THE NUTRITIONAL MANAGEMENT OF FOOD HYPERSENSITIVITY IN DOGS AND CATS: AN ASSESSMENT OF A PROTEIN HYDROLYSATE

by

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ABSTRACT

Adverse reactions to food are exceedingly common reasons for the presentation of cats and dogs to veterinarians. Of those cases, a relatively small number involve a truly immune-mediated reaction to the food substance. However, differentiating those that are from the more common food intolerances is usually difficult and often impossible. In addition, certain individuals with a true food hypersensitivity are difficult to manage with conventional diets. The identification and availability of nutritionally complete commercially prepared diets with a protein component that is truly novel to the patient under investigation is often the stumbling block to successful diagnosis and management of food hypersensitivity. The recent development of protein hydrolysate based pet foods for dogs and cats provides an exciting tool for more reliable diagnosis and management or food hypersensitivity in those species. Chapter 1 of this thesis describes the immunological and pathophysiological basis of food hypersensitivity and presents some of the key areas of recent research that have lead to a deeper, if still incomplete understanding of the aetiological mechanisms responsible. The development and maintenance of oral tolerance is discussed including the key roles that the resident antigen presenting cells in the mucosa play. From that description follows a presentation of some of the current hypotheses regarding mechanisms by which oral tolerance is lost or not established. These include the action of mucosal adjuvants, parasitism, IgA deficiency and alterations in mucosal permeability. Building on this discussion is an examination of the methods currently available to veterinarians for the diagnosis of food hypersensitivity, their clinical usefulness and limitations.

The importance of obtaining a complete and accurate dietary history is emphasized. The difficulties in doing so and the consequences of not doing so are discussed. As stated, the recent development of diets in which the protein content has been hydrolysed provides a new tool for the veterinarian. Some of the practical aspects behind producing hydrolysate diets are presented and the theoretical basis, especially the importance of the molecular weight of remaining polypeptide fragments, is emphasized. Finally recommendations as to their use and the role that they may play in the future are discussed.

Chapter 2 describes the initial assessment of 2 candidate hydrolysates, one made from fish and the other from chicken proteins. The method used for this initial experiment was high-performance size-exclusion liquid chromatography. The investigation revealed the chicken hydrolysate to have the more favourable molecular weight profile of the two. The finding that 92.9% of the hydrolysate was of a molecular weight less than 5kDa is supportive of its potential value in a hypoallergenic diet. The molecular weight profile was then compared with a selection of those published in the human medical literature. Cow's milk hydrolysates have been widely available and used for the past 2-3 decades. The chicken hydrolysate appeared to compare very favourably to a number of extensively hydrolysed human infant formulae that have been demonstrated experimentally and clinically to be truly hypoallergenic. Despite the extensive use of molecular weight profiles to compare hydrolysates, they remain unreliable as predictors of allergenicity.

Chapter 3 describes the experimental evaluation of the *antigenicity* of the chicken hydrolysate. Following successful immunization of dogs to the intact parent protein, an IgG inhibition ELISA was developed using sera from the immunized dogs. It was demonstrated that the hydrolysate retained some ability to bind IgG but that at equal

levels of antibody binding, the concentration of the hydrolysate solution required was 66 times greater than that of the intact parent protein. It is likely that this represents a clinically highly significant reduction in antigenicity.

Of the limitations of the inhibition ELISA study, perhaps the greatest from the perspective of hydrolysate diet analysis is its inability to differentiate the molecular weight of the IgG-binding fragments. This is important since if they are less than 6-10kDa, they are unlikely to participate in IgE-mediated allergic reactions. Chapter 4 describes the experiment chosen to determine the size of the remaining IgG-binding fragments, namely Western blotting. It was established that the major antigenic fraction remaining in the parent protein following SDS-PAGE separation was a c.69kDa protein consistent with chicken serum albumin. It was demonstrated by both the Western blotting and the HP-SEC that this antigen was absent from the hydrolysate. The actual size of the few remaining binding fragments in the hydrolysate was not, however, clearly elucidated.

It was concluded that the chicken hydrolysate assessed during this thesis is a promising candidate for inclusion as the peptide component of a diet for the diagnosis and management of food hypersensitivity in dogs and cats. In addition, the diet has theoretical promise for the prevention of food hypersensitivity during periods of mucosal inflammation such as idiopathic inflammatory bowel disease and acute gastroenteritis. Ultimately, clinical trials are required to conclusively demonstrate the value of the hydrolysate in the diagnosis and management of these disorders.

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CHAPTER 1

Food Hypersensitivity in Dogs and Cats

A Literature Review

1.1 Background and Definitions

The first reported adverse allergic reactions were described by Hippocrates (c.400BC) when he wrote of respiratory embarrassment and pruritus following goats' milk ingestion in certain individuals.[1] Since then, sporadic reports of acute allergic reactions have been recorded. However, it is interesting to note that such reports have not been very frequent, particularly when considering allergic diseases (asthma, hayfever, acute intestinal reactions) are unlikely to be overlooked given their relatively obvious clinical features. Indeed, it was not until 1922 that the first report of an adverse food reaction in animals was reported.[2] It has been speculated that allergic diseases in humans were uncommon prior to the 20th century and are becoming increasingly more prevalent.[3] This may also be occurring in domestic species, perhaps for the same epidemiological and immunological reasons.

It was von Pirquet who coined the term "allergy" in 1906.[4] Originally, the term was used to describe a state of "changed reactivity" irrespective of whether that state resulted in immunity or hypersensitivity and did not indicate any particular immunological mechanism. However, the term "allergic" is now commonly used to denote a specific type I hypersensitivity reaction. In light of this, the term "hypersensitivity" will be used in this dissertation and an attempt will be made to specify, if known, which specific immunological mechanism is being referred to.

With respect to adverse reactions to foods, a distinction is made between immune-mediated reactions and those of a non-immunological basis. Any clinically abnormal response

following ingestion of a food or food additive is referred to as an adverse reaction to food. Such reactions are then divided into those reactions that involve an immunological mechanism (food hypersensitivity) and those that do not (food intolerance). The preferred terminology for all adverse reactions to food in humans was established by the American Academy of Allergy and Immunology[5] and it has been recommended that these be adopted for use in reference to disease in animals.[6]

1.2 Significance

1.2.1 Prevalence

Reports vary on the prevalence of food hypersensitivity in animals and estimates range widely. Most prevalence figures have been published by dermatologists and, not surprisingly, focus on patients with dermatological signs. If the breadth of the clinical spectrum of adverse reactions to food was better understood, the disorder might be more frequently reported. As will be discussed below, it is becoming increasingly apparent that gastrointestinal signs associated with food hypersensitivity may be as common as dermatological signs. Because owners are more likely to intuitively trial different diets for gastrointestinal complaints prior to presentation to a veterinarian, many of these cases may be unrecorded because of adequate responses to dietary interventions by the owner. Also, many prevalence figures are collected from biased populations (e.g. referral populations) and cannot be extrapolated to the wider population. However, perhaps the greatest obstacle to defining the prevalence of food hypersensitivity in veterinary patients is establishing the diagnosis. The absence of widely agreed diagnostic criteria, the poor specificities and

sensitivities of objective *in vitro* allergy testing and the difficulty in performing properly conducted food trials are significant hindrances. Table 1.1 details a selection of publications describing the prevalence of food hypersensitivity in dogs and cats along with the criteria for the diagnosis.

Table 1.1 Recorded Prevalence of Food Hypersensitivity in Dogs and Cats

Reference	Study Population	Prevalence	Diagnostic Criteria	Comments
[7] Baker 1974	82 referral cases of non-seasonal pruritus	62% of study popn	Pruritus; elimination diet and 1 challenge	Improvements of as little as 10% significant on elimination
[8] Scott et al 1990	419 dogs and 111 cats presented for skin disease	6% of all canine and 4.5% of all feline dermatoses	Not stated	
[9] Carlotti et al 1990	33 dogs and 10 cats from practice population	6% of all canine and feline dermatoses. 15% of allergic dermatoses	History of pruritus, elimination diet and challenge trial	Reduction of pruritus by 70-80% on elimination diet.
[10] O'Dair et al 1996	20 cats with signs suggestive of allergic skin disease	0% of study population	History, elimination diet and re-challenge	
[11] Guilford et al 1998	128 cats with chronic pruritus, vomiting or diarrhoea	17% of study population	Elimination diet and 2 challenges	Highly selected referral population
[12] Guilford et al 2001	70 cats with chronic gastrointestinal signs	22.8% of the study population	Vigorous diagnostic work-up to eliminate other causes of gastrointestinal disease, elimination-challenge trails	Highly selected referral population
[13] Bell 1999	500 referral cases of chronic dermatitis	1% of case load	Pruritus, Elimination diet and staple diet challenge	Owners required to keep a diary of pruritus to assess response

No sex predisposition has been identified in the collective studies. Also, since numbers are small, breed predispositions are difficult to assess although Rosser (1993) reported that Labrador Retrievers, Cocker Spaniels, Collies, Springer Spaniels and Miniature Schnauzers were over represented.[14] Significantly perhaps, these breeds are also over represented amongst atopics. There is disagreement as to the effect of age on the development of food hypersensitivity. White (1986) and Rosser (1993) respectively reported that 19% and 33% of cases with elimination-challenge proven food hypersensitivity initially developed clinical signs at less than 1 year of age.[14,15] In contrast, Guilford et al (2001) found that age was not correlated with the development of chronic gastrointestinal signs associated with food hypersensitivity.[12]

1.2.2 Owner/General Public Perceptions

Whilst the true prevalence of food hypersensitivity may be relatively low, to the general public, food "allergy" is a major concern. This is reflected by the high number of people who believe they have a food hypersensitivity in contrast to those who are subsequently shown to have one. Approximately one third of American households were found to alter their eating habits because it was believed a member of the family suffered from food hypersensitivity.[16] In a more recent survey of 7500 households in the UK, approximately 20% of people reported a food "allergy". A subset of those reporting signs agreed to a double blind placebo controlled food challenge trial. Only 19.4% had signs confirmed, which was extrapolated to an overall prevalence in the general population of 1.4-1.8%.[17]

Since no such study has been performed in veterinary patients, it remains unknown whether a similar situation exits. However, it is the author's opinion that there are many owners who perceive an adverse reaction to food in their pet, which may indeed be food hypersensitivity, may be food intolerance or may not actually exist. Either way, food "allergy" remains a major concern for the pet owning public.

Further popular credence is given to the importance of food hypersensitivity by the recommendations of various veterinary authorities that the disorder be routinely considered as a differential diagnosis for chronic pruritic dermatoses and gastrointestinal diseases.[18,19,20] However, regardless of the actual existence of food hypersensitivity in a given patient, its presence or absence can be difficult to prove in an individual patient (see later). For these reasons and irrespective of the true prevalence in animals, food hypersensitivity remains an important consideration for owner and veterinarian alike.

1.3 Gastrointestinal Immunity

In order to understand the pathophysiologic mechanisms that lead to and operate during food hypersensitivity, an overview of gastrointestinal immunity is required.

1.3.1 Scope of Antigenic Challenge

Since the physiological role of the gastrointestinal tract (GIT) is to digest and absorb nutrients it has employed strategies to maximize its surface area (folds, villi, microvilli) and enhance absorption. It has been estimated that the surface area of the GIT in the dog is

approximately 200 times that of the skin. The GIT is continually bombarded with a vast quantity and diversity of antigens throughout life (food, bacteria and viruses). If a 30kg dog lives for 14 years and consumes on average 400gm of a reasonable quality diet each day; then in its lifetime it will have exposed its gastrointestinal mucosa to 365kg of food protein.

While most of the intra-luminal antigenic material is harmless, some represents a significant hazard and requires a rapid and specific response. However, given the importance of the physiological function of the GIT, only limited amounts of damage from inflammatory responses can be tolerated before health is compromised. As such, an effective method for identification and discrimination of antigens is essential and it is not surprising that the lymphoid tissues associated with the GIT comprise the largest component of the immune system.

Physiologic and Immunological Barriers to Antigen Absorption

Acid

The low pH of the gastric environment alters the 3-dimensional conformation of most large peptides, will render many non-antigenic and acts as a bacteriocidal. In addition, the low pH environment inhibits absorption of intact protein, whereas decreased stomach acidity has been shown to increase absorption.[21]

Enzymes

Digestive enzymes represent the most potent mechanism for destroying antigens intraluminally. Gastric pepsins, pancreatic proteases, enterocyte brush-border proteases and

enterocyte lysozyme activity all contribute to rapidly reducing the antigenic load of the soluble intraluminal antigens.

Mucus

Overlying the epithelium is the glycocalyx composed of complex glycoproteins and mucins. This mucus coat is an important barrier to potentially invasive pathogens, with organisms becoming trapped and passed out in the faeces.

Peristalsis

In the fasted state, migrating myoelectric complexes limit bacterial proliferation by "flushing" trapped particles out in the stool.

Bacterial Competition

The normal bacterial flora exhibit significant microbial antagonism. Competition for essential nutrients suppresses the growth of many potentially pathogenic bacteria and fungi. In addition, gut commensals may also produce a class of bactericidins termed colicins, which bind to susceptible bacteria producing a membrane channel destroying the cell.[22]

Secretory IgA

Secretory IgA represents the first line of specific defence at mucosal surfaces. Whilst the antigen specificity and binding capacity of IgA are the same as other antibody classes, IgA cannot activate complement through the classical pathway. Instead, it seems to have evolved as a method of specific antigen exclusion, binding to luminal antigen and preventing its absorption.[23]

The local production of secretory IgA is the result of specific antigen presentation and activation, proliferation and IgA commitment of B cells in the Peyer's patches. After activation and differentiation, these B cells migrate selectively to other mucosal sites where they undergo terminal differentiation into IgA producing plasma cells. The induction of IgA production is dependent on T helper cells although other cells can play a role. The role of cytokines in IgA production has been reviewed.[24] In both humans and mice, Th2 cytokines promote IgA expression by surface IgA+ B cells derived from Peyer's patches and encourage proliferation of IgA committed B cells in the absence of direct T cell contact. Transforming growth factor β (TGF- β) and interleukin 10 (IL-10) with influence from IL-4 and IL-5 have been shown to promote the isotype switch from IgM production to IgA. IL-6 appears to be more important for the maintenance of IgA production.

1.3.2 Anatomical and Functional Organisation

Epithelium

A single layer of epithelial cells lines the entire intestinal mucosal surface. These cells provide a very thin barrier for systemic protection against luminal contents in addition to their role in digestion and absorption of nutrients. In addition as will be discussed later, they have an important function in bi-directional signaling between themselves and traditional immune cells below them.

Lamina Propria

Immediately beneath the epithelium is the lamina propria, which is the loose connective tissue layer, rich in blood vessels and lymphatics that supports and nourishes the epithelium. Diffusely scattered within the lamina propria of the normal animal are various leukocytes.

Peyer's Patches

Peyer's patches (pp) are the lymphoid follicular aggregates discretely organized in the small intestine with their apical surface devoid of intestinal microvilli.

Mesenteric Lymph Nodes

Draining the PP via lymphatics are the mesenteric lymph nodes (MLN), which are identical in overall structure to other peripheral lymph nodes.

Cells

Enterocytes

The epithelial layer is composed of a one-cell layer of columnar epithelium. These epithelial cells are derived from the basal crypts and differentiate into villus, surface epithelium, goblet and neuroendocrine cells. The tight junctions between adjacent enterocytes are so tight that they prevent even small peptide fragments from passing through.[25] As such in the normal animal, antigen transport through tight junctions is quite rare, with only small ions and water diffusing through. Under certain disease conditions the tight junctions may be broken and permeability increased.[26,27]

Invasive bacteria and certain viruses can bind to the epithelial cell membrane and be internalised.[27] Such organisms tend to be associated with intestinal inflammation and

disease. However, the intestinal epithelial cells (IECs) of the healthy animal are generally proficient at excluding pathogens. IECs have a well-documented role in nutrient and solute uptake and whilst most protein is absorbed as very small peptide fragments,[28] some undigested macromolecules in the gut are able to cross the intestinal mucosa intact.

Recently the expression of MHC class II molecules has been identified on the luminal surface of canine enterocytes and has highest expression in the ileum.[29] Since the ileum has significantly higher bacterial counts than other areas of the small intestine, it is likely that this accounts for increased expression. This finding, together with the knowledge of the uptake of intact proteins by IECs and the intimate association between IELs, LPLs and IECs raises the possibility of IECs acting as antigen presenting cells. It is also possible that binding of peptides to apical MHC molecules may play a role in the pathogenesis of food sensitive enteropathies (see below).

Human IECs constitutively express the adhesion molecule E-cadherin, the ligand for which is located on activated IELs ($\alpha_E\beta7$ or CD103). This enables the selection of specific lymphocytes for residence there under normal circumstances. Other adhesion molecules, such as ICAM-1, can be up-regulated by IFN-γ and IL-1 and mediate adhesion of a broader range of lymphocytes through ICAM-1/LFA-1 interactions.[30] This results in the accumulation of a more heterogenous population of IELs during periods of inflammation and is in part involved in sensitization to innocuous antigens under such circumstances (see "General mechanisms for loss of tolerance" below).

IECs have also been shown to be an important source of cytokines including TGF-β, IL-6, IL-7, IL-10 and TNF-α.[31] Of all these cytokines, only TGF-β and IL-6 have an important effect on antibody secretion whereby they greatly enhance IgA secretion. Therefore IECs and their derived cytokines seem to play a significant role (along with T cell derived IL-2) in creating an environment appropriate for IgA responses.

M Cells

The follicle-associated epithelium (FAE) differs functionally from the vast majority of intestinal epithelium. Whereas small and large intestinal enterocytes are principally involved in the absorption of digested nutrients and resorption of fluids, the FAE is designed for the uptake of macromolecules, particles and even microorganisms. This occurs via transcellular transport by specialized cells lacking the microvilli characteristic of normal enterocytes. Instead these cells possess microfolds on their luminal surface and have hence been called microfold-cells or M-cells.[32] While enterocyte brush borders express hydrolytic enzymes, M-cell apical surface enzyme activity is reduced or absent.[33]

M-cells provide functional openings in the epithelial barrier through vesicular transport activity. The relative absence of goblet cells in the FAE ensures that there is minimal brush border glycocalyx, thus facilitating macromolecule and microorganism contact. Selection of luminal antigen for sampling and presentation by M-cells occurs both specifically and non-specifically. Hydrophobic particles and cationic molecules adhere much more avidly to M-cells than to enterocytes and such adherence is a means by which non-specific antigen ingestion can occur.[34] Several gram-negative bacteria and some viruses bind either preferentially or exclusively to M-cells.[33] Whilst secretory IgA does not adhere to the luminal

surface of IECs, binding has been demonstrated to the luminal surface of M-cells.[35] Weltzin et al (1989) have described transportation of intact IgA-antigen complexes across the M-cell.[35] In this manner, membrane bound IgA can promote specific, enhanced uptake of antigen by M-cells.

The M-cell basolateral surface is characteristically deeply invaginated, forming an intra-epithelial pocket.[36] This feature shortens the distance across which transcytotic vesicles must pass to the basolateral surface. This invagination also provides a space into which leukocytes can sit, ensuring a large membrane to membrane surface apposition and increasing the efficiency by which particles and soluble antigens can be handed onto professional antigen presenting cells (APCs).

In contrast with enterocytes, M-cells from most species lack the abundant cytoplasmic hydrolytic enzymes. As a result, proteins transported across the M-cell cytoplasm reach the basolateral surface mostly undigested. It has been estimated that 90% of all protein absorbed by M-cells reaches APCs intact.[33]

M-cells are not believed to constitutively express MHC antigens and they also lack cytoplasmic lysosomes. The lack of these features emphasizes the proposed role of M cells as conduits for antigen transfer to APCs and not as antigen processing or presenting cells themselves. However, whether these features are the same for all species remains to be determined. In rats at least, some expression of MHC class II antigens has been identified.[37] Clearly, whilst M-cells probably do not represent a large component of the antigen presentation function of the GALT, their exact role as APCs has yet to be determined.

Dendritic Cells

Dentritic cells (DC) are a heterogenous population of MHC class II expressing, bone marrow-derived cells that can be found located throughout peripheral as well as central lymphoid tissues. The major role of these cells is to capture antigen in the periphery and transport it to secondary lymphoid tissues for presentation to lymphoid cells. DCs may also function somewhat as immunoregulatory cells.

The exact lineage of DCs has yet to be defined and it seems that there is not a single DC lineage. The major source may be peripheral blood mononuclear cells, which when stimulated with IL-4 and granulocyte/macrophage colony stimulating factor (GMCSF) can develop into DCs without further cell division.[38] Recently, the *in vitro* behaviour of DCs has been described.[39] In the immature state they are highly endocytic and engulf large amounts of fluid into cytoplasmic vacuoles enabling them to accumulate antigens at very low concentration. In this state, most of the MHC class II is present in these vacuoles. After maturation, most of the MHC class II is expressed on the cell membrane surface and has a half-life of days. After stimulation with lipopolysaccharide (LPS) or TNFα, DCs cease to process antigen, discontinue MHC class II synthesis and increase expression of the cell surface B7 antigens, thus becoming powerful stimulators for antigen-specific T lymphocytes (see "Oral Tolerance" later).

In many tissues, their distribution appears to be random, although local inflammatory stimuli in mucosal tissues will increase the local density of DCs. In contrast in the skin, DCs' distribution appears to be much more organized. In secondary lymphoid tissues, DCs are noticeably concentrated in the thymus-dependent zones.

In the intestine of rats, DCs have been described within the epithelium, the lamina propria and in Peyer's patches.[40] In contrast, within the canine intestine they are mostly seen in the lamina propria, especially at the upper villous regions where they are often associated with CD4+ T cells that are probably recognizing antigen presented to them.[29,41] Within Peyer's patches (PP) they are found both in the T-cell areas of Peyer's Patches and in the sub-epithelial area underlying the dome where they receive antigens delivered by M-cells and transport them to T-cell areas.[42]

The main functions of DCs are as immunological sentinels and APCs. They are ideally placed to detect invading microbes and are continually exposed to intact antigen from the gut lumen. DCs remain dormant until cytokine signals received from activated surrounding cells or bacterial signals induce activation. This results in changes in membrane receptor expression and migratory behaviour. DCs will continue to phagocytose and present antigen locally if not activated but importantly, antigen presentation by mucosally derived DCs will not result in lymphocyte activation if they themselves are not activated.[43]

Another essential part of DC function is their transport of captured peripheral antigen to lymph nodes. DCs are continually migrating from peripheral tissues back to lymph nodes. This rate of migration may be related to their rate of antigen encounter since intestinal DCs turn over at a much more rapid rate than the dermal DCs, Langerhan's cells.[44] In the normal rat intestine, DCs spend a minimum of 48 hours traversing the intestine.[45] In the intestine, LPS induces a rapid increase in the numbers of DCs migrating in lymph.[42]

Whereas it is currently thought that immunoglobulin isotype switching by B lymphocytes from IgM to IgA in PP is mainly under T-lymphocyte control, there is evidence that DCs may have a direct influence on isotype switching.[46] Whilst the mechanism by which this occurs is yet to be described, it is likely that this is through the influence of cytokines secreted by activated T-lymphocytes. It has been shown that mucosal DCs can induce a Th2 pattern of cytokine secretion by T-lymphocytes, whereas splenic derived DCs induce a Th1 type profile.[47]

Lymphocytes

In addition to the organised tissues of the PP and mesenteric lymph nodes (MLN), the villus/crypt units of the intestine contain large numbers of scattered lymphocytes both in the epithelium itself and in the deeper layer of the lamina propria (LP). It has been estimated that the T cells found in the small intestinal epithelium account for as much as 60% of all the T cells in the body.[48] Thus, mucosal lymphocytes are numerically the most significant part of the immune system. Also, intraepithelial lymphocytes (IEL) are located between the epithelial cells adjacent to the basement membrane and therefore are the only lymphocyte population situated so close to antigen in the entire body.

T Cells

T-lymphocytes of both the αβCD4+ and αβCD8+ subsets can be found in the intestine and these particular subsets are distributed non-randomly. A third subset of T cells which bear the γδTCR are prominent. In addition, the IEL compartment contains a heterogenous population including unusual subpopulations of lymphocytes.

Lamina propria T cells in both dogs and cats have a CD4:CD8 ratio similar to that in peripheral organs (1.5 in dogs and 1.0 in cats)[29,49] but they differ in that they make very poor proliferative responses to antigen despite being continually exposed to it.[50] In the normal canine small intestine there is an increased T cell density towards the villus tip, probably reflecting the increased level of exposure to luminal antigens there.[29]

A large proportion of the CD4+ T cells actively secrete IL-4 and IL-10 and very little IL2 in the absence of inflammation.[51] This is taken as evidence that LP CD4+ T cells are of a Th2 phenotye and is consistent with the large amount of IgA produced in the intestine. As will be discussed later, this may be a simplistic view since enhancement of IgA production by IL2 has been demonstrated and the situation seems to change and even reverse in the presence of inflammatory stimuli.[31]

Approximately 10-15% of the cells in the normal epithelium are lymphocytes, of which, in dogs at least, >85% are CD8+ with few IEL expressing CD4+.[41,29] In addition to T-lymphocytes bearing the conventional αβ T-cell receptor (TCR), γδ-TCR cells are also present in significant numbers. Approximately 50-55% of canine IEL are CD4-CD8α-.[41] The precise identification of γδTCR cells in the feline IEL population has yet to be performed. However, Roccabianca et al (2000) recently reported that 40% of feline IELs lacked expression of CD4 and CD8 (double negative or DN cells).[49] Since γδTCR cells are not expected to express CD4 and may not express CD8, it is likely this population of double-negative cells identified in both cats and dogs are indeed γδTCR cells as has been shown to be the case in rats, mice and humans.

γδ-TCR cells comprise the majority of IEL in young or gnotobiotic animals, whereas exposure to antigen then leads to the preferential recruitment of αβ-TCR cells and a reduction in the number of γδ-TCR cells.[52,53] The relative proportions of each T-lymphocyte subset have been best described for murine intestinal lymphocytes (Table 1.2).

Table 1.2. Subsets of IEL in Mice Defined on the Basis of CD8 and TCR expression

Subset	TCR	Proportion
CD4+8-	αβ	<5%
CD4+8+αα	αβ	5-15%
CD4-8+αβ	αβ	10-40%
CD4-8+αα	αβ	20-40%
CD4-8+αα	γδ	20-60%

The relative proportions of each subset depends on the age and antigen exposure of the mouse.
(Adapted from D Guy-Grand, P Vassalli, Gut Intraepithelial Lymphocytes, Curr. Opin. Immunol.1993)[48]

Manipulations of γδ-T cells *in vivo* have caused only small changes in host resistance, usually noticeable only in immune-compromised animals or those under a very high pathogenic load. However, in some cases, experimental γδ-T cell deletion has led to dramatic changes in the type of immune response.[54,55] Taken together, these findings suggest that γδ-T cells are important immune-regulatory cells and play an important role in oral tolerance.

B Cells

As with T cells, the intestine is the largest pool for B cells particularly the LP, where at least 80% of all Ig-producing B cell lines are found and where 75-90% of these cells are secreting IgA.[56] B cells can be found within the basal pocket of M-cells where they are thought to initially encounter antigen. It is thought that the cells most likely to mediate MHC class II interactions with T cells in the M-cell pockets are the predominating (c.66%) memory B cells.[57] However, relative to T cells, B cells represent a minor component numerically, about 1% in the LP of cats as detected by flow cytometry.[49] The reason for this difference is not known.

It is the germinal centre within isolated lymphoid follicles or Peyer's patches which is of vital importance for the T cell dependent generation of memory B cells. Activation of T cells by dentritic cells in the extra-follicular areas is followed by T/B cell interactions at the edges of the follicles.[56] This generates so called germinal centre "founder cells" which produce IgM (and some IgG) that binds circulating antigen forming antigen-antibody complexes. It is these complexes which become deposited on the surface membranes of follicular dendritic cells where antigen is retained for prolonged periods to maintain memory B cell populations.[58] Most of these memory B cells migrate rapidly to extrafollicular compartments such as the Peyer's patch M-cell areas, where they can continue to present antigen.[56]

In addition, these germinal cell reactions also induce terminal development of a substantial number of intrafollicular Ig-producing plasmablasts and plasma cells, predominantly of the IgA isotype.

Mast Cells

In the canine intestine, mast cells are primarily located in the LP but can also be found in the submucosa, muscularis mucosa and serosa.[41] It is likely that intestinal mast cells in dogs and cats include classical "connective tissue" or typical mast cells (CTMC) and "mucosal" or atypical mast cells (MMC). These heterogenous subsets differ not just morphologically but also immunologically. In the rat, MMC proliferate during parasitic infections and are decreased in number with suppression of T cell responses, whereas the numbers of CTMC are unaffected.[59] These observations suggest that the MMC is associated functionally with the immune system, whereas the CTMC is independent.

In the last decade, histochemical sub-typing of human mast cells has been replaced by immunocytochemical sub-typing. This divides populations into those that contain both tryptase and chymase (MC_{TC}) and those that contain only tryptase (MC_T). In humans at least, MC_T are preferentially located at mucosal surfaces and are increased in number in allergic and parasitic diseases, suggesting that, like rodent MMC, they are acting as an arm of the immune system. MC_{TC} are found predominantly in submucosal and connective tissues and are not increased in numbers in areas of heavy lymphocyte infiltration. Whilst the biological role of these cells is unclear at present, it has been suggested that they may be associated with angiogenesis, fibrosis and may play a role in tissue repair.[59] Mast cells in different locations may respond differently to activating agents and modulatory drugs, even though their histochemical sub-type may be similar. An example of functional heterogenicity is in human mast cells. Mast cells isolated from the colon are much more sensitive to the inhibitory effects of sodium cromoglycate than lung mast cells.[59] Whether in

canine and feline mast cells such a difference in functionality exists and whether this nomenclature can be ascribed warrants further investigation.

Regardless of sub-type, all mast cells bear the high-affinity IgE membrane receptor FcεR1. Cross linkage of two or more IgE molecules to bring their receptors into juxtaposition initiates a cascade of biochemical events that results in degranulation. Histamine, proteases and heparin are released and synthesis of prostaglandin D_2 and leukotriene C_4 from their membrane associated arachidonic acid is stimulated.[60] IgE independent mechanisms can also cause degranulation and activation. Substance P interacts with mast cells directly to induce mediator release.[59]

It is now clear that mast cells are also a source of a number of important cytokines. In response to FcεR1 activation, mast cells have the potential to synthesize and secrete IL1, IL2, IL3, IL4, IL5, IL6, IFNγ and TNFα amongst others.[59] Of particular importance in regards to type I hypersensitivity, is the release of preformed IL4 on activation because of its role in B cell activation, up-regulation of MHC class II and isotype switching to IgE production.[59,61] The multifactorial role of mast cells in mucosal allergic disease is presented in Table 1.3.

Table 1.3. Actions of Mast Cells in Allergic Disease

Action	Mediators
IgE Production	IL4
Leukocyte Production	IL5, TNFα
Leukocyte Migration	IL5, IL6, PAF
Leukocyte Activation	IL5, IL6, PAF
Mucus Secretion	Histamine, LTC_4, PGD_2, Kinins
Vasodilation and Oedema	Histamine, LTC_4, PGD_2, Kinins
Smooth Muscle Constriction	Histamine, LTC_4, PGD_2, Kinins
Nerve Stimulation	Histamine, LTC_4, PGD_2, Kinins

Eosinophils

Eosinophils have been described in the lamina propria of normal cats and dogs and they generally appear in low numbers sporadically throughout the lamina propria.[41,49] Whilst traditionally associated with parasitic infections, some doubt has been cast on their role in the defence against helminth parasites since abrogation of the eosinophil response in mice has been shown not to appreciably affect the course of helminth infection.[62] The demonstration that eosinophils themselves can express the low- and high-affinity IgE receptors as well as receptors for IgA, indicates they may have a significant role in mucosal tissues following sensitization.[63] Eosinophils represent not only the targets but also the source of numerous mediators and cytokines including major basic protein, phospholipase, IL-2, IL-3, IL-4, IL-5, TNF-α, leukotrienes and prostaglandins.[62] However, despite their formidable armory of pre-formed and post-activation synthesized mediators, their role in the pathogenesis of food hypersensitivity remains largely unknown.

1.3.3 Antigen Presentation

There are three potential avenues for antigen entry into the lamina propria: 1) M cells, 2) through tight junctions between epithelial cells (paracellular) and 3) through IECs (transcellular). Paracellular transport is relatively unimportant under normal conditions. However, under disease conditions when the local cytokine milieu is altered, paracellular transit may increase.[26] This would inevitably result in increased presentation by resident LP APCs such as dentritic cells or might result in increased systemic delivery of antigen. It seems then that the nature, position and degree of activation of the APC involved in the handling of any given antigen is the primary determinant in whether the end result is tolerance or sensitivity.

M Cells

As discussed above, M-cells do not seem to function as antigen presenting cells but rather they seem to be efficient conduits for soluble molecules, macromolecules, particles and microorganisms. Following endocytotic uptake, transcytosis, transport and exocytotic release of luminal substances into the basal intercellular space, antigen is subsequently taken up again by leukocytes present in the deep basal invaginations. Cells identified in this location are lymphocytes, macrophages and dentritic cells. In mice and humans, equal numbers of T and B lymphocytes are present in the M cell pockets with a higher ratio of CD4+ to CD8+ lymphocytes than in other areas of the epithelium.[36,57]

Dendritic Cells

In the normal intestine, DCs acquire soluble protein antigens either as the result of trans-epithelial cell or M cell passage. Within a few hours they can migrate into the peripheral lymph carrying antigen in a form that can be presented to T cells in an MHC II restricted

CD4+ dependent manner. Rodent T cells are far more responsive to antigens presented by activated DC than by other APC.[64] However, DCs are able to do more than just efficiently present antigen to T cells. They are key modulators of the immune response by preferentially inducing Th1 or Th2 polarization of CD4+ T cells depending on their state of activation, exact lineage, maturation stage and importantly the local cytokine environment.[65] As previously mentioned, in contrast to splenic DCs, mucosal DCs preferentially induce a Th2 pattern of cytokine secretion by T-lymphocytes.[47]

Whilst DCs may be the only APC capable of activating naïve T cells *in vivo*, it has recently been suggested that they may also be involved in processing and delivery of antigen to B cells.[66] Since the activation of naïve B cells requires recognition of three-dimensional epitopes (i.e. native antigen) by its surface IgM, DCs would need to retain acquired antigen intact long enough to interact with a B cell. MacPherson et al (1999) have clearly demonstrated that LP derived DCs do indeed interact directly with B cells by forming clusters with several B cells.[66] These authors suggest that the DCs retain intact antigen intracellularly in stark contrast with the degradation of protein seen in macrophages. In addition, they demonstrated that when DCs were pulsed with antigen and added to B cells *in vitro*, both IgM and IgG were secreted. However, if the DCs were omitted and antigen was incubated with the B cells alone, only IgM was produced. It seems that DCs have important roles in the activation of B cells in that they can acquire and store native antigen, interact with B cells, release antigen to be recognized by specific B cells and can deliver signals to initiate antibody class switching. Thus, DCs may act as a common calling point or matrix upon which antigen specific T1 and B cells can interact.

B Lymphocytes

As previously stated, the M cell pockets contain equal numbers of B and T cells in humans and mice although this has not yet been well described for dogs or cats. B cells in this location probably represent local extensions of B-cell follicles towards the gut lumen to facilitate antigen presentation and promotion of mucosal immune responses. These cells have been shown in humans to be predominantly of the B-memory phenotype.[67] Memory B cells but not naive B cells have the capacity to present antigen directly to T cells, owing to the constitutive expression of the accessory molecules B7-1/CD80 and B7-2/CD86 (see "Oral Tolerance" below).[67] Signals through antigen receptors and CD40 antigen result in these two molecules being further up-regulated more rapidly and strongly on memory B cells than on naive B cells.

Internalization of antigen by B cells via the surface immunoglobulin is reported to be 10,000 times more efficient than the non-specific uptake performed by other APCs.[23] The unique anatomical localization of memory B cells beneath the surface of mucosa, their strong APC capacity together with their dominance over other APCs in M-cell pockets, may explain the prompt and robust mucosal antibody responses following secondary antigen exposure.

The germinal center reaction discussed above is important for T-cell dependent generation of memory B cells and antibody isotype switching. However, the exact cytokine profiles and other influences that determine isotype differentiation in B cells are not well described. TGF-β has been shown to be a crucial IgA switch factor whereas IL-2, IL-5 and IL-10 are important cytokines for clonal expansion of activated B cells supporting the idea that Th2 cells are required for mucosal immunity in the normal gut.[58]

Enterocytes

Since Prausnitz's and Küstner's first demonstration of transferable hypersensitivity in 1921 it has been known that some antigen reaches the systemic circulation intact.[1] However, it has not been until recently that IECs have been suggested as the main conduit for this antigen. Heyman et al (1990) used an *in vitro* cell line to demonstrate that around 10% of orally administered horseradish peroxidase is transported across colonic IECs intact.[68]

Following pinocytosis by enterocytes, soluble antigens are taken up into an endolysosomal pathway that includes MHC class II compartments.[69] All the conventional processing enzymes required for antigen processing and presentation are found in these compartments and it has been suggested that given their qualities and numbers, IECs are the most significant antigen presenting cells in the intestinal immune system.[30,69]

In man, increased expression of HLA-DR and HLA-DP is seen in inflammatory bowel diseases[70] and the rate and efficiency of antigen uptake and endocytic processing by IECs can be modulated by a number of inflammatory mediators.[71] However, whether IECs in dogs or cats can stimulate naive CD4+ T cells remains speculative. The finding that IECs do not normally express the co-stimulatory molecules B7-1 or B7-2 suggests that presentation to CD4+ T cells is more likely to result in anergy or deletion rather than activation.[71]

The additional finding that IECs preferentially present antigen to CD8+ IELs further supports the idea that IECs play a significant role in the induction of tolerance.[69] However, the observation that blocking MHC class I molecules does not inhibit IEC-induced CD8+

proliferation *in vitro* suggests that there are novel interactions between the two cell populations.[72] The exact nature of this interaction has yet to be elucidated in cats and dogs.

There are also indications that the class II molecule on IECs may be structurally different from that expressed by classical professional APCs, at least in mice.[71] In addition, it has been proposed that although epithelial class II molecules can function as a restricting element in antigen presentation, their main role may be in the selection of absorbed antigen and protection of the antigen from lysosomal degradation during transcytosis.[73] This is of particular interest when the administration of small peptides derived from protein hydrolysates is considered.

The IEC is anatomically and immunologically well positioned to participate in the regulation of mucosal T-cell responses. The presence of tight junctions between cells, the presence of novel antigen presentation mechanisms, the juxtaposition with CD8+ and γδ-TCR bearing T cells and the absence of normal co-stimulatory molecule expression all suggest that the outcome of antigen presentation by these cells differs from classical APCs elsewhere in the body. This outcome of antigen presentation is likely to depend on the degree of inflammation and activation with either induction of tolerance or sensitization being possible.

Liver

The majority of blood draining from the intestine perfuses the liver. Following feeding, significant amounts of intact antigen enter the portal circulation. Considering the liver is the largest source of reticuloendothelial cells in the body, the potential for hepatic antigen

processing and presentation is great. Indeed, by-passing the GALT by infusion of antigen directly into the portal venous circulation, still induces a state of systemic hyporesponsiveness with down-regulation of delayed hypersensitivity reactions and IFN-γ in association with increased production of the Th2 cytokines IL-4 and IL-10.[74] This may have relevance to patients affected with porto-caval shunts where decreased toleragenic antigen presentation coupled with increased systemic antigenic exposure may complicate their disease.

Holistic View of Enteric Antigen Presentation

In summary, a holistic view of antigen processing and presentation by the gut yields three main different pathways:

1. Intestinal Epithelial Cells

   Antigen entering by this route is likely to result in the generation of antigen specific or antigen non-specific suppressor T cells (see "Oral Tolerance" below). Alternatively, presentation to LP CD4+ T cells probably results in deletion or anergy of those antigen-specific T cells.

2. Lamina Propria Dendritic Cells

   Intact antigen that reaches the LP, presumably unaltered from IEC transcytosis, is likely to be picked up by resident DCs. This will result in the generation of T cell anergy or deletion due to the lack of co-stimulatory molecule expression (see "Oral Tolerance" below).

3. M cells

   Antigen absorbed through M-cells will be preferentially picked up by activated memory B cells or activated DCs and will result in the generation of antigen specific IgA via the germinal center reaction detailed above.

Teleologically, this image of antigen sampling and presentation seems ideal. The preferential transport of particulate antigens or intact organisms by M cells and resultant IgA response is in accordance with the need to exclude these antigens by a non-phlogistic process. Furthermore, tolerance to soluble antigen is preferable in the non-diseased state and this is provided by IEL and DC presentation. However, in the presence of inflammatory cytokines, presentation routes, permeability and co-stimulatory molecule expression by APCs will change, resulting in the appropriate generation of an antigen specific, antibody or cell-mediated immune response.

1.3.4 Lymphocyte Recirculation

It has been known for many years that T and B cells that have been activated in mucosal tissues such as Peyer's patches and mesenteric lymph nodes, will preferentially circulate back to mucosal sites, whereas lymphocytes activated in peripheral tissues tend not to enter the mucosae.[75] Following oral antigen challenge, large numbers of lymphocytes emigrate from Peyer's patches and the lamina propria via the efferent lymphatics and reach the systemic circulation after expansion and maturation within the mesenteric lymph nodes.[76] Recently the molecular basis of lymphocyte recirculation into GALT has been defined.[50] After activation in the GALT, lymphocytes decrease expression of the adhesion molecule L-

selectin; the ligand for which lies on high endothelial venules in peripheral lymph nodes. This down-regulation is accompanied by up-regulation of the mucosal adhesion integrin $\alpha_4\beta_7$ whose ligand, the mucosal addressin cell adhesion molecule (MAdCAM-1), is expressed by blood vessels in mucosal tissues. Expression of MAdCAM-1 by mucosal vessels is up-regulated during intestinal inflammation. The importance of the $\alpha_4\beta_7$ - MAdCAM-1 interaction is illustrated by β_7-knockout mice which have no lymphocytes in their Peyer's patches , lamina propria or epithelium.[77] These findings have led to the image of compartmentalization of the mucosal immune system and explain why systemically delivered antigen is a poor stimulus for mucosal immunity.

1.3.5 Oral Tolerance

As has been stated, the gastrointestinal arm of the immune system is constantly exposed to vast quantities of intact antigens. Given that the primary function of the gastrointestinal tract is digestion, it is vital for the organism as a whole that mechanisms exist to prevent the unnecessary inflammatory response to harmless food antigens and non-pathogenic microorganisms. The mechanism by which these antigens are recognized without the generation of a potentially harmful inflammatory response is termed "oral-tolerance". Oral tolerance is now well recognized as a form of peripheral tolerance in which mature lymphocytes are rendered non-functional or deleted by prior oral administration of antigen. Peripheral immunological tolerance is a fundamental property of the immune system in that it provides a method for discrimination between self and non-self. Thus, the immune system can recognize and protect against external pathogens and remain disinterested in self-antigens avoiding autoimmune disease.

Besredka (1909) is often credited with the first experimental description of oral tolerance by reporting that guinea pigs fed milk became refractory to an anaphylactic reaction induced by intracerebral injection of milk.[78] American author H.G.Wells is credited with the first series of oral tolerance experiments demonstrating that anaphylaxis to hen's egg could be prevented by prior feeding.[79] Unfortunately the phenomenon received little attention until recently when interest has been fueled by investigations into the pathogenesis of food hypersensitivity, the desire for oral vaccination vehicles and the potential for its manipulation in autoimmune diseases.

As has been mentioned, three major immunological consequences of oral antigen administration occur in the normal intestinal tract:

1. Antigen specific local IgA production and secretion
2. A state of hyporesponsiveness via T lymphocyte anergy or deletion
3. Cellular regulation via active suppression.

Tolerance to an orally administered antigen is a complex process that requires suppression of some responses and induction of others. A noteworthy aspect of all experimental models of tolerance induction is that dietary feeding of a pure antigen has not been observed to eliminate active immune responses against that antigen following vaccination. As such, the term "tolerance" seems, at least to this author, an inadequate term to encompass all aspects of the process. For most intents and purposes, the term "oral tolerance" is used more narrowly to specifically denote mechanisms other than IgA production and antigen exclusion.

Williamson et al (1999) have clearly demonstrated that although the end result of feeding soluble protein antigens is profound systemic unresponsiveness, the induction of oral tolerance is an active process.[80] In their model of single-dose feeding of ovalbumin (OVA) to mice, the initial events following absorption of antigen across the gut were activation and expansion of antigen specific T cells in the Peyer's patches and mesenteric lymph nodes. This process was greatly enhanced by increasing dendritic cell numbers. The initial clonal expansion of lymphocytes was not seen in the peripheral lymphoid organs during the first 3 days. In other models, both gut and systemic expansion of antigen specific cells has been seen. Notably, Gutgemann et al (1998) utilized a multiple low-dose feeding regimen, which as will be discussed, induces tolerance via the expansion of regulatory T cells.[81] The differences between these two studies probably emphasize that the process of tolerance induction is highly dependent on feeding protocols. Either way, oral tolerance is very much an active and complex process.

The mechanisms that make up oral tolerance are T lymphocyte deletion, T lymphocyte anergy and the generation of active suppressor cells. Which mechanism dominates within any given individual for any given antigen is determined by the nature of the antigen, antigen dose, the individuals genotype and age, nutritional status, oral flora and the presence or absence of adjuvants.[82,83]

Active Suppression

Active suppression of T and B cells responses is centered around the secretion of cytokines that inhibit certain proliferative responses. Different cell types respond differently to

specific cytokines such that cytokines normally associated with Th2 responses (IL-4, IL-10) inhibit Th1 responses.[84] Conversely, IL-12 is a potent inhibitor of Th2 responses whilst promoting Th1 responses.[85]

There has been particular interest in the potent immunosuppressive effects of the cytokine TGF-β in addition to its role in directing the mucosal antibody responses towards IgA. There is convincing evidence, through the use of anti- TGF-β monoclonal antibodies that neutralization of endogenous TGF-β is effective in preventing the development of tolerance.[86] It is now established that the TGF-β-producing cells generated in the intestinal mucosa following low-dose antigen feeding migrate to systemic lymphoid tissues and target organs such as the CNS in the experimental autoimmune encephalitis model in mice.[87]

The TGF-β secreting cells generated by low-dose antigen feeding have been shown to be predominantly CD8+ antigen-specific T lymphocytes.[88] These cells are generated rapidly and can be found in Peyer's patches 24-48 hours after feeding one dose of antigen. In addition, CD4+ have a role in the oral tolerance generated by low dose feeding in mice. Chen et al (1994) demonstrated that a low dose of antigen fed to mice suppressed Th1 but not Th2 cytokine responses.[89] In that model, secretion of the Th2 cytokines IL-4, IL-10 in addition to TGF-β was significantly increased. These CD4+ cells that preferentially produce TGF-β have been suggested to be a unique subset of T-helper cells and have been termed Th3 cells.[89]

CD8+ T cells bearing the γδ TCR have been suggested to be involved in oral tolerance mechanisms. Tolerance and suppression of IgE production has been transferred in mice by

transplanting very small numbers of TGF-β secreting splenic γδ T cells and has been prevented through the administration of anti-γδ antibodies.[90] Of potential importance to understanding the pathogenesis of hypersensitivity reactions is the finding that γδ CD8+ T cells are required for suppressing IgE antibody responses to OVA following repeated aerosolisation in mice and rats.[91] That CD8+ T cells could be involved in antigen recognition of exogenous antigens suggesting that some APCs in the gut are capable of processing exogenous antigen and presenting it via either MHC class I proteins or via other mechanisms. Certainly, macrophages have been demonstrated to be the exception to the rule that somatic cells do not present exogenous antigen via the MHC class I pathway.[92] However, given their relative scarcity in the lamina propria, it is unlikely that they represent the most quantitatively significant antigen-presenting cell for γδ T cells. In addition, most γδ T cells do not recognize peptides in association with MHC molecules. Even when a classical MHC antigen is recognized by γδ T cells, conventional class I and II processing pathways are not necessarily required.[93]

As mentioned above, IECs seem to preferentially present antigen to intraepithelial lymphocytes (IEL), many of which are γδ CD8+ T cells. Thus, the IECs and γδ CD8+ T cells may constitute an active suppressor system that down-regulates humoral and cell-mediated responses to food proteins.

Anergy

T cell anergy is defined as a state of unresponsiveness characterized by the absence of proliferation and IL-2 production.[83] The principal mechanism by which anergy is induced is by antigenic presentation in the absence of costimulatory signals.[94] The best studied and

apparently, most potent co-stimulatory molecules expressed on cell surfaces are the B7 proteins (B7-1 and B7-2), the ligands for which are the CD-28 and CTLA surface antigens found on virtually all T cells.[95] This has lead to the two-signal model of T cell activation. The first signal is provided by interaction between the TCR and the antigenic peptide fragment incorporated into the MHC molecule. The second signal or co-stimulatory signal provided by molecules such as the B7 proteins, is required for IL-2 production. In the absence of a co-stimulatory signal, T cells fail to make IL-2, become unresponsive to further stimulation with antigen and are termed anergic.

There are several candidates for such "toleragenic" APCs including dendritic cells and enterocytes. Whilst, as previously discussed, normal enterocytes usually express low levels of MHC class II, they do not normally express B7-1.[82] It seems that presentation of antigen by these cells would normally result in anergy of naïve CD4+ cells. Characterization of dendritic cells following isolation from the lamina propria has been hampered by the fact that the isolation procedure results in activation and expression of membrane molecules not normally expressed. Careful recruitment of DCs demonstrates the expression of no or low levels of B7-1 or CD40 costimulatory molecules.[80] Expansion of DCs prior to feeding greatly enhances T-cell anergic responses, thereby implicating DCs as important toleragenic APCs.[80]

Anergy is proposed to be the main mechanism that operates when evidence of active suppression cannot be found.[83] In experimental models, anergy is usually identified when moderate to high doses of antigen are administered.[94] Although anergic cells are functionally inert, they remain intact and it has been shown in experimental murine models that the

anergic state can be reversed through exposure to IL-2.[94] Following rescue from anergy, the lymphocytes recover their normal patterns of cytokine production. Anergy is contrasted with active suppression by experiments showing the inability to transfer tolerance, using lymphocytes rendered anergic in another individual by moderate to high-dose feeding.[96]

However, the concept of silent disinterest by an anergised T cell seems too simplistic. Anergic T cells can affect APC signaling to other T cells by inhibiting expression of co-stimulatory signals and inducing anergy in other T cells as well. This has lead to the term "infectious tolerance" to describe the effects of cell contact between anergic and naïve T cells;[97] although perhaps "contagious" would have been a more accurate description.

Clonal Deletion

Clonal deletion of antigen specific lymphocytes by apoptosis has been demonstrated to occur following oral administration of large doses of antigen.[94] The differentiation between anergy and deletion comes from attempts to reverse oral tolerance through administration of IL-2. In a murine model of oral tolerance to myelin basic protein (MBP), administration of IL-2 did not reverse the tolerant state suggesting that the MBP reactive lymphocytes had been deleted.[98] In support of the idea that active suppression rather than deletion is responsible for tolerance in low antigen feeding models, these authors found that IL-2 was successful in reversing low-dose induced tolerance, suggesting anergic or suppressive mechanisms. The deletional mechanism(s) responsible for oral tolerance after high-dose feeding remains to be determined but is likely in at least some circumstances to be mediated through engagement of the Fas receptor molecules on the target lymphocytes. Engagement

of Fas by the so-called Fas-ligand on the effector cell induces an apoptotic signal in the recipient cell.

Benson et al (1997) have reported an experimental model that demonstrates how anergy and deletion might work in tandem.[94] In their work, MBP TCR transgenic mice were fed high doses of MBP. As predicted, this induced an immediate reduction of MBP-specific transgenic T cells in the intestinal mucosa and concurrently in several other peripheral lymphoid organs. However, within 3 days the MBP-specific population was re-established in the periphery and was maintained at the pre-feeding levels. In accordance with the anergic models, proliferative responses in this new population were reduced at least initially. Thus, a single high-dose of antigen seems to induce immediate deletion of the antigen specific T cells, followed by a re-population with T cells of identical specificity but which have been rendered anergic. The route by which antigen is absorbed and by which antigen presenting cell it is presented in order to elicit deletion has so far been undetermined

Factors Affecting Oral Tolerance Induction

Antigen Dose

The primary factor that determines which mechanism of peripheral tolerance develops following oral administration of antigen seems to be the dose of antigen fed.

Low doses of antigen (<0.1mg/g bodyweight in mice) favour the generation of active suppression through the generation of regulatory cells that, on subsequent interaction with the antigen, secrete down-regulatory cytokines, notably TGF-β.[99] In contrast, high doses of antigen (>0.5mg/g bodyweight in mice) favour deletion, predominantly of Th1 lymphocytes

with no evidence of active suppression.[100] Induction of anergy may represent a point between these extremes. It is important to realize that these mechanisms do not operate to the complete exclusion of the other. More often, the lower amounts of antigen usually encountered under physiological feeding will be associated with a range of responses.

Age

Age at the time of the first antigen encounter is an important factor in determining whether tolerance or immunization develops. Feeding of antigen within the first week of life in mice does not result in tolerance but results in priming of both humoral and cell-mediated responses to that antigen.[101] Bailey et al (1993) have shown that piglets weaned onto soya at 3 weeks of age develop levels of soy-specific serum IgG comparable to those generated by active vaccination with adjuvanted soya.[102] Most authors ascribe this lack of tolerance to two differences between neonates and adults: the lack of a fully competent immune system and the increased mucosal permeability and immature digestive abilities of the neonatal gastrointestinal tract.

Intestinal Flora

The extent and nature of the intestinal colonization by bacteria can affect the outcome of oral administration of antigen. In conventional mice, antigen (OVA) feeding results in IgG and IgE antibody unresponsiveness for up to 3 months from just a single feed whereas in germ-free mice, IgG unresponsiveness lasts less than 21 days after feeding.[103] Interestingly, IgE antibody unresponsiveness lasts much longer than that of IgG in this model.

Route of Entry

It has been discussed earlier that soluble antigens in the gut lumen are sampled and presented primarily by IECs leading to active suppression of the immune response through CD8+ T cells. On the other hand, antigen that reaches the lamina propria intact is generally phagocytosed by resident DCs and presented to CD4+ T cells resulting in anergy or deletion. In contrast, particulate antigens and intact bacteria, viruses and parasites are sampled by M cells leading to active immunity and the generation of IgA. Kaneko et al (1998) demonstrated that administration of particulate antigens in an oil-in-water emulsion fails to induce oral tolerance and can elicit a significant rise in serum IgG levels.[104] It is speculated by these authors that entry of these antigens was practically exclusively via the M cells thus by-passing the toleragenic mechanisms and instead, stimulating an antibody response. It is presumed that the ensuing response was predominantly a secretory IgA response although only serum antibodies were assessed. A study of reovirus infection in mice supports this proposal.[105] Reovirus type I infects IECs, whereas type III viruses gain entry via receptors expressed on M cell membranes. Administration of reovirus type I results in systemic tolerance whereas type III virus administration results in an active IgA response.

As has been discussed in the antigen presentation section, the three main routes of antigen absorption and presentation result in different immunological events. Other than those proteins that interact specifically with IECs (e.g. cholera toxin, *E.coli* heat-stable enterotoxin) or M cells (e.g. Reovirus type III), the determinants of route of antigen entry and presentation remain unknown.

Since antigens presented by IECs are likely to result in the generation of TGF-β secreting cells, such antigens are more likely to result in active suppression than anergy or deletion. This is supported by studies of the B subunit of the cholera toxin (CTB), which binds to receptors on IEC membranes but does not stimulate an inflammatory reaction. Czerkinsky et al (1996) found that conjugating CTB to soluble antigens resulted in oral tolerance at doses 1000 times lower than unconjugated protein.[106]

An intriguing possibility is that small peptide fragments in the intestinal milieu might bind to the peptide-binding groove of MHC molecules expressed on the luminal surface of IECs. As previously mentioned, the role of class II molecules expressed on IECs may specifically be for the selection of absorbed antigen and protection from lysosomal degradation during transcytosis. The implications of this possibility will be discussed further in the hydrolysate section.

1.4 General Mechanisms for Loss of Oral Tolerance

Increased Intestinal Permeability

A variety of probes has been used to measure intestinal permeability including Cr^{52}-labelled EDTA, polyethylene glycol and various sugars. It has been established that intestinal permeability is usually increased following antigen challenge in sensitized individuals. OVA sensitized rats show uptake of unrelated intact proteins in addition to increased uptake of sugars following antigen challenge. This increased mucosal permeability is associated with mucosal mast cell degranulation.[107]

In another study, horseradish peroxidase (HRP) sensitized rats were used to assess the role of mast cells in altered transepithelial antigen transport.[108] In this study, it was shown that transepithelial antigen transport occurs in two distinct phases in the sensitized intestine. Sensitization enhanced the initial phase of antigen uptake and endocytic transport and this was in a mast cell-independent manner. However, the second phase of increased uptake, namely a paracellular pathway, was dependent on the presence of mast cells and occurred after mast cell activation. This was predictably a nonspecific increase in permeability resulting in increased permeability to unrelated proteins. These findings suggest that once an individual is sensitized, even small amounts of antigen within the lumen can be preferentially transported to the lamina propria where the subsequent activation of mast cells induces a non-specific barrier defect. Such a defect might contribute to the sustenance of existing sensitivity or the expansion of sensitivity to other food proteins.

The question of whether increased mucosal permeability might precede sensitization is animportant one. There is some indication that epithelial permeability is increased in sensitized animals not only after antigen challenge but also under baseline conditions. Uptake of ^{51}Cr-EDTA from the jejunum in OVA sensitized rats has been shown to be higher than in controls.[109] There is also speculation that mucosal damage from any source might lead to an increase in mucosal permeability and may contribute to sensitization. Whether or not this occurs is still undecided, as is the pathway by which antigen might cross the mucosa under such circumstances.

Some of the circumstances under which increased mucosal permeability occurs in dogs have been established. These include dietary sensitivity, gluten-sensitive enteropathy, small intestinal bacterial overgrowth, giardiasis and lymphocytic/plasmacytic inflammatory bowel disease.[110,111,112,113] In addition, normalization of permeability has been demonstrated in dietary intolerant dogs following a period of novel protein feeding.[110] However, most of these cases were demonstrated to have a degree of intestinal inflammation at the time of permeability testing. As will be discussed below, bacterial adjuvants have also been shown to increase permeability. Whether in these cases, any loss of tolerance is due to the increased permeability or the result of abnormal mucosal antigen processing and presentation is unknown. Even in those dogs in which there was no histological evidence of mucosal inflammation, co-stimulatory molecule expression was not quantitatively assessed.

The consequences of increased antigen absorption have not been clearly established. In normal individuals, small amounts of intact proteins readily gain access to the systemic circulation in an immunologically intact form.[114] These molecules do not normally cause

adverse reactions because of the systemic unresponsiveness associated with oral tolerance. However, were large amounts of antigen to reach the systemic circulation, the immune response elicited by conventional, non-mucosal antigen presenting cells would be expected to over-ride active suppression and/or reactivate anergic T cells. It has been shown that the Th1/Th2 bias of the immune response to antigen administered i.v. without adjuvant is dependent on the dose of antigen. Ismail and Bretscher (1999) demonstrated that low doses of antigen administered i.v. to mice generates a virtually exclusive Th1 response in splenic lymphocytes.[115] In contrast a higher dose induced either a mixed Th1/Th2 or a predominantly Th2 response and stimulated the production of specific antibodies. Were this to occur in an individual already predisposed to generating Th2 type responses (i.e. an atopic individual), this would likely result in the production of significant amounts of antigen-specific IgE and potentially lead to hypersensitivity.

It therefore seems likely that increased intestinal permeability contributes to the development of dietary hypersensitivity. However, it may be difficult or even impossible to separate the relative importance of mucosal permeability from changes in mucosal antigen presentation in the presence of inflammation.

Neonates

It is well established that most species in the first few days after birth have significantly increased intestinal permeability to macromolecules.[101] The duration of this effect is species specific but the principal causes are probably not. Significant differences between neonates and adults have been discussed above but the net result is that the absorption of macromolecules into the systemic circulation is increased.

Mucosal Adjuvants

Provoking gastrointestinal inflammation prevents the induction of tolerance. Adjuvants such as bacterial toxins may contribute loss of tolerance by prolonging the normally transient responsiveness to co-administered antigens. This is best illustrated by the demonstration that when antigens such as OVA are coupled to immune-stimulating complexes or bacterial toxins (e.g. cholera toxin) and administered orally, local and systemic immunity are likely to develop.[116]

Two of the most potent and hence most studied mucosal adjuvants are the cholera toxin (CT) produced by *Vibrio cholerae* and the heat-labile enterotoxin (LT) produced by certain enterotoxigenic strains of *E. coli* . Elson and Ealding (1984) showed that CT prevents the development of tolerance to unrelated proteins when they are fed together.[117] This is in contrast to the previously mentioned profound enhancement of oral tolerance that the isolated B-subunit of the toxin creates when conjugated to an unrelated protein.[106]

Adjuvant Enhancement of Mucosal Permeability

The A-subunit of CT has been shown to increase secretion of water and electrolytes into the small intestine.[118] Once the B subunit has bound to epithelial cells via specific receptors, the A subunit reaches the cytosol and binds to NAD to catalyze reactions culminating in elevation of intracellular cAMP. In epithelial cells, an increase in intracellular cAMP results in secretion of water and chloride ions into the intestinal lumen and hence the typical secretory diarrhoea associated with cholera and enterotoxigenic *E.coli* enteritis . Lycke et al (1991) have proposed that the adjuvanticity of CT and LT is the result of their ability to

increase absorption by the small intestinal luminal enterocytes.[119] Using fluorescent Dextran 3000 as a permeability marker, they showed that an increase in Dextran uptake into the serum was only observed when CT was used as an adjuvant. They also administered the Dextran in association with Keyhole Limpet Haemocyanin (KLH) and found that the uptake of Dextran correlated with an adjuvant effect as evidenced by significant anti-KLH antibody responses. It was concluded by these researchers that the adjuvant effect of CT is associated with its ability to increase mucosal permeability, thus increasing exposure of luminal antigens to the GALT. Whilst this may be the case, investigations such as these are not able to tease apart the effects of altered antigen processing independently from permeability. In addition, the use of Dextran as a marker does not necessarily predict the augmentation of uptake of protein antigens and the immunological relevance of Dextran's enhanced uptake remains to be determined.

In contrast with Lycke's conclusions, Nedrud et al (1991) found that oral immunization with CT does not necessarily enhance uptake of unrelated dietary antigens.[120] Using CT alone or in association with Sendai virus they demonstrated a predictable rise in virus specific antibodies. However, no increases in serum antibodies to the main dietary protein (soy) were observed in the CT treated group compared with the control group. However, in mice immunized orally with OVA plus CT, intestinal IgA and IgG titres against OVA were significantly increased. Oral pre-immunization with OVA *prior* to oral OVA-CT immunization prevented both serum and intestinal OVA-specific antibody increases. Snider et al (1994) found that CT did not increase the uptake of orally administered hen egg lysozyme (HEL) into the peripheral circulation despite consistent rises in HEL-specific IgG and IgE antibodies.[121] Thus, whilst the role of CT as a potent mucosal adjuvant has been

established, the use of CT as an oral adjuvant does not seem to increase responses against dietary antigens if there has been prior exposure to the antigen. This may have relevance to the common practice of introducing a novel dietary protein source to cats and dogs during periods of enteritis and suggests that the introduction of a hydrolysed protein source may be preferable (see below).

So whilst it is not entirely clear how CT might induce immunity rather than tolerance, it has been shown that CT given orally with KLH primes both Th1 and Th2 responses.[122] This priming of Th1 responses makes it impossible to suppress systemic Th1 responses following peripheral immunization. These changes in T cell activation are consistent with alterations in the antigen presenting cells involved.

These studies collectively suggest that CT must be inducing antibody production by a means other than simply increased antigen uptake.

Mast Cell Dependent Responses to Adjuvants

Kosecka et al (1999) demonstrated that in rats sensitized to OVA by intraperitoneal injection, luminal challenge results in mast cell dependent secretion of chloride as measured by short-circuit current in Ussing chamber experiments.[123] This response decreases to less than 50% by 14 days post-immunization and is predictably absent by 230 days due to the establishment of oral tolerance. In contrast, co-administration of *Bordetella pertussis* derived pertussis toxin (PT) results in prolonged sensitization with responses at 8 months the same as those observed at 7 and 14 days post-immunization. Since the half-life of IgE in murine tissues is probably not much more than 7 days,[124] continual production of specific IgE must

still be occurring in this model. This may be partly explained by the persistent antigen presentation function of DCs in the germinal centres of Peyer's patches.

In Koseka's model, when antigen was co-administered orally to the sensitized rats with PT, a 20-fold increase in chloride secretion resulted compared with a 2.5-fold increase in response to antigen alone. This difference in the magnitude of the response was due to enhanced antigen access to the lamina propria by mast cell dependent mechanisms.

Adjuvant Altered Antigen Presentation

Adjuvants provoke local secretion of the necessary inflammatory cytokines required to up-regulate B7-1, B7-2 and CD40 expression on DCs, which in turn promote activation and expansion of naïve T cells.[125] In experimental models, CT has been shown to enhance the antigen presentation of IECs in rats.[126] This enhancement was not due to an upregulation of MHC II expression but rather a dose-dependent increase in IL-1 and IL-6 secretion by the IECs. Pro-inflammatory cytokines such as IL-1, TNF, IL-6, IL-12, IFN-γ and IFN-β will upregulate expression of B7-1, B7-2 or both molecules on most cells that can express them. Furthermore, B7-1 and B7-2 expression may differentially affect Th1/Th2 cell development. Studies have indicated that Th2 priming is more dependent on B7 co-stimulation than Th1 cell development.[95,127] Studies such as these support the proposal that bacterial toxins may contribute to loss of tolerance by prolonging the normally transient responsiveness to co-administered antigens. Certainly, the potent adjuvant effect of CT may be attributed mostly to its ability to enhance the co-stimulatory ability of APCs, including so-called non-professional APCs such as IECs.

Depletion of CD8+ Intraepithelial Lymphoctyes by Adjuvants

Given the aforementioned role for CD8+ IELs in the establishment and maintenance of oral tolerance, it is reasonable to assume that any process depleting their population might lead to sensitization. Elson et al (1995) demonstrated the preferential reduction of CD8+ T cells from a population of splenic lymphocytes when cultured in the presence of CT.[128] Subsequently these investigators used murine lymphocyte transference models to demonstrate that antigen specific suppressor T cells generated by oral tolerance can be transferred to naïve mice and induce tolerance in the recipient. However, including CT in the oral inoculations inhibited the development of suppressor T cells in the donor mice. The authors proposed that CT and LT abrogate the induction of oral tolerance by depletion of the CD8+ IELs by a direct cytotoxic or apototic effect.

IgA Deficiency

Teleologically speaking, the main role for local intestinal IgA production seems to be to block antigen uptake limiting the absorption of potentially harmful compounds. Considering this, it is reasonable to assume that a selective deficiency of IgA would lead to increased antigen uptake and might overwhelm normal tolerance mechanisms in a similar way to that discussed under "increased permeability". This hypothesis is supported by reports of a higher prevalence of food hypersensitivity amongst IgA deficient dogs and human patients than the general population.[129,130]

Parasitism

There is an obvious similarity between the immune response elicited by infection with intestinal parasites and the type I hypersensitivity response in food allergic individuals. Immunity to intestinal nematodes has been shown to be largely dependent on Th2 lymphocytes promoting IgE synthesis through the production of IL4 and IL5. Given this similarity, the possibility of intestinal parasitism influencing the development of food hypersensitivity is an intriguing one. This is especially true of atopic individuals for whom three hypotheses exist:

1. Intestinal helminthic infection predisposes to sensitization to oral antigens.

   There are numerous epidemiological observations documenting the association between the prevalence of parasitism and allergic diseases in humans.[131] No data is available for veterinary species. However, the undeniable ability of most helminths to induce an IgE response to themselves may enhance the allergic state. This view is supported by the fact that IL4 released by helminth specific T cells and activated mast cells will enhance Th2 cell development in Th0 cells specific for unrelated antigens. It is also consistent with observations that signs of allergic disease occur more frequently in children seropositive for *Toxocara sp* than in seronegative children.[132] In addition, the proven antigenic cross-reactivity between certain allergens and parasites in humans indicates that in some circumstances, parasites may be able to prime and eventually enhance allergic reactions to oral antigens.[133]

2. Helminthic parasites prevent or modulate the development of food hypersensitivity.

This has been for some time the most favoured hypothesis given the greater volume of epidemiological observations suggesting an inverse relationship between parasitism and the prevalence or allergic diseases in the human population.[134] Such observations clearly contrast to those supporting the first hypothesis. Perhaps the most convincing immunological explanation stems from the observation that in association with helminth specific IgE there is an accompanying increase in serum IgG4.[135] IgG4 antibodies do not fix complement and bind weakly to Fcγ receptors and therefore antigen binding by IgG4 antibodies does not seem to be associated with harmful consequences. These antibodies have thus been termed "blocking antibodies".[135] It is now clear that the blocking activity results from direct competition for allergen binding between IgG4 in the serum and IgE bound to high-affinity Fcε receptors on effector cell membranes. This has been proposed as one mechanism by which immunotherapy produces desensitization since human patients receiving immunotherapy for insect venom and house dust mite sensitivity develop relatively high levels of IgG4 antibodies.[136] Since infection with intestinal parasites can induce isotype switching to IgG4 specific for unrelated antigens, the development of food allergen specific IgG4 antibodies as a result of intestinal parasitism may decrease or even prevent clinical signs.

3. Atopics are protected against intestinal parasitism

Again, this hypothesis is based on epidemiological observations that contrast with those supporting the previous hypotheses. There is some evidence that atopic asthmatics in tropical areas harbour fewer parasites than their non-asthmatic counterparts.[137] In addition, countries with a high prevalence of atopy are more likely to be Western, urban and industrialized with a concurrent low prevalence of parasitism. In accordance,

countries with a low atopic prevalence are likely to be rural, non-Western and with high rates of parasitism.[134]

So whilst the exact relationship between parasitism and allergic diseases is undetermined, it could be argued that genetically predisposed atopics would be more prone to develop food hypersensitivity following the additional allergenic stimulation provided by parasite infection. This heightened immune response could in turn be effective in reducing or eradicating the parasite burden.

Abnormal Cytokine Responses

Aspects of the normal cytokine environment in the intestinal mucosal system have been discussed above. It has been suggested that in the normal animal the increased exposure to dietary antigens that occurs at time of weaning, efficiently induces T cell anergy and T cell mediated suppression. This ensures that responses to food allergens that occur in early infancy, including IL-4 driven IgE production are down-regulated. This normal down-regulatory response is characterized by increased IFN-γ and IL-2 production consistent with a shift towards a Th1 response. Using a mouse model for OVA sensitization and Th2 dependent OVA-specific IgE production, van Halteren et al (1997) confirmed that oral tolerance induction abrogates the Th2-dependent IgE response.[138] Consistent with the suppressed IgE levels, elevated IFN-γ production by splenic lymphocytes was observed.

Recently, and in contrast with normal animals, Österlund et al (1999) have shown that TNF-α and IFN-γ production of peripheral blood mononuclear cells (PBMC) is significantly lower whereas IL-4 production is unaffected in infants with cow's milk allergy (CMA) than in

normal infants.[139] Indeed, the mean post-stimulation production of TNF-α from PBMCs taken from CMA infants did not even reach the level of spontaneous (pre-stimulation) production by those of normal individuals. The situation was similar although not as marked for IFN-γ. This is despite the fact that T cells derived from normal neonates show a pre-existing decreased capacity for IFN-γ production compared with normal adults. This is consistent with the view that a PBMC maturational defect is present in children with a genetic predisposition to atopy.

These two studies support the hypothesis that TNF-α and IFN-γ may have a role in the establishment and maintenance of oral tolerance. Certainly, lack of sufficient IFN-γ production might contribute to abnormal T cell responses and facilitate sensitization by prolongation of normal neonatal Th2-dependent food-specific antibody responses. The possible role of derangements in the production of both or either of these cytokines in veterinary patients with a predisposition to developing food hypersensitivity warrants further evaluation.

Summary

Rather than a single defined mechanism, it is likely that loss of tolerance and sensitization to dietary proteins in any given individual represents the combined effect of more than one mechanism. The separation of altered antigen presentation and up-regulation of co-stimulation mechanisms from increased mucosal permeability may be an artificial one. Defining the initiating event may prove impossible. In addition, the influence of an individual's immunological phenotype is great. An exaggerated Th2 response is expected in atopic individuals and it is hardly surprising that food sensitivity is more common amongst

atopics than non-atopics. Derangements in Th1 cytokine production may play a role in the development of sensitization in the neonate.

Whenever there is the expectation of increased permeability and/or altered antigen processing and presentation, in adult or neonate alike, it is sensible to consider the possibility of the development of sensitization to dietary proteins, transient or otherwise.

1.5 Pathophysiology of Food Hypersensitivity

Once sensitization has occurred, the consequences of allergen exposure in the intestine are still complicated and the underlying mechanisms that give rise to the intestinal signs not well understood. Most of the studies investigating the effects of allergen exposure on the intestine have centered around changes in mucosal transport (secretion and/or absorption), changes in mucosal permeability and changes in intestinal motility. In contrast, descriptions of changes associated with food allergen exposure in the skin probably follow a more classical pattern of type I hypersensitivity following the systemic absorption of intact allergen.

Type I

In people, the majority of food hypersensitivity reactions are thought to be of a type I nature.[140,141] To date, clear definition of the pathophysiologic mechanisms involved in animals is lacking. The fact that normal intestinal T cell responses are predominantly of the Th2 type goes some way to understanding why a type I hypersensitivity response might develop following sensitization in a non-atopic, regardless of the inciting circumstances.

Gastrointestinal Tract

Ion Transport

In vivo and *in vitro* experiments in sensitized animals have demonstrated changes in salt and water transport following oral or luminal challenge. In mice passively sensitized with β-lactoglobulin-specific monoclonal IgE, administration of β-LG by gavage resulted in fluid accumulation in the small intestine and diarrhoea.[142] Rats sensitized to OVA showed a significantly reduced net absorption of sodium, chloride, potassium and water within 40 minutes of adding OVA to a perfused segment of jejunum.[143] Similar experiments have documented elevated cAMP levels in the mucosa 2 minutes after OVA challenge stimulating chloride secretion.[144] Most of these experimental models have also evaluated the effect of mast-cell stabilizers on the subsequent result. A common finding is that the net increase in ion transport following antigen challenge is inhibited by doxantrazole.[141] Doxantrazole has been shown to inhibit mediator release from both intestinal mucosal and connective tissue mast cells in rats.[141] Such studies are consistent with the view that mast cells are responsible for the ion transport abnormalities that occur during intestinal hypersensitivity responses to allergens. Further studies are required to confirm that the same is true for naturally occurring food hypersensitivity in dogs and cats.

Permeability

The experimental and clinical observations discussed earlier suggest that intestinal epithelial and/or vascular permeability are increased following antigen challenge in sensitized patients. Some of the permeability changes appear to manifest not only following sensitization but also prior to challenge suggesting an underlying permeability defect. In addition, many of

the permeability changes are associated with indicators of mast cell activation or can be inhibited by doxantrazole.[107] These studies support the theory that mast cell activation is accompanied by an enhanced uptake of large and small molecules by the gut epithelium.

Motility

It is well known that the products of mast cell degranulation cause smooth muscle contraction. Histamine, serotonergic, prostaglandins and leukotrienes have all been shown to affect intestinal smooth muscle. Scott et al (1990), using jejunal sections from OVA sensitized rats, demonstrated that following antigen challenge, jejunal contraction was blocked by a serotonic antagonist (methysergide, a 5-HT_1 – receptor antagonist) and the cycloxogenase inhibitor indomethacin.[145] Whilst the antigen induced contraction was resistant to a combination of H-1 and H-2 receptor antagonists, doxantrazole (and interestingly cromoglycate) inhibited the response. In addition, the response could be passively transferred with serum containing OVA-specific IgE antibody. In an *in vivo* model, Diamant et al (1989) showed that the motor abnormalities are not isolated to the site of challenge.[146] They reported that jejunal administration of OVA in sensitized rats resulted in generalized alterations in the postprandial motility relative to non-sensitized controls. They reported a significant increase in the number of high-amplitude aborally-propagating clustered contractions, where the phasic contractile activity was superimposed on a sustained tonic elevation of intraluminal pressure lasting 5-10 seconds. In addition, whereas none of the control group demonstrated clinical signs, all of the sensitized animals developed diarrhoea shortly after challenge. These studies show that hypersensitivity reactions in the

intestine do affect motility and that the products of mast cells are largely responsible for those effects.

Skin

The events that follow degranulation of connective tissue mast cells in the skin have been well described.[59] The onset of signs may be very rapid, occurring within minutes of ingesting the responsible allergen. There are, however, a number of cases (possibly even the majority) of food hypersensitivity with dermatological signs, that do not show rapid deterioration on ingestion of the allergen (see below). In these cases, it is hard to imagine how mast cell degranulation could be central to their signs. It is conceivable that the role of other IgE-receptor-bearing cells is more important in these cases. Langerhans cells, the resident professional antigen presenting cells in the skin have been shown to be increased in numbers and possess IgE receptors on their surfaces in humans with atopic dermatitis.[147] These IgE molecules are speculated to play a role in the selection of antigen for subsequent presentation and have been shown to be up to 1000 times more efficient than non-IgE-bearing cells at presenting to Th2 lymphocytes.[148] It is speculative whether a similar situation exists in veterinary patients with food hypersensitivity but it does provide a mechanism for chronic dermatitis with delayed onset of signs in patients without demonstrable tissue mastocytosis.

As such, the histopathological changes seen in cases of food hypersensitivity are not pathognomonic but instead could be the result of several allergic diseases such as atopic dermatitis or flea allergy dematitis.[19,20] There is often a perivascular infiltrate that is rich in

mononuclear cells and occasionally neutrophils, whilst tissue eosinophilia and increased mast cells can be seen in some cases.[149]

Other Types

Whilst it is established that mast cells are central to the pathogenesis of dermatological and gastrointestinal signs in most models of food hypersensitivity, the image of a purely IgE/mast cell mediated process is misleading. In addition, as stated previously, there is uncertainty about the exact nature of the immunological reactions responsible for most cases seen in dogs and cats. As will be discussed later, the poor predictive value of *in vitro* and *in vivo* diagnostic tests for food hypersensitivity in dogs and cats that are reliant on IgE measurement or IgE-mediated effects suggests that perhaps other mechanisms are involved. Although type I reactions are clearly involved in mucosal hypersensitivity reactions such as asthma and hayfever, the presence of a so-called "late-phase response" has long been recognized in humans. Present evidence suggests that the allergen-induced late phase response has both a mast cell (type I hypersensitivity) and a T cell (type IV hypersensitivity) component.[150] This has lead to use of the term "chronic allergic inflammation" or "type IVa_2 hypersensitivity".[150]

As discussed above, mast cells contain or can synthesize a virtual pharmacopoeia of biologically active substances. In addition to the well described preformed granules, in response to activation, mast cells have the potential to synthesize and secrete many cytokines, including IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, INF-γ and TNF-α.[59] As previously mentioned, IL-4 activates B cells for antibody secretion and plays a pivotal role in the isotype switching to IgE production and importantly induces the development of T cells of

the Th2 phenotype.[84] IL-5 is a growth, differentiation and activation factor for eosinophils promoting endothelial adhesion and chemotaxis.[23] IL-6 has activities on a wide range of cellular processes including T cell activation, stimulation of antibody production by B cells and enhancement of IL-4 dependent IgE synthesis.[59] Thus, mast cells have profound influences on the development and maintenance of chronic allergic inflammation as the result of prolonged allergen exposure and clearly contribute to the accumulation and expansion of allergen-specific T and B cells at mucosal sites.

It is hypothesized then, that chronic food allergen exposure in sensitized dogs and cats may result in the establishment of chronic allergic inflammation (a type IVa_2 hypersensitivity) that was initially purely a type I hypersensitivity. The consequences of regional chronic mucosal inflammation might influence mucosal handling of other antigens in some individuals resulting in a polyclonal lymphocytic-plasmacytic infiltrate. Further study into the exact immunological nature of food hypersensitivity reactions in dogs and cats is required to ascertain if this occurs. If so, this might explain the delayed onset of clinical signs recorded in a large number of food hypersensitive veterinary patients following challenge.

Inflammatory Bowel Disease

The association between food hypersensitivity and inflammatory bowel disease is unclear. Investigations into the nature of the relationship are hampered by the lack of robust diagnostic criteria. The simple finding of lymphocytic-plasmacytic infiltrates is virtually meaningless unless attempts are made to exclude known causes of such infiltrates. In addition, many cases of intestinal disease associated with inflammatory infiltrates would be

expected to improve with the introduction of a low fat, highly digestible diet. Since the majority of recommended elimination diets fulfill these criteria, it is difficult to know how many cases truly involve dietary hypersensitivity.

However, there are some cases with histological evidence of inflammatory infiltrates where all attempts have been made to exclude known causes, in which food hypersensitivity is involved. Guilford et al (2001) found that in 16 cases of elimination-challenge proven dietary hypersensitivity, all 16 had histological changes in at least one region of the bowel.[12] These 16 cases had progressed through an extensive gastrointestinal investigation to eliminate other causes of chronic gastrointestinal disease. One cat had severe lymphocytic infiltrates throughout the gastric lamina propria with some areas showing evidence of obliteration of gastric glands. Of the other cats, 9 had histological changes that were consistent with a diagnosis of mild to moderate lymphocytic-plasmacytic enteritis, gastroenteritis, or enterocolitis. A variety of other leukocytes was identified in the infiltrates. All of these cases were reported to have responded completely to the elimination diet alone and offending foods were identified in all cases.

In humans cases of inflammatory bowel disease there is disagreement regarding the role that diet has to play. Some studies have shown a positive benefit to elemental diets in cases of Crohn's disease whereas others have not.[151] Evidence of decreased tolerance to dietary antigens has been demonstrated by the finding of increases in food-specific serum antibodies (IgG4) in patients with Crohn's disease.[152]

This is consistent with the understanding of the pathogenesis of food hypersensitivity. Regardless of the inciting agent or circumstance behind the mucosal inflammation, sensitization to a variety of food and microbial antigens is expected. Whether in the above cases reported by Guilford et al (1999), food was the only antigen to which the cats were hypersensitive is unknown. It is equally possible that eliminating the quantitatively most significant antigen source is sufficient to eliminate clinical signs. Accordingly, normal mucosal immunity is eventually established.

Atopy

Laboratory and clinical investigations in humans have demonstrated that food hypersensitivity plays a pathological role in a subset of patients, primarily infants and children with atopic dermatitis. Approximately 40% of infants and young children with moderate to severe atopic dermatitis have food hypersensitivity.[153] It is commonly reported that the concomitant occurrence of atopic dermatitis and food hypersensitivity in dogs and cats is uncommon.[20] However, other authors report that food allergy is often associated with other pruritic dermatopathies including atopic dermatitis.[154] Of the 51 dogs with food hypersensitivity investigated by Rosser (1993), 15 dogs also had atopic dermatitis.[155] Carlotti et al (1990) observed that 6 of 32 dogs[9], White (1986) found that 4 of 30 dogs,[15] Kunkle et al (1992) found that 2 out of 9[156] and Paterson (1995) observed that 9 out of 20 dogs with food hypersensitivity had concurrent atopic dermatitis.[157] Accumulating these figures gives a total of 142 cases of food hypersensitivity out of which 36 cases were atopic. This equates to 25.4% of cases of food hypersensitivity concurrently having atopic dermatitis. Clearly, this represents a strong argument for an atopic predisposition as a risk factor for the

development of food hypersensitivity. This is in general agreement with the hypothesis that a significant number of cases are due to a type I hypersensitivity.

Summary

Data from experimental studies and in naturally hypersensitive human patients indicate that the ingestion of an allergen in most sensitized individuals results in intestinal mast cell degranulation. Systemic absorption of intact allergen through a normal mucosa and especially through a diseased mucosa, results in the interaction of allergen-specific IgE bound to tissue resident mast cells. The subsequent release of a variety of mediators that act directly on the vascular enothelium, smooth muscle and epithelium results in the generation of the acute-onset clinical signs wherever the particular sensitizedmast cells are resident. In addition, the release of a variety of cytokines is postulated at least in some individuals with chronic allergen exposure, to result in the development of a late phase reaction comparable with that seen in human atopic asthmatics. Given the typical history of chronic allergen exposure in veterinary patients diagnosed with food hypersensitivity, this may explain some of the reasons for the poor diagnostic value of IgE-dependent assays (see below). Lastly, whilst the majority of patients affected with lymphocytic-plasmacytic enteritis require some form of pharmacological therapy to induce remission of clinical signs, there are some patients that are reported to respond to dietary management alone. This is supportive of the idea that a type IVa_2 food hypersensitivity has a part in the pathogenesis of those cases.

1.6 Clinical Signs

The clinical signs that have been attributed to food hypersensitivity are many and varied. Affected animals may have signs confined to one body system (e.g. skin or gastrointestinal tract) or may have signs relating to two or more body systems. No study has yet identified any sex predisposition and few have identified age or breed predispositions despite the predisposition of certain breeds to develop atopic dermatitis. This is in contrast to human studies where there is a strong association between young age, familial history of atopy and the development of food hypersensitivity.[158,140]

1.6.1 Cutaneous Signs

The spectrum of cutaneous clinical signs and their prevalences are listed in Table 1.4. Studies listed include only those with adequate investigations to eliminate non-food allergic diseases from the list of differentials and where the diagnosis was made on the basis of an elimination and challenge trial.

As can be seen from Table 1.4, there are no pathognomonic cutaneous signs but pruritus is the most commonly reported clinical finding for dogs and cats with food hypersensitivity. This is characteristically of a constant, non-seasonal nature. However, given the bias towards cases of food hypersensitivity to be investigated and reported by veterinary referral dermatologists, the prevalence of all clinical signs within the true total population of food sensitive individuals, cutaneous and otherwise, is almost certainly different. However, in those cases that present with dermatological signs as a manifestation of their hypersensitivity, pruritus is probably present in all those patients and is probably the only consistent finding.

Other signs reported less frequently include: pododermatitis, facial swelling, crusts and erosions, perianal furunculosis and urticaria.[9,11,140,158,159]

Table 1.4. Prevalence of Cutaneous Signs in Dogs and Cats with Food Hypersensitivity

Symptom	Reported Symptom Prevalence (reference)
Pruritus	97% (White 1986),[15] 100% (White 1989),[159] 100% (Rosser 1993),[14] 100% (Jeffers 1991),[160] 47% (Guilford 1998),[11] 100% (Paterson 95),[157] 100% (Carlotti 1990),[9] 80% (Harvey 1993)[161]
Erythema	50%(White 1986),[15] 7% (White 1989),[159] 63%(Rosser 1993),[14] 11% (Carlotti 1990),[9]
Papules (including feline miliary dermatitis)	21% (White 1989),[159] 37% (White 1986),[15] 43% (Rosser 1993),[14] 9% (Carlotti 1990)[9]
Hyperpigmentation	27% (Rosser 1993),[14] 7% (Carlotti 1990)[9]
Alopecia	64% (White 1989),[159] 24% (Rosser 1993),[14] 9% (Carlotti 1990)[9]
Seborrhea (sicca / oleosa)	10% (White 1986),[15] 4% (White1989),[159] 26% (Rosser 1993),[14] 21% (Carlotti 1990)[9]
Pyoderma	16% (White 1986),[15] 35% (Rosser 1993),[14] 16% (Harvey 1993)[161]
Otitis externa	6% (White 1986),[15] 78% (Rosser 1993),[14] 27% (Carlotti 1990),[9] 56% (Harvey 1993)[161]
Eosinophilic granuloma	14% (White 1986),[15] 14% (White1989),[159] 4% (Carlotti 1990) [9]
Lichenification	16% (Rosser 1993),[14] 7% (Carlotti 1990)[9]

It should be noted however, that many of the animals included in the above studies were also affected with other dermatological conditions including atopic dermatitis, flea dermatitis and flea allergic dermatitis, contact hypersensitivity and cheyletiellosis. A response to dietary elimination trials is gauged by a reduction in pruritus and other clinical signs might be expected to resolve more slowly. In addition, since the other listed cutaneous signs could easily be the result of other concurrent diseases, the actual prevalence of signs purely attributable to food hypersensitivity may be different.[19]

The distribution of lesions is varied and is often indistinguishable from that seen with atopic dermatitis.[20] Rosser (1993) reported that 80% of 51 food hypersensitive dogs were pruritic and/or had lesions around the pinnae or had concurrent otitis externa, whilst 61% chewed their feet and 53% were pruritic in the inguinal region.[14] In Rosser's (1993) study of 13 cats with food hypersensitivity, the ears were involved in 69% and the facial region in 62% with a generalised distribution of pruritus and lesions in 8%.[162] However, it is interesting to note the relatively high prevalence of otitis externa as a presenting complaint in a number of publications.

1.6.2 Gastrointestinal Signs

Not surprisingly, pruritus is less prevalent amongst animals with food hypersensitivity which present primarily for gastrointestinal signs. Guilford et al (1998) selected the study population (128) based on cats with the primary complaint of chronic vomiting, chronic diarrhoea or any combination thereof.[11] Of those confirmed as food hypersensitive, 45% also presented with pruritus, 14% with vomiting, 18% with diarrhoea and 23% with both pruritus and gastrointestinal disease. Paterson (1995) described 20 food allergic dogs with perennial pruritus that were diagnosed as being food hypersensitive.[157] Of those 20 dogs, 13 (65%) had evidence of gastrointestinal disease. Signs described included tenesmus, haematochezia, faecal mucus and increased frequency of defaecation (>3 motions per day). In a report of 8 cats within a colony of 26 that were diagnosed with food hypersensitivity, all 8 of the cats had vomiting as well as dermatological disease.[163] Both the vomiting and the dermatopathies were 100% responsive to an elimination diet. The results of the more

inclusive criteria of these studies suggests that gastrointestinal complaints are relatively commonly associated with food hypersensitivity.

In the development of an experimental model of food hypersensitivity in dogs using codfish as the antigen, Guilford found that 65% of dogs developed diarrhoea during oral challenge following sensitization.[164] The diarrhoea was usually of large bowel character and at least one of the dogs developed no other signs. Vomiting was seen in 20% of the dogs.

Neither Rosser,[14] Carlotti[9] nor White [15,159] included signs of gastrointestinal disease in the selection criteria of their cases. However, 6 of the 138 (4.3%) animals identified in those case series had concurrent gastrointestinal disease and Carlotti (1990) has suggested that 10-15% of animals with food hypersensitivity have signs of concurrent gastrointestinal disease.[9]

It has been suggested that animals with both dermatological and gastrointestinal signs are more likely to be affected by a food hypersensitivity than other causes of the chronic dermatosis. Indeed Griffin has observed that pruritic dogs that had more than 3 bowel motions a day were more likely to have food allergies than other causes of chronic pruritic dermatitis.[19]

Of importance is the observation that clinical signs specific to large bowel disease may be the only presenting sign. In an early experimental study in humans, passive sensitization of the rectal mucosa in a non-allergic individual with serum from a peanut-allergic patient resulted in erythema, oedema and friability of the rectal site after the volunteer ingested peanuts.[165] Similar experiments involving colostomies have demonstrated local allergic

reactions.[166] Such demonstrations suggest that either intact antigen is reaching the large bowel lumen or is being systemically absorbed and reacting with resident mucosal mast cells following haematogenous transit. Certainly, responses to elimination diets have been demonstrated in canine idiopathic colitis.[167] In addition, the possibility that food hypersensitivity might be responsible for anal pruritus in dogs and cats is feasible.

The role that food hypersensitivity plays in eosinophilic inflammatory bowel disorders (eosinophilic gastroenteritis, enteritis, enterocolitis, colitis) is unclear. Eosinophilic inflammatory bowel disorders are characterized by a mixed inflammatory infiltrate in which eosinophils predominate. The disorder appears less common than lymphocytic-plasmacytic enteritis. Whilst the diagnosis at present rests on the histological appearance it is likely that this description includes an heterogenous collection of aetiologies. The finding of the disease in a SPF cat suggests that parasitism and infectious agents are not always required as initiating events.[168] However, hypersensitivity to food proteins may be involved in only a few animals since dietary trials are reported to be unsuccessful in alleviating clinical signs in many affected patients.[169] This may be similar to the spectrum of eosinophilic diseases seen in people. Although 50% of all human patients presenting with eosinophilic inflammatory bowel diseases are shown to be atopic, food-induced IgE mediated reactions are implicated in only a few patients.[170] Whether this is because food is not truly the offending antigenic source for the infiltrate or whether the inflammatory process is too advanced and becomes less specific for the initiating antigens by the time a diagnosis is reached, remains unanswered.

In summary, it is difficult to establish the true prevalence of gastrointestinal signs in the feline and canine food hypersensitive population from the available publications. However, it is certainly higher than those figures commonly quoted by many referral veterinary dermatologists. Whilst it is hardly surprising that gastrointestinal signs should occur in patients with food hypersensitivity, it is perhaps that they are not *more* commonly seen in food hypersensitive patients that is surprising. The mechanisms by which a patient might experience dermatological or systemic clinical signs in the absence of gastrointestinal signs are unclear but they probably reflect differences in regional tolerance mechanisms and compartmentalization of the respective immune responses.

1.6.3 Other Clinical Signs

Whilst dermatological and gastrointestinal signs dominate the clinical presentation of food hypersensitivity in all species, a number of other organ systems can be affected. The human medical literature abounds with reports, both substantiated and otherwise, of clinical signs and disease syndromes linked to food hypersensitivities. In contrast, veterinary reports of clinical presentations pertaining to systems other than the skin and gastrointestinal tract are scarce.

Respiratory

The association between food hypersensitivity and onset of signs of respiratory disease in humans has long been recognized. Hill and Hosking (1984) found that in 100 children with cow's milk allergy, stridor was seen in 4%, tachypnoea in 1%, rhinitis in 21% and coughing or wheezing in 29% of patients following controlled challenge.[171] Similarly, James et al (1994) in a review of 323 children and adolescents with atopic dematitis undergoing double-blind placebo controlled food challenges (DBPCFC) for the evaluation of food

hypersensitivity, documented significant changes in pulmonary function studies in a significant number of patients.[172] Of these patients, 55% had a history of asthma and 45% presented with both asthma and allergic rhinitis. Food hypersensitivity was confirmed by DBPCFC in 205 (64%) patients and of those, 121 (59%) experienced respiratory signs ranging from nasal and laryngeal signs to wheezing in 34 (17%). Other studies have found similar prevalences.[173,174]

These studies suggest that ingested allergens circulate rapidly to the respiratory mucosa and activate mast cells. Cross-reactivity between epitopes on inhaled allergens and ingested allergens is relatively common amongst atopic individuals.[175] In other patients, inhalation of the food allergen is suspected as the inciting cause. In patients with the so-called "bird-egg" syndrome, patients have a concurrent egg yolk and bird feather hypersensitivity. In a study of 31 affected patients, a variety of signs resulting from contact with birds was reported ranging from rhinitis to severe dyspnoea and bronchospasm.[176] Symptoms following egg ingestion were usually confined to the skin but conjunctivitis and dyspnoea were seen in one patient. In this study it was demonstrated that the signs were predominantly due to sensitization to a 70kDa protein that was identified as chicken serum albumin, which is present in the inhaled dander and in egg yolk.

Given the high prevalence of atopic asthma in the human population it is perhaps not surprising that respiratory food hypersensitivity reactions should be so common. In Guilford's (1992) model of food hypersensitivity, one of the sensitized dogs developed a cough following oral challenge during a pilot study.[164] However, during the larger-scale investigations no signs of respiratory disease were observed and the cytological appearance

of aspirates collected by bronchoalveolar lavage after challenge were not statistically different. Baker (1974) reported laryngeal oedema and dyspnoea as being associated with food hypersensitivity.[7] Walton (1967) described acute asthmatic attacks in 2 of 82 dogs with food hypersensitivity.[177]

It has also been suggested by some authors that food hypersensitivity might play a role in some cases of feline allergic bronchitis.[178,179] Given that the clinicopathological findings in feline asthma are so similar to those in humans, it is interesting that definitive demonstrations of an association have not been published. This may be the result of the multifactorial nature of the disease, establishment of chronic inflammatory airway disease that is independent of inciting allergens, that such an association has not been carefully looked for or that the association does not exist. Hendrick (1981) reported persistent sneezing in 1 of 6 cats with eosinophilic enteritis.[180] However, whether that cat was truly affected with food hypersensitivity was unclear. It is certainly reasonable to imagine that systemically absorbed, intact allergens might interact with IgE bound to mast cells and other FcεR1 bearing cells resident in the respiratory tract of affected individuals.

Other Syndromes

A myriad of other clinical syndromes and signs has been attributed to food hypersensitivity in humans. Only sporadic and anecdotal reports of other clinical signs apparently associated with food hypersensitivity have appeared in the veterinary literature. Rosser (1993) reported two dogs with histories of seizures that had decreased seizure frequency during the feeding of the elimination diet and one dog had a seizure within 2 hours of challenge with its previous diet.[14]

1.7 Food Allergens

1.7.1 Definition

Allergens are by definition antigens that react with specific IgE antibodies and are capable of eliciting mast cell degranulation.[181] It is probable that most allergens are responsible for inducing primary sensitization and the T cell directed switch to IgE synthesis. However in some cases it is feasible that allergens are recognized as such because of their ability to cross-react with IgE antibodies generated in response to another antigen with some degree of structural homology.

1.7.2 Biochemistry

Foods contain an enormous variety of proteins, most of which would be considered antigenic and yet only a few have been shown to be allergenic. To date, characterization of the offending food allergens in hypersensitive veterinary patients has not been published. All of the information regarding the biochemical properties of food allergens therefore is derived from human or experimental studies. There are undoubtedly species differences in the recognition of antigens due to MHC restriction and the actual sites on an antigen that an individual will respond to will vary. For instance, whilst beef is the most common allergen in veterinary species, it is not a common cause of allergy amongst people living in North America despite it being a significant source of protein in their diets.[182] However, it is generally thought that the biochemical properties that make a particular substance an allergen are not species specific and that in general, significant homology might be preserved in the recognition of allergens.

Although the majority of the known food allergens are naturally occurring food proteins or glycoproteins, there is evidence that non-protein molecules can function as allergens. Certain carbohydrates, free of proteins, such as pneumococcal polysacharides and highly cross-linked dextran, have been demonstrated to induce allergic reactions in man.[183,184]

Mast cell degranulation requires simultaneous bridging of two IgE molecules on the mast cell membrane. This requirement for divalency places a minimum size limit on molecules that can stimulate IgE-mediated reactions. Most publications refer to this lower limit as being 10kDa although smaller peptides could act as haptens.[185,186] However, there is debate over the exact figure. As will be discussed below when protein hydrolysate diets are considered, the actual limit seems to be significantly smaller. In contrast, proteins of greater than 70kDa are unlikely to be efficiently absorbed intact through the enteric mucosa.

Food allergens can be considered separately from other allergens because of a number of unique features that they must possess namely: resistance to heat, acid treatment and enzymatic hydrolysis. The resistance of food allergens to degradation from low pH conditions has been demonstrated by Astwood et al (1996).[187] Amongst others, peanut allergen (Ara h 2), soy allergen (Gly m 1) and milk β-lactoglobulin show that in contrast with the non-allergenic proteins of the source foods, these substances are resistant to acid digestion. These features are necessary for allergens to reach the intestinal tract in an immunologically active form.

1.7.3 Foods

Whilst human patients have reportedly incriminated a multitude of food substances, additives, colourings and preservatives as being responsible for food allergies, double blind placebo controlled food challenge studies show that 80% of all food reactions are to a relatively small number of proteins. In children the most important are derived from egg, peanut, milk, soy and wheat, whereas in adults peanuts, fish, shellfish and tree nuts account for 85% of hypersensitivities.[140]

Analysis of veterinary publications in which the offending allergen has been identified through elimination and single ingredient challenge trials yields a total of 194 individual food hypersensitivities (Carolotti 1990, Guilford et al 2001, Rosser 1993, Paterson 1995, White 1989, Hirt 1998, Jeffers 1996, Mueller 1998, Reedy 1994, Elwood 1994).[9, 12, 14, 157, 159, 163,188, 189, 190,191] Since several of these animals were identified as having multiple food sensitivities, the actual number of animals is significantly less. The allergens are presented in Table 1.5.

The figures presented in Table 1.5 are consistent with the widely held belief that dietary allergens are usually dietary staples of affected animals. Accordingly, beef is identified as the most common allergen in almost all reports. Since most of the case series are from patients resident in North America, this is consistent with the feeding patterns there. The finding that dairy products are the second most commonly identified allergen is less easily explained given that they form a relatively small part of most animals' diets. It is possible that the allergen(s) responsible for some (or even all) of the beef hypersensitivities is/are present in dairy products. An obvious candidate would be bovine serum albumin. Another explanation is that there is cross-reactivity between proteins. A third possibility is that dairy

products are inherently more allergenic than other proteins. Indeed, whilst it might be tempting to conclude that the likelihood of allergenicity is correlated with the extent of exposure this is not always the case. The best illustration of this point is the prevalence of peanut hypersensitivity in North America where, whilst ubiquitous, it does not represent a quantitatively significant part of the protein intake there.

Table 1.5. Prevalence of Individual Food Allergens Amongst 194 Individual Canine and Feline Hypersensitivites.

Allergen source	Number of reported sensitivities (%)
Beef	59 (30.4%)
Dairy / Milk	29 (14.9%)
Chicken	19 (9.8%)
Egg	15 (7.7%)
Soya	14 (7.2%)
Wheat	12 (6.2%)
Wheat Gluten	9 (4.6%)
Cereal	8 (4.1%)
Corn Gluten	4 (2.1%)
Fish	3 (1.5%)
Corn	3 (1.5%)
Rice	3 (1.5%)
Tuna	2 (1%)
Pork	2 (1%)
Sardines	1 (0.5%)
Viscera	1 (0.5%)

1.8 Diagnosis

An adverse reaction to food is suspected when there is a temporal association between the ingestion of a food and the onset of clinical signs. However, even when an obvious temporal association is not identified on presentation, this alone cannot be the basis for exclusion of consideration of food hypersensitivity in patients presenting with appropriate clinical signs. It is often the case therefore, that an investigation into the presence or

absence of food hypersensitivity is initiated because such a diagnosis appears on the list of differentials for chronic gastrointestinal or dermatological diseases.

It is also important to distinguish between the diagnosis of an adverse reaction to food and the diagnosis of food hypersensitivity. Given that in order to diagnose the latter involves demonstration of an immunological basis to the clinical signs and the difficulty associated with doing so, it is not surprising that a convincing diagnosis of food hypersensitivity in veterinary patients is rarely made.

1.8.1 History

The value of a medical history is dependent on the accuracy and depth of recollection of the animal's owner in addition to the ability of the clinician to extract the pertinent information. The points that should be established whenever possible include:

- The food suspected of provoking the reaction
- The length of time between ingestion and the onset of clinical signs
- The exact nature and repeatability of clinical signs
- Dietary history including all significant protein sources fed during the disease period and preferably at least 6 months prior to the onset.

As highlighted above, the proteins responsible for dietary hypersensitivity are usually quantitatively significant components of the staple diet, which is helpful in generating a list of likely candidates for dietary allergens.[9,12]

To assist identification and evaluation of food hypersensitive patients, a number of principles should be kept in mind when assessing the patient history. These are presented in Table 1.6. Whilst the usefulness of these principles has not been established in veterinary investigations, they are consistent with current understanding and conventional wisdom.

Table 1.6. General guidelines to consider when evaluating potential food hypersensitivity

Patient history is notoriously inaccurate.
Food allergy may be more common in young animals, especially with atopic dermatitis.
Relatively few foods are responsible for the vast majority of allergic reactions.
Food hypersensitivity to more than one ingredient may be relatively common, especially in cats.
True food hypersensitivity generally involves "classical" signs affecting the skin or gastrointestinal tract.
Pruritus is almost a required sign for food hypersensitivity associated dermatoses.
The timing of food ingestion and vomiting may range from minutes to greater than 12 hours.
The nature of diarrhoea associated with food hypersensitivity is variable. A significant number of food hypersensitive patients have signs of only large bowel disease.
The finding of both dermatological signs and gastrointestinal signs is strongly suggestive of food hypersensitivity.
Episodic signs are seen in a significant number of individuals.

1.8.2 Physical Examination

There are no pathognomonic features that distinguish food hypersensitivity from other diseases in a physical examination. As discussed above, the range of dermatological signs described are broad and overlap significantly with those of other allergic, parasitic and bacterial dermatoses. In animals presented with gastrointestinal signs, physical examination might reveal evidence of chronic intestinal disease such as weight loss or a dull coat but mostly a physical examination is unremarkable.

1.8.3 Elimination Diets/Novel Proteins

The initial step in the diagnosis of food hypersensitivity is the conduct of a carefully designed dietary elimination-challenge trial. This requires removal of the previous diet and introduction of a novel protein source. The identification of what is truly a novel protein for any given individual is entirely dependent on the accuracy and extent of the dietary history obtained. Inappropriate use of a previously fed protein would be expected to result in no improvement if the animal is sensitized to that protein and would lead to the incorrect exclusion of food hypersensitivity. Given the relative frequency of sensitization to multiple foods, this may be a major factor in diagnostic failure in veterinary practice.[12]

It is clear that the use of diets that include single protein and carbohydrate sources is preferable to multiple protein or carbohydrate sources. This has lead to the term "select protein diets" to identify those that have been formulated with use as an exclusion diet in mind. It has been suggested that home-prepared diets are superior to commercially prepared select protein diets on the basis of assured antigenic purity.[19] An often quoted example of this is the finding of Rosser (1993) that following successful elimination challenge trials using a home cooked lamb and rice diet, introduction of a commercial lamb and rice diet resulted in recrudesence of pruritus in 4 (14%) cases.[14] Seven further cases developed vomiting and/or diarrhoea. Whilst the gastrointestinal signs could feasibly be the result of food intolerance rather than hypersensitivity, the recrudescence of the original presenting clinical sign (pruritus) is convincing evidence for hypersensitivity to the commercial diet. There are other reports of animals being tolerant to a home-cooked fresh protein source but being hypersensitive to a commercial select protein made from the same protein.[15] White (1986) proposed that leaching of metals from the can into the food might lead to a metal

hypersensitivity but this has not been tested. More likely as explanations are the presence of unknown antigens (e.g. more than one protein source) and altered antigenicity from cooking (e.g. Maillard compounds, exposure of hidden epitopes or development of "neo-antigens"). However, it has long been known that metal ions such as Ni^{2+}, Co^{2+}, Cu^{2+} and Cr^{3+}, are haptens with a high immunogenic potential.[192] Up to 10% of the Caucasian population are reported to be affected by Ni^{2+}- contact sensitivity resulting in a delayed-type hypersensitivity and *in vitro* stimulation of PBMC from sensitized individuals by metal ions leads to strong proliferative responses.[192] It is interesting to consider the possibility that prolonged exposure to metal ions from canned food might play a role in the genesis or exacerbation of delayed intestinal hypersensitivity reactions.

In a survey of veterinarians in the American Academy of Veterinary Dermatologists in 1992, homemade diets were recommended as the initial elimination diet more often than commercial diets.[193] However, 90% of those diets recommended were determined by the authors to be nutritionally inadequate for adult maintenance. If an elimination diet is to be fed for several weeks, nutritional deficiencies might become clinically significant.

The advantages of using a commercially prepared select protein diet are: convenience, nutritional completeness and balance, high digestibility and competitive costs relative to home-prepared diets.

Recently, the use of the term "hypoallergenic" has been introduced by pet-food manufacturers to describe certain select protein diets. This claim is almost never accompanied by any evidence to suggest that indeed the diet is hypoallergenic. More often,

such dietary proteins are non-traditional but intact proteins sources such as duck, venison, rabbit, lamb and fish. However, what may be novel and therefore non-allergenic to one individual may be the sole offending protein for another individual. It is suggested therefore that the use of the term "hypoallergenic" be reserved for diets where there is *in vitro* or *in vivo* evidence of reduced ability by the food to participate in immune-mediated reactions. Only elemental or hydrolysate diets can genuinely make this claim.

The length of time an elimination diet needs to be fed before dietary hypersensitivity can be excluded is controversial. Rosser (1993) found that only 13 of 51 dogs (25%) showed partial or complete improvement within 3 weeks of commencing the elimination trial.[14] Ten weeks of feeding an elimination diet was required in 3 out of 51 dogs subsequently demonstrated to have food hypersensitive dermatitis before an improvement was noted. However, Rosser describes that 18 of the 51 dogs were also diagnosed with additional hypersensitivities (eg. atopy or flea-allergic dermatitis) but does not detail if any or all of the 13 aforementioned dogs were amongst those. Rosser (1993) concluded that elimination diets should be fed for at least 10 weeks but it is not clear from the data that such a need has been established. In a series of 20 dogs diagnosed with food hypersensitivity, Paterson (1995) reported that 11 of the 20 dogs responded within 4 weeks.[157] In the remaining 9 dogs, responses took as long as 8 weeks to be significant. However, in 8 of these 9 dogs, concurrent atopic dermatitis was diagnosed on the basis of intradermal skin testing.

In 14 cats with food hypersensitivity, White and Sequoia (1989) found that all 14 cases demonstrated a reduction of pruritus by 80-90% within 3 weeks.[159] In concordance with these findings, Jeffers et al (1991) studied a population of 13 food hypersensitive dogs and

found that all 13 dogs demonstrated at least a 50% reduction in pruritus within 3 weeks.[160] Harvey (1993) reported 25 dogs with food hypersensitive dermatitis.[161] All 25 of those cases responded within 3 weeks of the elimination diet. In a further study of 43 cases of food hypersensitive dermatitis in dogs and cats by Carlotti (1990), all but 8 dogs responded within 3 weeks.[9] Of the remaining 8 dogs, 7 responded within 5 weeks and one within 8 weeks. However, the endpoint for success was defined as complete cessation of pruritus and significant and convincing responses may have allowed identification of food hypersensitive cases prior to that point.

It is important to note that none of these publications describes the exclusion criteria for other cases presenting with similar signs shown not to have food hypersensitivity. It is therefore possible that truly hypersensitive individuals that could have been identified by more prolonged elimination trials were not included. In addition, it is not recorded whether the elimination period was conducted in the veterinary hospital or at home. This is important because of the difficulties in avoiding accidental ingestion of food substances (other than the prescribed diet) that might lead a misleading prolongation of clinical signs. It is then impossible after reading these publications, to conclude with certainty the required duration of the elimination diet.

In contrast, Guilford et al (2001) evaluated 70 cats with chronic gastrointestinal complaints but excluded 15 cases during the diagnostic work-up.[12] Of the remaining 55 cats, 16 cases of food sensitivity were confirmed. Of those cats, all responded completely within 7 days of the elimination trial. Elimination trials were continued for a total of 4 weeks but no further cases

were identified after those that responded within the first week and were deemed to be not food sensitive.

In summary then, it seems very likely that the significant majority of food hypersensitive individuals will demonstrate a significant improvement on an appropriate elimination diet within 3 weeks. This is especially the case for animals presenting with gastrointestinal signs. In rare cases, extension of the diet to a period of 10 weeks might be necessary but this has still to be clearly determined. Reasons for the requirement of extended elimination diets in some individuals is not known but may be due to mechanisms other than type I hypersensitivity since the half-life of IgE in tissues is not that long. Another explanation is the role that dentritic cells might play in the prolonged retention of antigen in Peyer's patches (see above). Recently it has been reported that intestinal plasma cells in humans responsible for humoral immunity, may have a life-span of more than 1 year.[194] This has been proposed as a mechanism for sustaining persistent antibody responses and may explain the persistence of clinical signs in some animals.

1.8.4 Hydrolysates

The problems associated with identifying a truly novel protein are to a large degree avoided when the protein in the diet has been hydrolysed, thus destroying the antigenic determinants. Furthermore, the feeding of a hydrolysate would be expected to avoid sensitization to the novel protein were there to be an underlying persistent defect in the establishment of oral tolerance in the individual being studied. The production and suggested uses of protein hydrolysates are discussed in detail below.

1.8.5 Food Challenge Trials

Once an improvement has been noted on an elimination diet, confirmation of the dietary association is required. This involves reintroduction of the original diet, either in its entirety or sequentially as individual ingredients to identify the offending substance. Most authors recommend that the initial challenge consist of the entire diet to avoid unnecessary challenge trials in an animal that is not actually food hypersensitive. The importance of this was emphasized by Guilford et al (2001).[12] They demonstrated that in addition to 16 truly food sensitive cats that recrudesced on challenge trials, the signs of 11 cats also resolved on the elimination diet but failed to recrudesce on subsequent challenge. If the diagnosis of food sensitivity had relied solely on a response to the elimination diet, these cats would have incorrectly been diagnosed as food sensitive.

Recrudescence of clinical signs is usually rapid and the time needed for challenge feeding is less debated than that for elimination. It is generally agreed that for both gastrointestinal and dermatological signs, convincing recrudescence occurs within 14 days of challenge with the great majority responding within 7 days.[12, 15, 159, 9, 160, 162, 161] However, Hirt and Iben (1998) in a study of food hypersensitivity in 8 related cats found that it took 8 weeks on the original diet for the vomiting that they had originally presented with to recrudesce.[163] All of the cats had various gastric, duodenal or gastroduodenal inflammatory infiltrates which is not a consistent finding in type I mediated food hypersensitivity.[6] It is speculated therefore that the pathogenesis of the immune-mediated reaction may have been a delayed type IV, type IVa_2 or mixed hypersensitivity. On the basis of this study alone, it is conceivable that prolonged challenge trials are required to confirm food hypersensitivity in some animals.

Food challenges can be performed openly (i.e. the owner and the veterinarian are aware of the challenge ingredients), single-blind (the owner is ignorant but the veterinarian is still aware) or double-blind (both the owner and the veterinarian are ignorant of the challenge ingredients). The gold standard for the diagnosis of food hypersensitivity in humans is double blind placebo-controlled food challenge (DBPCFC).[140] In these studies, the challenge ingredient is placed into gelatin capsules and ideally fed in association with an elemental diet. Several studies in the human literature comparing the predictive value of open and single blind challenges to DBPCFC highlight the need for DBPCFC.[195] Whilst no such studies have yet been performed in veterinary patients and owners, it is likely that the situation is similar, especially given the significant difference between results obtained by single blind and DBPCFC. Whereas some authors lament their absence from food hypersensensitivity investigations,[196] others claim that their use is unnecessary.[154] Since there is no experimental evidence to suggest that veterinary investigations are immune to bias, this author does not share the latter view.

Having said that, open challenge trials are certainly the method most commonly employed in veterinary practice. Either way, the usefulness of any elimination-challenge trial relies not just on owner compliance and veterinary objectivity but the availability and reliability in providing a novel or truly hypoallergenic diet.

Recommendations vary as to the time required for the "wash-out" period on a novel protein source between challenges, ranging from 3 days[6] to 14 days,[154] Simply, the time between challenges should be sufficient to return the animal to its pre-challenge stage.

Selection of foods to be tested by oral challenge is determined mainly by diet history but may be influenced by findings of *in vitro* or *in vivo* tests. Again, recommendations as to the way in which the challenge food is fed vary from feeding a small amount of the test diet in conjunction with the elimination diet[196] to feeding solely the test ingredient during the challenge period.[154,197] Given the theoretical concerns about sensitization developing to proteins fed in tandem with the offending allergen as a result of the resulting changes to mucosal tolerance, the latter recommendation appears more worthy.

Difficulties, failures and misleading results are relatively commonplace when feeding trials are conducted. Possible reasons for poor results include:

- Poor compliance with the prescribed diet (owner or patient).
- Accidental ingestion of non-prescription food stuffs ("dining out")
- "Hidden" protein sources (hide chews, flavouring agents in vitamin or anthelmintic treatments)
- Concurrent unresolved diseases (atopic dermatitis, flea allergic dermatitis, pyoderma, inflammatory bowel diseases)

1.8.6 Other Tests for Food Hypersensitivity

Because veterinarians are confronted with a relatively high number of food hypersensitivity candidates and oral elimination-challenge tests have a number of difficulties, it has been tempting to look for more immediate and objective assays. Even if improvement and recrudescence are demonstrated following elimination and re-challenge of the staple diet, the

time and commitment to individual protein challenge testing may seem overwhelming. The possibility of screening an individual for reactivity to a large number of foods by an *in vitro* or *in vivo* assay seems very attractive.

Skin Tests

The basic principle of skin testing is to introduce a small amount of a well-characterized allergen extract into the epidermis. In skin prick tests (SPT), this is achieved by pricking the skin without drawing blood, with an instrument coated with the allergen extract (e.g. with a fine hypodermic needle). This introduces a very small amount of extract into the superficial dermis. Alternatively, intradermal skin testing (IDST) can be performed whereby 0.02 to 0.05ml of the extract is injected into the dermis. Whichever method is employed, if allergen-specific IgE is present and bound to resident mast cells, the allergen will cross-link the IgE molecules triggering degranulation and producing a visible wheal. Negative and positive controls are similarly introduced. Intuitively, it would be expected that the sensitivity of these assays would be greatest for those patients in which dermatological signs are present.

The value of skin testing in evaluating veterinary patients for food hypersensitivity is generally reported to be low[7,19,20,198] but has been critically assessed only a small number of times. Jeffers et al (1991) evaluated 13 food hypersensitive dogs using IDSTs and determined the sensitivity to be 10%, specificity to be 95%, positive predictive value to be 60% and negative predictive value to be 62%.[160] These investigators used a commercial source for the intradermal extracts and compared the results with open challenge trials. Kunkle and Horner (1992) evaluated 28 dogs with chronic pruritic dermatitis with an

elimination diet and staple diet challenge and identified 9 dogs with food sensitivity.[156] The authors concluded that the sensitivity and specificity for IDST were 33% and 50.5% respectively. However, no attempt was made to identify through specific challenge trials the offending allergen(s) in 8 of those dogs. In light of the lack of elimination-challenge trials, it is hard to assess the validity of the findings from this study. Despite that, their study has been touted as evidence against the use of IDSTs and SPTs.[20]

In Guilford's experimental model of food hypersensitivity, IDSTs were found to have a sensitivity for pruritus, vomiting and diarrhoea of 77, 100 and 80% respectively and a specificity of 25, 29 and 30%. For SPTs the sensitivity for pruritus, vomiting and diarrhoea was 30, 50 and 33% whereas the specificities were 58, 67 and 60%.[164]

In humans, SPTs are highly reproducible and utilized to elicit supportive screening evidence of suspected IgE-mediated food allergies.[195] However, a positive SPT denotes only the presence of allergen-specific IgE bound to mast cells, it does not mean that the patient will develop signs when ingesting the food. Vanto et al (1999) evaluated 301 infants suspected to be milk hypersensitive.[199] SPTs showed a specificity of 91%, a sensitivity of 69%, a positive predictive value of 79% and a negative predictive value of 85% when compared to results of DBPCFCs. In another study of 143 infants evaluated for milk hypersensitivity, SPTs were found to have a sensitivity of only 14%, a specificity of 98%, a positive predictive value of 91% and a negative predictive value of 51%.[200] In this study, skin patch testing was more sensitive but less specific, whereas in the former, patch testing was both less specific and less sensitive. In an evaluation for wheat hypersensitivity in 39 infants, SPTs had a sensitivity of 23%, a specificity of 99%, a positive predictive value of 99% and a negative predictive value

of 50%.[201] In all three of these studies, patch testing varied from being more sensitive and less specific, to less sensitive and more specific.

These results contrast with the findings of Sampson and Albergo (1984) [202] and Bock and Atkins (1990).[203] These authors concluded that in general, the positive predictive values of SPTs are less than 50% whereas negative SPT responses virtually exclude IgE mediated reactions with a negative predictive value of greater than 95%.

Given the wide variation in predictive values published by different investigators and for different allergens in the human literature, it is reasonable to suggest the same might be true in veterinary allergology. In order for an IDST or SPT to be specific, the test allergen must be identical to the ingested allergen. Given that the veterinary publications reporting on SPTs and IDSTs used commercially prepared allergens, it is conceivable that different results would have been established had fresh solutions been made from the actual diet constituents being fed. Some allergens may exist as haptens, some may require modification during cooking or manufacturing (e.g. maillard compounds) and some allergens may be denatured during production of the test solution. Furthermore, the optimum concentration and method of preparation for test solutions has yet to be determined. The use of serial titrations of the antigen has been recommended to determine the optimum concentration.[204] It has been suggested that use of too much antigen will result in inhibition of IgE cross-linking and decreased mast cell degranulation. It may be that the strength of the skin test reaction at a standard dilution is not as important as the determination of the dilution limit at which a reaction still occurs.

For these reasons and in light of the precedents in human allergology, it may be premature to conclude that SPTs and IDSTs are of no value in evaluating veterinary patients. Careful evaluation of individuals where convincing elimination-challenge trials have identified the offending allergen (or source food substance) and use of fresh preparations from that source will clarify the role that skin testing can play in the evaluation of food hypersensitivity.

Serum IgE

A similar situation exists for the use of serum food-specific IgE in veterinary patients to that of IDSTs and SPTs. Most authors report that evaluation of serum IgE is no value in the investigation of food hypersensitivity.[19,20,198] However, Blackmore (1994) is more enthusiastic and reports that he encourages the assays.[196] Jeffers et al (1991) determined the sensitivity of an anti-IgE ELISA to be 23.8%, specificity to be 86.6% with a positive predictive value of 40% and a negative predictive value of 60.9%.[160] In contrast, Mueller and Tsohalis (1998) evaluated 8 dogs with elimination-challenge diagnosed food hypersensitivity. 6 of the 8 were found to be hypersensitive to beef alone, one was hypersensitive to beef and "dairy" and one to chicken.[189] In none of the dogs was food-specific IgE identified whereas in the control group of 8 non-food hypersensitive dogs, 3 mild positive reactions were identified. Given the high proportion of beef allergic dogs in this study and the false beef-positive findings in the control group, the possibility of reagent or test allergen deficiencies has to be entertained. To further cloud interpretation of the study findings, chicken-specific IgE was not evaluated in any of the dogs, including the chicken hypersensitive dog. Zunic (1998) evaluated 48 cases of allergic dermatitis of which 12 were found to be hypersensitive to beef on the basis of elimination and challenge trials.[205] Of those dogs, only 2 demonstrated weak positive beef-specific IgE using a commercial immunodot strip test.

Guilford et al (2001) found that 6 out of 24 cats that were shown not to have a food hypersensitivity had food specific IgE greater than twice that of the negative control serum (defined as a positive result).[12] Of the 12 food sensitive cats, 7 had positive results. Of those 7 cats, 3 had positive results to antigens they were not clinically hypersensitive to. However, this population was selected on the basis of gastrointestinal signs and none of the cats were reported to have had dermatological signs. In Guilford's experimental model of food hypersensitivity in dogs, ELISA determination of serum IgE had a sensitivity for pruritus, vomiting and diarrhoea of 62, 50 and 60% and a specificity of 42, 38 and 40%.[164]

In another experimental model of food hypersensitivity using 14 atopic spaniel/basenji-type puppies with a genetic predisposition for atopic diseases, Ermel et al (1997) evaluated serum IgE using an enzyme-labeled immunodot assay.[204] Following sensitization these investigators recorded a high sensitivity for the IgE assay but did not attempt to assess specificity.

So whilst the exact predictive values for serum IgE assays remains obscure, it can be concluded that because of the apparently high incidence of asymptomatic sensitization, a positive IgE assay is not evidence for clinical food hypersensitivity in the absence of a positive elimination-challenge trial. It is generally accepted that for the validation of laboratory assays such as IgE measurement, positive and negative controls are required. Given that canine or feline, food-specific IgE antibodies are unavailable, positive controls are not utilized. This is unfortunate and impacts on the conclusions that can be drawn from

these studies. As for skin testing, the role for serum food-specific IgE in the diagnosis of type I food hypersensitivity has yet to be fully determined.

Gastroscopic Food Sensitivity Testing

Gastroscopic food sensitivity testing (GFST) is a procedure that involves the application of a small volume of a food extract solution onto or into the gastric mucosa during gastroscopy. The area of mucosa is then observed for erythema, blanching, oedema, petechiation. In some cases, systemic signs such as bronchospasm, hyperventilation, urticaria and hypotension may be identified. The use of GFST has been evaluated in both dogs and cats. In Guilford's model of food hypersensitivity in dogs, GSFT was found to be highly specific for vomiting and diarrhoea (95 and 100% respectively) but poorly sensitive.[164] Elwood et al (1994) evaluated GFST in 7 food hypersensitive dogs and described the technique as specific but of limited sensitivity.[191]

Using 6 spaniel/basenji-type atopic and food sensitive dogs, Guilford et al (1994) investigated the diagnostic accuracy of GFST in elimination-challenge proven cases of food hypersensitivity.[206] As long as the dogs were evaluated following a 4-week period of feeding on the elimination diet, the sensitivity was 100%, the specificity was 81% with a positive predictive value of 50% but a negative predictive value of 100%. The effect of the feeding period on the elimination diet on the results was suggested to be due to an increase in tissue histamine content during the period. This seems a plausible explanation since repeated allergen exposure would be expected to exhaust mast cell reserves over time. The diagnostic value was poor if the GFST was performed prior to elimination diet feeding.

These findings have great significance for future investigations and suggest that performing a GFST without a period of feeding on an elimination diet is of questionable value.

Guilford et al (2001) investigated GFST in 70 cats with chronic gastrointestinal diseases.[12] Following a 3-day period off their staple diet, a variety of food proteins and additives were evaluated but resulted in only one reaction in a cat shown not to be hypersensitive to the test antigen. Possible reasons for this difference between cats and dogs were suggested to be differences in gastric mucosal permeability, different mast cell behaviour and a difference in the pathogenesis behind the hypersensitive reaction.

Thus, GFST shows some promise in the evaluation of dogs for food hypersensitivity with a possible role in identifying antigens to be excluded from the patients diet. However, since the sensitivity is greatest following a period on a successful elimination diet and since it is an expensive procedure to consider at times other than during gastroduodenoscopy for other diagnostic purposes, its use is currently limited.

Role of Testing for Food Hypersensitivity

It is important to distinguish between the role that elimination-challenge trials play and that of other tests in the evaluation of patients with suspected food hypersensitivity. Since none of the tests described above has sufficient sensitivity and specificity to diagnose food hypersensitivity, they cannot be used as screening tests for such. Their place should be confined to patient(s) with an adverse reaction to food, which has been established through elimination-challenge trial(s), to better define the nature of that reaction. Only elimination-

challenge trials can determine the presence of an adverse reaction to food. As has been discussed above, defining the nature of that reaction, and in particular distinguishing between hypersensitivity and intolerance, using the fore-mentioned tests is neither easy nor certain and may not be clinically relevant

1.9 Management of Food Hypersensitivity

1.9.1 Avoidance

The success of avoidance of a food allergen is initially dependent on finding a novel protein source. As previously mentioned, in the absence of demonstrated hypoallergenicity, all diets should be considered potentially allergenic and selection is based upon a thorough diet history. Practically, once a diagnosis of food hypersensitivity has been made from elimination and previous diet challenge, owners and veterinarians will often elect to maintain feeding with the elimination diet rather than pursue individual food substance challenges. This has the immediate appeal of simplicity but has the obvious disadvantage of limiting the patient's diet to one food resulting in unnecessary avoidance.

Whilst there may be some advantages to the feeding of a home cooked elimination diet in the diagnosis of food hypersensitivity, the use of commercial diets are more attractive in the long term. Reasons include owner convenience, compliance and the concern regarding the frequency of nutritional deficiencies with home-cooked diets.[193]

1.9.2 Sacrificial Proteins

There are few reports in the veterinary literature of neo-sensitization to newly introduced novel proteins.[7] Reedy (1994) described a cat with clinical evidence of a food hypersensitivity based on a response to an elimination diet although no challenge trials were performed initially.[190] Following a 2 year period of remission on the elimination diet the clinical signs reoccurred (pruritus, crusting dermatitis) and subsequently resolved on a new elimination diet. Subsequent recrudescence was demonstrated on reintroduction of the original elimination diet. Other investigators have observed similar findings (Guilford unpublished personal communication).

As discussed above, there is strong theoretical and some experimental evidence to suggest that sensitization to dietary proteins occurs in the context of gastrointestinal inflammation of a variety of causes. In some cases of food hypersensitivity, significant mucosal inflammation can be present.[12,163] It is reasonable to consider the possibility that novel proteins introduced into such a milieu will have only a transiently beneficial effect due to eventual sensitization. Patients with an underlying defect in oral tolerance, especially those predisposed to the development of type I hypersensitivities (i.e. atopics), would be considered at risk for the development of further dietary sensitization. This has been suggested in cases of inflammatory bowel disease and has lead to the concept of "sacrificial proteins".[169] Using this concept, the first novel protein fed during the initial treatment phase is discarded after a period of time proposed to be long enough for the resolution of the inflammatory changes. Subsequently, a second novel protein is fed and the first is avoided in the future for fear of sensitization. It is still unclear how valuable this dietary approach is although it has significant theoretical appeal.

In patients in which rapid resensitization is suspected, the use of a rotation diet is recommended. In these patients, successive novel or non-allergen (established following challenge trials) proteins are fed. The period of feeding for each protein source is planned to be shorter than the period required for sensitization to the diet. Establishing how long that period is for any given patient is likely to be based on trial and error. The risk of developing multiple dietary sensitivities in such a scenario is unknown. Clearly, the use of a truly hypoallergenic diet such as a hydrolysate diet to which sensitization is unlikely or impossible would negate the need for this approach.

The requirement for life-long avoidance in veterinary patients has not been established. In human allergology, it is well established that the majority of food hypersensitivities are transient and will resolve following a sufficient period of elimination.[140] It is not well understood why the re-establishment of tolerance should be delayed. Retention of activated memory B and T cells within the lamina propria and Peyer's patches probably accounts for part of the phenomenon. However, it is feasible that rapid re-development of tolerance following withdrawal of the allergen might develop in some cases. As discussed above, the diagnosis of food hypersensitivity is dependent on the demonstration of improvement following elimination in association with deterioration following re-introduction of the staple diet. Guilford et al (2001) found that the clinical signs of 14 of 70 cats presented for evaluation of chronic gastrointestinal disease resolved on the elimination diet but did not recrudesce on challenge with the staple diet.[12] These were diagnosed as not being food sensitive. Whilst some of these cats may not have been challenged with the offending food substance due to an incomplete diet history, it is interesting to conjecture that some of the

cats may have rapidly re-established oral tolerance. Either way, it is possible that some of those cases did indeed have a food hypersensitivity.

1.9.3 Pharmacological Therapy

Without doubt, the majority of animals with food hypersensitivity will respond completely to the elimination of the offending allergen from the diet. In addition, distinction should be made between those animals where food hypersensitivity is the only etiopathogenesis and those animals with other concurrent diseases (e.g. atopic dermatitis, flea allergy dermatitis, pyoderma). In animals with concurrent diseases, pharmacological intervention may be required to completely normalize the animal. It is generally accepted that pharmacological therapy is indicated in all moderate-to-severe cases of inflammatory bowel disease as an adjunct to dietary therapy.[169] However, in animals with dietary hypersensitivity in which the associated clinical signs are dermatological in nature, the role of pharmaceuticals is less clear. The use of some form of antipruritic therapy to provide initial relief during an elimination trial has been suggested for severely affected individuals.[198]

Corticosteroids

Traditionally, the dermatological signs associated with food hypersensitivity have been thought of as being poorly responsive to corticosteroids.[19,154] Despite this, the use of corticosteroids in the management of food hypersensitivity has been suggested for those cases where identification of a suitable diet is not possible or for initial symptomatic relief of pruritus.[198,149] Harvey (1993) reported on the response to oral prednisone (0.5 to 1.1mg/kg daily) as being complete in 12 and partial in 6 of 25 dogs with pruritus associated with food hypersensitivity.[161] Rosser (1993) similarly found that the pruritus associated with food hypersensitivity responded completely in18 of 51 dogs, partially in 20 dogs and was non-

responsive in the remaining dogs to prednisone.[14] Doses used were 0.5mg/kg once to twice daily. The use of corticosteroids for cases of food hypersensitivity where gastrointestinal signs are prominent has not been critically evaluated. Whether or not corticosteroids are efficacious in the short or long term for food hypersensitivity, their long-term use cannot be considered optimal.

H1 blockers

Similarly to corticosteroids, H1-blockers have been suggested for the initial symptomatic relief of pruritus.[198] However, they are reported to be generally ineffective for the prevention of gastrointestinal signs,[149] suggesting either the presence of mast cell functional heterogenicity or the relative unimportance of histamine in the pathogenesis of clinical signs.

Sodium Cromoglycate

The use of the mast cell stabilizer sodium chromoglycate has been extensively studied in humans for the management of food hypersensitivity but results are conflicting.[195] In one DBPCFC cross-over trial in 10 children with atopic dermatitis and food hypersensitivity, oral cromoglycate failed to influence the development of food associated pruritus or the results of DBPCFC.[207] Limited experiences of sodium cromoglycate in veterinary patients suggests that there is no improvement in gastrointestinal manifestations of food hypersensitivity.[6] In rats, cromoglycate has been shown to be effective in preventing histamine release from cutaneous mast cells but not intestinal mucosal mast cells.[208] It is likely that this is part, if not all of the explanation for the poor responsiveness of food hypersensitivity to the oral use of cromoglycate.

1.9.4 Hyposensitization

Hyposensitization therapy consists of the repeated administration of gradually increasing doses of the offending allergen, usually parenterally, in an attempt to reduce sensitivity to the allergen. Hyposensitization therapy has broad-ranging effects including increases in IgG4 concentrations (the so-called "blocking antibody"), decreased T cell IL-4:IFN-γ ratios (indicating a shift from a Th2 dominated response to a predominantly Th1 response), T cell anergy and deletion, decreases in mast cells and mast-cell associated mediators and resolution of tissue eosinophilia.[209,210] The main indications for hyposensitisation in the management of food hypersensitivity have been suggested as being:[211]

1. The hypersensitivity reaction may be so severe that the risk of exposure to the allergen and the subsequent development of anaphylactic reactions warrant hyposensitisation.
2. The food in question may be so common and unavoidable within the patients environment that exposure is unavoidable.
3. A very small number of patients are sensitized to such a wide range of commonly accessible proteins that it is difficult or unreasonably expensive for alternative diets to be prescribed.

However, very few placebo-controlled clinical trials have been carried out. Efficacy still remains unknown and deaths have occurred during therapy.[212] Experience with immunotherapy for food hypersensitivity in animals is extremely limited and anecdotal. For

these reasons, hyposensitisation cannot be recommended at present as a reliable alternative to dietary management.

1.9.5 Probiotics

Probiotics are defined as microbial food supplements consisting of cell preparations or cell components that beneficially influence the host by altering the intestinal microbial balance.[213] The influence of luminal bacteria on the development of oral tolerance has been discussed above. Recently, their effectiveness in modulating food hypersensitivity responses has been described. In a double-blind cross-over study in 8 milk hypersensitive and 7 normal subjects, the effect of feeding probiotic bacteria on subsequent milk induced mucosal responses was studied.[214] These investigators found that feeding of milk in hypersensitive individuals increased the expression of receptors for complement, IgG and IgA on splenic neutrophils and mononuclear cells. This effect was prevented by the feeding of *Lactobacillus GG*. In contrast, in normal subjects the feeding of *Lactobacillus GG* resulted in increased expression demonstrating that probiotic bacteria can modulate the immune responses to dietary proteins in healthy and hypersensitive individuals.

Of particular interest are the findings of Majaama et al (1997) from their evaluation of infants with atopic dermatitis and hypersensitivity to cow's milk.[215] They found that infants given an extensively hydrolyzed whey formula supplemented with *Lactobacillus GG* experienced a significant improvement in clinical signs in comparison to controls fed the hydrolyzed formula alone.

To date, there are no publications detailing the use of probiotics in veterinary patients with food hypersensitivity. Should their use continue to show promise in human allergology, it is likely that their efficacy will be explored in veterinary patients.

1.10 Protein Hydrolysates

It is amongst the frustration of diagnosis and management of food hypersensitivity that the idea of feeding elemental and hydrolyzed formulae arose. Simply speaking, the theory behind such diets is to reduce the antigenic proteins to sufficiently small molecular fragments as to prevent recognition by the recipient's immune system. Biologically derived protein hydrolytic enzymes are used for the hydrolysis reaction, which occurs under specific and controlled physical conditions. The reduction in antigenicity is to a large degree dependent on the degree to which the hydrolysis proceeds, so disrupting the antigenic components.

The antigenicity of a protein is determined by its primary, secondary and tertiary structure. Reducing the antigenicity can be achieved by:

1. Disrupting the 3-dimensional structure of the protein
2. Altering the structure of amino acid side chains
3. Cleaving peptide bonds

Broadly speaking, the methods for producing a food product with reduced antigenicity fall into the categories of heat treatment, pH manipulation, hydrolysis and filtration.[216]

1.10.1 Heat Treatment

The effectiveness of heat treatment depends on the inherent lability of the protein to increased temperatures.[217] Amongst milk proteins for example, casein is relatively heat stable and can withstand temperatures of 130°C for over one hour without significant denaturation.[218] In contrast, whey proteins are much more heat-labile, denaturing at between 50°C and 80°C.[219]

Accordingly, in humans it has been shown that whilst heat treatment may reduce the number of whey protein antigens it has little effect on the casein antigens despite heating at 121°C for 15 minutes.[217] Thus, heat treatment used alone to reduce the allergenicity of a milk product is of no use in those individuals sensitized to the casein component.[220]

It can be assumed that there are a significant number of heat-stable allergens since many of the food allergies identified in domestic animals include reactions to protein components in commercial diets (dry or canned) which are subject to heat treatment during manufacturing. Alternatively it may be that some proteins increase in allergenicity with heat treatment. The effect of heat treatment is mostly to change the 3-dimensional conformation of the protein.[221] Whilst this may disrupt some allergens, it may equally uncover previously hidden allergenic determinants. Other reactions occurring at high temperatures include the Maillard reactions, which involve the reactions between certain amino acids and reducing sugars to produce compounds called melanoidins, which give a characteristic brown colour. Melanoidins can either be more or less allergenic than the original protein by acting as haptens or by reducing peptide absorption respectively.[222] These findings may explain some

of the observations pertaining to differences between home-prepared elimination diets and commercial diets.

1.10.2 pH Treatment

Alterations in the pH of the solution can be used to further reduce the antigenicity of a protein in addition to the conformational changes that occur at high temperatures. Even small adjustments in pH can alter ionic interactions between amino-acid side chains and result in conformational changes.[223]

In an alkaline environment at high temperatures the side chains of lysine, cysteine and phosphoserine can interact to form lysinoalanine and other related compounds disrupting any epitopes within which they may be contained.[217]

Most food allergens are usually quite resistant to moderate acid treatments, particularly those acid concentrations simulating stomach acid conditions.[185] For example the 65-kDa concanavalin A-reactive glycoprotein, one of the major peanut proteins allergenic to humans is stable at pH 2.8.[224]

In summary, there is potential for reducing antigenicity by combining heat and pH adjustments during production. However, heat treatment and pH adjustments alone cannot be relied upon to significantly reduce the allergenicity of parent compounds and as discussed, some reactions may actually increase allergenicity.

1.10.3 Hydrolysis

The most reliable way of reducing the antigenicity of a protein molecule is to directly cleave peptide bonds with the aim of reducing the molecule into smaller fragments. If the disruption occurs within an antigenic peptide sequence then it will be immunologically inactive. Alternatively, since antigenic determinants rely to some degree on the 3-dimensional structure of the peptide, disruption of the surrounding amino acids may lead to a sufficient change in 3-dimensional conformation that loss of antigenicity occurs.

Hydrolysis of proteins is achieved by using food grade proteolytic enzymes. The resultant hydrolysate varies in composition according to the composition of the parent compound, the specificity of the proteolytic enzymes chosen, the method by which the hydrolysis is conducted and any further processing of the resultant product.

Proteases are broadly categorised into either endopeptidases or exopeptidases according to the specificity of sites of action along the peptide chain. Endopeptidases lyse the protein at sites along the chain wherever the enzyme meets the amino acid configuration or sequence specific to its action. Exopeptidases attack the peptide chain from one end, again at specific sites. Table 1.7 lists some examples of food grade hydrolytic enzymes.

The selection of enzymes is therefore important since the specific site at which the particular enzymes act determines the likelihood of degradation of the particular epitopes responsible for the hypersensitivity reactions. Since the amino acid sequence and 3-dimensional structure of the individual epitopes are rarely known, trial and error with *in vitro* evaluation is usually the method by which a particular hydrolytic enzyme is selected.

Table 1.7. Food Grade Protein Hydrolytic Enzymes

Enzyme	Source
Bromelain	Pineapples
Ficin	Figs
Papain	Papaya
Fungal proteases	Aspergillus sp.
Bacterial proteases	Bacillus sp.
Pepsin	Porcine or other mammalian stomachs
Trypsin	Mammalian pancreas

Adapted from Yung-Hsiung Lee, Food-processing approaches to altering allergenic potential of milk based formula, Journal of Pediatrics Nov 1992, S47-50 [217]

1.10.4 Ultra-filtration

Hydrolysates usually contain residual amino acid sequences that were resistant to the hydrolysis along with traces of the enzymes used in the hydrolysis process. The hydrolysate will therefore contain a variety of fragments, which may range from single amino acids to large molecular weight polypeptides depending on the degree of hydrolysis. Removal of the larger fragments via physical separation or molecular filtration can have a significant influence on the "quality" of the finished product. Currently, ultra-filtration of the hydrolysate is the most widely used method to remove the large molecular weight fragments.

The size of the filter and the efficiency of the filtration process determine the success of ultra-filtration.

1.10.5 Problems with Hydrolysates

The most significant problem that manufacturers of hypoallergenic hydrolysate formulae face is persistent immunogenicity. Although a particular process may significantly reduce the allergenicity of the product, it does not abolish the risk of producing an immune-mediated reaction. In the initial stages of an enzymic hydrolysis, it is common for previously hidden antigenic sites to become exposed and for the product to increase in allergenicity, which is only reduced with further hydrolysis.[225] In extremely hypersensitive children, the reactions to hydrolysate formulae can be life threatening.

As the number of so-called hypoallergenic formulae appear on the market for the use in allergic human patients, so the number and range of reported hypersensitivity reactions, even anaphylaxis, increases.[226,227,228,229,230,231]

It has been shown that only very small amounts of intact allergenic epitopes are required to elicit significant and even fatal IgE mediated responses in sensitized individuals.[212] The best guarantee of producing a truly non-allergenic diet resides in the production of amino acid residue products. Unfortunately, the widespread use of elemental products is cost prohibitive. Furthermore, such products cannot be easily fed enterally due to their exceptionally high osmolarity.

Several other problems are often encountered once the reduction of the parent compound to small fragments has been achieved. Preserving palatability is difficult with the more extensively hydrolysed products.[232] Peptides and amino acids can exert many taste sensations. The sweet taste of the D-amino acids such as aspartamine has long been known.[233] However, bitterness offers the greatest hurdle to palatability. The bitter taste sensation of peptides is directly related to their hydrophobicity, which is in turn a product of their amino acid composition.[233,234]

Naturally occurring proteins usually exist with most of their hydrophobic residues such as leucine and phenylalanine concealed in the interior of their 3-dimensional structure [235] where they are unable to interact with the taste buds. When a protein is hydrolysed, the peptide fragments that contain hydrophobic side chains are exposed and can be tasted. Thus, as hydrolysis proceeds, bitterness tends to increase.[236] As the peptide fragments decrease in size to less than 1kDa or even free amino acids, bitterness declines.[233] This is because free amino acids do not interact well with the protein receptor responsible for the bitter taste sensation. Most bitter peptides are in the range of 500 to 5000Da.

From a product manufacturing perspective, protein hydrolysis creates challenges. Osmolarity increases significantly with increasing hydrolysis and diarrhoea is therefore not uncommon when extensively hydrolysed diets are fed. Moreover, small peptides cannot stabilize lipid droplets and therefore emulsions are difficult to achieve. The physical functional capacities of proteins, such as the emulsifying, foaming and gelling capacities can be improved by carefully controlled, limited hydrolysis.[217] Extensive hydrolysis, however, practically destroys all the functional capacities of proteins.

1.10.6 Nutritional Evaluation

With the recent flood of hydrolysate infant formulae on the human market, it has been recommended that the nutritional value of such formulae be demonstrated by several different means. Data on longitudinal growth, biochemical indices of protein metabolism and plasma amino acid profiles as well as metabolic balance studies have been recommended to demonstrate nutritional value.[237]

Reduced growth and various biochemical abnormalities have been observed in newborn human-infants fed from birth to one month with various hydrolysates when compared to breast fed controls.[238] Nitrogen absorption as well as nitrogen utilization appears to be reduced when compared to conventional formulas.[239] Alterations in calcium and phosphorus absorption and differences in the serum amino acid profiles can occur between those fed whey-protein and those fed a whey hydrolysate.[239]

Numerous studies comparing enteral amino acid or peptide absorption have shown that peptides are better absorbed than free amino acids.[240,241] Malliard compounds are less well absorbed and may further reduce amino acid availability. In summary, the digestibility and nutritional value of hydrolysates cannot be assumed to be the same as the parent protein. The main disadvantages of protein hydrolysates are given in Table 1.8.

Table 1.8. Disadvantages of Protein Hydrolysates

• Potential for persistent residual antigens • Potential for new antigens (inactivated enzymes) • Bitter taste • Higher osmolarity • No protein precipitation in the stomach ("hunger response") • Limited stability of emulsions • Altered biological value • Altered absorptive qualities

1.10.7 Immunological Evaluation

Non-clinical testing programmes provide manufacturers with the opportunity to characterize various molecular and immunologic properties of these hydrolysates and their corresponding final product forms. The antigenicity and allergenicity of a hydrolysate are partly but not wholly dependent on the molecular weight of the remaining peptides. As stated previously, the smaller the resulting fragments are, the less likely that residual epitopes will remain. Physicochemical analyses of hydrolsyates provide data relating to the extent of protein hydrolysis and peptide molecular weight distribution and allow manufacturers to characterize various hydrolsyates. This is often the starting point in selection of a candidate hydrolysate for inclusion in a hypoallergenic formula.[242] However, it is difficult to determine the limit under which the remaining peptide fragments are small enough to prevent immunological recognition.

As discussed above, for the initiation of type I hypersensitivity reactions, cross-linking of two or more IgE molecules bound by FcεR1 IgE receptors on the surface of mast cells is required. This establishes a minimum limit on the size of proteins that can act as allergens.

The most commonly quoted figure for this limit is 10kDa.[185,182] However, work by Van Beresteijn et al (1994) and more recently by van Hoeyveld et al (1998) suggests that the limit may be much smaller.[243,244] Van Beresteijn et al (1994) utilized a mouse-rat heterologous passive cutaneous anaphylaxis test and an anaphylactic shock test in mice with whey protein as the sensitizing antigen. Using two ultra-filtrated whey protein hydrolysates, one with a molecular cut-off of 5kDa and the other with a cut-off of 3kDa, demonstrated that the minimum molecular mass for peptides to provoke type I hypersensitivity reactions is between 3kDa and 5kDa. Van Hoeyveld et al (1998) demonstrated that for skin reactivity, peptides of greater than >1.4kDa are needed. The minimum molecular mass for simple IgE binding appeared to be somewhere between 1.4kDa and 0.97kDa.

Ensuring that a hydrolysate has no peptides greater than 3kDa or even 1kDa would ensure the greatest chance of eliminating any residual allergens. However, the expense involved in extensive hydrolysis and ultrafiltration make this, at least for pet food manufacturers, an unrealistic objective. In contrast, hypersensitivity reactions have been identified in even the most hydrolyzed formulae.[227] Suggested explanations include the presence of a hapten effect or reassembly of old or new epitopes either *in vivo* or *in vitro* during or subsequent to formulation. Importantly, it should also be realized that the presence of fragments of greater than 5kDa or even greater than 10kDa does not guarantee allergenicity. As detailed above, reduction of epitopes is dependent on the specificity for the proteolytic enzymes as to whether any given epitope is cleaved and rendered non-antigenic.

Immunochemical analyses can semiquantitatively estimate hydrolysate reactivity with preformed antibody. The use of ELISA and RAST assays to assess residual antibody

binding is widespread. The ability of hydrolysate-based products to induce an immune response can be evaluated by using animal models such as the passive cutaneous sensitivity test[245] or laboratory animal hyperimmunization.[246] It is therefore, the responsibility of the manufacturer to choose the appropriate combination of non-clinical tests and use them to document product consistency, thus helping to ensure consistent clinical performance.

Ultimately however, controlled clinical studies are necessary to demonstrate conclusively the biologic efficacy of these formulations in their target species in order to truly claim their hypoallergenicity.

1.10.8 Clinical Evidence of Efficacy

To date, the introduction of commercial hydrolysate formulae onto the veterinary market is so recent as to prevent the accumulation of significant clinical experience. Instead, veterinary clinicians must peruse the human literature for insight. There are numerous reports demonstrating the efficacy of cow's milk-based hydrolysates in eliminating the clinical signs of hypersensitivity in all but a small number of children.[247,248,249] In addition to their role in managing milk hypersensitivity, hydrolysate formulae have demonstrated efficacy in preventing the development of cow's milk hypersensitivity in children pre-disposed to the development of atopic diseases.[250]

The use of hydrolysate formulae in cases of diarrhoea not associated with hypersensitivity has also been suggested. Stimulation of enterocyte adenylate cyclase by bacterial enterotoxins such as the cholera toxin contributes to the associated secretory diarrhoea. As mentioned above, adenylate cyclase activity has also been shown to be elevated following

oral exposure of allergens to the sensitized intestine and seems to play a role in the pathogenesis of diarrhoea in sensitized individuals.[144] In an experimental model of induced cAMP production in rats, the feeding of intact casein and whey was shown to increase cAMP production whereas a whey/casein hydrolysate inhibited production.[251] This suggests that small peptides may play a physiological role in the regulation of nutrient absorption and secretions by intestinal epithelial cells through the modulation of cyclic AMP production. It may also explain the decreased diarrhoea reported with the feeding of peptide-based formulae.

1.10.9 Tolerogenic Peptides

Perhaps some of the most interesting work on protein hydrolysates has been the recent discovery of so-called tolerogenic peptides in partially hydrolyzed formulae. Fritsche et al (1997) investigated whether oral tolerance can be induced with protein hydrolysates.[252] The authors investigated both a partially hydrolyzed and an extensively hydrolyzed formula based on β-lactoglobulin (β-LG). Predictibly, they were able to demonstrate that whilst both formulae had reduced β-LG-specific IgE and IgG binding abilities, the extensively hydrolyzed formula is significantly less able to bind than the partially hydrolyzed formula. However, the partially hydrolyzed formula was able to induce dose-dependent, antigen specific tolerance when administered before and during experimental sensitization whereas the extensively hydrolyzed formula was not. The determination of the specificity of the induced tolerance was established by the concurrent feeding of an unrelated antigen (OVA). Subsequent investigations into the peptides demonstrated that they were around 4.5kDa.[253] The significance of these findings is that it introduces the possibility of inducing tolerance in a sensitized patient even when the patient is sensitized to the parent protein. As long as

there are no peptides large enough to induce an IgE-mediated response to the peptides but there are sufficiently large fragments to be antigenic, the establishment of tolerance to the parent protein may be hastened. In addition, if the feeding of a hydrolysate was to be considered as a prophylactic measure in patients "at risk" of developing a food hypersensitivity, the inclusion of some low molecular weight antigens might be advantageous. In concordance, it was suggested by Fritsche et al (1997) that for "at risk" infants, the feeding of partially hydrolyzed formulae may be more suitable for allergy prophylaxis than extensively hydrolyzed formulae.

Hashimura et al (1993) provided more evidence for the importance of size in this phenomenon. He reported oral tolerance induction feeding mice with a casein hydrolysate in which the peptide fragments were smaller than 6kDa.[254] However, Michael (1989) had earlier demonstrated that oral administration of hydrolyzed bovine serum albumin with fragments of around 13kDa did not tolerize mice against bovine serum albumin.[255] It is possible, however, that despite the larger fragments, destruction of the relevant tolerizing peptides had occurred in Michael's experiment.

Importantly in the studies of Pecquet et al (2000), the measures of residual antigenicity of the various fractions demonstrated that antigenic and toleragenic sites might be distinctly located suggesting that allergenicity and tolerogenicity could be uncoupled.[253] As discussed above, feeding of low doses of antigen has been claimed to favour the generation of active suppressor T cells, whereas higher doses favour clonal anergy or deletion. Which of these mechanisms is most dominant remains uncertain although anergy appears to be the most accepted mechanism for food antigens. Whether the mechanisms involved in the induction

of oral tolerance are the same for intact proteins and peptides is unknown at present. It is possible that peptides are processed differently by antigen presenting cells resulting in unconventional responses. Certainly, these findings raise the intriguing possibility that hydrolysates may have a role in the future for not simply avoiding hypersensitivity reactions but for preventing them in at risk individuals as well.

1.11 Indications for the Use of Hydrolysates

The suggested indications for the use of hydrolysate diets are presented in Table 1.9.

Elimination Diets

Clearly, the primary role for the use of hydrolysate diets is as elimination diets. Increasingly, feline and canine patients are being exposed to a wide variety of protein sources as the range of commercial diets and their source proteins grows. The identification of a truly novel protein in patients presented for evaluation of dietary hypersensitivity can be difficult. Protein hydrolysate diets obviously allow greater confidence in the instigation of an elimination trial when a dietary history is either uncertain or reveals prior exposure to multiple proteins. In addition, in patients at risk for the subsequent development of sensitization to novel proteins, or in cases when there is sensitization to multiple proteins the obliteration of antigenic epitopes ensures that the hydrolysate diet can be fed without concern.

Established Intestinal Inflammation

Given the current understanding of the aetiopathogenesis of food hypersensitivity presented above, the feeding of a protein hydrolysate diet during periods of mucosal inflammation is preferred over the feeding of intact protein. One uncontrolled trial of the feeding of a soy

hydrolysate diet in 6 dogs with refractory inflammatory bowel disease, demonstrated a positive response compared with their previous diets.[256] It is conceivable that temporary sensitization to food proteins during and following a mucosal insult from any cause, might lead to prolongation of the clinical signs. The feeding of a hydrolysate diet during recovery from intestinal disease would be expected to abrogate such an effect. Further studies are required to elucidate the role that such diets might play in the management of inflammatory gastroenteropathies of various causes.

Table 1.9. Indications for the Use of Hydrolysate Diets

Standard elimination diet
Difficulty identifying a novel protein
Suspicion or demonstration of multiple sensitivities
Refractory food hypersensitive dermatitis
Refractory food hypersensitive gastroenteritis
Induction and management of inflammatory bowel disease
Prevention of food hypersensitivity in "at - risk" individuals
Feeding during periods of intestinal mucosal inflammation or increased permeability

1.12 Conclusion

Whilst food hypersensitivity remains an uncommon disease in veterinary patients, it is of great interest to the general pet-owning public. In addition, food hypersensitivity is recommended to be included in differential lists for animals presenting with chronic pruritic dermatoses and chronic gastrointestinal complaints; both common presentations. It is therefore the case that food hypersensitivity is frequently considered.

Recent developments in the understanding of mucosal immunity have shed light on the mechanisms that underlie the development of food hypersensitivity. The circumstances under which the GALT might possibly be induced to respond to a normally innocent antigen in a phlogistic manner include: exposure to microbial adjuvants, intestinal parasitism, non-infectious causes of mucosal injury, idiopathic inflammatory bowel diseases, IgA deficiency and abnormal cytokine expression. The likelihood that the resulting type of immune-response is a Th2-biased, IgE-mediated type I hypersensitivity appears to be predictably increased in atopic individuals. However, in veterinary allergology, the exact mechanisms that operate in any given hypersensitive individual are at present usually ellusive. It is speculated that chronic oral exposure to an allergen, regardless of the inciting cause of the hypersensitivity, will eventually lead to a type IVa_2 hypersensitivity in the majority of individuals. If this is the case, it may explain why diagnostic tests that rely on the role of IgE in the pathogenesis of the response to the allergen such as skin prick tests, have such low sensitivity and specificity in veterinary patients.

Whatever mechanism operates in a given individual, the ability to diagnose food hypersensitivity and ultimately manage it effectively is dependent on the ability of the clinician to identify a novel antigen source and effectively manage an elimination trial. With the increased practice of variety-feeding by owners, identifying a truly novel diet becomes more troublesome. The advent of hydrolysate diets significantly assists the clinician in both diagnosing and treating both easily managed and recalcitrant patients.

The efficacy of a hydrolysate diet is highly reliant on the degree of hydrolysis and the resulting destruction of antigenic epitopes. It follows then that evaluation of both of these

aspects is required before a candidate hydrolysate could be accepted as a reasonable dietary component for the purposes of diagnosis and management of dietary hypersensitivity.

The following chapters of this thesis describe the attempts to assess the biochemical and immunological characteristics of a protein hydrolysate and to validate its potential inclusion as the peptide component of a hypoallergenic diet.

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CHAPTER 2

Molecular Weight Profiles of Two Protein Hydrolysates

Introduction

Hydrolysates vary widely in the degree to which the hydrolysis process has reduced the intact protein into peptide fragments. Prior to selection of a given hydrolysate and inclusion in a commercial diet, a significant amount of information is required. This information can be broken broadly into 3 categories:

1. Biochemical Characteristics
2. Residual Antigenicity
3. Nutritional Value and Palatability

The starting point in the selection process is usually the assessment of the biochemical characteristics. In evaluating a hydrolysate from this standpoint, the question being asked is, "to what degree has the hydrolysate been hydrolysed?" If the resulting hydrolysate has not been reduced into sufficiently small fragments then it will be rejected. Alterations can then be made to the starting protein, method of hydrolysis, selection of enzymes and most significantly of all, duration of hydrolysis.

There are two methods commonly used to determine the extent to which a hydrolysate has been hydrolysed.

1. Degree of Hydrolysis (DH)

   The DH of a hydrolysate can be expressed either as a percentage or as a semi-quantitative category. One of the most commonly used methods for quantification of

primary amino groups is the trinitrobenzenesulfonic acid method or TNBS assay.[1] Less exact semi-quantitative methods use the categories 'extensive', 'moderate' and 'partial' hydrolysis. 'Extensive' has been used to describe hydrolysates with 'few' peptides greater than 7kD. 'Moderate' has been used to describe hydrolysates with most peptides less than 15kD whilst 'partial' is used to denote hydrolysates that have 'many' peptides in the 15-40kD range.[2] van Beresteijn et al (1995) used the average degree of hydrolysis of the protein component.[3] This they defined as the increase of the number of primary amino groups compared with that of the parent protein as determined by the 0-phthaladialdehyde method.[4]

2. Molecular Weight Profiles

The molecular weight profile of a hydrolysate more accurately and completely describes the extent to which the parent compound has been reduced into smaller fragments, and provides the most reliable biochemical standard by which hydrolysates can be compared. Molecular weight distributions are generally calculated by liquid chromatography and expressed as percentages within certain molecular weight ranges.[5,6,7] Unfortunately as yet, there are no widely accepted standard weight range groupings by which published data can be easily compared.

<u>High Performance Size-Exclusion Chromatography (HP-SEC)</u>

The principle of chromatographic separation processes is based on the difference between the surface interactions of the analyte and solvent molecules. In the case of a polymeric solute, differences between analyte molecules may allow separation according to those interactions. For HP-SEC, the analyte (solute) is dissolved in a solvent and is

injected through a column, which is packed with spherical particles with a known and specific porosity and diameter range. The spherical particles are termed the stationary phase since they remain unchanged within the column. As the solution flows out of the column, molecules that have little interaction with the solid phase will leave the column prior to those which interact greatly and are therefore retained within the column for a longer period.[8]

There are many reasons why different molecules interact differently within the column.[9] In the case of size exclusion chromatography (SEC), a porous material is used as the column packing. Molecules in the solution, which are too large to diffuse into the pores, are excluded, and they are transported by the mobile phase through the column within the shortest possible time. However, molecules that are small enough to penetrate the pores will move into them in association with the solvent molecules (mobile phase). On this basis, polymeric substances can be sorted according to their molecular size and the molecular weight distributions or profiles can be qualitatively and quantitatively assessed.

As the mobile phase remains within the pores, passive diffusion is the only way in which molecules can escape and consequently they escape much more slowly. For SEC, there must be no interaction between the stationary phase and the solvent or solute other than the passive pore diffusion. Molecules as small as the solvent are eluted last.

As molecules exit the column they must be detected and recorded. Ideally, molecules would be detected individually as they exited. Practically, however, a small volume of solution is analysed at each point in time and the ability to accurately separate out closely

related molecules is in part related to the volume of the detector cell. UV detectors are the most commonly used type of detector. UV detectors measure the absorbance of the eluent. Radiation absorbance is dependent on the radiation wavelength (energy), the chemical composition of the eluent (molar absorptivity), the molar concentration and the length of the cell. The photodetector measures transmitted light, which is converted to a logarithmic relationship proportional to concentration.

Calibration of the column is required using a test mixture of compounds of known molecular weight. The size of molecules selected usually ensures that:

a. One component is completely excluded

b. Several components partly penetrate the pores

c. One component completely penetrates the stationary phase.

In an ideal case, a graph of log molar mass against retention time is a straight line that characterizes the column.

Objectives

The primary objective of this study was to determine the molecular weight profiles of two hydrolysates, one produced from chicken liver and heart and the other from fish. The generated profiles were then to be used as indicators of the suitability of the hydrolysates for further study as the peptide component of a hypoallergenic diet. The questions of bitterness and effects of osmolarity had already been answered by feeding trials conducted by Hills Pet Nutrition Inc.®.

Materials and Methods

To provide consistency and expertise in high performance liquid chromatography (HPLC), the Dairy Research Institute of New Zealand (DRI) were commissioned to perform the chromatography. The DRI have considerable experience in the molecular weight profiling of milk protein hydrolysates. The candidate hydrolysates were supplied in a dry powdered form by Hills Pet Nutrition Inc.®.

Peptides and amino acids in the hydrolysates were separated according to their molecular size using high performance size-exclusion chromatography (HPSEC). The equipment initially used was a Shimadzu[i] LC-10Ai® automated solvent delivery system with a TSK[ii] G2000SW XL gel filtration column. This is a 30cm long, 7.8mm internal diameter stainless steel column with a high-resolution 125Å silica based exclusion gel.

One hundred and sixty mg of each sample was dissolved in 5mls Milli-Q water, filtered through a 0.45µm micropore filter and then a 250µl sample of the filtrate was diluted into 4.75mls of mobile phase (36% acetonitrile + 0.1% trifluroacetic acid (TFA) in Milli-Q water) to make a total running sample volume of 5mls.

Elution was performed at ambient temperature and at a flow rate of 0.4ml/min. Peptides were detected as the solution exited the column by monitoring the absorbance at 205nm using a Shimadzu SPD-10AVP® high sensitivity UV-Vis detector.

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[i] Shimadzu Scientific Instruments, Auckland, New Zealand
[ii] Tsukishima Kikai Ltd, Tokyo

Three separate groups of purified proteins, peptides and amino acids with known molecular weights were used as molecular weight standards to calibrate the column (Table 2.1.) and absorbance was measured every second. Time for elution from the column was then plotted against molecular weight of each standard. The relationship between retention time of the peptides within the column and the molecular weight was plotted logarithmically and the square of the correlation coefficient (r^2) was calculated. A value for r^2 greater than 0.95 was deemed acceptable for reasonable resolution of the molecular weight ranges. Automatic data processing was performed using a Shimadzu C-R7A Chromatopac® data processor.

Table 2.1. Molecular Weight Standards for HPSEC Column Calibration

Peptide	Molecular Weight (Da)
G-3-PD	36000
Carbonic Anhydrase	29000
Trypsinogen	24000
Soy Trypsin inhibitor	20100
Lysozyme	14300
Apoprotein	6500
Insulin	5730
Insulin β-chain	3400
Bacitracin	1420
Glutathione/oxidised	612
Glutathione/reduced	307
Phenylalanine	165

The molecular weight standards for the TSKSW XL column ran within 42 minutes under the above stated conditions. The running times for the standards are given in Table 2.2 and the calibration graph for the standards is show in Figure 2.1. The correlation between retention time and molecular weight was calculated to be 0.9928887 with the $r^2 = 0.985828$. This correlation was considered suitable for column calibration.

Table 2.2. Molecular Weight Standards Running Times TSKSW XL column

Peptide	Molecular Weight	Running Time (Minutes)
G-3-PD	36000	23.1167
Carbonic Anhydrase	29000	23.3667
Trypsinogen	24000	24.1333
Soy Trypsin inhibitor	20100	24.6
Lysozyme	14300	25.5333
Apoprotein	6500	28.3833
Insulin	5730	29.6
Insulin β-chain	3400	32.15
Bacitracin	1420	33.5167
Glutathione/oxidised	612	38.6833
Glutathione/reduced	307	41.3
Phenylalanine	165	41.6

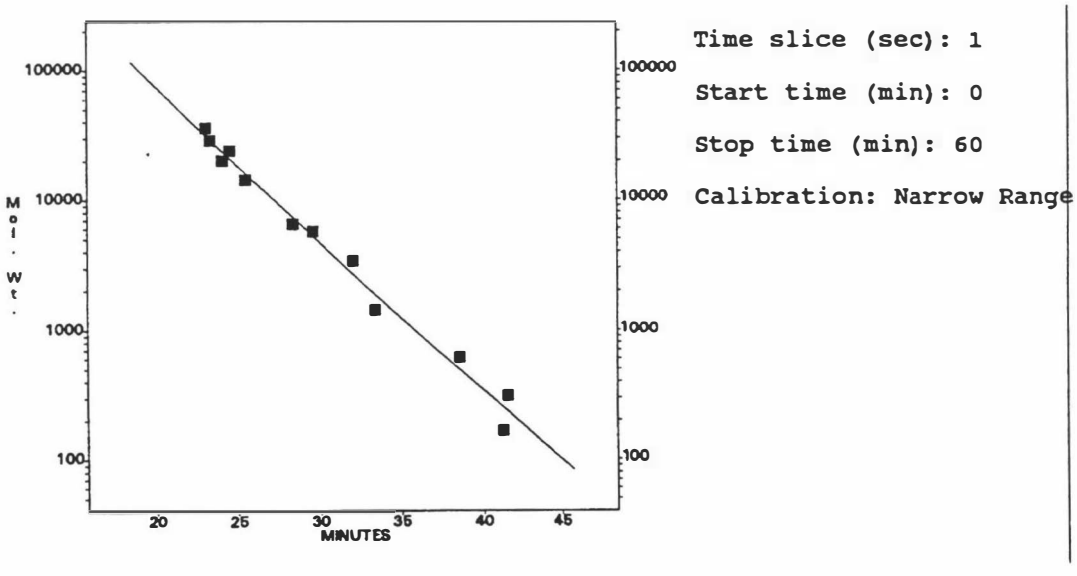

Figure 2.1. Plot of molecular weight against retention time for molecular weight standards using a TSKSW XL gel filtration column

Following calibration, samples of both the chicken hydrolysate and the fish hydrolysate were run through the column. Molecular weight distribution curves were generated and accumulative percentage curves were plotted following complete sample elution.

To improve the resolution of the lower molecular weight fragments, it was decided to repeat chromatographic separation of the chicken hydrolsyate on another HPSEC column. The column chosen was a TSK G2000 SW column. Running times for the same molecular weight standards are presented in Table 2.3 and are plotted graphically in Figure 2.2.

Table 2.3. Molecular Weight Standards Running Times TSK G2000 SW column

Peptide	Molecular Weight	Running Time (Minutes)
G-3-PD	36000	14.8333
Carbonic Anhydrase	29000	15.1167
Trypsinogen	24000	15.0833
Soy Trypsin inhibitor	20100	15.65
Lysozyme	14300	16.55
Apoprotein	6500	18.0667
Insulin	5730	18.6333
Insulin β-chain	3400	20.0333
Bacitracin	1420	20.7833
Glutathione/oxidised	612	23.85
Glutathione/reduced	307	25.5333
Phenylalanine	165	25.35

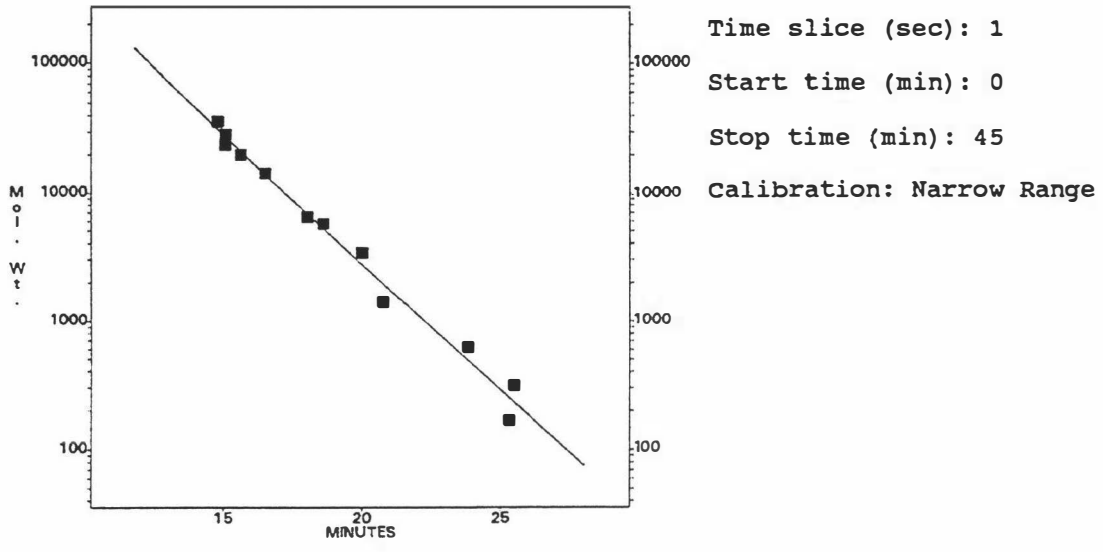

Figure 2.2. Plot of molecular weight against retention time for molecular weight standards using a TSK SW gel filtration column

The correlation between retention time and molecular weight was calculated to be 0.993238 with the r^2 = 0.986521. This correlation was considered suitable for column calibration. Following calibration, a sample of the chicken hydrolysate was run through the column. A molecular weight distribution curve was generated and an accumulative percentage curve was plotted following complete sample elution

In order to assist conjecture about whether the candidate hydrolysates might be useful as the peptide component of hypoallergenic pet food, the molecular weight profiles of the hydrolysates were compared with those of previously published commercially produced human infant whey protein or casein hydrolysate formulae.[3] These human infant formulae were selected for the comparison because, van Bernsteijn et al (1995) had shown the formulae possessed reduced allergenicity as determined by IgE and IgG ELISA inhibition assays.[3] The percentage of each candidate hydrolysate within the specific molecular weight

ranges published by van Bernsteijn et al (<3kDa, 3-5kDa, 5-10kDa and >10kDa) were compared by manually plotting the percentage from the accumulative percentage curves.

Results

Molecular weight profiles and the accumulative percentage curves of the chicken and fish candidate hydrolysates run on the TSKSW XL column are presented graphically in Figures 2.3 and 2.4 respectively. The molecular weight distribution and peptide concentration of the candidate hydrolysates determined with the TSKSWXL column are presented in Table 2.4.

Both hydrolysates were extensively hydrolysed with the chicken hydrolysate (H1) demonstrating the greatest degree of hydrolysis. The first significant peak on the chromatogram of the chicken hydrolysate corresponds to a molecular weight range of 40 – 100 daltons. This probably represents a small quantity of the smallest free amino acids: alanine (89Da) and glycine (75Da). The next peak was between 200Da and 1kDa and probably represents most of the free amino acids and some 2-4 amino acid peptide fragments. An even distribution of polypeptide fragments were then seen with a final smaller peak between 20kDa and 40kDa contributing less than 10% of the total hydrolysate polypeptides.

The fish hydrolysate (H2) was less extensively hydrolysed. The first molecular weight peak was noted between 20 and 60Da whilst the first major peak was noted between 80 and 150Da. Further peaks were noted between 400 – 1500Da, 2 – 4kDa and 4 – 40kDa.

Resolution of the low molecular weight fragments was poor using the TSKSW XL column.. The relatively few individual peaks are unusual for profiles of this degree of hydrolysis.[3,10]

The molecular weight profile and the accumulative percentage curve of the chicken hydrolysate run on the TSK SW column are presented graphically in Figure 2.5. The molecular weight distribution and peptide concentration of the chicken hydolysate determined with the TSK SW column are presented in Table 2.5. The TSK SW column provided better separation of the lower molecular weight fragments than the TSKSW XL column.

The molecular weight distribution and peptide concentration of the candidate hydrolysates are compared with those of 8 hypoallergenic infant whey formulae in Table 2.6.

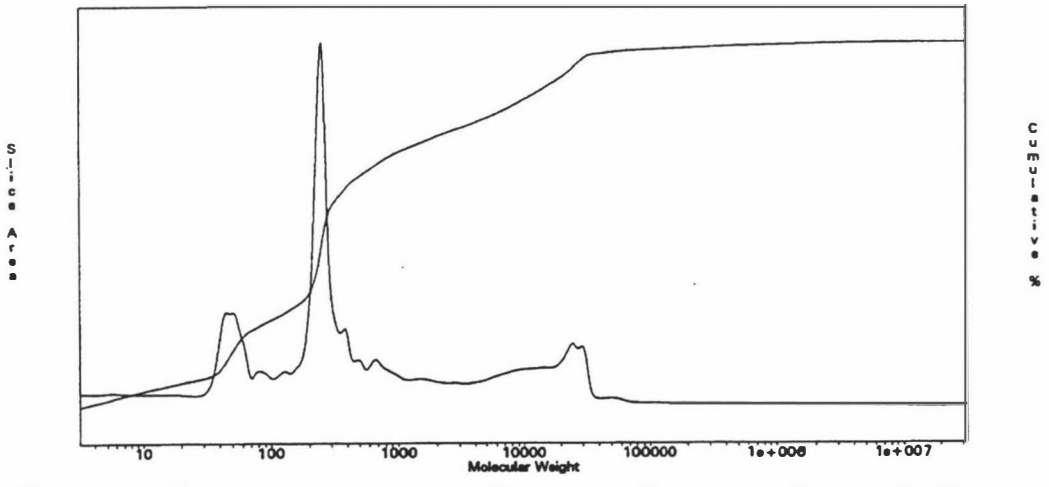

Figure 2.3. Chromatographic separation of peptides in a chicken hydrolsyate.

HPSEC separation performed using a TSKSW XL gel filtration column. Absorbance was measured at 205nm. Molecular weight (x-axis) in daltons plotted against absorbance at 205nm (y-axis). Accumulative % curve plotted following completion of sample elution.

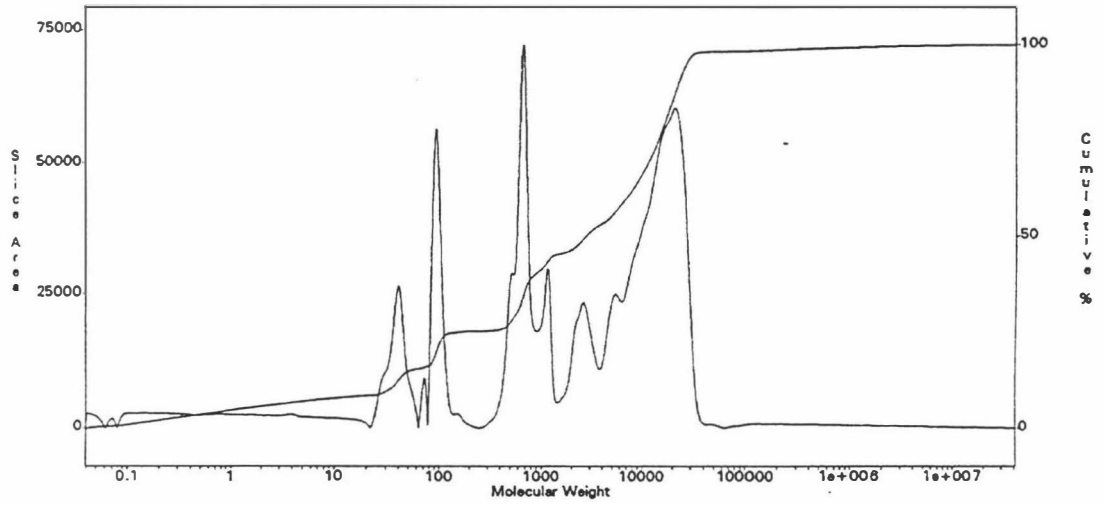

Figure 2.4. Chromatographic separation of peptides in a fish hydrolsyate.

HPSEC separation performed using a TSKSW XL gel filtration column. Absorbance was measured at 205nm. Molecular weight (x-axis) in daltons plotted against absorbance at 205nm (y-axis). Accumulative % curve plotted following completion of sample elution.

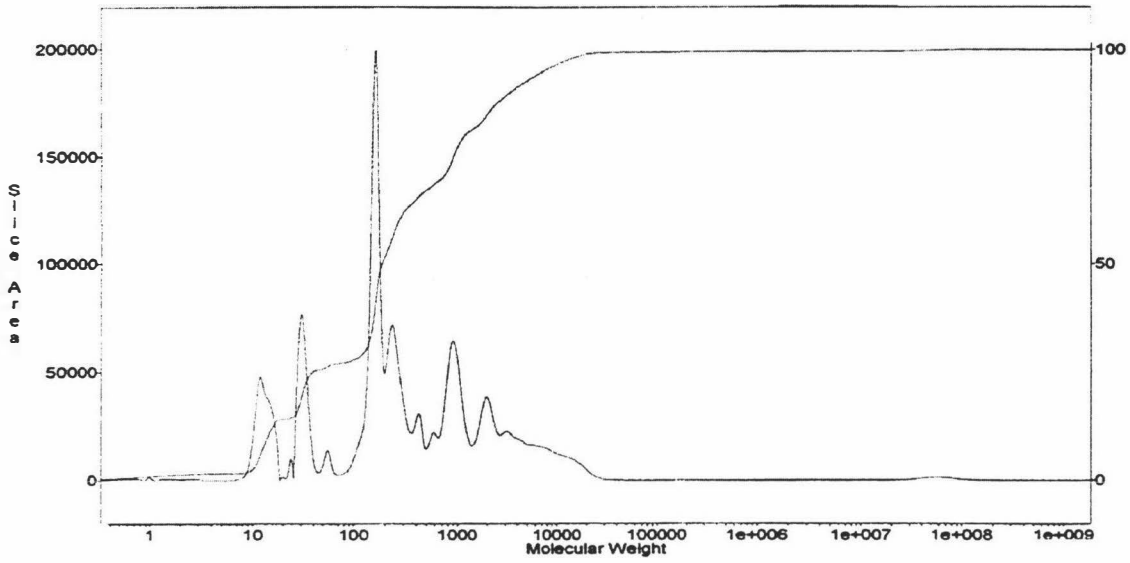

Figure 2.5. Chromatographic separation of peptides in a chicken hydrolysate.

HPSEC separation performed using a TSKSW gel filtration column. Absorbance was measured at 205nm. Molecular weight (x-axis) in daltons plotted against absorbance at 205nm (y-axis). Accumulative % curve plotted following completion of sample elution.

Table 2.4. Molecular Weight Distribution of Two Candidate Hydrolysates (TSKSW XL column)

MW Range (Kda)	% of Total Sample within MW Range	
	Chicken	Fish
< 3	76	51
3 – 5	3	5
5 – 10	4	10
> 10	17	34

Table 2.5. Molecular Weight Distribution of Chicken Hydrolysate (TSK SW column)

MW Range (Kda)	% of Total Sample (w/w)
< 0.5	67.6
0.5-1	10.8
1-3	10.8
3-5	3.7
5-10	4.0
>10	3.1

Table 2.6. Molecular Weight Distribution of 2 Hydrolysates and 8 Hypoallergenic Infant Formulae.

MW Range (kDa)	H1 %	H2 %	IF 1* %	IF 2* %	IF 3* %	IF 4* %	IF 5* %	IF 6* %	IF 7* %	IF 8* %
< 3	76	51	59.3	46.1	43.6	33.4	52.6	47.0	25.6	76.8
3 – 5	3	5	26.4	27.6	30.2	22.4	32.6	30.1	17.7	18.4
5 – 10	4	10	12.0	22.6	21.7	17.9	13.4	19.6	16.1	4.3
> 10	17	34	2.3	4.0	4.5	26.3	1.4	3.3	40.6	0.5

H1 = Chicken Protein Hydrolysate H2 = Fish Protein Hydrolysate
IF = Commercially available hypoallergenic milk whey or casein infant formulae
* E.C.H. van Berensteijn et al, J Allergy Clin Immunol 1995;96:365-74[3]

Discussion

The objective of this experiment was to evaluate and compare two candidate hydrolysates and ultimately to select one of the hydrolsyates for further evaluation as the potential peptide component of a hypoallergenic diet. Describing the molecular weight distribution of the peptide fragments provides a means of initial objective comparison without having to employ *in vivo* investigations or time consuming and expensive immunological assays. As previously stated, HPSEC has become the standard means by which such comparisons can be made.

There was clearly better separation of the lower molecular weight fragments using the TSK SW column. This was probably due to accumulative retention of peptides within the silica gel over time in the older column despite regular cleaning procedures.

Both candidate hydrolysates were extensively hydrolysed with the chicken hydrolysate (H1)

demonstrating the greatest degree of hydrolysis. Using the TSK SW column, 96.9% and 92.9% of the chicken hydrolysate was determined to be composed of amino acids or peptides less than 10kDa and 5kDa, respectively.

As can be seen from Table 2.6, these figures compare very favourably with some of the human infant formulae published by van Bernsteijn et al (1995).[3] However, both hydrolsyates appeared to have significantly larger amounts of fragments larger than 10kDa than most of the infant formulae. Comparing the hydrolysates with the infant formulae obviously requires caution. A superdex-75 HR 10/30 column was used by van Bernsteijn et al (1995) with potassium phosphate and sodium sulphate as the elutants. The different methodology used by van Bernstein to that used in the present experiment hampers direct comparisons. In addition, analysis of complete food preparations containing fats and carbohydrates or preparations containing other additives may cause problems with solubility of samples and should be interpreted with care. Furthermore, there will be differences in the antigenic components of the hydrolysates. Nevertheless, it is reasonable to assume that chicken and, to a lesser extent, the fish hydrolysates will demonstrate degrees of reduced allergenicity.

The limitations of molecular weight profiling of hydrolysates have been stated above. General assumptions about the reduction of antigenicity can be made, whereas accurate predictions about the *in vivo* allergenicity cannot. The reduction in allergenicity of individual proteins following hydrolysis is determined mainly by the molecular structure of the antigenic epitopes and enzyme specificity rather than the DH or MW distribution.

Summary and Conclusion

The theory behind hydrolysate diets is to reduce the allergenic proteins to sufficiently small molecular fragments as to prevent recognition by the recipient's immune system. Assessment of allergenicity can only be inferred biochemically and molecular weight profiling is currently the preferred method with which to do so. The molecular weight profiles of two candidate hydrolysates, one from chicken and the other from fish protein were evaluated and the hydrolysates were found to be extensively hydrolysed. Of the two, the chicken hydrolysate was more hydrolysed.

Both hydrolysates compared favourably with the MW profiles of two human infant hydrolysate formulae which have been shown to be hypoallergenic on the basis of IgE and IgG ELISA inhibition assays.

On the basis of the MW profiles and the comparisons with the infant formulae, it was concluded that both hydrolysates were suitable candidates for further study as the peptide components of hypoallergenic diets. It was expected that the chicken hydrolysate, being the more extensively hydrolysed, would demonstrate the greatest reduction in allergenicity.

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CHAPTER 3

In Vitro Assessment of Antigenicity of a Protein Hydrolysate

Introduction

The molecular weight profile of a hydrolysate describes the extent to which the parent compound has been reduced into smaller fragments, and provides the most reliable biochemical standard by which hydrolysates can be compared. Molecular weight distributions are generally calculated by either size exclusion chromatography (HP-SEC) or gel electrophoresis (e.g. SDS-PAGE) and expressed as percentages within certain molecular weight ranges. These are relatively simple and repeatable techniques.

The degree by which the antigenicity of a parent protein has been reduced by hydrolysis can be inferred from the molecular weight profile. However, this is by no means an exact indicator. Indeed, antigenicity may increase with partial hydrolysis due to exposure of previously hidden epitopes. Furthermore, even with extensively hydrolysed formulae, small peptide fragments (less than 10kD), which may be too small to cross link IgE molecules, may act as haptens when combined with other hydrolysis fragments, other exogenous proteins or self-antigens.

ELISA techniques have been used extensively to evaluate the residual antigenicity of protein hydrolysates and complete enteral formulae[1,2,3,4,5]. An ELISA will assess the ability of a hydrolysate to bind to antibodies specific for the parent protein. If a patient is hypersensitive to the parent protein then the patient's serum IgE antibodies can be used. Alternatively, serum from experimentally sensitised subjects is used and either IgG or IgE antibodies can be utilised. Inhibition ELISA is the preferred method.

Objective

The objective of this study was to develop an inhibition ELISA to describe the residual antibody binding of a chicken protein hydrolysate using polyclonal canine serum IgG antibodies specific to the parent protein.

Materials and Methods

Sensitization of Dogs and Collection of Serum

All dogs used in the study were obtained from the Animal Health Services Centre, Jennersmead Farm, Massey University Palmerston North. The dogs were housed on the Jennersmead property for the duration of the sensitization period and were fed an AFFCO approved diet containing no chicken protein[1]. For the production of chicken protein-specific IgG antibodies, three 5-6 month old entire New Zealand Heading dogs (2 female, 1 male) were used. Unsensitized control samples were collected from 2 entire female, 20 week old Border Collie cross dogs. None of the dogs in the study had been fed chicken protein at any stage of their life. The experimental design was approved by the Massey University Animal Ethics committee.

The study dogs were sensitized to the intact protein using the technique described by Williams[6]. One hundred mg of powdered purified protein (supplied by Hills Nutrition Inc) was dissolved in 20mls of ddH_2O to make a stock solution of 5mg/ml. Twenty mls of the stock solution was added to 10mls of 10% alum solution. The pH of the solution was adjusted to 6.5 using 1 N NaOH. The solution was then centrifuged at 480g for 15

minutes in a refrigerated centrifuge. The supernatant was aspirated and discarded and the precipitate was re-dissolved by vortexing with 0.0001% methiolate in saline to make a total volume of 30mls. Centrifugation followed by aspiration of the supernatant and re-suspension in 0.0001% methiolate in saline was repeated to reach a final concentration of 200μg/ml. Protein binding capacity was assumed to be 300mg protein per gram of alum. The vaccine was stored in plain 10ml vacutainers® at 4°C for 2 weeks. After that time the solution was discarded and fresh solution was prepared.

Baseline serum samples were collected prior to the first sensitization injection. One ml (200μgm of intact protein) was injected subcutaneously on 4 occasions 7 days apart. Blood samples were collected on days 0, 7, 14, 21 and 28. Samples from the control dogs was collected on day 28. All samples were collected from the jugular vein without sedation using a 22-guage needle and were placed into 10ml plain Vacutainers®. Blood was allowed to clot at ambient temperature, was centrifuged at 3200rpm for 10 minutes and serum was separated and stored at -86°C in 1.8ml appropriately labeled Nunc Cryo Tubes® until use.

Evaluation of Serum IgG by Indirect ELISA

The canine anti-chicken protein IgG antibody titres were determined by basic indirect ELISA. Nunc, Maxisorb® and Polysorb® ELISA microtitre plates were used during chequer board optimization titrations but Maxisorb® plates were used for all further assays. A stock solution of chicken antigen was prepared using the powdered product

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[i] Tux Dog Biscuits

used for the sensitisation procedure. This was dissolved in 0.2M carbonate buffer[ii] pH9.6 to produce a stock solution concentration of 5mg/ml.

Wells were coated with 100µl of appropriately diluted chicken antigen solution overnight at 4°C. Following incubation, the contents of the wells were discarded by inversion. Washing cycles consisted of the wells being individually filled with phosphate buffered saline plus polyoxyethylene (20) sorbitan monolaurate[iii], pH7.4 (PBS+T20), and allowed to rinse for 3 minutes followed by inversion and slapping onto disposable paper towels. This rinse procedure was repeated 3 times.

Wells were blocked initially with 150µl of 1% bovine serum albumin[iv] in carbonate buffer and incubated for 1 hour at 37°C. Following the initial optimization, the blocking protein was changed to 150µl of 1% human serum albumin (HSA)[v] in carbonate buffer Washing cycle was repeated. 100µl of canine serum diluted in PBS+T20 was added to the wells and incubated for 1 hour at 37°C.

Washing cycle was repeated. 100µl of rabbit anti-dog IgG horseradish peroxidase conjugate (IgG-HRP)[vi] diluted 1:1000 in PBS+T20 was added and the plate was incubated for 1 hour at 37°C. Washing cycle was repeated. One hundred µl of tetramethylbenzidine (TMB) in acetate buffer pH5.2[vii] was added to the wells and allowed to incubate at ambient temperature for 15 minutes.

[ii] Carbonate-Bicarbonate Buffer Capsules, Sigma-Aldrich Product No.C3041
[iii] Phosphate Buffered Saline, pH 7.4, with Tween 20, Sigma-Aldrich Product No. P3563
[iv] BSA, Sigma-Aldrich Product No. 05477
[v] HSA, Sigma-Aldrich Product No. 05420
[vi] Sigma-Aldrich Product No. A6792

The reaction was stopped with 50µl of 2M sulphuric acid. Optical densities were measured at 450nm on an automated ELISA plate reader[viii]. All ELISA determinations were carried out in duplicate and the results were averaged.

Chequer board titrations were performed to optimize the working dilution of test serum and solid phase antigen. Chicken protein dilutions of 1:10, 1:20. 1:40, 1:80, 1:160, 1:320, 1:640, 1:1280, 1:2560, 1:5120 were run against pooled week-4 and control serum dilutions of 1:10, 1:20. 1:40, 1:80, 1:160, 1:320, 1:640, 1:1280.

To compare Maxisorb® with Polysorb® plates, pooled week-4 serum and pooled control serum dilutions of 1:10, 1:20. 1:40, 1:80, 1:160, 1:320, 1:640, 1:1280 were run against antigen dilutions of 1:40 and 1:80.

Results

Optimization

The absorbance value for each serum's antigen control (antigen negative) was subtracted from the test well to give the serum's corrected value. These corrected values are given in Table 3.1. and are represented graphically in Figure 3.1.

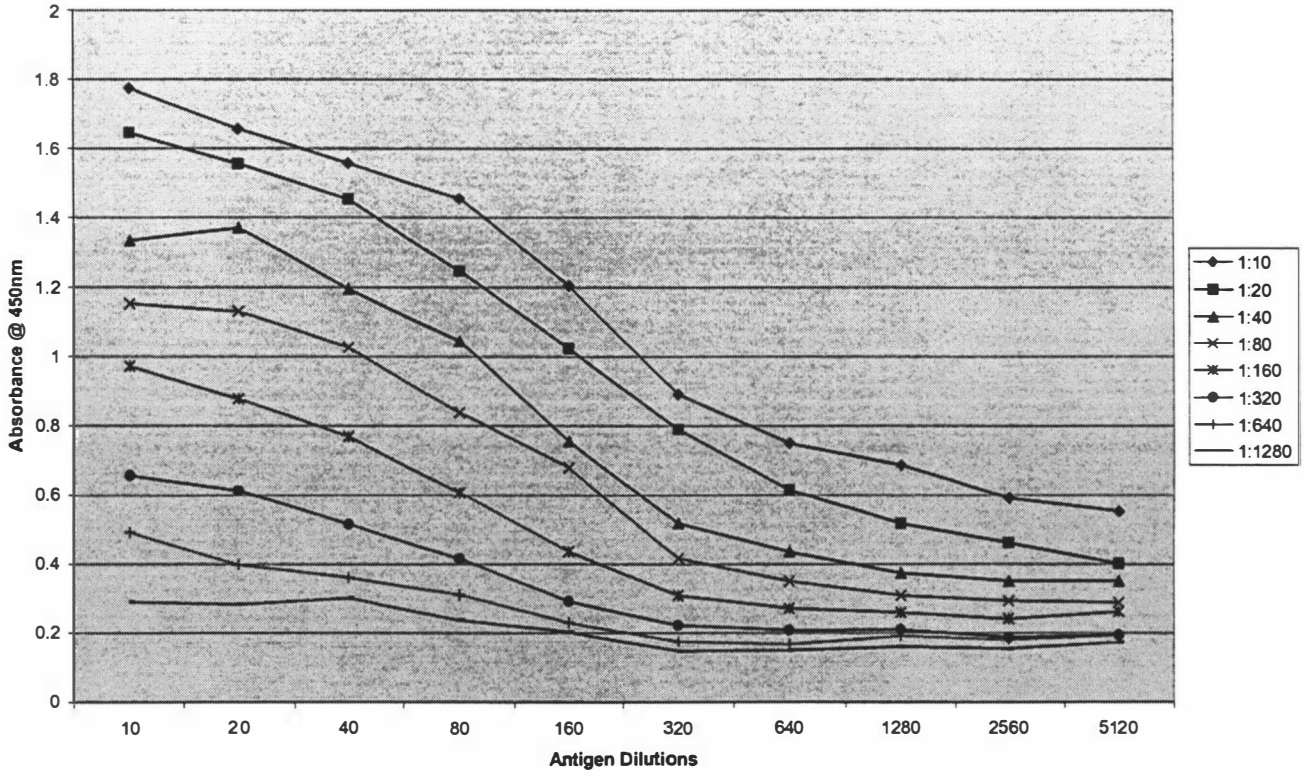
[vii] Imunopure® TMB, Dihydrichoride, Pierce, IL, USA
[viii] ELISA kinetic plate reader (SLT 340 ATC), SLT Lab Instruments

Table 3.1. **Corrected Absorbance Values for Serum/Antigen Optimization** (Read @ 450nm) BSA Block Nunc® Plate

Serum Dilutions	Antigen Dilutions									
	1:10	**1:20**	**1:40**	**1:80**	**1:160**	**1:320**	**1:640**	**1:1280**	**1:2560**	**1:5120**
1:10	1.774	1.656	1.557	1.454	1.204	0.891	0.75	0.686	0.591	0.553
1:20	1.645	1.555	1.453	1.245	1.023	0.789	0.615	0.517	0.461	0.401
1:40	1.333	1.37	1.194	1.044	0.755	0.518	0.436	0.374	0.35	0.35
1:80	1.152	1.129	1.026	0.838	0.679	0.416	0.35	0.309	0.292	0.288
1:160	0.972	0.877	0.768	0.606	0.435	0.308	0.271	0.259	0.241	0.261
1:320	0.656	0.612	0.515	0.415	0.291	0.222	0.208	0.209	0.187	0.193
1:640	0.491	0.397	0.361	0.31	0.229	0.174	0.167	0.191	0.18	0.193
1:1280	0.29	0.283	0.301	0.237	0.202	0.146	0.149	0.159	0.153	0.172

Antigen Controls	
1:10	0.200
1:20	0.183
1:40	0.179
1:80	0.180
1:160	0.167
1:320	0.180
1:640	0.204
1:1280	0.198
Average	0.186

Figure 3.1. **Graph of Absorbance vs Antigen Dilution for Serum/Antigen Optimization.** Nunc Plate, BSA Block.



The chequer board titration of parent chicken-protein against the canine serum IgG showed a decrease in absorbance with both increasing serum dilution and decreasing antigen concentration.

However, strong background values for the antigen and antibody control wells resulted in low corrected absorbance figures for all but the most concentrated serum. This was taken to suggest non-specific antibody binding either due to residual unbound areas of the wells or due to antibody-bovine albumin interactions.

The latter of these two was felt to be the most likely. Food specific antibodies are uniformly found in the serum of dogs and these dogs had previously been on diets that were rich in bovine proteins. It was speculated that low levels of serum antibodies specific to bovine serum albumin might have been binding to the blocking agent. On this basis, human serum albumin (HSA) was chosen as the blocking agent for all the subsequent experiments.

From the first optimization curves it could be seen that maximum antigen binding was seen at a dilution of between 1:40 and 1:80.

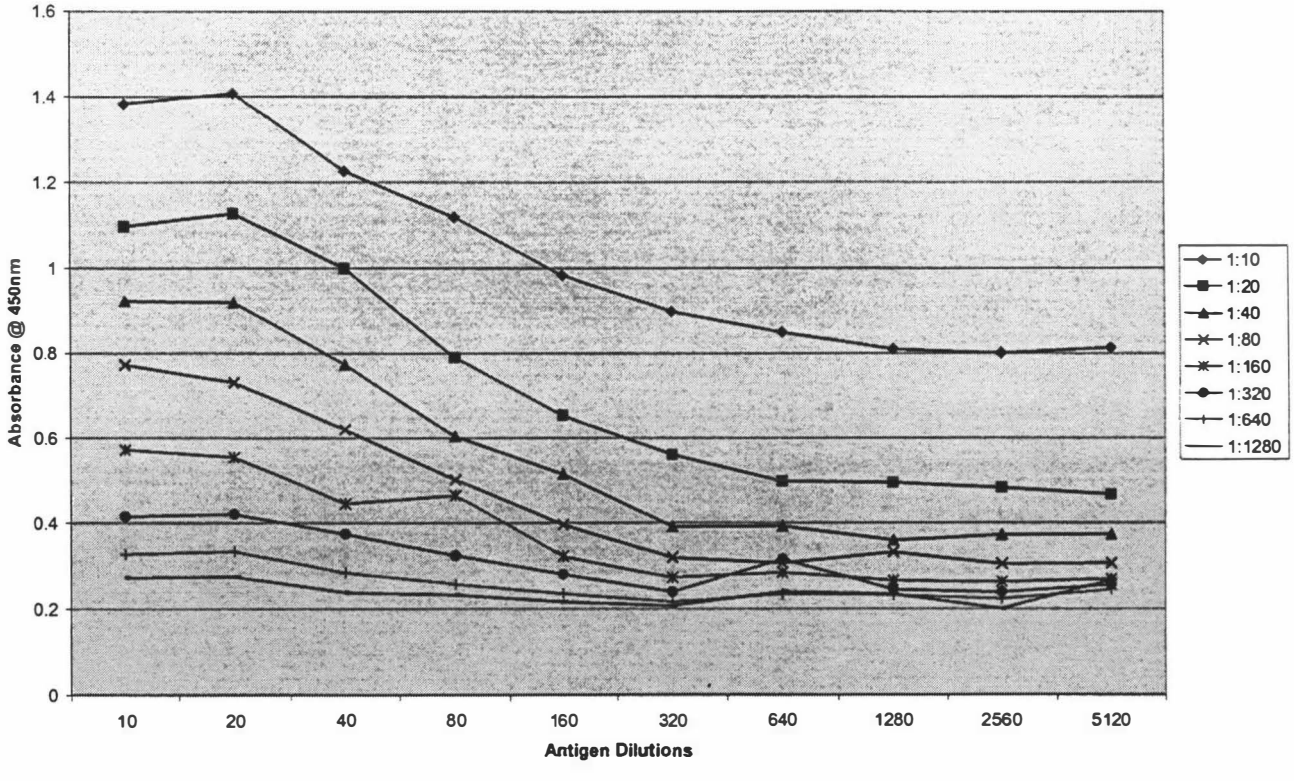
A second optimization experiment was performed using the same conditions but substituting HSA for BSA. The absorbance figures are given in Table 3.2. and these are represented graphically in Figure 3.2.

Table 3.2. Corrected Absorbance Values for Serum/Antigen Optimization (Read @ 450nm) HAS Block Nunc® Plate

Serum Dilutions	Antigen Dilutions									
	1:10	**1:20**	**1:40**	**1:80**	**1:160**	**1:320**	**1:640**	**1:1280**	**1:2560**	**1:5120**
1:10	1.383	1.407	1.226	1.118	0.982	0.897	0.849	0.809	0.8	0.812
1:20	1.096	1.126	0.998	0.789	0.653	0.56	0.497	0.494	0.482	0.466
1:40	0.921	0.918	0.772	0.603	0.515	0.391	0.392	0.359	0.372	0.373
1:80	0.772	0.73	0.619	0.501	0.396	0.319	0.306	0.332	0.305	0.306
1:160	0.572	0.554	0.444	0.464	0.323	0.272	0.284	0.265	0.262	0.269
1:320	0.415	0.42	0.374	0.324	0.28	0.239	0.315	0.244	0.238	0.255
1:640	0.327	0.333	0.284	0.257	0.235	0.215	0.233	0.231	0.223	0.244
1:1280	0.273	0.274	0.237	0.232	0.216	0.206	0.24	0.233	0.2	0.267

Antigen Controls	
1:10	0.187
1:20	0.173
1:40	0.161
1:80	0.169
1:160	0.167
1:320	0.18
1:640	0.205
1:1280	0.202
Average	0.1805

Figure 3.2. Graph of Absorbance vs Antigen Dilution for Serum / Antigen Opimization. Nunc Plate, HSA Block.



Using the human rather than bovine serum albumin resulted in significantly lower and acceptable background values. However, as can be seen, the resulting absolute absorbance values were also significantly lower. This was taken to indicate that antigen binding to the Nunc® plate was poor and two further optimisation experiments were performed using Nunc Maxisorb® and Nunc Polysorb® plates. For these experiments, 1:40 and 1:80 antigen dilutions were used since plateau effects were again seen above these concentrations.

The absorbance values for the Polysorb® and Maxisorb® plates are shown in Table 3.3 and Table 3.4 and are graphically represented in Figures 3.3 and 3.4.

Table 3.3. Absorbance Values (@450nm) for Nunc Maxisorb® Plate

Serum Dilutions	Antigen Dilutions		Controls
	1:40	**1:80**	
1:10	2.02	1.602	0.14
1:20	1.437	1.262	0.14
1:40	0.914	0.701	0.114
1:80	0.626	0.505	0.105
1:160	0.426	0.307	0.104
1:320	0.202	0.294	0.104
1:640	0.267	0.224	0.005
1:1280	0.234	0.164	0.064

Table 3.4. Absorbance Values (@450nm) for Nunc Polysorb® Plate

Serum Dilutions	Antigen Dilutions		Controls
	1:40	**1:80**	
1:10	1.14	1.079	0.705
1:20	0.916	0.83	0.3
1:40	0.404	0.507	0.22
1:80	0.346	0.344	0.19
1:160	0.336	0.229	0.161
1:320	0.3	0.16	0.145
1:640	0.235	0.139	0.141
1:1280	0.209	0.153	0.101

Figure 3.3. Graph of Absorbance vs Serum Dilution for Serum/Antigen Optimization. Nunc Maxisorb® Plate, HSA Block.

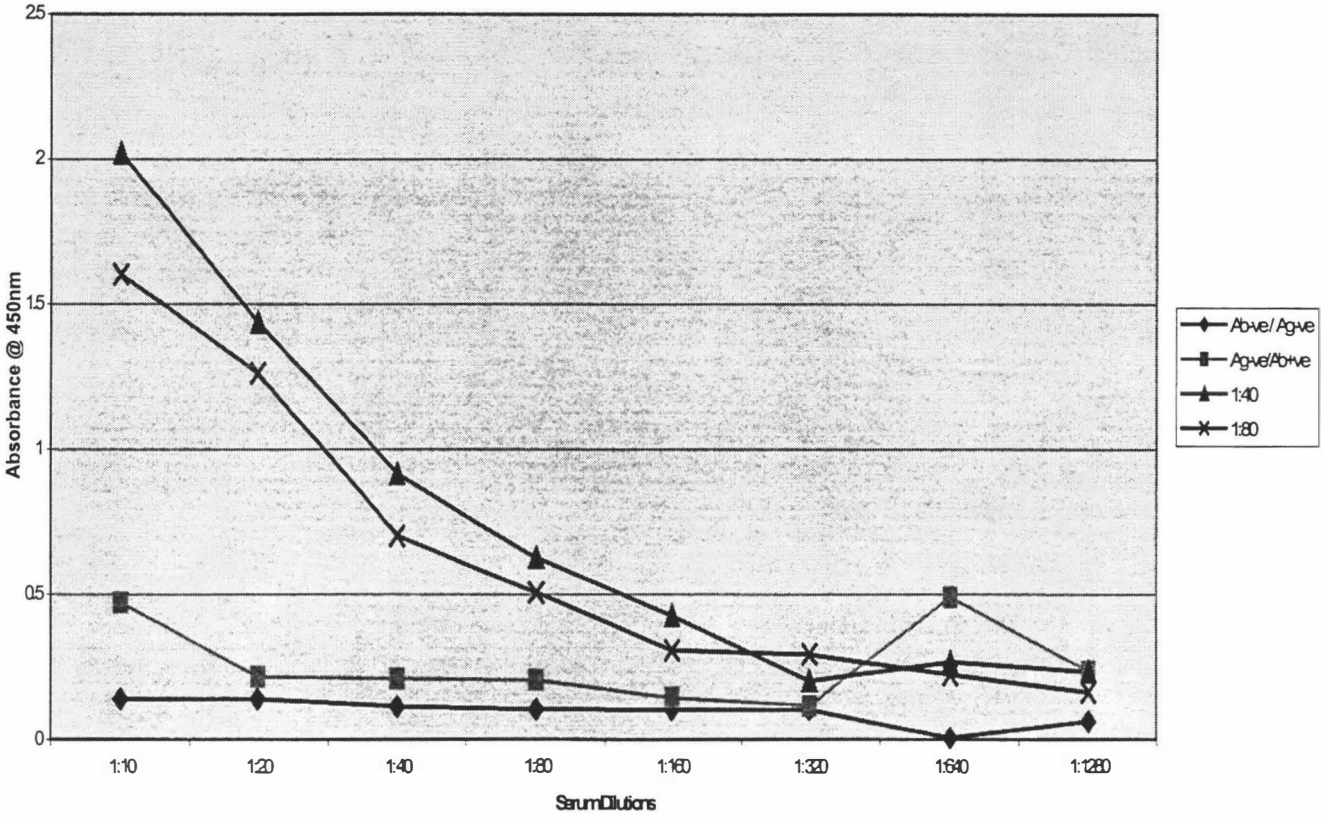
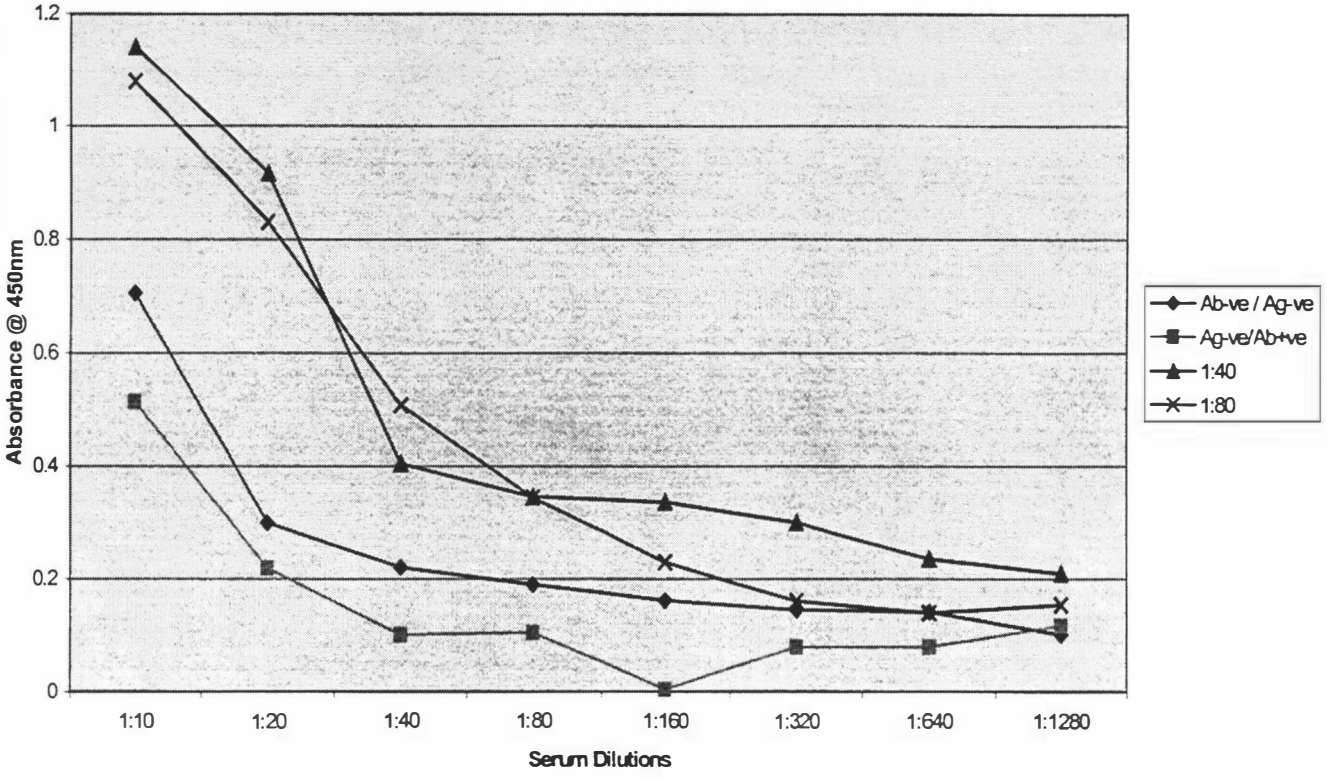


Figure 3.4. Graph of Absorbance vs Serum Dilution for Serum/Antigen Optimization. Nunc Polysorb® Plate, HSA Block.



As can be seen from the above data, the Nunc Maxisorb® plate gave excellent separation of the serum + antigen wells verses the antigen control wells with minimal background binding. In contrast, the Nunc Polysorb® plate showed reduced antigen binding (as demonstrated by the lower absolute absorbance values) and poorly separated the test wells from the controls.

On the basis of these optimization procedures, Nunc Maxisorb® plates and HSA block were used for subsequent assays.

A repeat chequer board optimization assay was performed using the new conditions.

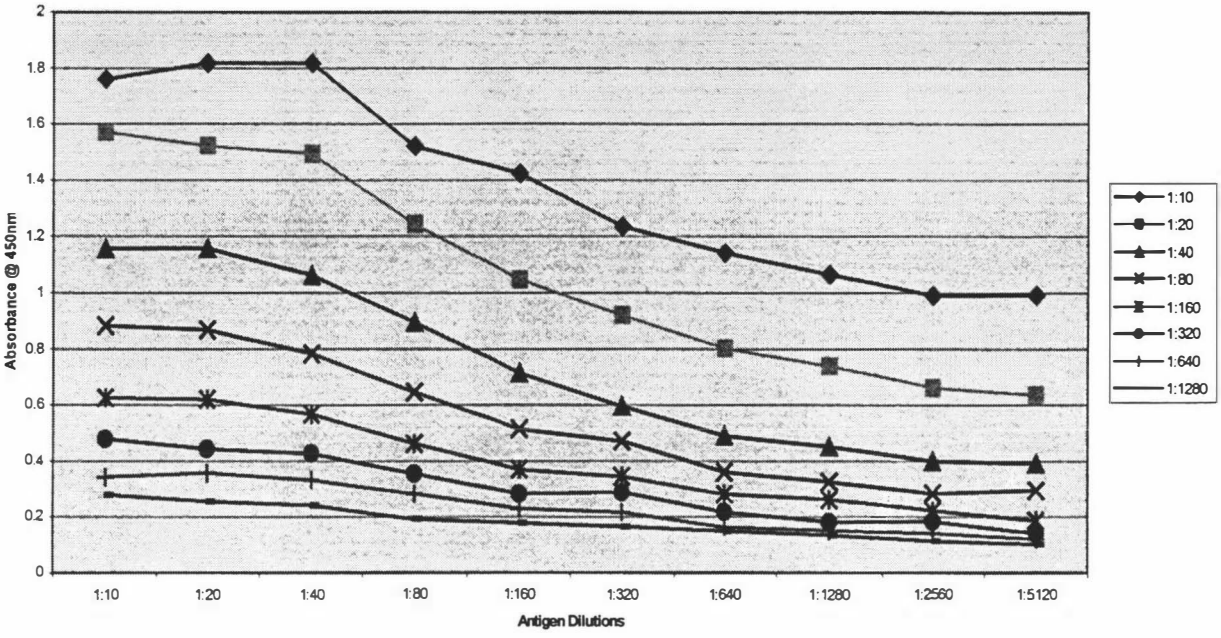
The results of this assay are given in Table 3.5 and represented graphically in Figure 3.5.

Table 3.5. Corrected Absorbance Values for Serum/Antigen Optimization (Read @ 450nm) HAS Block Nunc Maxisorb® Plate

Serum Dilutions	Antigen Dilutions									
	1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560	1:5120
1:10	1.758	1.816	1.816	1.519	1.423	1.234	1.141	1.064	0.989	0.99
1:20	1.569	1.522	1.492	1.241	1.045	0.92	0.801	0.738	0.66	0.635
1:40	1.155	1.157	1.062	0.895	0.713	0.595	0.489	0.45	0.399	0.391
1:80	0.883	0.867	0.782	0.644	0.514	0.47	0.36	0.325	0.283	0.294
1:160	0.625	0.619	0.567	0.461	0.369	0.343	0.281	0.261	0.222	0.186
1:320	0.478	0.442	0.426	0.354	0.282	0.288	0.216	0.181	0.183	0.141
1:640	0.342	0.357	0.332	0.282	0.229	0.215	0.163	0.15	0.141	0.12
1:1280	0.278	0.254	0.24	0.191	0.178	0.165	0.149	0.134	0.113	0.102

Antigen Controls	
1:10	0.110
1:20	0.115
1:40	0.109
1:80	0.117
1:160	0.105
1:320	0.098
1:640	0.102
1:1280	0.1
Average	0.106571

Figure 3.5. Graph of Absorbance vs Antigen Dilution for Serum/Antigen Optimization. Nunc Maxisorb® Plate, HSA Block.



Given the above results, the optimum antigen dilution was determined to be 1:40 to 1:80 whereas the optimum serum dilution was determined to be 1:10 to 1:20.

Evaluation of Canine Protein Specific IgG Titres

Following optimization, samples of sequential week pooled canine serum and pooled control serum at a dilution of 1:20 and an antigen dilution of 1:40 were assayed to describe the rising protein specific titres. For this assay, a new batch of Nunc Maxisorb® small plates was used. Absorbance values for the 4th week serum were too high to register suggesting different binding characteristics than the large, older plates.

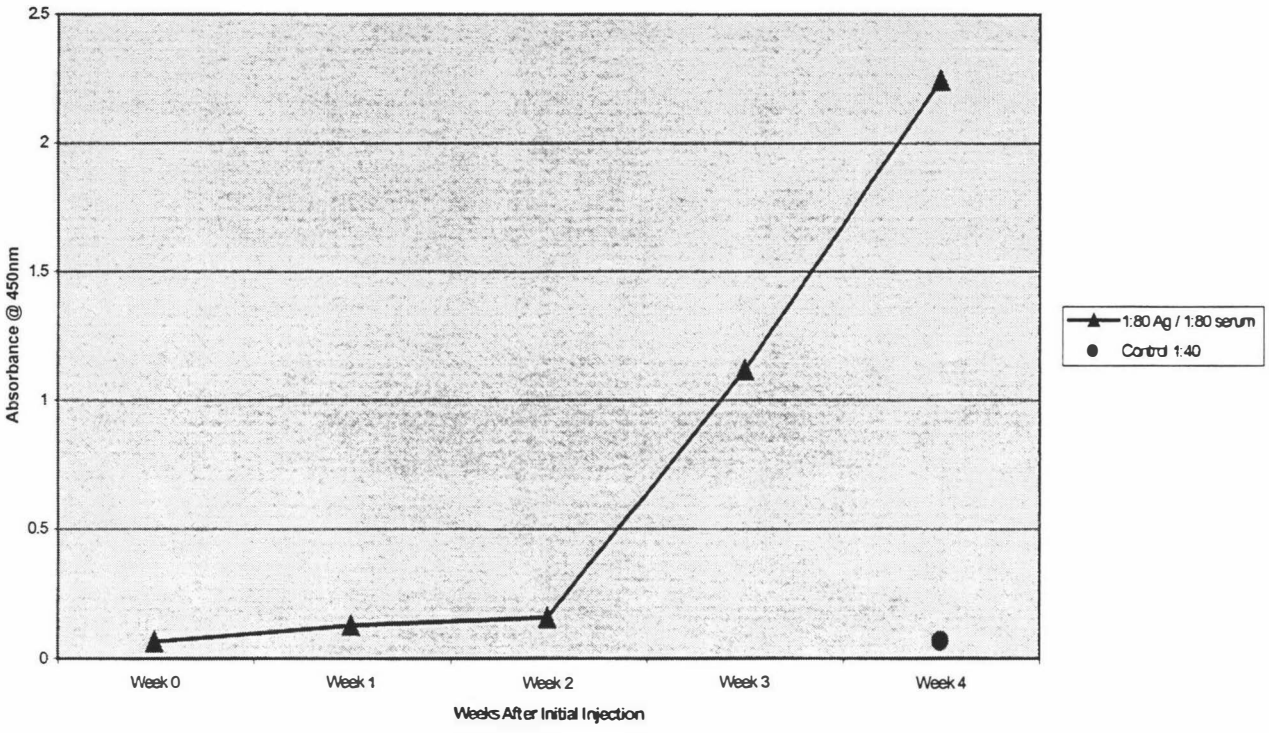
The antigen/antibody optimizations were repeated using a smaller range of antigen/serum dilutions. The new antigen and serum optimum dilutions were 1:80 and 1:80 respectively. Serum from vaccinates and control dogs were assayed. Control samples were run individually to determine if any of the three dogs had produced antibodies that bound to the solid phase. Samples were run in duplicate and averaged prior to correction.

The corrected absorbance values are given in Table 3.6 and are graphically represented in Figure 3.6.

Table 3.6. **Corrected Absorbance and Control Values for Sequential Titres** (Read @ 450nm) HSA Block Nunc Maxisorb® Plate

Samples	Average Corrected Value	Antigen Controls
Week 0	0.0635	0.213
Week 1	0.1265	0.183
Week 2	0.156	0.171
Week 3	1.116	0.187
Week 4	2.243	0.203
Control 1	0.0675	0.104
Control 2	0.0485	0.102
Control 3	0.227	0.139

Figure 3.6. Graph of Absorbance @450nm versus Time following Sensitisation to Chicken Protein.
HSA Block Nunc Maxisorb® Plate. 1:80 solid phase dilution



IgG antibodies against the parent protein began to rise between 2 and 3 weeks after the first vaccination. High levels were detected by the fourth week consistent with successful immunization.

Inhibition ELISA

Optimization

The IgG binding capacity of the hydrolysate was determined by inhibition ELISA. Intact protein and hydrolsyate were dissolved in distilled water to concentrations of 5g/l (stock solutions). A solid phase antigen dilution of 1:80 was used. Human serum albumin (HSA) was used as a blocking agent. In 2ml glass Vacutainers®, serial dilutions of the intact protein solution were incubated for 1 hour at 37°C with serial dilutions of pooled

week 4 serum. Solutions without the competing antigen were used as positive controls and solutions containing control animal serum were used as negative controls.

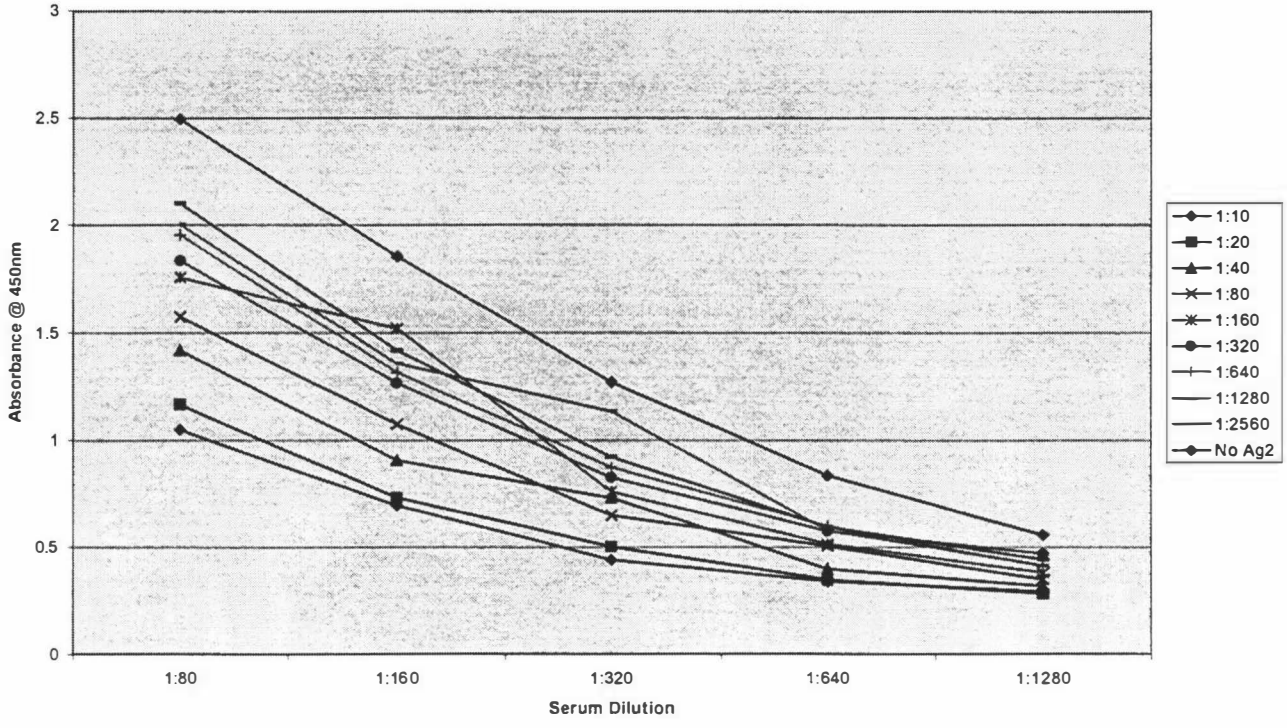
Residual unbound antibodies were determined using the indirect ELISA method described above.

The corrected absorbance data is presented in Table 3.7 and is displayed graphically in Figure 3.7.

Table 3.7. Corrected Absorbance and Control Values for Inhibition Optimization (Read @ 450nm) HSA Block Nunc Maxisorb® Plate

Inhibitor Dilutions	**Serum Dilutions**				
	1:80	**1:160**	**1:320**	**1:640**	**1:1280**
1:10	1.05	0.695	0.439	0.339	0.294
1:20	1.166	0.732	0.502	0.346	0.282
1:30	1.419	0.907	0.731	0.398	0.316
1:40	1.574	1.073	0.648	0.505	0.348
1:80	1.755	1.518	0.759	0.515	0.38
1:160	1.834	1.265	0.826	0.575	0.468
1:320	1.953	1.314	0.874	0.6	0.412
1:640	2.002	1.356	1.133	0.578	0.415
1:1280	2.102	1.417	0.921	0.595	0.442
No Ag	2.494	1.852	1.268	0.837	0.556

Figure 3.7. Graph of Absorbance versus Serum Dilutions for Optimization of Inhibition ELISA Nunc Maxisorb® Plate, HSA Block, Whole Chicken Protein Inhibitor1:80 solid phase dilution.



The chequer board titration of intact protein dilutions against the canine serum IgG showed a decrease in absorbance with both increasing serum dilution and decreasing antigen concentration. A serum dilution of 1:80 demonstrated the best separation of absorbance with inhibiting antigen variation.

In order to describe the difference between the antibody binding of the hydrolysate and the intact protein, an inhibition ELISA was performed using the solid phase dilution of 1:80, pooled week 5 serum dilution of 1:80 and variable competing antigen concentrations. Human serum albumin (HSA) was also used as a competing antigen

(5g/l stock solution) to control for non-specific antibody-to-competing antigen interactions.

The corrected absorbance data is presented in Table 3.8 and is displayed graphically in Figure 3.8. All figures represent the average of duplicate assays.

Figure 3.9 shows the above data but with increasing inhibitor concentrations (calculated) as the x-axis values.

Table 3.8. **Corrected Absorbance and Control Values for Inhibition ELISA** (Read @ 450nm) HSA Block Nunc Maxisorb® Plate. 1:80 solid phase dilution , serum Dilution 1:80

Inhibitor Dilutions	Protein	Hydrolysate	HSA	Control Serum
1:5	0.245	2.0265	2.3625	0.234
1:10	0.7745	2.1855	2.4055	
1:20	1.1205	2.1855	2.548	
1:40	1.308	2.2705	2.481	
1:80	1.7095	2.3475	2.502	
1:160	1.8345	2.46	2.5254	
1:320	2.0495	2.4245	2.525	
1:640	2.169	2.5375	2.504	
1:1280	2.3805	2.609	2.628	

Inhibitor Negative	2.465

Figure 3.8. Inhibition of Canine IgG Binding to Intact Chicken Protein by a Chicken Hydrolysate, Intact Chicken Protein and HSA
(Nunc Maxisorb® Plate, HSA Block, Whole Chicken Protein Inhibitor 1:80 solid phase dilution.)

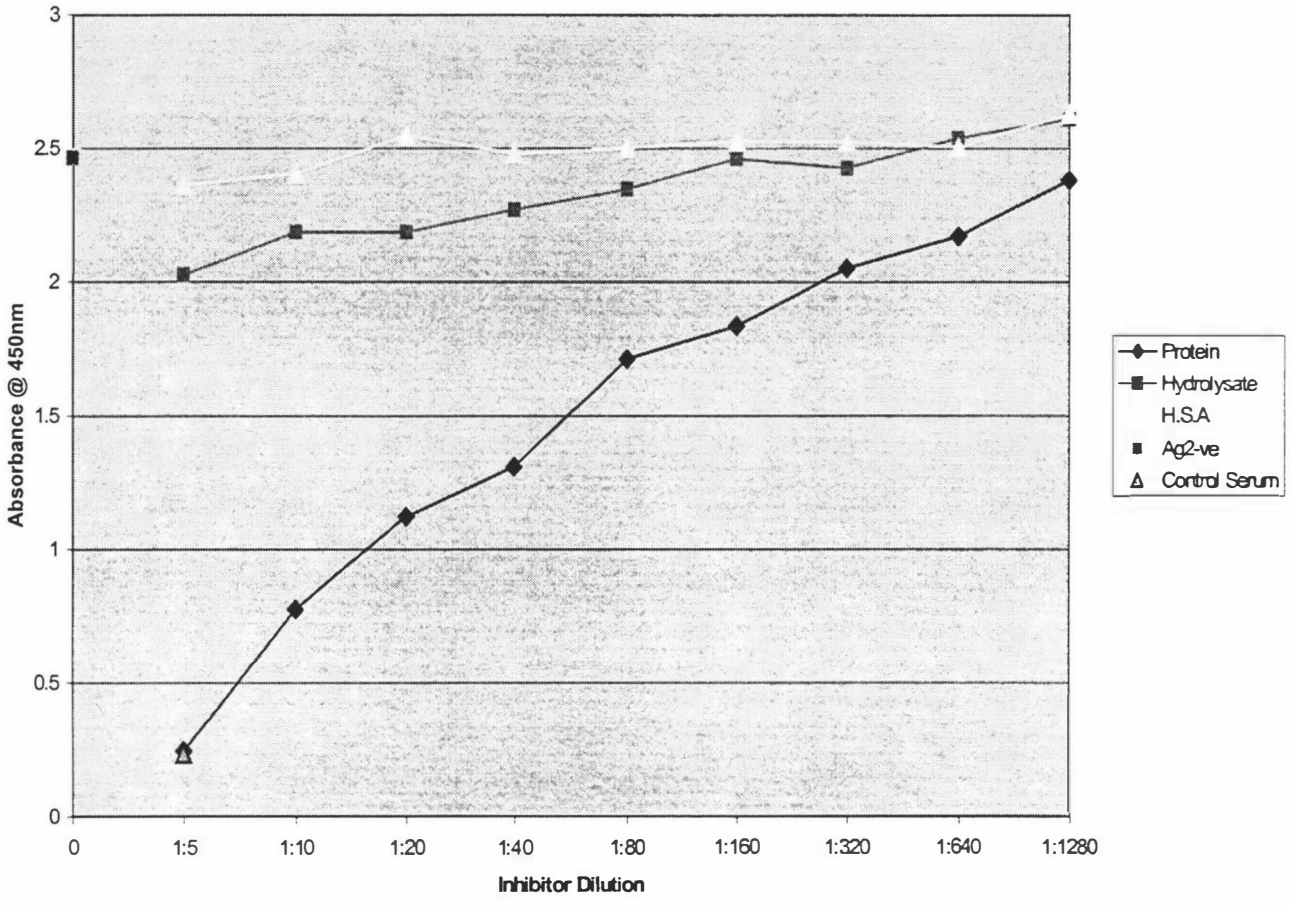
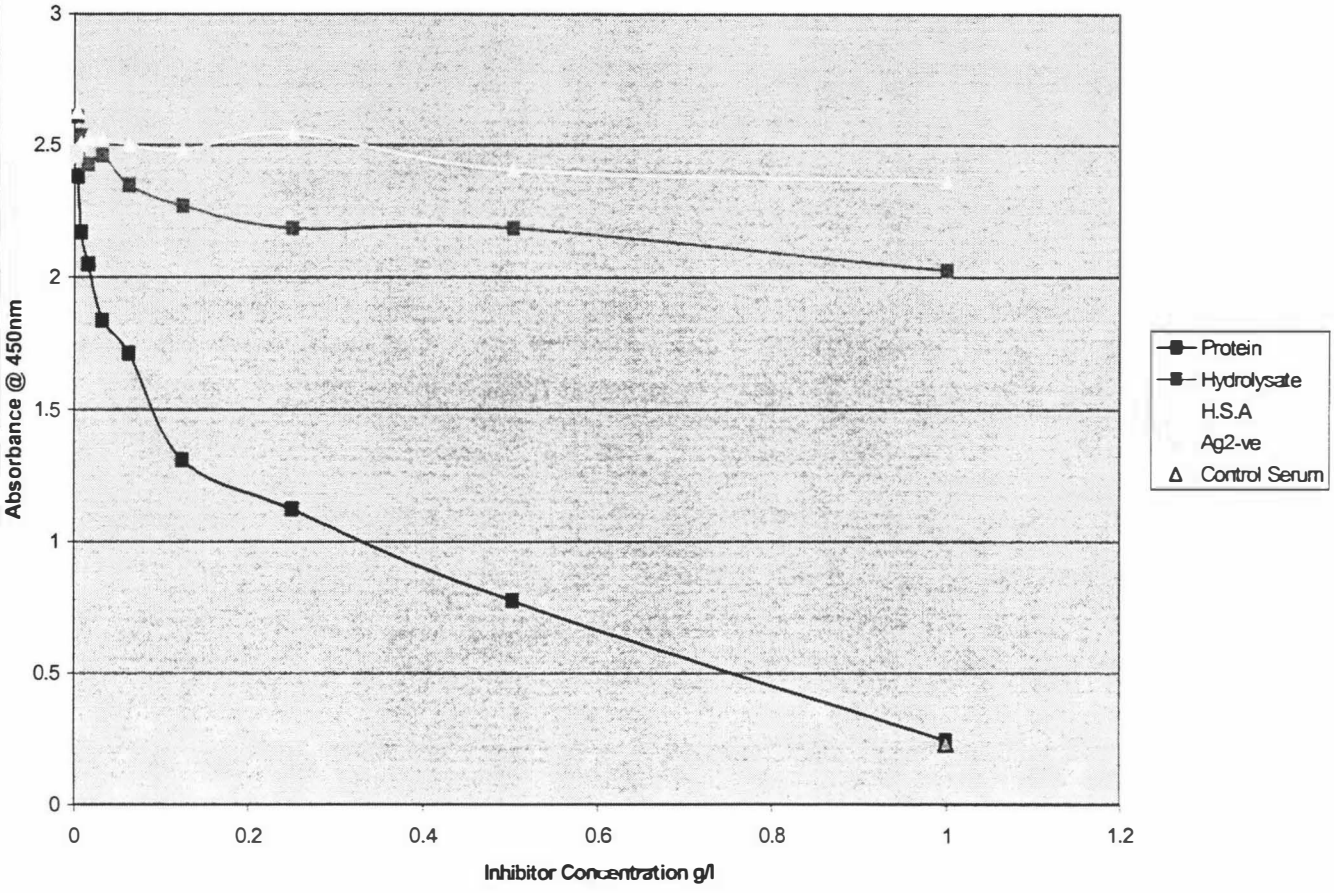


Figure 3.9. Inhibition of Canine IgG Binding to Intact Chicken Protein by a Chicken Hydrolysate, Intact Chicken Protein and HSA
(Nunc Maxisorb® Plate, HSA Block, Whole Chicken Protein Inhibitor 1:80 solid phase dilution.)



Percentage inhibition values were calculated in relation to the maximum inhibition (absorbance value for control serum) and the minimum inhibition (value for wells containing no inhibiting antigen (Ag2-ve)). For this purpose, an inhibitor concentration of 1g/l was chosen because that was the concentration at which an almost 100% inhibition for the intact protein could be attained. These values are presented in Table 3.9.

Table 3.9. Percentage Inhibition of Antibody Binding at Inhibitor Concentration of 1g/l

Inhibiting Antigen	Percentage Inhibition
Intact Protein	99.5
Hydrolysate	27.2
HSA	13.6

Discussion

Inhibition ELISAs detect the ability of one protein to bind to specific antibodies. In this experiment, indirect and inhibition ELISAs were successfully developed to detect canine serum IgG specific for the intact parent protein.

The subsequent ELISAs demonstrated that the immunisation protocol was successful thus indicating that the parent intact protein is immunogenic.

Optimisation demonstrated that the Nunc Maxisorb® plates were superior in both binding the solid phase protein (yielding higher absolute absorbances) and binding the blocking agent (better separation between the test and control wells). The difference between the older and newer plates is speculated to be due to storage although side-by-side comparisons to control for day-to-day variations were not performed.

Whilst all measurements were made in duplicate and variability between wells was low, the repeatability of the assay was not determined. Given that the purpose of developing the ELISA was to compare the binding inhibition between the parent protein and the hydrolysate simultaneously and under identical conditions, the repeatability of the absolute values was not thought to be important. However, the repeatability of the relative inhibition values could have been evaluated to strengthen validity of the results.

The standard approach for describing the percentage inhibition is to calculate the value relation to the maximum inhibition (absorbance value for control serum) and the minimum inhibition (value for wells containing no inhibiting antigen (Ag2-ve)). The inhibiting antigen concentration chosen for this is the concentration at which an almost 100% inhibition for the intact protein could be attained. This was the method used for the calculations presented here. This technique makes two assumptions.

1. The maximum inhibition attainable is expressed by the absence of protein specific antibodies.

Whilst the maximum inhibition was almost reached in this experiment (99.5%), other published inhibition ELISA values have not. Comparing inhibition percentages between publications might be more immunologically accurate if the highest inhibition attainable by the intact protein is used as the maximum inhibition.

2. The minimum inhibition is represented by the absence of an inhibiting protein.

This will probably over-estimate the minimum inhibition possible by a protein at a given concentration. The presence of protein molecules in the solution will inevitably interact with the antibodies in solution in ways independent of specific antibody-epitope binding and prevent some specific antibody binding to the solid phase. For instance, it has been shown that some proteins evaluated for use as blocking agents (such as porcine skin gelatin) exert their blocking effects through protein-protein interactions with the solid phase protein[7]. It is proposed that such non-immunological interactions might occur in solution between antibodies and non-antigenic fragments in the parent protein solution and in the hydrolysate solution. It therefore seems reasonable to control for these non-

specific interactions by using a protein solution of the same inhibitor concentration used to make the calculations but using a protein for which there will be no specific antibodies in the test serum.

In these experiments, human serum albumin was used as the blocking agent and as a control for these non-specific interactions. As can be seen from the data, the HSA resulted in an inhibition of 13.6% higher than the control serum value at the concentration of 1g/l. It may be that the use of such a technique would provide a better control (minimum inhibition) than using inhibitor-negative values.

Although inhibition ELISA techniques have been used extensively in the human medical field to assess the residual antigenicitiy of protein hydrolysates, there are no established absolute values to predict significant residual allergenicity. In addition, since individual patients recognise a specific set of epitopes, often in a MHC-restricted fashion that may be different for another patient, only general statements of reduced allergenicity rather than specific predictions of "anallergenicity" should be used. However, some comparisons can be made with human infant hydrolysate formulae that have been evaluated using inhibition ELISAs and have clinically proven hypoallergenicity in hypersensitive patients. Such work is almost exclusively confined to cow's milk protein hydrolysates and cow's milk allergic patients.

Percentage inhibitions (inhibitory binding potencies) for several hypoallergenic infant formulae have been published[2,1,4]. Often these have been performed using specific milk proteins as comparison inhibitors rather than whole milk whey or casein. Published values for a variety of hypoallergenic formulae, their parent proteins and the inhibition comparison are presented in Tables 3.10-13. As can be seen from the published data, the

percentage inhibition of the chicken hydrolysate tested here compares very favourably with those of established hypoallergenic human infant formulae. On this basis, it is reasonable to suggest that the chicken hydrolysate might be truly hypoallergenic for individuals sensitised to the parent protein. In addition, the development of hypersensitive reactions would be much less likely in individuals fed the hydrolysate than the parent protein.

Table 3.10. ELISA Inhibition Results Using Several Commercial Hypoallergenic Hydrolysate Formulae and Pooled Serum from 215 Milk Allergic Patients. Reference 2

Product[a]	Parent Protein Source	Solid Phase Proteins	Inhibition of IgE Binding (%) @ inhibitor [1gm/l]
Whey Concentrate	Whey	αLa	99
Formula 5	Whey	αLa	72
Formula 6	Whey	αLa	68
Formula 7	Whey	αLa	93
Formula 8	Whey	αLa	69
Formula 9	Whey	αLa	49
Formula 10	Whey	αLa	83
Formula 11	Whey	αLa	85
Formula 12	casein	αLa	43
3000da hydrolysate	Whey	αLa	37
Whey Concentrate	Whey	βLg	100
Formula 5	Whey	βLg	52
Formula 6	Whey	βLg	33
Formula 7	Whey	βLg	84
Formula 8	Whey	βLg	60
Formula 9	Whey	βLg	25
Formula 10	Whey	βLg	72
Formula 11	Whey	βLg	83
Formula 12	Casein	βLg	19
3000da hydrolysate	whey	βLg	17

[a]Original formulae were not named in the publication for reasons of confidentiality. All products were reportedly designated as "hypoallergenic" by the manufacturer. The 3000da hydrolysate was one manufactured by the author's institute.

Table 3.11. ELISA Inhibition Results Using Several Commercial Hypoallergenic Hydrolysate Formulae and Pooled Serum from Two Rabbits Sensitized to the ABBOS Peptide.
ABBOS used as solid phase and formulae used as inhibitors**
Reference 1

Product	Parent Protein Source	Solid Phase Protein	Inhibition of IgE Binding (%) @ inhibitor [1gm/l]
Formula 5	Whey	ABBOS	24
Formula 6	Whey	ABBOS	46
Formula 7	Whey	ABBOS	52
Formula 8	Whey	ABBOS	45
Formula 9	Whey	ABBOS	16
Formula 10	Whey	ABBOS	40
Formula 11	Whey	ABBOS	62
Formula 12	Casein	ABBOS	25
Heat Treated BSA	Bovine Serum Alb.	ABBOS	91
ABBOS	Bovine Serum Alb.	ABBOS	92

** ABBOS is a 19-amino acid peptide found in BSA

Table 3.12. ELISA Inhibition Results with Alfare®[a] Hydrolysate, using sera from 5 milk allergic patients. Reference 4

Patient	Inhibition of IgE Binding (%)
1	86
2	51
3	96
4	97
5	75

[a] Ultrafiltrated whey hydrolysate, Nestle, Vevey, Switzerland

Table 3.13. Mean RAST Inhibition Results for Several Commercial Hypoallergenic Hydrolysate Formulae Using Serum from 10 Milk Allergic Patients. Reference 3

Product[a]	Parent Protein Source	Inhibition of IgE Binding (%)
Cow's Milk	Cow's Milk	92
Nutramigen®	Casein	17
Pregestimil®	Casein	16
Alfare®	Whey	5
Pregomin®	Soy-collagen	46
Good Start H.A®	Whey	43

Whilst ELISAs will reliably detect residual antigenicity in the hydrolysate, the molecular weights of the remaining epitopes responsible for the antibody binding are unknown. Since cross-linking of IgE on mast cell membranes is required for the classical type I hypersensitivity reaction, remaining antigens must be *divalent* in order to be clinical *allergens* and this practically limits the size of food allergens. There is significant variation in the quoted size limit for allergenicity and whilst it is commonly reported to be greater than c.10kDa, peptides in the range of 3.4kDa-6.5kDa have been shown to give clear passive cutaneous anaphylactic reactions[8]. However, whether such peptides were truly binding with and cross-linking membrane-bound IgE as individual peptides was undetermined. It is possible that the peptides were interacting in solution prior to injection or *in vivo* following injection and combining to reform epitopes or establish new ones. Alternatively, these peptides may be causing degranulation through IgE independent mechanisms. So whilst the minimum possible size of allergens holds great importance when evaluating a protein hydrolysate as a candidate for inclusion in a hypoallergenic diet, the exact figure remains to be established.

The difference between *in vitro* IgE binding and *in vivo* allergenic activity has been well demonstrated by van Beresteijn et al[5]. Using experimentally whey-sensitized mice, they demonstrated IgE binding (through an inhibition ELISA) but the absence of an experimental anaphylactic reaction by a whey protein hydrolysate with a MW cut-off of 3kDa. Hence, ELISA techniques may tend to overestimate *allergenicity* by simply evaluating residual antibody binding.

Finally, as stated in the introduction, it is possible that during the hydrolysis procedure, previously hidden epitopes are exposed. It may be that sensitisation to the intact protein may not have resulted in the formation of antibodies to these epitopes. Therefore, whilst there may not be pre-formed antibodies in a patient sensitised to the parent protein, there may be immunogenic peptides and possible allergens that could result in sensitisation following introduction of the hydrolysate diet. Clearly, ELISA techniques using serum from patients sensitised to the parent protein will not detect such epitopes and may, in such a circumstance, under-estimate antigenicity or allergenicity.

To more fully describe the residual *allergenic* activity of a protein hydrolysate, further evaluation is required:

1. Description of the molecular weights of the residual antigens.
2. Determination of the *immunogenicity* of the hydrolysate by experimental sensitisation of subjects.

The attempt to describe the antigens in the chicken parent-protein and the residual antigens in the hydrolysate using Western blotting techniques is described in Chapter 4.

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[1] Van Beresteijn ECH, Detection by Competitive Enzyme-Linked Immunosorbant Assay of a Bovine Serum Albumin Peptide in Infant Formulas Based on Hydrolysed Cow's Milk Protein

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CHAPTER 4

An Attempt to Identify and Describe the Antigenic Fragments in a Chicken Protein Hydrolysate

Introduction

Molecular weight profiles have been used extensively to describe the molecular weight distribution of the peptide and polypeptide fragments in a hydrolysate sample following the hydrolysis procedure. Whilst this gives an impression of the potential for residual antigenic fragments it does not directly assess antigenicity.

However, inhibition ELISA methods do not illuminate in any way the actual size of the remaining antigens. This information is of value to a manufacturer of a potential hypoallergenic diet because if those remaining antigens are less than 6 to 10kDa in size, there is an excellent chance that they will not be able to participate in any IgE-mediated reactions. Alternatively, if any residual antigens were large enough to be considered potential allergens this information could then be potentially used as a guide to the need for and degree of ultrafiltration in the manufacturing of a hypoallergenic diet based on the hydrolysate. Inhibition ELISAs are widely used to quantify this residual antigenicity.

Methods to describe the molecular weight of the antigenic polypeptides remaining in a hydrolysate include immunoblotting,[1] skin prick testing,[2] murine passive cutaneous anaphylaxis testing,[3] lymphocyte proliferation,[4] and laboratory animal immunisation.[5] Whatever method is used, the starting point is to separate the hydrolysate into fractions of known molecular weights. This can be done using methods such as size-exclusion chromatography or gel electrophoresis.

Once the sample has been separated, individual fractions can be assayed for their antigenic or allergenic activity. The use of single fractions in assays such as skin prick testing or lymphocyte proliferation is time consuming and expensive. Separation of samples using gel electrophoresis and assessment of antibody binding using Western

blotting is a simple and relatively inexpensive method for describing the molecular weight of the remaining antigenic fragments.

In order that the size of migrating molecules can be determined in a gel electrophoresis assay, all the molecules in the sample must have similar shapes because shape affects mobility through the gel as well as size. Secondly, since the movement force is the potential difference across the gel, the native charge of the protein molecule will influence the tendency to migrate in the electric field. Native charge on a protein is partly related to but not wholly dependent on the size of the molecule. Thus standardisation of both shape and charge is required before a meaningful correlation between migration distance and molecular weight can be established.

To achieve these goals, proteins are denatured with sodium dodecylsulfate (SDS) and reduced with 2-mecaptoethanol (2ME). SDS binds to the protein causing it to unfold, whereas the reducing agent 2ME reduces the intramolecular and intermolecular disulfide bonds. The binding of SDS by the protein confers a net negative charge, overwhelming any native charge the protein may have had and the denatured protein will migrate through a gel of known percent acrylamide in the presence of an applied electric field towards the positive electrode (anode). The amount of SDS bound to the molecule and thus the net charge is dependent on the size of the protein and not the shape or native charge.

Phase 1 Objectives

The objectives of this study were to use Western immunoblots to identify the molecular weight of the major antigens present in a commercially prepared powdered chicken

protein and to identify the molecular weight of the remaining antigens in a hydrolysate produced from the protein.

Materials and Methods

Gel Electrophoresis

The samples were migrated on sodium dodecyl sulfate polyacrylamide gels (SDS-PAGE) using a modification of the method described by Laemmli.[6] Initially, samples were migrated on mini-gels using a Mini-PROTEAN 3 electrophoresis gel.[1] Separation was achieved using a 12% running gel. A 20ml stock solution of the resolving gel was prepared, which was sufficient to make 5 gels at one time. Both reduced and non-reduced samples were run at the same time.

Resolving gel

7.5mls of 1.5M Tris (pH8.8) was mixed with 12.6mls 30%:0.44% acrylamide-bisacrylamide and 9.9mls dH_2O. To initiate polymerisation, 150µl ammonium persulfate (APS) and 30µl N,N,N',N'-Tetramethylethylenediamine (Temed) were added. The solution was then poured into the gel frame, butanol poured on top to prevent drying and oxidisation and the gel was allowed to set at room temperature. Following polymerisation, the stacking gel was poured.

Stacking gel

Five mls of 0.5M Tris (pH6.8) was mixed with 3.33mls 30%:0.44% acrylamide-bisacrylamide and 11.68mls dH_2O. This was followed by the addition of 100µl APS and 40µl Temed.

Sample loading buffer

SDS (0.05gm 5%) was dissolved in 150µl 15% glycerol, 50µl 1M Tris (pH6.8), 60µl 0.003% bromophenol blue and 720µl dH_2O. For reduced samples, 20µl 2% 2-mercaptoethanol was also added. This was omitted from the non-reduced samples. Buffer was added to the samples at a ratio of 5:1.

Molecular Weight Standards

Molecular weight markers used were Bio-Rad Kaleidoscope Pre-stained Standards®[i] as displayed in Table 4.1.

Table 4.1. Molecular Weight Standards

Protein	Calibrated MW (Daltons)
Myosin	204,000
β-galactosidase	134,000
Bovine serum albumin	84,000
Carbonic anhydrase	41,000
Soybean trypsin inhibitor	31,700
Lysozyme	18,900
Aprotinin	7,500

Samples

Stock solutions of both the intact parent protein and the hydrolysate were prepared by dissolving powdered purified protein (supplied by Hills Nutrition Inc., Topeka, KS) in dH_2O by vortexing. Stock solutions were stored at 4°C for 1 week then discarded. A loading buffer to sample ratio of 1:5 was used for all experiments. Once samples were mixed with the loading buffer they were heated in a water bath at 80°C for 5 minutes then loaded.

[i] Biorad Laboratories, Richmond, CA, USA

Initial migration runs resulted in very poor resolution and marked lateral movement of the samples as the migration distance increased. This was interpreted to be due to large amounts of small, ionised particles in the samples impeding peptide migration. To correct this, further samples were dialysed individually prior to addition of the buffer. This was achieved by placing the samples onto 0.025μM millipore filters and floating on dH_2O for 10mins. Samples were then aspirated, made up to the original volume with dH_2O and mixed with the appropriate buffer solution. On each occasion, 15μl samples were run.

A variety of concentrations of protein solutions were used to optimise the separation procedure. Total amounts ranged from 10μg to 100μg per lane initially. The range of sample amounts loaded per lane resulted in a range of band resolutions and densities as expected. Sample quantities of less than 25μg per lane resulted in poor band density whereas increasing the mass of protein above 50μg per lane did not improve density but resulted in poorer resolution. Above 75μg of sample per lane resulted in significant amounts of protein retention within the sample loading well that hindered migration of the sample into the stacking gel. For future experiments using the mini-gels, sample weights of 50μg per lane were used.

Running Conditions.

The samples were migrated using a Bio-Rad Mini-Protean II[ii] at 80V through the stacking gel then at 160v through the running gel. Running buffer was prepared from 15g Tris, 72g glycine and 5g SDS powder and made up to a total volume of 500mls in dH_2O. After electrophoresis, gels were then either stained Coomassie brilliant blue G-250

[i] Biorad Laboratories, Richmond, CA, USA
[ii] Biorad Laboratories, Richmond, CA, USA

followed by methanol de-staining or transferred to polyvinylidene difluoride (PVDF) membrane paper for blotting.

Western Blotting

The migrated chicken proteins and hydrolysate polypeptides were electro-transferred from the polyacrylamide gel to PVDF membrane[iii]. The blotting buffer consisted of 48mM Tris mixed with 39mM glycine and 0.375g SDS, which was then dissolved in dH_2O and 200mls of methanol and then the resulting mixture was made up to a total volume of 1litre. The pH was measured and the solution was discarded if the pH was outside of the range 9.0-9.3. Initially the SDS-PAGE gel was equilibrated by soaking in blotting buffer twice, for 10 minutes with agitation. The PVDF membrane was soaked in methanol for 1 minute, rinsed twice in dH_2O then equilibrated in blotting buffer twice, for 10 minutes with agitation.

The gel and membrane were juxtaposed between 10 sheets of Whatman 3M® filter paper pre-soaked in blotting buffer. Each layer was placed individually and care was taken to avoid the trapping of bubbles between layers. Electro-transfer was achieved in a horizontal, semi-dry transfer cell (Trans-blot SD, Bio-Rad). A range of voltages and times were tested to optimise protein transfer. SDS-PAGE gels were initially run in duplicate and one was stained with Coomassie blue and the other was blotted. After completion of the blotting procedure the blotted gel was then stained to evaluate for residual protein and was compared with the unblotted gel. Protein transfer for the molecular weight range 10kDa to 100kDa was adequate when the voltage was at 15V for 60 minutes. Following blotting, the membrane was soaked in 2% milk powder in

[iii] Polyscreen® PVDF Transfer Membrane

phosphate-buffered saline with polyoxyethylene(20) sorbitan monolaurate (Tween-20) at 4°C for 12 hours to block further protein binding sites.

Test Serum

The immunized serum used in these experiments was harvested from dogs sensitized by subcutaneous injections, to the unhydrolyzed chicken protein. The experimental protocol is described in Chapter 3. All serum was stored at -80°C until usage.

Developing Western Blot

Serum was used as a pooled sample with equal volumes of each dog's serum. The required dilution of serum was estimated from previous ELISA experiments using the same serum and protein solutions. Thus, a range of serum dilutions from 1:40, 1:80, 1:160 and 1:320 in phosphate PBS+T20 were used with a conjugate dilution (rabbit anti-dog IgG horseradish peroxidase conjugate[iv]) of 1:1000 in PBS+T20.

After blocking, the membrane was rinsed twice in PBS+T20 with agitation for 2 minutes. The membrane was then soaked in the serum solution with agitation for 2 hours at ambient temperature. The membrane was then washed 3 times in PBS+T20 with agitation for 3 minutes. The conjugate solution was added and soaked with agitation for 2 hours at ambient temperature. A further washing cycle of 3 washes in PBS+T20 with agitation for 3 minutes was performed.

Detection of bound antibodies was performed using 4-Chloro-1-Naphthol (4C1N)[v]. Triethanolamine buffered saline was used as the buffering solution. This was prepared

[iv] Sigma-Aldrich Product No. A6792
[v] 4C1N tablets, Sigma-Aldrich Product No C6788

by adding 7.5g NaCl, 2.8ml triethanolamine[vi] and 17ml of 1M HCl to 800ml dH_2O. Following pH assessment (pH 7.5) the solution was made up to 1 litre with dH_2O. To produce the developing solution, 30mg of 4C1N was dissolved in 10ml ethanol to produce a stock solution. A 2ml sample of stock solution was added to 10ml of triethanolamine buffered saline (pH 7.5). Immediately prior to use, 5µl of 30% hydrogen peroxide was added.

The membrane was covered in the 4C1N developing solution with gentle agitation for 45 minutes. To stop the reaction, the membrane was rinsed once in PBS-T20 and then was allowed to dry.

Results

SDS-PAGE clearly demonstrated the presence of multiple discrete protein bands in the intact protein sample. The molecular weights of these proteins varied from about 180kDa to at least as small as 7.5kDa. Few, if any, bands that were present in the reduced sample were absent from the non-reduced sample. However, despite equal quantities of protein being loaded there was a noticeable difference in the amount of protein that had migrated in the non-reduced lane. A large amount of the sample was between about 100kDa and 130kDa. Only faint residual bands were evident in the hydrolysate lanes, which was consistent with the findings of the high-performance size exclusion chromatography where most of the hydrolysate is composed of fragments less than 10kDa. There was no discernible difference between the reduced and non-reduced lanes of the hydrolysate.

[vi] Sigma-Aldrich Product No. T-1377

A photograph of a mini SDS-PAGE gel following Coomassie blue staining prepared for sample quantity optimization is presented in Figure 4.1.

A photograph of a large SDS-PAGE gel following Coomassie blue staining is presented in Figure 4.2.

Figure 4.1. **SDS-PAGE of intact chicken protein with 25μg, 50μg or 75μg or protein loaded per well. Gel stained with Coomasie blue.**

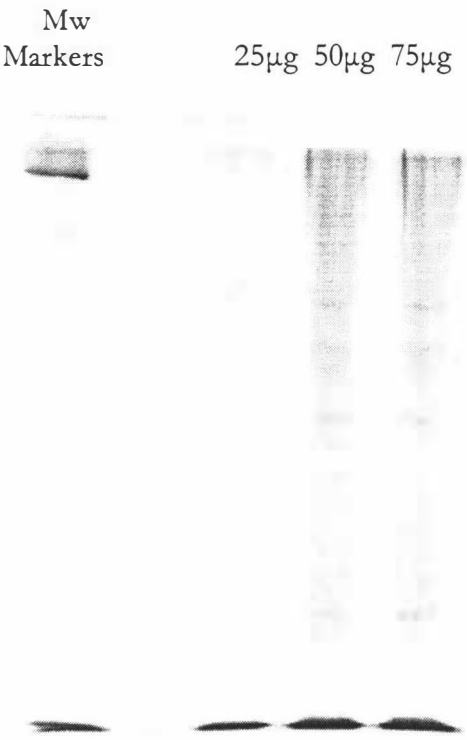
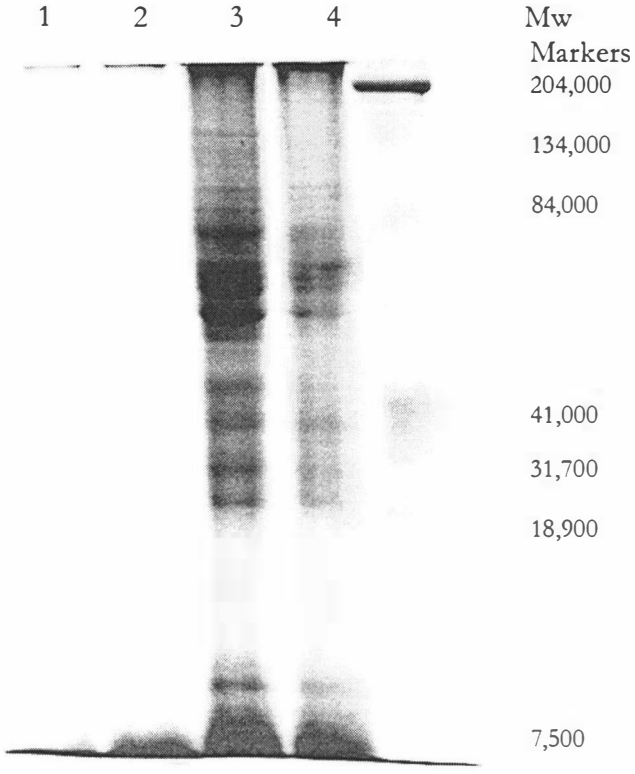


Figure 4.2. SDS-PAGE of intact chicken protein and chicken hydrolysate



Lane 1 = hydrolysate non-reduced, Lane 2 = hydrolysate reduced sample, Lane 3 = intact protein reduced, Lane 4 = intact protein non-reduced. 50μg protein loaded into each well.

Western blots made from the mini-gels produced consistent antibody binding results that appeared independent of the serum dilution used. A strong immunoreactive band was apparent between 65 and 70kDa in both the reduced and non-reduced intact protein samples. Some fainter bands were visible between 30Kda and 60Kda but these were indistinct. No immunoreactive bands were visible in the hydrolysate lanes.

Discussion

The appearance of the hydrolysate sample once subjected to SDS-PAGE was consistent with the HP-SEC profile. The majority of the sample mass lay close to or within the dye front. This observation was expected because a 12% polyacrylamide gel is not suitable for clearly separating peptides smaller than 10kDa under the above running conditions.[7] However, both the SDS-PAGE and the HP-SEC demonstrated the presence of some polypeptides within the 10kDa to 40kDa range in which antigens and potentially allergens may still be intact. Given the faint appearance of immunoreactive bands within this molecular weight range in the intact protein there remains a possibility that there could be residual antigenic material of this molecular weight within the hydrolysate.

Given that there seemed to be no improvement in sensitivity with increasing serum concentration it was hypothesised that to increase sensitivity the amount of antigen would have to be increased. However, optimisation of sample quantity had demonstrated that there was a 50μg limit to the amount of protein that could be loaded into the wells without interfering with sample migration into the stacking gel. This is thought to be due to the presence of large, poorly soluble particles suspended in the sample. To overcome this, two steps were taken. Firstly, large gels were used and secondly, brief centrifugation of the sample prior to adding the buffer and use of only the supernatant was investigated. The use of a larger gel was also expected to give a more accurate measurement of the molecular weight of the major antigen.

Phase 2: Objectives

To increase the sensitivity of the immunoblotting procedure by increasing antigen quantity using larger polyacrylamide gels and discarding undissolved protein fragments.

Materials and Methods

Gel Electrophoresis

Samples were migrated on 16cm gels using Bio-Rad PROTEAN II electrophoresis gel equipment. Separation was achieved using a 12.5% resolving gel.

Resolving gel (12.5%)

Thirteen mls of 1.5M Tris (pH8.8) was mixed with 20.78mls 30%:0.44% acrylamide-bisacrylamide and 16.22mls dH_2O. Prior to polymerisation, the acrylamide solution was degassed to prevent bubbles forming within the gel matrix. This was performed using a sealed Venturi system with tap pressure for 20 minutes. To initiate polymerisation, 250µl APS and 50µl Temed were added. The solution was then poured into the gel frame, butanol poured on top to prevent drying and oxidisation and the gel was allowed to set at room temperature. Following polymerisation, the stacking gel was poured.

Stacking gel (5%)

2.5mls of 0.5M Tris (pH6.8) was mixed with 1.66mls 30%:0.44% acrylamide-bisacrylamide and 6.48mls dH_2O. 50µl APS and 20µl Temed were then added. 1.5mm spacers were used.

Sample loading buffer and running buffer

These were identical to those used for the mini-gels.

Molecular Weight Standards

A different batch of Bio-Rad Kaleidoscope Pre-stained Standards® to those used for the mini-gels were used. The molecular weights are displayed in Table 4.2.

Table 4.2. Bio-Rad Kaleidoscope Pre-stained Standards®

Protein	Calibrated MW (Daltons)
Myosin	217,000
β-galactosidase	126,000
Bovine serum albumin	73,000
Carbonic anhydrase	43,500
Soybean trypsin inhibitor	31,600
Lysozyme	18,000
Aprotinin	7,500

Samples

A ratio of sample to loading buffer of 4:1 was used. Stock solutions of the hydrolysate and intact protein were prepared using dH_2O to produce a concentration of 5mg/ml. Samples were vortexed extensively then centrifuged at c.600rpm in a microcentrifuge[vii]. The supernatant was then aspirated and the sediment was discarded. Total sample volumes for the intact protein were 100µl sample and 25µl buffer. To increase the quantity of the higher molecular weight fragments present in the gel from the hydrolysate samples, the hydrolysate volume added was 150µl to 38µl buffer. This resulted in approximate sample quantities for the intact protein and hydrolysate of 500µg and 750µg

[vii] Micro 12 Compac Microcentrifuge, Fisher Scientific, USA

respectively. The exact quantities could not be calculated because of the unmeasured loss of sample mass following centrifugation and aspiration of the stock solutions. However, the amount discarded is estimated to have been less than 1% of the total mass. Samples were dialysed as previously described prior to addition of the loading buffer.

Running Conditions.

The samples were migrated using a Bio-Rad Protean II PowerPac 1000 [viii] at 25amp through the stacking gel then at 35amp through the resolving gel. The frame was mounted in a water cooler which was maintained at a constant temperature of 12°C. Running time was approximately 7 hours. After electrophoresis, gels were then either stained in Coomassie blue followed by ethanol de-staining or transferred to PVDF membrane for blotting.

Western Blotting

The blotting technique was identical to the mini-gels described above. Blotting was performed at 20V for 90 minutes.

[viii] Biorad Laboratories, Richmond, CA, USA

Developing Western Blot

Conditions used were identical to those described for the mini-gel blots. A pooled serum concentration of 1:80 was used. The blot was photographed immediately after developing then stored overnight in dH_2O to allow colour intensification.

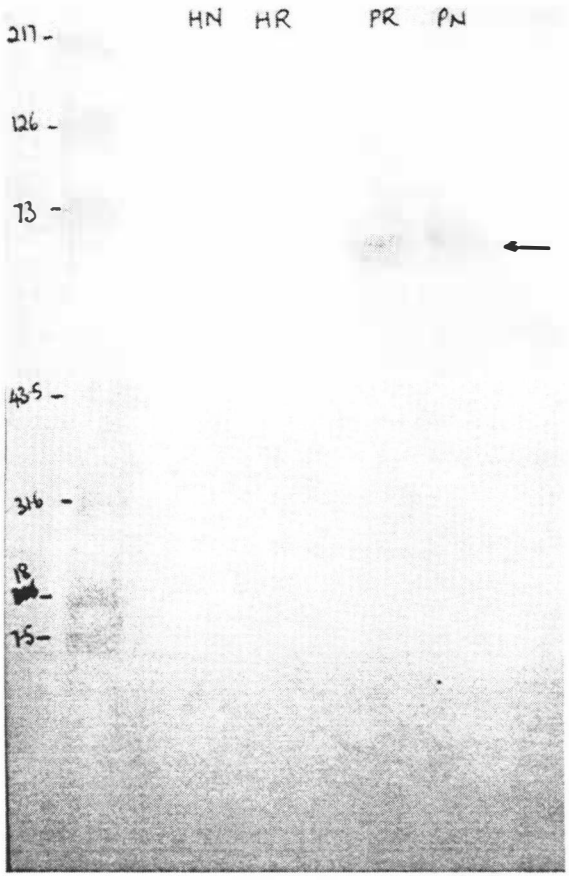
Migration distances were measured from an enlarged image of the blot and plotted against the molecular weight to produce the standard curve. The migration distance of the immunoreactive peptide in the intact protein lanes was then measured and plotted manually on the curve to give an estimated molecular weight.

Results

A photograph of a Western blot taken from a large gel is presented in Figure 4.3. As can be seen, an immunogenic band was present in both the reduced and non-reduced intact protein lanes.

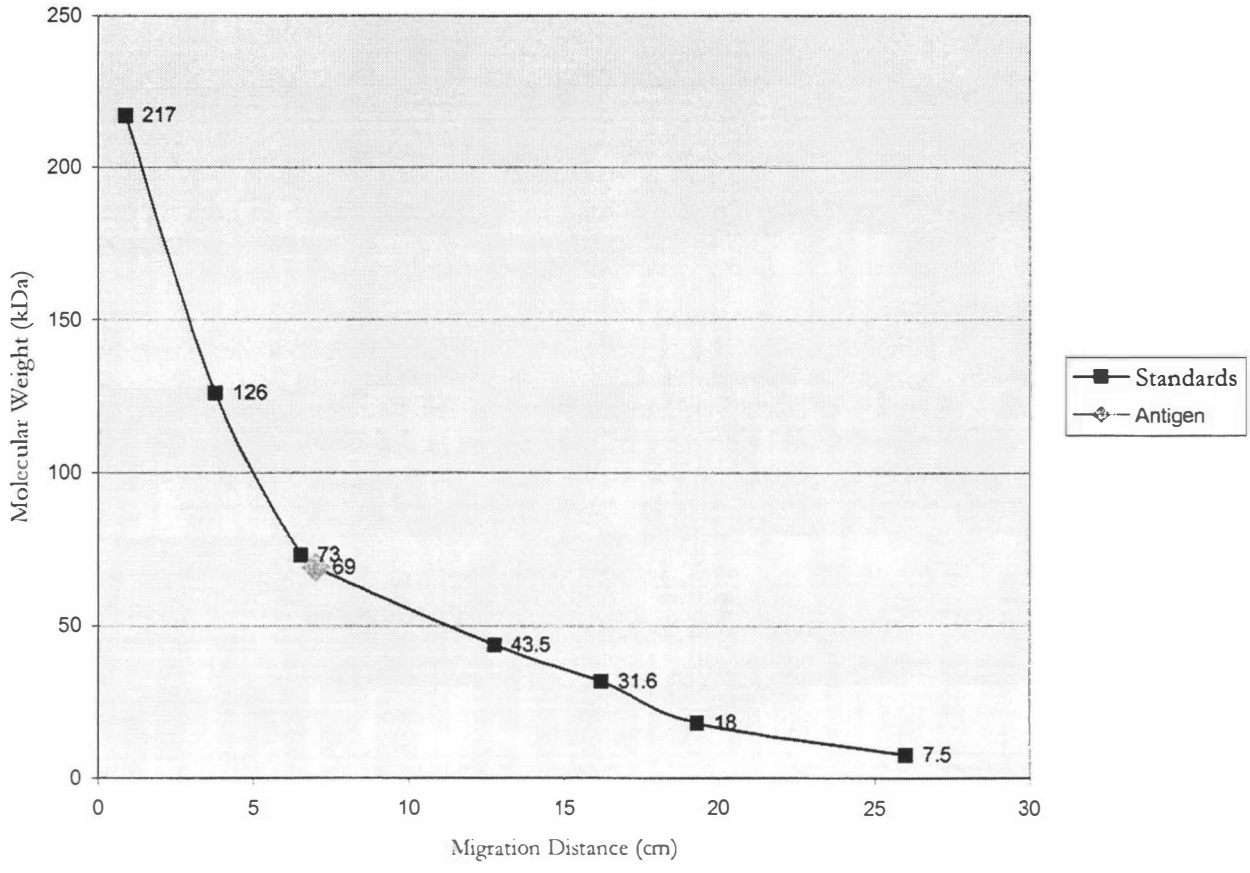
The graph depicting migration distances of the molecular weight standards is presented in Figure 4.4. The migration distance of the immunoreactive peptide in the intact protein lanes was then plotted on the curve and found to be consistent with a molecular weight of about 69kDa. Given the width of the antigenic band the molecular weight range was from 68kDa to 70kDa.

Figure 4.3. Canine IgG binding to intact chicken protein and chicken hydrolysate isolated on SDS-PAGE immunoblot.



HR= reduced hydrolysate sample, HN= non-reduced hydrolysate sample, PR= reduced intact protein sample, PN= non-reduced intact protein sample

Figure 4.4. Graph to show Migration of Molecular Weight Standards and Plot of a Major Antigen in Intact Chicken Protein



As for the mini-gel blots, fainter bands were evident in the 30kDa to 60kDa range. Unfortunately, all identified bands faded rapidly following developing and the fainter bands were not able to be recorded photographically. In addition, rather than intensifying the colour of the substrate reaction, storage overnight in dH_2O resulted in marked fading. No immunogenic bands were identified in the hydrolysate.

Discussion

The objectives of this study were to use Western immunoblots to identify the molecular weight of the major antigens present in a commercially prepared powdered chicken protein and to identify the molecular weight of the remaining antigens in a hydrolysate

produced from the protein. The molecular weight of the main antigen within the intact chicken parent protein appeared to be a peptide of 68-70kDa. The identified 68-70kDa antigen was apparent in both the 2ME-reduced and non-reduced lanes. The fact that the antigen remained capable of binding antibody despite SDS denaturing and 2ME reduction is significant. This suggests that despite its size, the binding epitope(s) is/are linear and not conformational. It also demonstrates that the epitope(s) is/are robust and relatively heat resistant. These qualities are consistent with those found in most food allergens.[8,9]

Given the absence of any apparent antibody binding to 68-70kDa antigen peptides in the hydrolysate lanes (and the molecular weight profile of the hydrolysate) it can be stated that this particular antigen appears to be absent from the hydrolysate. The apparent absence of this peptide is a promising feature of the hydrolysate given the proposed use of the hydrolysate in a hypoallergenic diet.

The molecular weight of the remaining antigenic fragments in the hydrolysate could not be determined by the methods used in this experiment. Several possible explanations for the inability to detect antigen in the hydrolysate are presented below:

1. Residual antigens <10kDa.

   If the antigens remaining in the hydrolysate are significantly less than 10kDa then they will not have been resolved well by the 12.5% gel used in these experiments. The smallest marker (7.5kDa) was very close to the dye front at the end of the electrophoresis and appeared faintly on the membrane after electro-transference. Transference from out of the gel onto the membrane is influenced by the molecular weight of the individual proteins. Larger molecular weight proteins will leave the gel

more slowly than smaller ones. Hence for any given blotting time, proteins will be present in various positions that may range from still retained in the gel to having passed entirely through the membrane. It may be that fragments smaller than the 7.5kDa marker that still retained antigenic activity may have continued to migrate through the membrane and were not retained on it during the transfer.

2. Failure of antigen transference.

Western blotting techniques are generally considered extremely sensitive detection methods. Detection limits using polyclonal serum, HRP-conjugated secondary antibody and 4C1N substrate are reported to be around 1-2ng of protein per band.[10] Confirmation of adequate protein transfer to the membrane can be achieved by either temporary staining of the membrane to identify the presence of transferred proteins or by including coloured markers, the method used here. In these experiments, transference of the coloured markers was clearly achieved and as such, sample transference is expected to have adequately occurred. However, the amount of protein transferred from the hydrolysate lanes in the molecular weight range of interest (i.e. >10kDa) may have been too low for detection.

3. Incorrect conjugate or serum dilutions.

The dilutions of the conjugate and serum were extrapolated from the concentrations used in the ELISA experiments and may not have been optimal. This seems unlikely, however, because of the successful detection of the chicken antigens in the gels containing the parent protein. Whilst it is unlikely that increasing the serum concentration would improve sensitivity, it is conceivable that increasing the conjugate concentration might do so.

4. Conjugate/substrate deficiencies.

The conjugate used in these experiments was the same as that used successfully in the inhibition ELISA experiments. The conjugate had been dispensed into 20µl aliquots and stored at –86°C until use. However, the conjugate had been stored for c.18 months prior to usage in both the ELISA and Western blot experiments. TMB, the substrate used in the ELISA experiments is reported to be a very sensitive substrate for use with HRP. 4C1N has been demonstrated to be a less sensitive conjugate than stabilised TMB by a factor of at least 3.[11] Furthermore, fading of the reaction product is common. Deterioration of the conjugate with time might be apparent sooner with one substrate that with the other. This could be due to the difference in reactions between the TMB and the 4C1N and the fact that the ELISA reaction product is a soluble molecule as opposed to the insoluble (solid phase) reaction product formed by the 4C1N.

5. Conformationally dependent epitopes.

It may be that the remaining antigenic fragments have retained a 3-dimensional epitope conformation that is preserved during the ELISA protocol but is disrupted during the heating or SDS denaturing. If this is the case then it is likely to be a large molecular weight fragment and therefore represents a very small percentage of the total sample mass. The manufacturing process responsible for the incorporation of the hydrolysate into a commercial diet includes a heating phase (Lynn Huffaker, Hills Nutrition Inc., Personal communication) that will exceed the SDS-PAGE conditions. It would be expected therefore that the complete diet would be less likely to contain residual intact antigens from the hydrolysate than the hydrolysate on its own.

Given that the ELISA experiments had demonstrated excellent antigen-antibody reactions with the intact protein, it remains undetermined exactly why the Western blotting procedure failed to produce clear, distinct and lasting results. The reasons considered are similar to those considered for the lack of bands visible in the hydrolysate lanes of the gel, namely: incorrect conjugate or serum concentrations, conjugate or substrate deficiencies or that in contrast with suspicions, the problem is solely one of loss of intact epitopes during the SDS-PAGE procedure. If this last possibility is the reason then the antigen is reasonably labile and is not a good candidate for a food allergen. Again, if it is the case that the most antigenic component in the intact chicken protein is a large, confomationally dependent antigen then that might explain why chicken is underrepresented in reports of food allergies.

The powdered intact protein is prepared from a combination of chicken liver and heart tissues. The resulting product would be considered to be a relatively heterogeneous immunologic compound. Consequently, speculation over what the 68-70kDa protein might be is difficult. However, the SDS-PAGE separation demonstrated that there was a significant amount of protein present in the sample around the 65kDa to 75kDa range. It is reasonable to suggest that the protein then is a ubiquitous protein in the source tissues.

Chicken serum albumin is a 70kDa protein found also in egg yolk where it is termed α-livetin.[12] As previously stated, allergy to chicken meat is rare in both veterinary and human patients relative to other meat allergens. However, some attention has been given to the immunologic and clinical aspect of the so-called "bird-egg" syndrome.[13] Patients

with this allergy exhibit dermatological and respiratory symptoms after ingestion of egg yolk and/or inhalation of bird- and chicken-derived materials (e.g. feather dander). This has recently been shown to be due to the presence of α-livetin in both feather dander and yolk.[14] Given that chicken serum albumin has previously been shown to be a food allergen, to retain allergenicity after heating, SDS denaturing and 2ME reduction, it is speculated that the antigen identified here may be chicken serum albumin. Further immunoblotting experiments using monoclonal antibodies to chicken serum albumin would test this hypothesis.

Conclusion

The major antigen present in the intact chicken protein sample is a 68-70kDa protein that seems to posses qualities expected of a food allergen. It is speculated that this protein is chicken serum albumin. This antigen was undetectable in the hydrolysate.

It has been demonstrated using inhibition ELISA techniques that the hydrolysate does have some residual antigenicity. The molecular weight range of the remaining antigenic peptide fragments could not be detected with the techniques used in this experiment. This is speculated to be due to the low concentrations of these peptides, technical shortcomings, or the loss of epitopes during the SDS-PAGE procedure. Further immunoblotting experiments are required to determine the size of these antigenic fragments.

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CHAPTER 5

Summary, Conclusions and Future Directions

SUMMARY AND CONCLUSIONS

The use of protein hydrolysates in commercially prepared diets for dogs and cats provides a new means by which food hypersensitivity can be diagnosed and managed. However, the variety of potential proteins, enzymes and hydrolysis conditions available to manufacturers means that hydrolysates vary greatly in their nutritional, physicochemical and immunological characteristics. The experiments presented in this thesis have described a protein hydrolysate from both physicochemical and immunological perspectives.

The use of high-performance size-exclusion chromatography described in Chapter 2 allowed the generation of molecular weight profiles of 2 candidate hydrolysates, one produced from fish and the other from chicken. The finding that 92.9% of the chicken hydrolysate was composed of peptides smaller than 5kDa and that only 3.1% was composed of fragments larger than 10kDa suggested that the hydrolysate was a suitable candidate for further study. In contrast, 56% of the fish hydrolysate was composed of fragments smaller than 5kDa whilst 34% was composed of fragments larger than 10kDa. In addition, the chicken hydrolysate compared favourably with the profiles of established hypoallergenic human-infant milk hydrolysate formulae. On the basis of these findings, the chicken hydrolysate was selected for further evaluation.

The development of an inhibition ELISA was chosen to assess the residual antibody binding ability of the chicken hydrolysate. To achieve this end, polyclonal IgG was raised in dogs by sensitizing them to the intact parent protein. The sensitization procedure was successful in producing high levels of protein-specific serum IgG. The ELISA inhibition assays demonstrated that whilst the hydrolysate did retain some ability

to bind serum IgG, this was greatly reduced when compared with the intact protein. Indeed, at equal levels of antibody binding (27.2% inhibition), the concentration of the hydrolysate solution required was 66 times greater than that of the intact parent protein. It is likely that this represents a clinically highly significant reduction in antigenicity.

In Chapter 4, an attempt was made to identify the molecular weight of the fragments that were capable of binding IgG. This was felt to be important because there is a limitation to the minimum size at which a peptide can act as an allergen. This is due to the requirement for cross-linking of IgE molecules bound to the high affinity receptor FcεR1 on the surface of cells such as the mast cell. Whilst there is considerable debate about the exact size, it is generally believed to be somewhere between 6 and 10kDa. Samples of the intact protein and hydrolysate were subjected to SDS-PAGE and demonstrated the heterogeneous nature of the parent protein source and the presence of high molecular weight fragments. In contrast, the SDS-PAGE supported the description of the hydrolysate by HP-SEC in finding that there were no peptide bands larger than 10kDa visually detectable following Coomassie blue staining. Western immunoblotting after SDS-PAGE separation allowed identification of the major antigen in the intact protein sample, which was found to be a 69kDa protein consistent with the size of chicken serum albumin. The molecular weight of the IgG-binding fragments within the hydrolysate were not successfully identified.

In summation, the chicken hydrolysate evaluated during the experiments presented in this thesis, shows promise as the peptide fraction of a diet to be prescribed for the diagnosis and management of food hypersensitivity and as an adjunct to conventional therapy in the management of idiopathic inflammatory bowel disease.

FUTURE DIRECTIONS

Further attempts to describe the remaining antigenic fragments within the chicken hydrolysate could be valuable in that they might allow identification of large molecular weight fragments that could be effectively eliminated through a post-hydrolysis ultra-filtration stage. This might result in the production of a diet with almost zero antigenicity with relatively little added expense depending on the filtration requirements. Whilst it is reasonable to extrapolate from the ELISA and Western blotting experiments performed using canine IgG to make assumptions about feline responses, they remain assumptions. Similar experiments using feline IgG would be required to confirm the same degree of reduction in antigenicity in that species.

The ideal assessment of a protein hydrolysate diet is to demonstrate that it does not elicit clinical signs in animals hypersensitive to the parent protein. Since spontaneously occurring food hypersensitivity to chicken is uncommon amongst cats and dogs it is unlikely that this will ever be adequately tested in clinical cases. Experimental sensitization of dogs to dietary proteins has proved difficult to maintain for long periods after oral exposure to the food protein to which the dogs have been sensitized. An alternative approach is to harvest sera from dogs and cats with naturally occurring chicken hypersensitivity and to perform serum IgE ELISA inhibition and Western blotting experiments. These experiments will determine if the *antigens* used during experimental sensitization procedures are a true reflection of the *allergens* to which naturally sensitized individuals respond. More practical however, would be to assess the response of animals with other more common forms of dysregulated mucosal immunity such as idiopathic inflammatory bowel disease. In that model, the hypothesis would be that the feeding of a protein hydrolysate would have a steroid-sparing effect or more

reliably induce remission than an unhydrolyzed diet derived from the same protein source. Such investigations are currently underway.