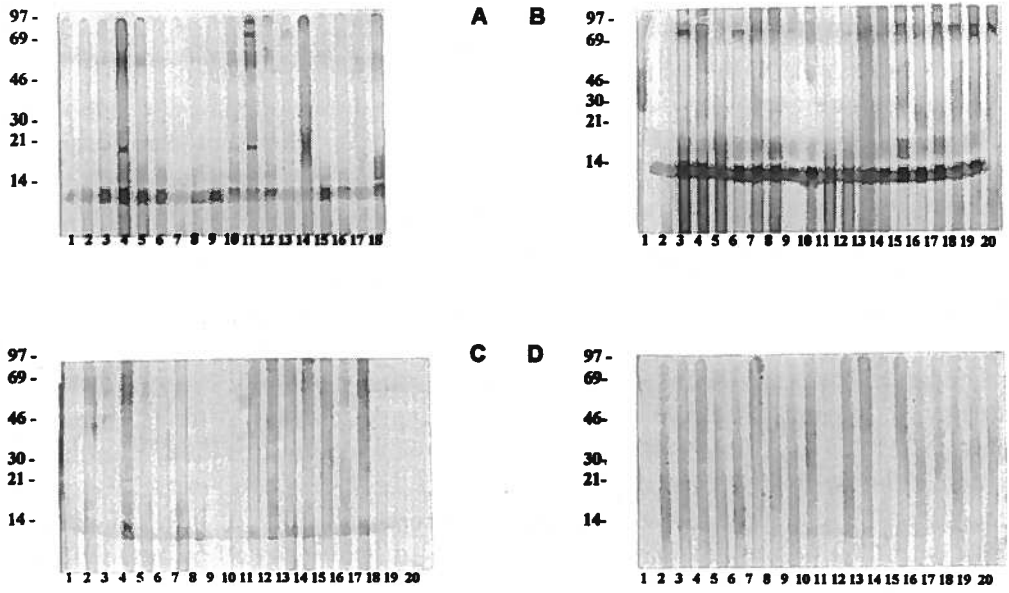


Figure 6. Immunoblot analysis of IgG and IgE antibodies against cow's milk-proteins present in sera from cow's milk allergic patients (blot a and c for IgG and IgE, respectively) and sensitized BN rats (blot b and d, respectively). Molecular weights are indicated on the left in kD. **Blot A**: *Lane 1 to 18*, sera from cow's milk allergic patients. **Blot B**: *Lane 1*, blanc; *lane 2 to 19*, sera from rats orally sensitized with cow's milk-proteins; *lane 20*, serum from a rat orally sensitized with hen's egg white-proteins. **Blot C**: *Lane 1*, blanc; *lane 2 to 19*, sera from cow's milk allergic patients; *lane 20*, serum from a non-allergic subject. **Blot D**: *Lane 1*, blanc; *lane 2 to 19*, sera

Using a previously reported oral sensitization protocol [Knippels *et al.* Clin Exp Allergy, in press], we sensitized Brown Norway (BN) rats with different food allergens by daily gavage dosing. The allergen sources used were a total hen's egg white (HEW) protein extract and skimmed cow's milk (CM). Following the induction treatment, sera were collected for analysis of allergen specific antibodies. The sera with the highest levels of specific antibodies against either HEW-proteins or CM-proteins as determined by ELISA and PCA tests were subsequently used in immunoblotting experiments to investigate whether the BN rat produced a profile of antibodies to HEW-proteins and CM-proteins similar to that observed in sera from food allergic patients.

Immunoblotting experiments with rat sera demonstrated specific-IgE antibodies against both HEW-proteins and CM-proteins while only very few sera were positive in the PCA-tests. These results suggest that immunoblotting experiments are more sensitivity compared to PCA-tests to determine specific IgE antibodies. Previously we also demonstrated a higher sensitivity for specific IgE detection of an ELISA compared to PCA-tests [Knippels *et al.* Clin Exp Allergy, in press].

The profile of allergens recognized by the immune system of the BN rat appeared comparable to the profile of allergens recognized by allergic-humans. Specific IgG antibodies in sera from hen's egg-allergic patients and rats orally exposed to HEW-proteins recognized a rather broad yet similar spectrum of proteins. Although egg white is a complex mixture of more than 20 proteins, particularly the specific IgE antibodies in sera from hen's egg-allergic patients and rats orally exposed to HEW-proteins recognized the same proteins (mainly ovotransferrin, ovalbumin, ovomucoid, and to a lesser extent, lysozyme) and no reaction was observed against any other protein present in the HEW-protein extract. The same phenomenon was observed when the pattern of protein recognition by antibodies in sera from rats orally sensitized to CM-proteins and antibodies present in sera from milk allergic patients was compared. The induced antibodies were mainly directed against β-lactoglobulin and, to a lesser extent, against the caseins. Although cow's milk contains more than 30 proteins [10], no reaction was observed against any other protein present in CM. These studies indicate that upon daily intra-gastric dosing with HEW-proteins or CM-proteins, the specific protein recognition of induced antibodies in the BN rat is comparable to that observed in sera from allergic patients. The same was described for BN rats intraperitoneally sensitized with CM which produced a profile of IgE antibodies to milk proteins similar to that observed in humans [2]. Our results obtained with sera from allergic patients are in accordance with human data in literature since it is known that β-lactoglobulin is the most common allergen recognized by CM allergic patients [11,12,13,14], although reactions are observed against other proteins from CM as well. In addition, ovotransferrin, ovalbumin, ovomucoid, and lysozyme have been claimed to be the major allergens for hen's egg allergic humans [15,16,17,18]. Although the induced antibodies in the BN rat apparently react to relevant proteins compared to the human situation, it remains to be elucidated

whether the induced specific antibodies in the rat react to the same epitopes as the antibodies in the sera from patients. Furthermore, it should be recognized that the observed differences in responses to the different food proteins as observed for the experimental animals as well as for patients will probably be due to a combination of factors such as the dose of allergen, known to influence the outcome of oral sensitization [4,5], and the allergenicity of the proteins used.

The present studies indicate that enterally exposed BN rats and young patients demonstrate IgE antibody responses to a comparable selection of proteins upon exposure to different protein mixtures and further support that the BN rat may provide a suitable animal model for food allergy research and research on allergenicity of food proteins. To characterize the developed rat model in more detail, additional studies were performed to investigate local and systemic immune-mediated effects upon enteral challenge and to study mechanisms involved in sensitization [Knippels *et al.* in preparation].

Acknowledgments

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CHAPTER 6

Immune mediated effects upon oral challenge of ovalbumin sensitized Brown Norway rats; further validation of a rat food allergy model

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Submitted for publication

Background: Although several *in vivo* antigenicity assays using parenteral immunization are operational, no full validated enteral models are available to study food allergy and allergenicity of food proteins. *Objective*: To further validate a developed enteral Brown Norway (BN) rat food allergy model, systemic and local immune-mediated reactions were studied upon oral challenges. *Methods*: The animals were exposed to ovalbumin (OVA) by daily gavage dosing (1 mg OVA/rat/day) for 6 weeks, without the use of an adjuvant, or by intraperitoneal injections with OVA together with $AL(OH)_3$. Subsequently, effects on breathing frequency, blood pressure, and gastro-intestinal permeability were investigated upon an oral challenge with 10 to 100 mg OVA *in vivo*. *Results*: In both parenterally and orally sensitized rats, an increase in gut permeability (increased passage of β-lactoglobulin as bystander protein) was determined between 0.5 and 1 hour after an oral OVA challenge was given. An oral challenge with OVA did not induce a clear effect on the respiratory system or blood pressure in the majority of the animals. However, some animals demonstrated a temporary decrease in breathing frequency or systolic blood pressure. *Conclusions*: Upon oral challenge with OVA of orally and parenterally sensitized animals, local effects were observed in all animals whereas systemic effects were observed at a low frequency. The relevance of the findings is discussed in a perspective to human clinical observations.

Introduction

Type I or IgE-mediated allergic reactions are known to play a major and primary role in food allergy [1]. In genetically predisposed (atopic) patients, food allergy is generally caused by an overproduction of IgE directed to common dietary proteins [2]. Several (bio)technological techniques can be applied to reduce the antigenicity of food proteins or to synthesize new proteins or protein products for application in food. However, it is of importance to evaluate the (residual) antigenicity of new or modified protein products. Unfortunately, no full validated oral animal models to study food allergy and the allergenicity of food proteins are available yet.

We have previously reported an oral sensitization protocol, without the use of an adjuvant, to sensitize Brown Norway (BN) rats to food allergens [Knippels *et al.* Clin Exp Allergy, in press]. In later studies, we confirmed that, using this sensitization protocol, the BN was the most suitable strain for inducing specific-IgE responses compared to Wistar, Hooded Lister and PVG rats which all were found not to produce measurable levels of antigen specific-IgE in the applied enteral induction protocol [Knippels *et al.* submitted]. Results from other studies [3,4,5] also indicated that the BN rat is a most suitable rat strain for sensitization studies. In addition, we demonstrated that the induced antibodies in orally sensitized BN rats recognise the same proteins when compared to antibodies in sera from food allergic patients [Knippels *et al.* in

preparation]. In food allergic humans, anaphylactic reactions may occur upon ingestion of the food allergens, which may result in the various manifestations that most often involve the digestive, cutaneous, respiratory, or cardio-vascular system [6,7,8]. The local effects on the gastro-intestinal tract often include a secretory response of the epithelium and, frequently, increased permeability to macromolecules [9,10,11]. Effects on the respiratory and cardio-vascular system may become life threatening and may include edema of the upper airway, severe asthma, and circulatory collapse [6,8]. Direct systemic and/or local immune mediated effects are difficult to investigate experimentally in man and there is a need for an *in vivo* animal model which would permit investigations into the induced effects and the underlying mechanisms. To further validate our enteral food allergy model, we investigated several possible immune mediated effects upon an oral challenge with ovalbumin of previously sensitized BN rats. Possible effects on the respiratory system, blood pressure, and permeability of the gastro-intestinal barrier were studied.

Materials and Methods

Animals and maintenance

Young male Brown Norway (BN) rats were obtained from Charles River (Sulzfeld, Germany). The rats were housed in an animal room maintained at 23 ± 3°C, with a light/dark cycle of 12 h, and a relative humidity of 30-70% during the experiment and for at least 10 days prior to study initiation. The animals were housed in stainless-steel wire cages in groups of four and had free access to food and tap-water. The rats were bred and raised on a commercially available ovalbumin (OVA) and cow's milk-protein free rodent diet (SDS Special Diet Service, LAD1 (E) SQC, Witham, England). Pre-study blood samples were always tested for OVA and cow's milk-protein specific antibodies to ensure the use of immunologically naive animals with respect to the antigens under investigation. All animal studies were approved by an independent ethical committee.

Experimental design

Animals, 4-6 weeks old at study initiation, were exposed to ovalbumin (OVA, Fluka Chemie, Buchs, Switzerland, purity: 70 %) by gavage dosing. Gavage dosing was performed daily during 6 weeks, using an 18-gauge stainless steel animal feeding needle (1 mg OVA in 1 ml of tap-water per animal per day). Control animals received a daily gavage dosing with 1 ml of tap-water during 6 weeks. After the induction period, the animals were not exposed to OVA for one week. Blood samples were obtained from the orbital plexus under light CO_2 anaesthesia at day 0, 35, 42 and 49. Positive control animals were injected intraperitoneally (ip) with 0.5 ml of a 0.2 mg /ml OVA solution

centrifuged (Heraeus Minifuge T, Osterode, Germany) for 20 min. at 2000g and 4°C to obtain sera. The sera were stored at -20°C until analyses for anti-OVA specific IgE titers by Enzyme Linked Immunosorbent Assays (ELISA) to confirm anaphylactic sensitization.

Antigen-specific IgE ELISA's were performed as described previously [Knippels *et al.* Clin Exp Allergy, in press]. To determine the antibody titer of the test sera a pre-study serum pool was used as negative control. The pooled preserum was measured at a 1:4 dilution. The average extinction in negative control wells, to which three times the standard deviation was added, provided the reference value taken to determine the titer in the test sera. Each test serum was titrated starting at a 1:4 dilution and the reciprocal of the furthest serum dilution giving an extinction higher than the reference value was read as the titer. All analyses were performed in duplicate. The serum pool derived from the ip immunized animals was used as a positive control sample. Positive and negative control samples were incorporated for each 96-wells plate. Only test animals that were clearly anti-OVA IgE antibody positive (anti-OVA ^{2}log IgE titer 5 or higher) were used in subsequent challenge studies.

At day 50 for the orally sensitized rats and day 29 for ip sensitized rats, these animals received an oral challenge with either 2 ml of an 5 to 50 mg/ml OVA-solution in tap-water or 2 ml of tap-water by gavage dosing. Prior to challenge, the animals were fasted for 24 hr. Upon oral challenge, the occurrence of immune-mediated effects was studied.

Evaluation of immune-mediated effects

Upon oral challenge, clinical signs were monitored and the possible occurrence of respiratory effects, effects on blood pressure, and effects on gastro-intestinal permeability were studied in test and control animals.

Determination of respiratory functions

To determine possible changes in the respiratory system, respiratory functions were measured for a period of 6 hr following challenge. Animals sensitized by daily gavage dosing with OVA or by ip sensitization were orally challenged with 2 ml of a 10, 25, 40 or 50 mg/ml OVA-solution in tap-water. Control animals received a daily gavage dose of tap-water or ip injections with saline and received an oral challenge with 2 ml of a 10, 40 or 50 mg/ml OVA-solution in tap-water according to the scheme below.

Number of animals	Parenteral sensitization	Oral challenge
3	OVA	OVA (20 mg)
5	OVA	OVA (50 mg)
3	OVA	OVA (80 mg)
8	OVA	OVA (100 mg)

Respiratory frequency was assessed using a plethysmograph with a separate head and body chamber and matched pressure transducers. Each plethysmograph was provided with a pressure transducer which sensed changes created by in- and expiration and transmitted amplified signals to a polygraph recorder, so allowing determination of respiratory frequency and pattern. Rats were restrained in modified Batelle tubes (Batelle, Geneve, Switzerland) with a water-wetted silicone diaphragm to give an air tight seal between head and neck at the one side and thorax and abdomen at the other. The tube was placed in the body chamber with the open end of the tube fitting into the front chamber. Breathing frequencies were determined by means of recording the pressure signal in the volume-calibrated body chamber. Prior to challenge, the respiratory function was measured constantly during 15 min. and immediately upon an oral challenge with OVA constantly during the first 10 min. and thereafter for periods of 30 sec. once every 5 min. for a total period of 6 hours.

Determination of blood pressure

To determine possible changes in blood pressure, blood pressure of the animals was repeatedly measured during a period of 7 hr following challenge. The OVA sensitized animals were orally challenged with 2 ml of a 5 mg/ml OVA-solution in tap-water. Control animals received ip injections with saline and an oral gavage dosing with either 2 ml of a 5 mg/ml OVA-solution in tap-water or 2 ml of tap-water according to the scheme below.

Number of animals	Parenteral sensitization	Oral challenge
16	OVA	OVA (10 mg)
3	control	OVA (10 mg)
3	control	water

One intraperitoneally sensitized animal was used to perform an Active Systemic

a bulk dose but in 3 consecutive doses within a time period of 3 ____

During the sensitization period, the rats were trained to get used to the measurement equipment in order to avoid stress during the experiments. Before challenge, individual baseline blood pressures of the animals were determined on two separate days. Prior to blood pressure measurements, the animals were placed under a heating light (30-32^{0}C) for 20 min. Thereafter, they were restrained in a small tube that was maintained at 20^{0}C. An inflatable pressure cuff was put around the tail and a distal taped sensor was used to record the systolic blood pressure. Sensor and cuff were controlled by a sphygmomanometer (Nacro Biosystems, type PE-300) which was connected to a reader (Nacro Biosystems, Type MK-II). Blood pressures were recorded at intervals during a period of 7 h.

Determination of gut permeability

To determine possible changes in gut permeability, the uptake of a bystander protein was determined following challenge. Animals sensitized by daily gavage dosing or by ip sensitization were orally challenged with 2 ml of a 50 mg/ml OVA-solution in tap-water or 2 ml of tap-water. Control animals received a daily gavage dose with 1 ml of tap-water and received an oral challenge with 2 ml of a 50 mg/ml OVA-solution in tap-water or 2 ml of tap-water according to the scheme below.

Number of animals	Daily gavage dosing	Oral challenge
12	OVA	OVA (100 mg)
4	OVA	water
4	water	OVA (100 mg)
4	water	water
Number of animals	Parenteral sensitization	Oral challenge
12	OVA	OVA (100 mg)

The animals received an additional intra-gastric dose of β-lactoglobulin (β-LG, 1 ml of a 100 mg/ml solution in tap-water; obtained from Sigma Chemicals Co., St. Louis, USA, purity 90%) 30 minutes after the oral challenge with OVA. Blood samples were collected from the orbital plexus under light CO_2 anaesthesia at 0.5, 1, 2, 3 and 5 hr after the β-LG administration or by exsanguination from the abdominal aorta at sacrifice 8 hr after the β-LG administration. Sera were prepared and stored as described and used for the quantification of β-LG by ELISA. Some sera were also used to perform immunoblots to detect the presence of β-LG.

An indirect competitive ELISA was used to detect β-LG in sera of rats obtained after challenge. For the detection of β-LG, 96-wells microtiter plates (Flat-bottomed, Maxisorp, NUNC, Roskilde, Denmark) were coated for 20 h at 4°C with 100 µl/well of a 100 ng/ml solution of β-LG, in carbonate buffer, pH 9.6. The plates were washed five times with Tris Buffered Saline (TBS; 50 mM Tris-HCl, 150 mM NaCl) containing 0.05% Tween 20 (Merck, Hohenbrunn, Germany). Subsequently, the plates were blocked by adding 300 µl/well phosphate-buffered saline (PBS) containing 1% polyvinylpyrollidone (PVP-40, mol wt. 40.000, Sigma Chemicals Co., St. Louis, USA) and 1% Tween 20 buffer. After 2 h incubation at room temperature, the plates were washed. Samples of 100 µl of 1:2 dilutions of each rat test-serum or standard (0-250 µg/ml β-LG in rat serum in TBS-Tween 20) were pre-incubated with 100 µl anti-β-LG (Sheep anti-bovine β-LG, Instruchemie, Hilversum, The Netherlands) diluted 1:1000 in TBS-Tween 20 for 30 min at 37°C. After pre-incubation, the samples were added to the wells (200 µl/well) and incubated for 20 h at 4°C. After incubation and subsequent washing, 200 µl/well of peroxidase conjugated donkey anti-sheep IgG (Instruchemie, Hilversum, The Netherlands) diluted 1:13.000 in TBS containing 0.05% Tween-20 and 4% Polyethylene Glycol 6000 (Fluka Chemie, Buchs, Switzerland) was added. The plates were incubated for 1.5 h at room temperature, and after subsequent 7 times washing, an enzyme substrate solution of 3,3',5,5'-tetramethylbenzidine (TMB; Sigma Chemicals Co., St. Louis, USA) was added (100 µl/well; 6 mg/ml DMSO). Plates were developed at room temperature for 5 to 15 min. and 100 µl/well of 2N H_2SO_4 was added. Optical densities were read spectrophotometrically at 450 nm with an ELISA plate reader (Microplate Reader, Bio-rad Laboratories, Richmond, USA). All analyses were performed in duplo and the absorbances of the samples were compared with the absorbance curve obtained from the β-LG standards included for each plate. Negative control serum samples and blancs were also incorporated at each 96-wells plate.

In addition to the ELISA's, β-LG detection in sera of rats was also performed using immunoblotting. SDS-PAGE was performed essentially according to Leammli *et al.* [12] using 12% Tris-HCl polyacrylamide gels. Serum samples from the gut permeability study were diluted 1:2 in 63 mM Tris-HCl, 2% (w/v) SDS, 20% (v/v) glycerol, 0.01% (w/v) bromophenol blue and, 1% (w/v) dithiothreitol, pH 6.8, and boiled for 10 min. A rainbow marker (Amersham International plc, UK) with molecular weights of 200, 97, 69, 46, 30, 21 and 14 kD was used as reference. Electrophoresis was performed for 15 min at 80 V followed by 1 h at 160 V.

After SDS-PAGE, the separated proteins were transferred to a polyvinylidenedifluoride membrane (PVDF; Immobilon-P Transfer membrane; Millipore Corporation, Bedford, MA, USA) using a semi-dry electrophoretic apparatus as described by Towbin *et al.* [13]. The membranes were blocked with 3% (w/v) BSA in 50 mM Tris-HCl, pH 7.4, 150 mM NaCl. After 1.5 h blocking at room temperature, sheep anti-β-LG, diluted 1:500 in washing solution (0.1% Tween 20, 1% BSA) was added and incubated overnight at

methanol, 0.6 ml of 4% 3,3-diaminobenzidinetetrahydrochloride in TBS and 20 µl of 30% H_2O_2) during approximately 5-20 min.

Results

Specific IgE antibodies in rats sensitized to ovalbumin

Pre-study serum samples were always tested for OVA-specific antibodies. No anti-OVA antibodies were detected in the pre-study serum samples from any of the animals. These sera were pooled and used as negative control in the ELISA's. In case the animals were also used to determine possible changes in gut permeability, pre-study serum samples were also tested for cow's milk-protein specific antibodies. No anti-cow's milk-protein antibodies were detected in the pre-study serum samples of these animals. OVA-specific IgE antibodies were demonstrated in the day 28 sera from all positive control animals (n=47; ^{2}log IgE titer 7.3 ± 1.7 [mean ± SD], range 3-11). A pool of these sera was used as positive control in the ELISA's.

Day 42 serum samples obtained from the orally OVA dosed rats were assayed for the presence of anti-OVA IgE by ELISA. The animals with a ^{2}log anti-OVA IgE titer of at least 5 in the ELISA (20 out of 28 animals; ^{2}log IgE titer 6.6 ± 1.13, range 5-8) were used to investigate the possible occurrence of immune-mediated effects upon oral challenge. True negative control (not OVA-exposed) animals, confirmed to be anti-OVA IgE negative, were always included in subsequent studies.

Effects on the respiratory system

Upon oral challenge with OVA of non-sensitized and orally or parenterally sensitized animals, the possible occurrence of respiratory effects was investigated in individual animals. No changes in breathing frequency were observed in non-sensitized animals upon challenge (n=16 in total; n=12 for animals challenged with 100 mg OVA; see figures 1a and 1b). The breathing frequency in these animals was around 2.5 Hz and tended to decrease slowly over the measurement period. An oral challenge with OVA did not induce a clear effect on the respiratory system in the majority of the sensitized animals. However, some animals demonstrated a temporary decrease in breathing frequency. In parenterally sensitized rats, a temporary decrease in breathing frequency was observed upon oral challenge with 100 mg OVA in 1 out of 8 animals (Fig. 1c). About 1 min after challenge, a drop in breathing frequency was observed from around 2.5 Hz to 1.6 Hz. The breathing frequency returned to normal rates within 10 min. A similar, yet somewhat retarded pattern was observed in 1 out of 7 orally sensitized animals with an anti-OVA IgE titer of ≥5 and challenged with 100 mg OVA by gavage (Fig. 1d). A clear drop in frequency from about 2 to 1.15 Hz was observed about 10 min after challenge and recovery occurred slowly and only after about 1 hr breathing

frequency returned to normal rates (2 Hz).

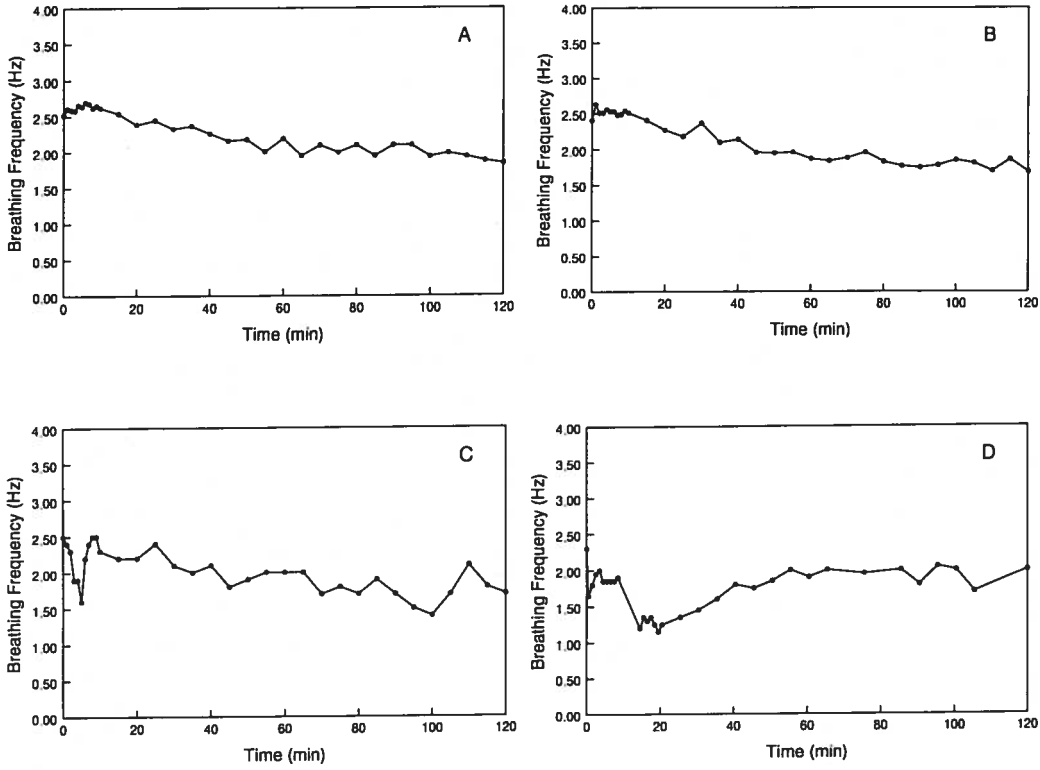

Figure 1. Breathing frequency of non-sensitized rats orally challenged with OVA (a and b; n=16 in total; n=12 for animals challenged with 100 mg OVA), one rat ip sensitized with ovalbumin and orally challenged with 100 mg ovalbumin by gavage (c) and a rat sensitized by daily gavage dosing with ovalbumin and challenged orally by gavage with 100 mg ovalbumin (d). No clear effect on the respiratory system was observed in the majority of sensitized and challenged animals. Breathing frequency measurements (in Hz) were performed for a period of 30 seconds once every 5 min for 7 hours and prior to challenge.

Effects on blood pressure

Systolic blood pressure was measured in control and intraperitoneally sensitized rats upon oral challenge with either tap-water or 10 mg OVA. No changes in blood pressure occurred in control animals upon OVA challenge (n=3) as well as in control animals challenged with tap-water (n=3). The individual blood pressure pattern of a representative control animal is presented in figure 2a. The mean blood pressure of the control animals was 107.5 mm Hg with a 95% confidence interval of 96.2-111.8 mm Hg.

intraperitoneally sensitized animal was challenged intravenously with OVA and blood pressure was measured during 25 min (Fig. 2b). Upon challenge, a critical drop in blood pressure (to 10 mm Hg) was observed within 5 to 8 min. which lasted for at least 15 min. After 20 min, the blood pressure had returned to normal levels.

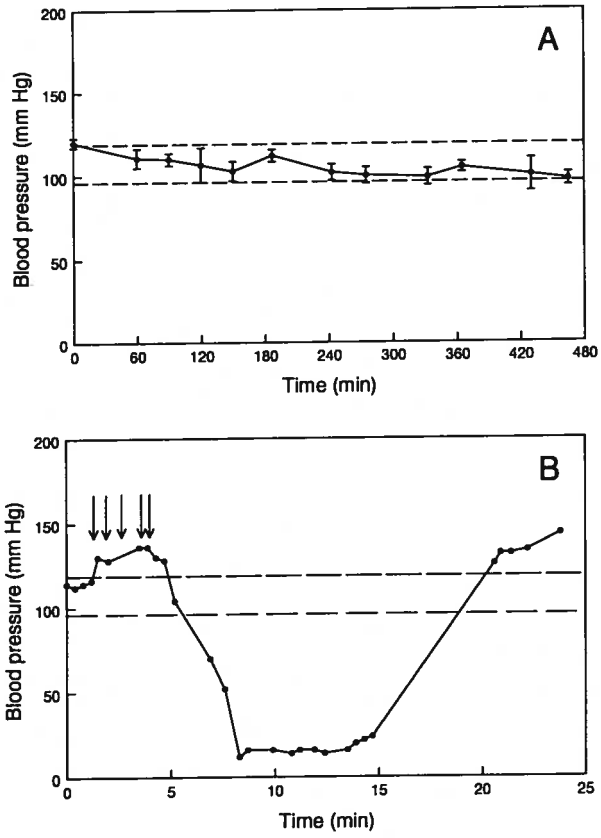

Figure 2. Blood pressure of a rat ip sensitized with ovalbumin and challenged orally with water (a) and a rat ip sensitized with ovalbumin and challenged intravenously with ovalbumin (b). The arrows in figure b indicates the time-points of the repeated intravenous challenges with ovalbumin. The data are presented as mean of repeated blood pressure measurements (mm Hg) per time interval of approximately 2 minutes for individual rats. The broken lines indicate the upper and lower limit of the 95% confidence range of blood pressure values of control animals.

In orally challenged sensitized animals, the observed effects on blood pressure were not as dramatic as seen in the intravenously challenged positive control animal. However, in 7 out of 16 animals tested, blood pressure levels dropped beneath the lower 95% confidence limit. There were two different patterns that could be distinguished. In a few animals (n=3), the blood pressure dropped approximately 4 hours after challenge and returned to normal levels within 2 hours (Fig. 3a and 3b for 2 representative examples). In other animals (n=4), an almost continuous decrease in blood pressure was observed (Fig. 3c and 3d for 2 representative examples).

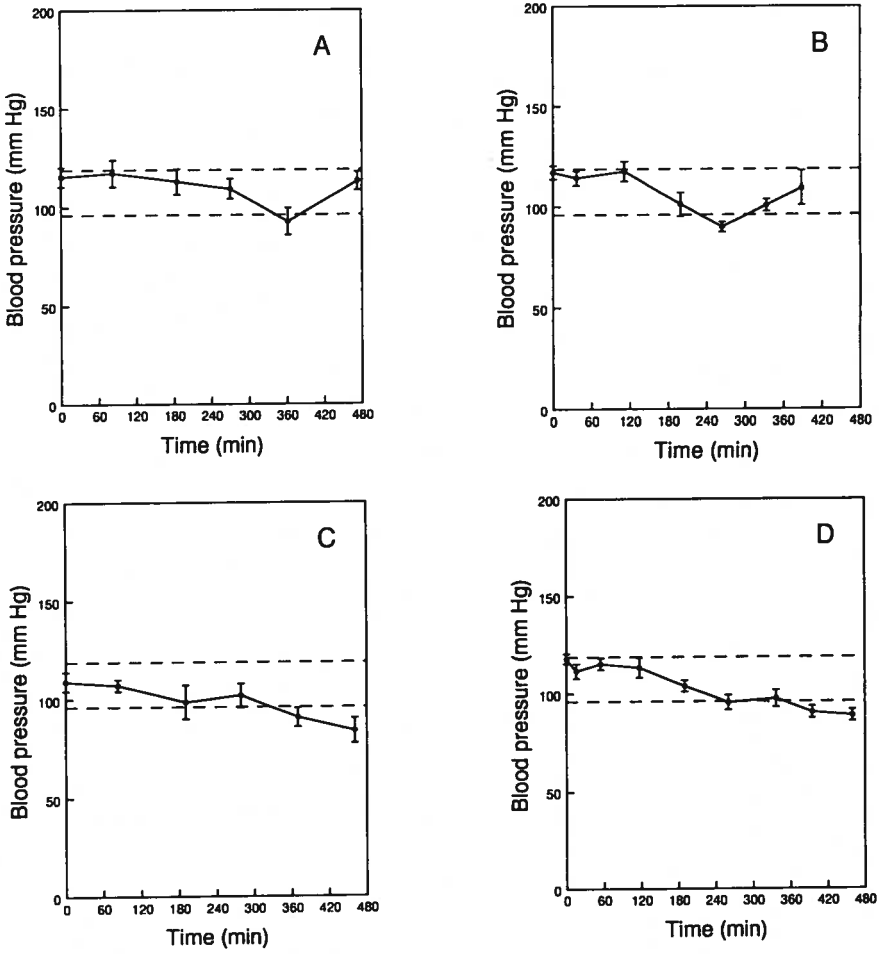

Figure 3. Blood pressure measurements in 4 representative rats ip sensitized with OVA and challenged with 10 mg OVA by gavage. Blood pressure either dropped approximately 4 hours after challenge (a and b) and returned to normal values or an almost continuous decrease in blood pressure was observed after challenge (c and d). The data are presented as mean of repeated blood pressure measurements (mm Hg) per time interval of approximately 2 minutes for individual rats. The broken lines indicate the upper and lower limit of the 95% confidence range of blood pressure of control animals.

Effects on gastro-intestinal permeability

Upon an oral challenge of sensitized and non-sensitized rats with either ovalbumin (OVA) or water, and a subsequent gavage dose with β-lactoglobulin (β-LG) 30 minutes later, the amount of β-LG was measured in sera obtained 0.5, 1, 2, 3, 5 and 8 hours after the β-LG administration. The concentrations of β-LG in sera from control, parenterally

Time (hr)	0	0.5	1	2	3	5	8
ip sensitized[1]	<LOD	1.7-4.6	0.9-2.4	<LOD-1.6	<LOD	<LOD	<LOD
orally sensitized[2]	<LOD	0.05-0.13	0.07-0.09	<LOD-0.05	<LOD	<LOD	<LOD
control[3]	<LOD	<LOD-0.02[3]	<LOD	<LOD	<LOD	<LOD	<LOD

LOD: Limit of detection (0.01 µg/ml)
* sensitized or non-sensitized control animals were intra-gastrically challenged with either ovalbumin (100 mg/animal) or tap-water. Half an hour later, an intra-gastric dose of β-lactoglobulin (β-LG) was given as a bystander protein. At the indicated time-points following β-LG administration, the levels of β-LG in sera were determined by ELISA.
1: 12 animals ip sensitized and intra-gastrically challenged with ovalbumin (OVA).
2: 9 animals orally sensitized and intra-gastrically challenged with OVA (i.e. 9 out of 12 animals with anti-OVA IgE titers of ≥5).
3: 4 non-sensitized animals challenged with water, 4 non-sensitized animals challenged with OVA, and 4 orally sensitized animals challenged with water; one animal in the latter group demonstrated a β-LG level of 0.02 µg/ml 0.5 hr after β-LG administration. β-LG levels remained below LOD of 0.01 µg/ml in all other serum samples from the control animals.

In one sensitized control animal challenged with tap-water, β-LG was detectable in serum obtained 0.5 hr after the β-LG administration (0.02 µg/ml). In all other serum samples from control animals either challenged with OVA or tap-water, β-LG levels were below the limit of determination of 0.01 µg/ml. In orally sensitized animals with anti-OVA IgE titers of ≥5 and challenged with OVA (n=9), β-LG was detectable in sera obtained 0.5 hr after the β-LG administration (0.1 ± 0.03 µg/ml (mean ± SD); range: 0.05-0.13). In sera obtained from parenterally sensitized animals at 0.5 and 1 hr after β-LG administration, a significant amount of β-LG was detectable (2.9 ± 1.56 µg/ml; range: 1.7-4.6 and 1.6 ± 0.9 µg/ml; range: 0.9-2.4, respectively). These data indicate an increase in permeability of the intestinal epithelium to proteins upon challenge of sensitized animals. Moreover, immunoblotting experiments indicated the presence of traces of the entire β-LG protein (18 kD) in the sera obtained after challenge and β-LG administration of sensitized animals (Fig. 4), whereas no β-LG could be detected in sera obtained before β-LG administration or in control animals upon challenge and β-LG administration. This indicates that at least a part of the β-LG absorbed upon intestinal anaphylaxis was absorbed as a macromolecule.

Figure 4. Detection of β-lactoglobulin by immunoblotting in sera of 2 parenterally (lane 1 to 4) and 2 orally (lane 5 to 8) OVA sensitized rats, orally challenged with OVA followed by a gavage dose of β-lactoglobulin. The molecular weight is indicated on the left in kD. *Lane 1 to 8*, β-lactoglobulin in serum obtained before challenge (*lane 1, 3, 5, 7*) and 30 min after the β-lactoglobulin administration (*lane 2, 4, 6, 8*); *lane 9*, purified β-lactoglobulin.

Discussion

Oral antigen exposure of food allergic patients may result in many different clinical signs or physiological reactions. Most frequently, effects on gastro-intestinal physiology are induced. However, in some patients, effects on the respiratory system and/or cardio-vascular effects are noted. In this study we showed comparable respiratory, circulatory, and gastro-intestinal effects upon challenge of ovalbumin (OVA) sensitized Brown Norway (BN) rats.

Upon an oral OVA challenge, gut permeability was increased as evidenced by an increased uptake of a bystander protein (β-lactoglobulin; β-LG). One hour after an OVA challenge followed by a dose of β-LG 30 min later, the amount of β-LG in the sera of previously sensitized rats was significantly higher when compared to non-sensitized animals. This effect was more pronounced in ip sensitized animals when compared to orally sensitized rats. Several models of intestinal hypersensitivity to food proteins have shown that antigen challenge of the sensitized intestine causes alterations in ion transport, permeability, and motility [10,14,15], and mediators released in anaphylactic reactions such as histamine, platelet-activating factor, prostaglandins, leukotrienes, and some newly formed cytokines have been shown to alter mucosal function in experimental models [9,16,17,18,19,20,21,22,23,24]. Up to now, it has however not been fully revealed whether the increased macromolecular passage is mainly due to transcellular or paracellular transport. However, our finding from immunoblotting experiments that a significant amount of intact β-LG is present in sera of sensitized animals together with the findings of Scudamore *et al.* [25] who showed that the release of rat mast cell protease-II, a known rat mucosal mast cell mediator, increases epithelial permeability via a paracellular route, suggests an increased epithelial permeability through the paracellular route, although an increased permeability via the transcellular route cannot be excluded. The observed difference in magnitude of increased serum β-LG levels in orally and ip sensitized animals in the present study may be due to several

Saunders *et al.* [26] showed that stress may impair the barrier function of the intestine. The oral challenges in our study were given by gavage dosing which may have caused stress resulting in an increased permeability. This may particularly have affected the ip sensitized animals, since these received a first gavage dosing when challenged, whereas the orally sensitized animals were exposed to OVA by daily gavage dosing for 42 days during the sensitization period. As such, the orally sensitized rats will have been used to gavage dosing to a certain degree. However, the absence of β-LG in sera from OVA and β-LG dosed non-sensitized animals, or only water and β-LG dosed animals, proves that stress alone does not account for any increased passage of β-LG through the gastro-intestinal barrier. Rioux *et al.* [27] reported a marked and progressive diminution of mucosal mast cell degranulation upon repeated oral challenges in sensitized rats. This phenomenon may also have contributed to the less pronounced effect observed in the orally sensitized animals.

In addition to studies on local effects, the possible occurrence of systemic effects upon an oral challenge were investigated by monitoring respiratory functions and blood pressure. An oral challenge with OVA did not induce a clear effect on the respiratory system or blood pressure in the majority of the animals. However, some animals demonstrated a temporary decrease in breathing frequency or systolic blood pressure. These observations indicate that systemic effects can be induced in orally and ip sensitized animals upon oral challenge. In literature, a drop in breathing frequency below 70% of the normal breathing frequency is referred to as an indication of severe respiratory effects [28]. Although, we only observed severe respiratory effects in a few animals, this low incidence is in agreement with observations from food allergic patients, of whom only about 10% is reported to react with respiration problems [8,29]. In several animals, a decrease in systolic blood pressure was observed although no dramatic drop in blood pressure resulting in circulatory collapse was observed. This may have been due to the amount of immuno-reactive protein that reached the circulation to elicit systemic anaphylaxis since an intravenous challenge in both ip and orally sensitized animals resulted in a drastic drop in blood pressure within minutes. Again, the rather low incidence of cardio-vascular effects upon oral challenge of the rats is in accordance with the human clinical practice.

The studies reported here show that upon an oral challenge of both orally and parenterally sensitized BN rats, local immune mediated effects, as studied by changes in gut permeability, are observed. An oral challenge with OVA did not induce a clear effect on the respiratory system or blood pressure in the majority of the animals. However, some animals demonstrated a temporary decrease in breathing frequency or systolic blood pressure. In humans, food induced fatal anaphylactic reactions are more frequently observed in patients allergic to peanut-proteins when compared to egg-proteins [30]. Therefore, studies are in progress to investigate the sensitizing potential of peanut-proteins and the possible occurrence of local and systemic immune-mediated

effects upon oral challenge with peanut-proteins using the rat model described.

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CHAPTER 7

Summarizing Discussion

Humans rather frequently suffer from more or less severe allergic reactions after consumption of dietary proteins [1-3], in which Type I or IgE mediated allergic reactions are known to play a major and primary role [4]. People with atopy are considered to have a hereditary trait (the atopic constitution) associated with a greater risk of development of IgE mediated allergies. Although genetic factors play a major role in the development of allergic reactions other factors, like the introduction of new allergens and air pollution are also thought to play a role in the recent increase in the prevalence of allergic diseases [5-7]. Our knowledge on the pathophysiological mechanisms involved in the development of food allergy as well as in the development of immune mediated effects upon challenge has greatly increased over the past decades. Nevertheless, many questions have remained unanswered. Because tools for research into these issues are rather lacking, new models suitable for mechanistic studies will be of great value.

Several (bio)technological techniques can be applied to reduce the antigenicity of food proteins to produce for instance hypoallergenic infant formulas. Biotechnological techniques are also available to synthesize for instance new proteins or new biological varieties for applications in food. For such biotechnologically derived protein products (novel foods), allergenicity may also pose a major concern. For safety reasons, it is of importance to evaluate the residual antigenicity of modified protein products, to screen on possible cross-reactivity to prevent reactions in previously sensitized individuals, and to test for sensitizing properties of new and/or modified protein products. Although well validated models to determine the allergenic potential of new dietary proteins are not available yet, several methods may currently be applied to generate some relevant information with respect to the antigenicity and allergenicity of proteins. Several *in vitro* assays, like for instance immunochemical analyses and mast cell and basophil degranulation test, are available to determine antigens. However, these *in vitro* analyses are not directly suitable to study the allergenicity of new proteins, since antibodies or sera obtained from already sensitized subjects are needed. To determine the antigenicity of proteins, several well validated assays are operational. These assays are based on parenteral application of the test protein to laboratory animals, in which the guinea pig is the most regular test species. Although the information from antigenicity assays may be of major relevance, it must always be recognized that such assays only provide information on the antigenicity of proteins. In general, any protein that may be recognized as an antigen (foreign protein) will induce a humoral and cellular immune response upon injection and will most likely give a positive testing result in such assays. Whether a protein has a high or low potency of inducing food allergic reactions in (susceptible) humans can not be concluded or predicted based only on the results of these parenteral antigenicity assays. Natural barriers such as the gastro-intestinal acid denaturation and digestion and the mucosal/epithelial layers, which all are known to prevent, reduce, or in any other way influence the contact between food antigens and the

local and systemic immune system [8,9], are not modelled or taken into account in such assays. A refinement may be achieved by the use of *in vitro* models for gastro-intestinal digestion and physico-chemical information in combination with antigenicity assays. Because also the possibilities for human research are very limited, animal models suitable for food allergy research or for research on the allergenicity of food proteins would be of great value.

This thesis

Several attempts to develop animal models for food allergy research have been conducted in the past. Although some of the attempts to develop enteral sensitization and/or challenge protocols for laboratory animals were rather successful or at least promising, these efforts hardly resulted in structured approaches aimed at the development of well validated enteral allergenicity models. The overall aim of the work presented in this thesis was the development and validation of an enteral rat model for food allergy research and research on the allergenicity of food proteins.

In Chapter 2, we reported an oral sensitization protocol to sensitize Brown Norway (BN) rats (high IgE-responder strain) to ovalbumin (OVA), a well defined chicken egg white allergen. Daily intra-gastric dosing with OVA, without the use of an adjuvants, resulted in OVA-specific IgG as well as OVA-specific IgE responses whereas upon *ad libitum* exposure through the drinking water, OVA-specific IgG but no OVA specific IgE was detected. The cellular response was examined by determination of delayed-type hypersensitivity (DTH) responses. Both sensitization protocols (intra-gastric and *ad libitum*) sensitized for DTH, yet the DTH responses were stronger in the *ad libitum* exposed rats. The more pronounced DTH response in the *ad libitum* exposed animals in combination with the strong OVA-specific IgG response and the absence of an OVA-specific IgE response suggest a dichotomy in T cell function as is also observed in humans and mice [10-12], with a Th1-like response in the *ad libitum* exposed animals and a Th2-like response in the daily gavage dosed animals. Although there is no proven explanation for the observed differences in responses between *ad libitum* and gavage dosed animals, a difference in gastro-intestinal digestion efficiency with a resulting difference in potential immunological response to ingested protein may have played a role. Intra-gastric administration of proteins may interfere with normal gastric functions such as the gastric emptying rate [13] and as such may affect the digestive breakdown. An altered digestive breakdown of food proteins may result in a different spectrum of digestive fragments, which possible may have resulted in a Th2-like response and subsequent IgE production. Although the pathophysiological mechanism in oral sensitization are not yet fully clear, intra epithelial lymphocytes (IEL), which are continuously in contact with luminal antigens, may play a crucial role in controlling

recently been demonstrated that γδ T cells produce either type 1 or type 2 cytokines *in vivo* [15]. Since these γδ T cells produce these cytokines with rapid kinetics and upon first encounter with the antigens, these T cells may be one of the sources for cytokines to skew the immune response towards a Th1 or Th2 response [16,17]. The described oral sensitization models may provide interesting tools to investigate pathophysiological mechanisms such as the role of γδ T cells and the dichotomy in oral sensitization, since *ad libitum* exposure to OVA resulted in a Th1-like response whereas intra-gastric exposure to OVA resulted in a Th2-like response.

It is known that oral exposure to food antigens may easily induce an immunological tolerance. Repeated low dose feeding was shown to induce immune deviation (active suppression mediated by regulatory T cells), whereas progressively higher feeding regimes induced T cell anergy (lymphocytes which are alive, but fail to display functional responses) [18]. Although these findings were mostly observed in mice, oral tolerance induction is also described for rats [19]. However, oral tolerance is not the normal response observed upon oral administration of proteins to rats [20-23]. These observations indicate that it is very important to control the dietary protein exposure in the development of oral sensitization models in order to avoid the possibility of tolerance induction or the use of immunologically non-naive animals. In Chapter 3, we described some studies on the importance of prevention of dietary pre-exposure to antigens in sensitization research. Animals initially bred on a soy-protein containing diet were fed a soy-protein free diet for at least 12 months. Upon prolonged feeding of the soy-protein free diet, these animals as well as their first generation of offspring bred on the soy-protein free diet still demonstrated the presence of anti-soy-protein IgG antibodies. Despite the fact that the parental generation was not exposed to certain dietary antigens during a prolonged period, a non-hereditary transfer apparently occurred to the first generation of offspring resulting in continued specific antibody expression against dietary proteins, although the offspring never directly encountered these antigens via its diet. No specific antibodies were detectable in the second generation of offspring and later generations, which became fully responsive to oral exposure to the antigens. These results indicate that besides the age of the animals, the dose of antigen, the presence of adjuvant, and the frequency of administration, which all may influence the immune response upon oral antigen exposure [23-26], special caution should be paid concerning the diet when (oral) sensitization studies are performed with animals and not only the test animals must be bred and raised on a specified antigen-free diet but also their parental generations in order to avoid any problems in (oral) sensitization studies. The question how to explain this continued expression of soy-protein specific antibodies is discussed in Chapter 3. At present, we have no indication whether, and if so, to what extend, the observations described for rats in Chapter 3 and for guinea pigs by Pahud *et al.* [27] can be extrapolated to the human situation. Yet, this observed phenomenon may have important implications, for instance with respect to the introduction of novel

foods. Since theoretically, protective antibodies to newly introduced proteins may be absent, the chance of getting orally sensitized to these proteins may be changed. Furthermore, the observed phenomenon may provide new insights into the development of the adoptive immune responses in young animals and infants.

In humans food allergy is more common in atopic humans who have a genetic predisposition to react with an elevated production of IgE antibodies to generally harmless substances, although IgE mediated allergies are observed in non-atopic humans as well. Orally induced OVA-specific responses were therefore compared between rat strains with different genetically-based strain-specific characteristics of the immune system (Chapter 4). Responses in Wistar and Hooded Lister rats were characterized by an OVA-specific IgG but no OVA-specific IgE response and strong DTH reactions, suggesting a Th1-like response. Piebald Virol Glaxo (PVG) rats showed the lowest induction of OVA-specific IgG antibodies, the absence of specific IgE antibodies and a weak DTH response. Responses in the BN rats, a high immunoglobulin responder strain which to a certain degree resembles atopic humans in their (genetic) predisposition to react with an overproduction of IgE to antigens, were characterized by strong OVA-specific IgG and IgE responses, and weak DTH reactions (Th2-like response). In humans, the T-cell system in atopics and normal individuals responds in a qualitative different fashion to environmental allergens [28-32]. T-cell cloning studies have revealed that atopics have a preference for establishment of T-memory for allergens that is of the Th2 subset, being dominated by CD4+ T-cells secreting IL-4 and IL-5. Corresponding T-cell responses in normal individuals tend to be of Th1 (INF-γ secreting) phenotype. The differences in response pattern between the various rat strains may give a perspective in using strains representative for specific human populations. Moreover, the profile of allergens recognized by the immune system of the BN rat upon daily intra-gastric administration with either a total hen's egg white-protein extract or cow's milk-proteins, appeared comparable with those recognized by food allergic patients (Chapter 5). All together, the BN rat seems a most suitable rat strain for oral sensitization studies and the specificities of the induced antibody responses resemble those found in food allergic patients.

In sensitized individuals, anaphylactic reactions occur upon ingestion of the allergen. Food-allergen specific IgE antibodies are bound to the high affinity IgE receptors (FcεRI) present on mast cells throughout the body tissues and basophils in the circulation. Upon renewed contact with the food-allergen, the allergen binds to the Fab region of cell-associated IgE and subsequently cross-links membrane-bound IgE molecules. Cross-linking of several IgE molecules will result in an intra-cellular signal causing degranulation of the mast cells and basophils [33]. The release of basophil and mast-cell mediators results in the various manifestations that in case of food allergy most often involve the digestive, cutaneous, respiratory, and cardio-vascular system [34-36]. In chapter 6, we showed that upon an oral challenge of OVA sensitized BN rats, local

macromolecular passage is mainly due to transcellular or paracellular [illegible]
been shown that antigen challenge in egg albumin-sensitized rats results in the release of rat mast cell protease-II (RMCP-II, a known rat mucosal mast cell mediator) and disruption of the epithelial basement membrane [37]. Our finding that a significant amount of intact β-lactoglobuline is present in sera of sensitized and challenged animals together with the findings of Scudamore *et al.* [38] who showed that the release of RMCP-II increases epithelial permeability via a paracellular route, suggests an increased epithelial permeability in our animals via a paracellular route, although an increased permeability via the transcellular route can not be excluded. An oral challenge with OVA did not induce a clear effect on the respiratory system or blood pressure in the majority of animals. However, some animals demonstrated a temporary decrease in breathing frequency or systolic blood pressure. Although the low incidence may be due to the amount of immuno-reactive protein that reaches the circulation to elicit systemic effects, the low incidence of respiratory and cardio-vascular effects upon oral challenge are in agreement with observations from food allergic patients [36,40]. All together, the results indicate that the oral rat model for food allergy exhibits some of the clinical characteristics of food allergy. In humans, food induced fatal anaphylactic reactions are more frequently observed in patients allergic to peanut-proteins when compared to egg-proteins [41]. It would be interesting to investigate whether oral sensitization of BN rats with peanut-proteins and subsequent oral challenge would result in more pronounced immune-mediated effects when compared to the results obtained with OVA sensitized rats. This will be one of the issues addressed in future studies using the animal model described and characterized in this thesis.

Possible applications of the enteral BN rat model

Mechanistic studies

We demonstrated that the BN rat can be orally sensitized to food proteins, without the use of an adjuvant. This model provides an important refinement when compared to the parenteral animal models since the gastro-intestinal tract, known to influence the ultimate allergenicity of food proteins [8,9,42,43], is taken into account in this model. The physiological more relevant route of sensitization offers an interesting possibility to study mechanisms involved in oral sensitization. The occurrence of responders and non-responders as also observed in humans suggests further similarities with the human situation in factors involved in the sensitization phase. In addition, as marked similarities in local and systemic effects upon challenge were demonstrated in the sensitized rats and human patients, the model offers an important tool in research with respect to processes occurring during elicitation of clinical effects.

Studies on allergenicity of food proteins

Although we demonstrated that oral sensitization could be achieved to different food proteins, it remains to be further studied whether this rat model is sufficiently able to distinguish between strong and weak food allergens. However, the results obtained until now suggest that this model can be used to obtain important additional information on the possible allergenicity of food proteins. The choice of the appropriate animal model to study the possible allergenicity of proteins is very important as is demonstrated by an example in which an animal model did not predict allergenicity of the Brazil nut 2S globulin protein which was assessed by PCA in mice fed the antigen orally [44]. This study reported that the 2S albumin protein did not elicit an IgE response in the mouse strains used under specific conditions, and led to the conclusion that the 2S gene was a strong candidate for genetic engineering into crop plants to enhance the nutritional quality of derived products. Brazil nut 2S protein was engineered into soybean. Since it is known that Brazil nut causes anaphylactic reactions in a small number of individuals [45,46], a RAST was used in conjunction with immunoblotting to assess whether an allergenic protein from Brazil nut had been transferred to soybean. A positive RAST was observed with sera for 8 out of 9 Brazil nut sensitive individuals [47]. The results showed that the gene obtained from Brazil nut probably encoded for a major Brazil nut allergen. Since it is known that oral antigen exposure to mice most easily results in tolerance induction [48-52], the mouse was not the most appropriate animal model. Although speculative, a more appropriate animal model, like possibly the described BN rat model, might possible have predicted the allergenicity more accurately.

The presented enteral BN rat model requires further validation with respect to the sensitivity and specificity in studies on the allergenicity of food proteins for humans. Until further validation data are available, most profit is to be expected from a combination of assays and model systems. Using models for human gastro-intestinal digestion in combination with operational *in vivo* antigenicity assays to investigate the residual antigenicity of absorbed fragments, it is possible to study several aspects which are of major relevance in terms of the allergenic potency of dietary proteins. This approach provides the possibility to take into account the influence of the gastro-intestinal digestion and the combination of these procedures with the routine antigenicity assays may provide an important refinement of the currently available antigenicity assays. In addition, application of combinations of the *in vitro* gastro-intestinal model systems under both human and laboratory animal conditions, extended with *in vivo* oral animal studies, may be very helpful in the extrapolation of animal data to man. On a case-to-case basis, evaluation of the antigenicity, process and acid stability, digestibility, residual antigenicity of digested and absorbed protein (fragments), and sensitizing properties in animal feeding studies may be performed, all together finally enabling an optimal, yet probably still not perfect evaluation of the allergenic potency of dietary protein products.

the offending food is avoided is mostly recommended. However, in some cases, a full elimination diet is not feasible and a supporting drug treatment is needed. In addition to research on the allergenicity of food proteins, the described rat model may also be a useful tool to study possible applications of drugs in the treatment of food allergy.

Immunoprophylaxis and Immunotherapy

Current research in the field of atopy is directed for an important part towards treatment of established disease. A potentially more effective and achievable goal may be the prevention of initial Th2 cell sensitization to environmental allergens during infancy [reviewed in 53]. The ultimate goal would be active *in vivo* intervention in order to prevent "failure" in individuals who are genetically at high risk of developing atopic disease. Several studies have shown that CD4+ Th2 cells play an important role in the pathophysiology of allergic diseases. T cell clones from atopic donors, specific for environmental allergens, were shown to have a Th2 phenotype with high levels of IL-4 and IL-5 and little or no IFN-γ, whereas T cell clones from non-atopic donors produced IFN-γ and no or little IL-4 upon stimulation with antigen [28-30]. These data suggest that the feature distinguishing the allergen-responder status of atopic individuals from that of non-atopic individuals is the nature of the T cell subsets that dominate their respective allergen-specific T-memory pools. Theoretically, the induction of appropriate allergen-specific signals into the natural T cell selection process during the early stages of infancy, may tip the equilibrium towards selection of a Th1-like immunity and a subsequent "block" for allergic sensitization. Delivering soluble antigens through mucosal surfaces is an effective way of modulating the function of both CD4+ and CD8+ T cells [49,54-56]. The potential efficacy of this approach has been demonstrated via the successful induction of oral tolerance to intact Der p1 allergen from house dust mite by feeding with a single T cell epitope [57,58]. Preliminary results obtained at our laboratory showed that oral pre-exposure of BN rats to OVA- derived peptides modulates the immune response upon subsequent oral exposure to OVA. Intra-gastric administration of OVA derived peptides for two weeks diminished or abrogated the induction of OVA-specific IgE responses upon subsequent daily gavage dosing with OVA for 42 days in the majority of animals. Although additional research and confirmance of the preliminary results is needed, these results indicate that the enteral BN rat model may provide a useful tool in studying possible immunoprophylaxis and immunotherapy.

Concluding remark

All together, the enteral BN rat model presented in this thesis shows sufficient similarities with human observations to be considered a valuable research tool. It may

provide an important possibility for research on mechanisms and factors involved in oral sensitization and elicitation of clinical effects. In addition, it may be of value in studies on the allergenicity of food proteins and in research with respect to prophylactic and therapeutic interventions in food allergy. Continuation of the research with a concomitant generation of broad experience with the model will reveal additional insight into possible applications and limitations.

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Samenvatting

Overgevoeligheidsreacties op voedingsmiddelen komen regelmatig voor. Voedselovergevoeligheid is een verzamelnaam voor niet-toxische ongewenste reacties op voedsel. Voedselovergevoeligheid kan worden onderverdeeld in voedselallergie en voedselintolerantie, waarbij men spreekt van een voedselintolerantie als de patiënt klachten krijgt van een bepaald voedingsmiddel zonder dat het afweersysteem daarbij een rol speelt, zoals vaak het geval is bij lactose (melksuiker) of aardbei. Indien het afweersysteem een duidelijke rol speelt in de reactie op voeding spreekt men van een voedselallergie. Alhoewel veel mensen denken een voedselallergie te hebben, zal na onderzoek blijken dat er in sommige gevallen sprake is van een voedselintolerantie of een aversie, waarbij in het geval van een aversie het voedingsmiddel niet verdragen wordt ten gevolge van psychologische redenen. In een groot deel van de gevallen is een relatie tussen gerapporteerde klachten en het gebruik van bepaalde voedingsmiddelen echter niet vast te stellen en spelen andere oorzaken dan voedselovergevoeligheid waarschijnlijk een rol bij het ontstaan van de klachten. Slechts ongeveer 2% van de bevolking lijdt aan een voedselallergie, waarbij de reacties van het afweersysteem gericht zijn tegen een deel van het voedingsmiddel, meestal eiwit. De voedingsmiddelen waarvoor voedselallergie het meest frequent wordt waargenomen zijn koemelk, kippenei, pinda, soja, vis, schaal- en schelpdieren en in mindere mate ook vruchten en noten. Voedselallergie komt voornamelijk voor bij jonge kinderen en met name bij die individuen die een erfelijke (atopische) aanleg hebben om allergieën te ontwikkelen. Bij een voedselallergie reageert het afweersysteem met de aanmaak van een bepaald type antilichaam (het zogenaamde IgE) tegen voedingseiwitten. Dit antilichaam wordt in gezonde mensen niet of nauwelijks aangemaakt tegen voedingseiwitten. Het IgE wordt afgegeven aan het bloed en circuleert door het lichaam totdat het wordt gebonden aan bepaalde cellen. Wanneer het lichaam opnieuw in contact komt met hetzelfde voedingseiwit (allergeen), zal het allergeen binden aan het IgE op het oppervlak van de die cellen. Als gevolg van deze binding worden deze cellen geactiveerd, hetgeen het vrijkomen van allerlei stoffen tot gevolg heeft. Deze stoffen zorgen voor de klinische verschijnselen van een voedselallergische reactie zoals o.a. diarree, het opzwellen van de slijmvliezen van de keel en mondholte, huiduitslag, braken, astma en in het ergste geval een zogenaamde systemische anafylactische reactie, hetgeen kan resulteren in een shock of zelfs overlijden. Allergische reacties vinden doorgaans plaats binnen enkele minuten tot uren na het eten of drinken van de voedingsmiddelen die de allergenen bevatten. Ondanks het feit dat de reacties tegen de voedingseiwitten erg heftig kunnen zijn, nemen de productie van IgE en de klinische verschijnselen in veel gevallen af voor het vierde levensjaar. Sommige voedselallergieën, zoals pinda allergie, blijken echter vaak veel langer aan te houden en kunnen levenslang een gevaar opleveren. Indien de diagnose voedselallergie is gesteld is de beste remedie het vermijden van het betreffende

ne producten). Naast de bekende traditionele voedingsmiddelen die bij sommige mensen aanleiding geven tot voedselallergische reacties tegen eiwitten in deze produkten kunnen tegenwoordig met behulp van biotechnologische technieken ook nieuwe eiwitten of nieuwe biologische variëteiten (zoals bijvoorbeeld transgene soja) gemaakt worden om gebruikt te worden in voeding. Deze biotechnologisch ontwikkelde producten (novel foods genaamd) kunnen in principe, net zoals de traditionele voedingsmiddelen, voedselallergie veroorzaken. Om veiligheidsredenen is het daarom van belang dat deze nieuwe of veranderde eiwitten getest worden op hun vermogen om allergieën te veroorzaken.

Eén van de problemen met betrekking tot onderzoek naar de mechanismen die een rol spelen bij het ontstaan van klachten bij een voedselallergie en onderzoek naar het voedselallergie inducerend vermogen van voedingseiwitten is het gebrek aan geschikte gevalideerde modellen. Aangezien orale toediening van eiwitten in de meeste gevallen niet leidt tot een ontsporing van de afweerreactie wordt onderzoek meestal verricht met diermodellen waarbij de eiwitten ingespoten worden in combinatie met stoffen die de afweerreactie stimuleren. In deze modellen wordt de normale route van blootstelling, namelijk via het maag-darmkanaal, waarvan het bekend is dat dit een grote invloed heeft op het allergie inducerend vermogen van eiwitten, buiten beschouwing gelaten. Het doel van het in dit proefschrift beschreven onderzoek was om een nieuw diermodel te ontwikkelen voor onderzoek naar de mechanismen die een rol spelen bij voedselallergie en voor onderzoek naar het voedselallergie inducerend vermogen van voedingseiwitten.

In **Hoofdstuk 1** wordt een algemene inleiding gegeven over ongewenste effecten van voedingsmiddelen en in het bijzonder voedselallergie met de daarbij voorkomende immunologische responsen. Tevens worden de huidige beschikbare testen en diermodellen beschreven om onderzoek te verrichten naar de potentiële allergeniciteit van voedingseiwitten.

Hoofdstuk 2 beschrijft het door ons ontwikkelde nieuwe diermodel in de rat. Indien de voedingseiwitten via het drinkwater aan de ratten wordt gegeven ontstaan er reacties die als normaal beschouwd worden en die we ook bij gezonde mensen zien. Wanneer het voedingseiwit echter volgens een speciaal protocol met een maagsonde aan de dieren wordt toegediend dan ontwikkelen de dieren IgE reacties zoals die ook bij allergische mensen optreden.

Studies met het model kunnen verstoord worden als dieren per ongeluk de onderzochte eiwitten in hun eten hebben gehad. In **Hoofdstuk 3** is echter beschreven dat zulke verstoringen ook op kunnen treden als de moeders van de gebruikte dieren de eiwitten per ongeluk in het dieet hebben gehad.

In **Hoofdstuk 4** is onderzocht of ratten van verschillende stammen andere reacties laten zien. Dit blijkt zo te zijn en uit de resultaten valt af te leiden dat de zogenaamde Brown Norway (BN) rat, die ook voor de studies in Hoofdstuk 2 en 3 is gebruikt, inderdaad de meest geschikte stam is. Behalve dat de BN rat geschikt lijkt om

vergelijkbare reacties op eiwitten te bestuderen, is in **Hoofdstuk 5** aangetoond dat deze dieren in het onderzochte model ook op dezelfde eiwitten reacties laten zien zoals we die bij allergische patiënten zien. In het model kan dus mogelijk ook onderscheidt gemaakt worden tussen eiwitten die bij de mens problemen veroorzaken en eiwitten die bij de mens minder vaak of bijna nooit allergieën veroorzaken.

Daarnaast blijkt uit resultaten zoals beschreven in **Hoofdstuk 6** dat na het toedienen van het allergeen aan de gevoelig gemaakte ratten tot vergelijkbare reacties leidt zoals we die ook bij de mens zien: effecten op de doorlaatbaarheid van de darmwand, effecten op de bloeddruk en effecten op de ademhaling. Frappant was dat, evenals in de mens, de effecten op bloeddruk en ademhaling slechts bij een deel van de dieren wordt waargenomen.

In het laatste hoofdstuk, **Hoofdstuk 7**, worden de resultaten zoals gepresenteerd in dit proefschrift bediscussieerd tegen de achtergrond van de huidige literatuur over allergische reacties tegen voedingseiwitten en worden mogelijke toepassingen van het ontwikkelde rattenmodel aangegeven. Verder onderzoek in de toekomst moet uitwijzen of het ontwikkelde rattenmodel inderdaad in staat zal zijn om onderscheid te maken tussen sterke en zwakke voedingsallergenen.

Concluderend kan worden vermeld dat de resultaten van dit proefschrift aangeven dat het ontwikkelde model voldoende vergelijking vertoond met bevindingen in mensen en zodoende een belangrijk middel voor onderzoek levert. Het lijkt een goed model te zijn om onderzoek te verrichten naar mechanismen die ten grondslag liggen aan het ontstaan van een voedselallergie en de klinische verschijnselen en naar preventie of behandeling van voedselallergie in mensen. Verder onderzoek zal moeten uitwijzen of het model ook in staat is om onderscheidt te kunnen maken tussen eiwitten die bij mensen vaak allergieën veroorzaken en eiwitten die bij de mens zelden of nooit tot problemen zullen leiden, en zodoende gebruikt kan worden om een voorspelling te geven omtrent de eventuele allergeniciteit van voedingseiwitten, met name ook het vermogen van biotechnologisch ontwikkelde nieuwe eiwitten om voedselallergie te induceren.

Curriculum Vitae

De schrijver van dit proefschrift werd geboren op 5 juli 1968 te Sittard. In 1987 behaalde hij het VWO diploma aan het St. Thomascollege te Venlo. In hetzelfde jaar begon hij met de studie Biologie aan de Universiteit Utrecht. Deze studie werd in augustus 1993 succesvol afgerond door het behalen van het doctoraal examen met als specialisatierichtingen Immunotoxicologie (onder leiding van dr. G.F. Houben en prof. dr. W. Seinen; RITOX te Utrecht) en Immunolgie (onder leiding van dr. D. Dooijes en prof. dr. H Clevers; AZU te Utrecht). Van oktober 1993 tot oktober 1997 was hij werkzaam als assistent in opleiding (AIO) in dienst van de Universiteit Utrecht en werd hij gedetacheerd bij TNO Voeding te Zeist. Het onderzoek dat in dit proefschrift wordt beschreven werd verricht onder leiding van dr. G.F. Houben, dr. A.H. Penninks en prof. dr. W. Seinen. Vanaf 1mei 1998 is hij werkzaam als postdoctoraal onderzoeker bij de afdeling Immuno-, Inhalatie- en In vitro Toxicologie, van de divisie Toxicologie, bij TNO Voeding te Zeist.

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Zonder jullie steun was het voor mij niet mogelijk geweest.


..BWWN..
NI! NI!
CHOOO!
THIS FOOD IS ALLERGIC TO ME.