# STUDIES ON FELINE IgE

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## DECLARATION

I certify that all the work reported in this thesis
was undertaken by me with the exception of the immunisations
and the IDSTs of the normal cats in chapter 3, and
the quantification of HSA-specific IgG and IgA in chapter 6.

## DEDICATION

A maman, papa, Christophe, Colette et les enfants
To Prof., Jenny and Jemma
with love and thanks

# ACKNOWLEDGEMENTS

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# LIST OF ABBREVIATIONS

| | |
|---|---|
| *A.* | *Ascaris* |
| AA | Arachidonic acid |
| AD | Atopic dermatitis |
| Asc-1 | *Ascaris suum* antigen |
| *B.* | *Brugia* |
| BSA | Bovine serum albumin |
| CAPS | (3-(cyclohexylamino)-1-propanesulfonic acid) |
| CD | Cluster determinant |
| CD23 | Low affinity receptor for IgE (FcγRII) |
| CFA | Complete Freund's adjuvant |
| CNBr | Cyanogen bromide |
| CO | Cyclooxygenase |
| con-A | Concanavulin A |
| CSA | Cat serum albumin |
| DBPCFC | Bouble-blind placebo-controlled oral food challenge |
| DEAE | Diethylaminoethyl |
| DF | *Dermatophagoides farinae* |
| DGLA | Dihomo-gamma-linolenic acid |
| DLA | Dog leucocyte antigen |
| DNP | 2,4-dinitrophenyl |
| DP | *Dermatophagoides pteronyssinus* |
| DS | Donkey serum |
| EFA | Essential fatty acid |
| ELISA | Enzyme-linked immunsorbent assay |
| ES | Excretory/secretory antigens |
| Fc | Fragment crystallizable |
| FcεRI | High affinity receptor for IgE |
| FcεRII | Low affinity receptor for IgE |
| FcγRI | High affinity receptor for IgG |
| FcγRII | Low affinity receptor for IgG |
| FcγRIII | Low affinity receptor for IgG |
| Fig | Figure |
| GLA | gamma-linolenic acid |
| GM-CSF | Granulocyte/macrophage-colony stimulating factor |
| H1, H2 | Histamine receptors |
| HC | Heavy chain |
| HD | House dust |
| HDM | House dust mite |
| HLA | Human leucocyte antigen |
| HPLC | High performance liquid chromatography |
| Hr | Hour |
| HRF | Histamine releasing factor |
| HSA | Human serum albumin |
| IDST | Intradermal skin test |
| IEP | Immunoelectrophoresis |

| | |
|---|---|
| IFN | Interferon |
| Ig | Immunoglobulin |
| Il | Interleukin |
| IU | International unit |
| Ka | Constant of affinity |
| KDa | Kilodalton |
| L-2 | Larvae second stage |
| LA | Cis-linoleic acid |
| LC | Light chain |
| LPR | Late phase reaction |
| MHC | Major histocompatibility complex |
| MW | Molecular weight |
| NE | Nitrogen equivalents |
| NK cells | Natural killer cells |
| NRS | Normal rabbit serum |
| OD | Optical density |
| PAF | Platelet activating factor |
| PBS | Phosphate-buffered saline |
| PCA | Passive cutaneous anaphylaxis |
| PHA | Phytohemagglutinin |
| PNP substrate | p-nitrophenyl phosphate disodium hexahydrate |
| PG | Prostaglandin |
| PK | Praustniz Küstner test |
| PNU | Protein nitrogen unit |
| RAST | Radioallergosorbent test |
| RAU | Relative antibody unit |
| RIA | Radioimmunoassay |
| S-200 | Sephadex 200 |
| S | Sedimentation coefficient |
| SASS | Saturated ammonium sulphate solution |
| SDS-PAGE | Sodium dodecyl sulphate polyacrylamide gel |
| *T.* | *Toxocara* |
| TBS | Tris-buffered saline |
| TEMED | N,N,N',N'-tetramethylenediamine |
| TGF | Transforming growth factor |
| Th-0 | T-helper class null cells |
| Th-1 | T-helper class 1 cells |
| Th-2 | T-helper class 2 cells |
| TGF | Transforming growth factor |
| TNF | Tumor necrosis factor |
| Tris-HCl | Tris (hydroxymethyl) amino methane-hydrochloride |
| w/v | Weight per volume |

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# ABSTRACT OF THESIS

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Feline reaginic antibody has been shown to be present in the serum of some cats with allergic skin disease or parasitic infections. However few studies have attempted to characterise feline IgE and the production of antisera specific for feline IgE has yet to be reported. The involvement of this isotype in feline allergic diseases is thus unclear.

The first aim of these studies was to purify and characterise feline IgE to produce specific polyclonal antisera and investigate anitigen-specific IgE responses in cats. Cats naturally or experimentally infected with *T. cati* were immunised with dinitrophenylated ascaris antigen (DNP-Asc). All cats developed immediate skin reactivity to DNP coupled to bovine serum albumin (DNP-BSA) and the sera of the nine cats had a heat labile homocytotropic antibody detectable by Prausnitz-Küstner (PK) tests. Reagin-rich fractions were prepared from these sera by a variety of techniques and used for the preparation of antisera in rabbits. Resultant antisera were passed through an immunoabsorbent column of heated normal cat serum. An immunoabsorbent column prepared with the putative anti-IgE serum removed PK reactivity from the cat sera, and the reactivity was recovered following acid elution. The antiserum failed to detect any recognised immunoglobulin in cat sera but precipitated with a heat labile protein with γ-1 electrophoretic mobility in the sera of parasitised cats. These findings support the contention that the antiserum is specific for feline IgE.

Ten normal cats were immunised with *Dermatophagoides farinae* (DF) antigen and intradermal skin tests (IDSTs) were performed weekly. Sera from the latter were also assessed for DF-specific IgE by ELISA and PK tests. Detectable DF-specific IgE was induced in all of the 10 cats, however levels were found not to be correlated with the development of positive IDSTs nor with the levels of IgE as assessed by PK tests.

Sera from 10 cats with symptoms consistent with atopy, from 15 normal household cats and from 11 laboratory maintained cats were assessed for allergen-specific IgE and IgG to DF by ELISA. Although DF-specific IgE was detectable in all the atopic cats, there was no significant difference between the levels in this group and in the clinically normal household cats. However levels in both these groups were significantly higher than those in the laboratory maintained cats.

The influence of vaccination and endoparasitism on the IgE response to a food antigen was assessed in 34 cats. Seventeen kittens experimentally infected with *T. cati* and 17 parasite-free kittens were dosed with human serum albumin (HSA) daily for 3 weeks. Seven cats from both groups were given two injections of a live attenuated viral vaccine. The group of parasitised cats had significantly higher levels of HSA-specific IgE, IgG and IgA than did the group of parasite-free cats. Vaccinated cats had also higher levels than non vaccinated cats but only in the group of parasite-free cats. None of the cats developed clinical signs of food allergy.

The findings from these studies are strongly suggestive of the existence of a heterogeneity of IgE in cats, and imply that endoparasitism and vaccination have an immunomodulatory role on the antigen specific antibody response.

# CHAPTER I. INTRODUCTION

## I. IMMUNOGLOBULIN E

### I.1. IMMUNOGLOBULIN E (IGE) IN MAN AND DOGS

#### 1. Early studies on human reaginic antibodies

The presence of reaginic antibodies in the serum of allergic individuals was first described by Prausnitz and Küstner in 1921. They showed that an intracutaneous injection of fish antigen into a sensitive individual gave a wheal and erythema reaction. Subsequently they showed that the injection of serum from Küstner, who was allergic to fish, into Prausnitz's skin followed by the intradermal injection of the fish antigen at the same site on the next day resulted also in skin reactivity. Therefore they concluded that the reactivity to the allergen could be transferred to a normal individual by injecting serum from the sensitive individual. These observations suggested that atopic patients have an unusual type of antibody that mediates immediate hypersensitivity reactions.

The active principle in the serum capable of mediating immediate hypersensitivity reactions in response to antigenic challenge was called reagin (Coca and Grove, 1925).

In 1959, Ishizaka et al. suggested that the bridging of two cell-bound antibody molecules by antigen to form a biologically active complex on the cell surface may be responsible for the induction of allergic reactions and that the biologic activities of antibody molecules are associated with the Fc portion of the molecules (Ishizaka and Ishizaka, 1959; Ishizaka et al., 1962).

#### 2. Characterisation of human reaginic antibody

By 1960, evidence was accumulating that supported the concept that reaginic antibodies belonged to the IgA class which at that time was the latest of the immunoglobulin classes to be isolated and characterised. However, Ishizaka and Ishizaka (1966) reported that the reaginic activity was associated with a previously undescribed immunoglobulin class. They showed that reaginic antibody titres, determined by PK reactions using sera from ragweed-sensitive patients, did not truly parallel the levels of IgA. They also showed that anti-IgA antibody was not able to neutralise the PK reactivity in sera from allergic individuals. They then prepared

antibodies specific for reaginic antibody and were able to detect allergen-specific antibody in the sera of allergic patients by radioimmunoelectrophoresis. The adsorbed antiserum did not give a precipitin band with IgG, IgA, IgM and IgD but the skin-sensitising activity of the sera of allergic patients was removed by the addition of this antiserum (Ishizaka and Ishizaka, 1966). Therefore they suggested that the reaginic activity was associated with a previously undescribed immunoglobulin class designated γE because of the erythema that the allergen provokes following the injection into the skin of allergic individuals (Ishizaka et al., 1966a). They also showed that the levels of allergen-specific antibody closely paralleled the levels of this isotype (Ishizaka et al.,1966b). Furthermore, antigenic analysis of γE revealed antigenic determinants which are not shared by any immunoglobulin of the other classes or subclasses. This immunoglobulin therefore represented a distinct immunoglobulin class (Ishizaka et al., 1966b&1967).

Simultaneously, Johansson and Bennich (1967) discovered a myeloma protein that was not recognised by antisera to any of the known Ig classes. They termed this protein IgND. Later the myeloma protein and γE were found to share the same antigenic structure and physicochemical properties (Bennich et al., 1969).

In 1971, the direct demonstration of the cytophilic properties of IgE was achieved by Ishizaka and collaborators. They showed that it bound to basophils and mast cells and sensitised these cells for anti-IgE induced release of histamine and serotonin (Ishizaka et al., 1971). The minimum concentration of human IgE required for sensitising human skin for a positive PK reaction was estimated to be 0.2 ng/ml (Ishizaka and Ishizaka, 1975). Ishizaka and Ishizaka (1978) using antibodies specific for IgE-receptors on rat mast cells showed that bridging of IgE-receptors can also activate mast cells for mediator release.

## 3. Structure and physicochemical properties of IgE

### 3.1. Structure of immunoglobulins

The basic subunit of all immunoglobulin molecules consists of 4 polypeptides, 2 identical heavy chains (HC) and 2 identical light chains (LC). Each of these chains consists of an amino-terminal variable (V) region and a carboxy terminal constant (C) region. The carboxy terminal constant region domains of each chain are defined by 2 isotypes of Ig LC (κ and λ) and 5 isotypes of Ig HC (α, δ, ε, γ and μ). In man, the α and γ classes comprise 2 and 4 subclasses respectively. The heavy chains (HCs) of the

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subclasses share many common antigenic determinants. The variable regions of each Ig HC and light chain (LC) pair to form the antigen binding site of the molecule whereas the immunoglobulin HC constant regions are responsible for the effector functions common to antibodies of a given isotype (Honjo, 1983). Antibodies of different isotypes differ significantly in their abilities to fix complement, to bind to heavy chain-specific receptors (Fc receptors) present on a wide variety of cell types, to cross mucosal and placental barriers, and to form polymers of the basic 4-chain immunoglobulin molecule.

## 3.2. Structure of IgE

Human IgE has a molecular weight of approximately 190 kilodaltons (KDa) with a reported range from 184.5 to 196 KDa (Johansson and Bennich, 1967; Bennich and Johansson, 1971; Kochwa et al, 1972; Ishizaka, 1988). Epsilon heavy chains have an estimated molecular weight ranging from 69.8 to 72.5 KDa (Bennich and Johansson, 1971; Kochwa et al, 1972; Ishizaka, 1988) and are composed of five domains (C$\varepsilon$1-C$\varepsilon$5), all rich in carbohydrate.

The 2 heavy chains are connected with each other by 2 inter-HC disulphide bonds. IgE is split into Fc and Fab fragments by papain, and susceptible to pepsin digestion which yields F(ab')$_2$ fragments. The 2 constant-terminal domains of IgE share many common amino acid sequences with those of IgG (Helm et al., 1991). However, in contrast to IgG, IgE is bent and the distance between the N- and C-terminals of the IgE molecule in solution is no more than 7 nm, compared with the 17.5 nm implied by a planar structure (Zheng et al., 1991).

The principal binding site for the high affinity receptor of mast cells has been shown to be located on C$\varepsilon$3 in both the mouse and man (Presta et al., 1994).

## 3.3. Physicochemical properties of IgE

One of the classical properties of reagins, first demonstrated by Coca and Grove (1925), is their heat sensitivity. Reaginic antibody is inactivated by heating at 56°C for 4 hours (Loveless, 1940; Ishizaka et al., 1967). The loss of affinity of IgE for tissue by heating has been ascribed to structural changes in the CH3 and CH4 regions of the molecule (Bennich and Dorrington, 1972). On the other hand the antigen-binding capacity which resides in the Fab portion is preserved.

Sensitivity to reduction-alkylation is another property of reaginic antibody. Ishizaka and Ishizaka (1968a) showed that the ability of IgE antibodies to sensitise

human skin for PK reactions was lost following reduction in 0.1 M mercaptoethanol and subsequent alkylation.

Therefore, it appears that the Fc portion of the IgE molecule is essential for binding to target cells and that the disulphide bonds are essential for the biological characteristics of the molecule whose sensitisation persists for a long time. IgE does not have the capacity to activate either complement pathway.

Since IgE is larger than IgG, it is eluted earlier following gel filtration. When an IgE-rich fraction was filtered through a Sephadex G-200 column, it was detected in the ascending portion of 7S peak, which is similar in position to that of IgA and IgD (Ishizaka et al, 1966a; Ishizaka and Ishizaka, 1967).

Using sucrose density gradient ultracentrifugation, Anderson and Vannier (1964) found that the average sedimentation coefficient of human reaginic antibodies was 7.8S.

IgE has $\gamma$-1 mobility upon immunoelectrophoresis at pH 8.6 and because of its lower concentration and its slower diffusion, its precipitin line is closer to the well than the precipitin line of IgG.

It elutes at a higher molarity from diethylaminoethyl (DEAE) ion-exchange columns when compared with the bulk of IgG and it was not found in the IgG fraction that was excluded from DEAE using 0.005M phosphate buffer pH 8.0 (Ishizaka et al, 1966a; Ishizaka and Ishizaka, 1967).

Unlike IgG, IgE is still soluble in 40 % saturated ammonium sulphate (Isersky et al., 1974). A low proportion (7 to 8 %) of IgE binds to protein A (Johansson and Inganäs, 1978).

IgE antibody does not cross the placenta but can be synthesised by the human foetus as early as the 11th week of gestation (Miller et al., 1973).

### 3.4. Physicochemical properties of canine IgE

In the dog, preliminary characterisation of IgE was first undertaken by Patterson et al. in 1963. These workers observed that the canine reagin shares a number of characteristics with the human counterpart. It was found to be soluble in 33% saturated ammonium sulphate, but precipitated by 50%. It migrated with the $\beta$ globulins upon electrophoresis, was heat labile, was capable of sensitising normal recipients for cutaneous and systemic anaphylaxis and was incapable of inducing a passive cutaneous anaphylaxis (PCA) reaction in guinea pigs.

In 1967, Rockey and Schwartzman showed that canine reaginic antibody had a sedimentation coefficient of 8-9S, was mercaptoethanol sensitive and was recovered on Sephadex G200 before the elution of the bulk of the 7SγG globulin.

The term IgE for the canine reaginic antibody was subsequently introduced by Schwartzman et al in 1971. They induced anti-DNP reaginic antibody in atopic dogs with ragweed hypersensitivity by immunisation with dinitrophenylated ragweed antigen. They showed that the induced and spontaneous reaginic antibodies were immunochemically very close to each other but different from the other classes of immunoglobulins.

Halliwell et al. (1972) demonstrated the antigenic similarity between canine IgE and human IgE. They showed that the PK titre to ragweed was decreased by absorption of canine reaginic serum with anti-human IgE and that anti-human IgE was able to elicit reversed cutaneous anaphylaxis in the dog. More recently, Peng et al. (1996) directly demonstrated the cross-reactivity of dog IgE with human IgE by Western blot analysis using an anti-human IgE. Moreover, canine reaginic antibody from dogs suffering from allergic disease is able to sensitise both human and equine basophils (Prélaud et al., 1993; Sainte-Laudy and Prost , 1996).

It was in 1973 that Halliwell successfully isolated, purified and further characterised canine IgE from dogs infected with *Toxocara canis* (*T. Canis*) (Halliwell, 1973a).

Canine IgE antibody was found to have a molecular weight of 196 KDa for the whole molecule and of 73 KDa for the ε chain, which is closely similar to the molecular mass of human IgE (Peng et al., 1993a).

A proportion of canine IgE binds to protein A as does human IgE but it does not bind to protein G (Peng et al., 1991&1997).

## 4. Metabolism of IgE

IgE has a short half-life in serum of only 2 to 3 days and has the shortest survival, highest fractional catabolic rate and lowest synthetic rate of the 5 major classes of immunoglobulin (Waldman, 1969). However IgE can persist in the skin for 2 to 3 weeks and the average half-life for loss of sensitisation at passively sensitised skin sites was reported to be 13 days (Cass and Anderson, 1968).

## 5. Levels of total serum IgE

### 5.1. In man

In man, total serum IgE concentrations are highly variable. Usually the level of total IgE in normal individuals is very low with a range of around 20-200 ng/ml (Zetterstrom and Johansson, 1981). The level is usually lowest in neonates and rises gradually in early childhood to reach to adult levels before 7 years of age (Johansson et al., 1968). Elevated IgE concentrations are found in patients suffering from IgE-mediated allergic disease or that are heavily parasitised (Johansson et al., 1968; Patterson et al., 1973; Falk and Bolle, 1980). In patients with allergic conditions, the range may be 133 to 5850 ng/ml with a mean of 1191 ng/ml, whereas much higher concentrations of IgE up to 140,000 ng/ml were found in patients with parasitic infections (Bennich and Johansson, 1971). Nevertheless, although elevated IgE levels are a common feature of atopic individuals, normal serum levels of IgE can be seen in some atopic patients, especially those with "pure" atopic dermatitis, lacking allergic respiratory disease (Jones et al., 1975). IgE levels were also found to be increased in patients with non-IgE mediated asthma and with nonallergic rhinitis (Henderson et al., 1971).

Although many studies have shown that total serum IgE concentrations tend to be higher in allergic people as compared with nonallergic individuals, the diagnostic value of total serum IgE levels is limited (Klink et al., 1990). The diagnostic sensitivity and specificity of total IgE determinations is highly dependent on the cut-off level (Wittig, 1980). Klink et al (1990) concluded that there is no single level that clearly distinguishes different groups with a level of precision that is clinically meaningful.

### 5.2. In dogs

In normal dogs, the level of total IgE is higher than in man and was found to range from 2 to 410 µg/ml. However, serum total IgE concentrations were not significantly different in atopic, parasitised and normal individuals (Vriesendorp et al., 1975; Nimmo Wilkie et al., 1990; Hill and DeBoer, 1994). The reason is obscure, but it has been hypothesised to be the result of inhibited *T. canis* larvae in the muscle tissue of most dogs which could lead to polyclonal activation of IgE-synthesising plasma cells (Schwartzman, 1984).

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## 6. Receptors for IgE

The most important biological property of IgE is its ability to bind to cell receptors via the Fc region. Mast cells and basophils are not the only cells which have membrane receptors that bind to the Fc portion of IgE. B and T lymphocytes, macrophages, eosinophils, platelets, Langerhans' cells, follicular dendritic cells and natural killer also express receptors for IgE.

Receptors for IgE molecules can be divided into 2 distinct groups. The first type of receptor is characterised by having a very high affinity for IgE and is called the high-affinity receptor (FcεRI). In contrast, the second one binds IgE with much lower affinity and is called the low-affinity receptor (FcεRII).

### 6.1. The high-affinity receptor FcεRI

The high affinity receptor for IgE is expressed on the surface of mast cells and basophils and also on the surface of Langerhans' cells (Grabbe et al., 1993), monocytes (Maurer et al, 1994), dendritic cells (Osterhoff et al., 1994) and eosinophils (Capron and Capron, 1994).

FcεRI expressed by mast cells and basophils has a tetrameric structure. It is composed of an α chain which includes the IgE-binding site, one β chain with 4 transmembrane domains and 2 disulphide-linked γ chains (Metzger, 1992). This receptor belongs to the immunoglobulin superfamily. The IgE receptor binding site is located on Cε3 and the IgE molecule adopts a bent conformation to bind to FcεRI with its convex surface facing the membrane (Zheng et al., 1991). FcεRI binds monomeric IgE with a very high affinity ($Ka = 10^{10}$/M). The binding of IgE with the cell receptor is reversible and does not involve covalent bonding.

The cross linking of several surface-bound IgE molecules, by antigen or other molecules such as lectins (eg concanavalin A (con-A) and phytohemagglutin (PHA)), stimulates degranulation. Surface-bound IgE molecules can also be bridged by anti-IgE antibody. Antibodies to the IgE receptor can activate cells in the absence of IgE (Basciano et al., 1986). Receptor bridging (directly or indirectly via bridging of IgE molecules) is the critical event (Basciano et al., 1986), and results in the formation of aggregates, patches and caps, with subsequent internalisation of antigen-IgE-IgE receptor complexes (Furuichi et al., 1986). A very small number of FcεRI bridges can be sufficient to provide an activating signal to basophils and mast cells (Pruzansky and

Patterson, 1988). Most studies suggest that bridging of 100 or fewer IgE molecules will completely activate the cell for histamine release. Since mast cells/basophils appear to have approximately 100,000 receptors per cell (see below), it appears that bridging of less than 1% of the total IgE molecules on the cell surface activates biological events in the cell and the release of histamine (DeLisi and Siraganian, 1979a&b; Maeyama et al., 1986).

Surface expression of FcεRI on mouse mast cells can be influenced by the level of IgE (Hsu and Mac Glashan, 1996). Also the level of FcεRI expression on the surface of circulating human basophils (Malveaux et al., 1978) and on bone marrow basophils in mice (Lantz et al., 1997) exhibits a strong positive correlation with the serum concentration of IgE. Moreover, the administration of IgE *in vivo* can significantly upregulate FcεRI expression on mouse basophils, and genetically IgE-deficient mice have very low expression of basophil FcεRI (Lantz et al., 1997). Cytokines are other factors that may regulate FcεRI surface expression. Indeed, Il-4 has been shown to up-regulate levels of mRNA for the FcεRIα-chain in human eosinophils (Terada et al., 1995) and can up-regulate expression of FcεRI on the surface of human mast cells (Toru et al., 1996).

FcεRI expressed on antigen-presenting cells such as monocytes and Langerhans' cells exhibits profound differences, in terms of structure and function, from that expressed on mast cells and basophils (Bieber, 1994). This receptor is trimeric and composed of an IgE-binding α chain and 2 disulphide-linked γ chains.

## 6.2. The low-affinity receptor FcεRII

A second receptor for IgE, FcεRII (CD23), is found on other reticulo-endothelial cells including B and T cells, macrophages, eosinophils, platelets, Langerhans' cells, follicular dendritic cells and natural killer cells. FcεRII has a lower affinity for IgE ($Ka = 10^{-7}/M$). It is not a member of the immunoglobulin supergene family but is classified as a type II integral membrane protein. It is composed of a C-type lectin domain in its extracellular sequence and a cytoplasmic N-terminus. Although CD23 is a C-type lectin, it does not bind to the carbohydrate component of IgE. The binding site for FcεRII has been mapped to Cε3 (Nissim et al., 1993). However, it is distinct from the FcεRI site.

CD23 is spatially associated with the major histocompatibility complex (MHC) class II molecule human leucocyte antigen (HLA)-DR on the surface of B cells (Bonnefoy et al., 1988). CD23 bearing normal murine B cells are very efficient in IgE-

dependent antigen presentation to T cells and in adhesion of B cells to each other. This observation implies that B cell CD23 is capable of internalising IgE immune complexes (Kehry and Yamashita, 1988).

In addition it is a multifunctional lymphokine, being an autocrine growth factor for the differentiation of activated B cells, and acting as a cofactor for the Il-4 induced heavy chain switching to IgE expression (Holgate et al., 1993). It has been shown to act synergistically with a suboptimal concentration of Il-4 to induce IgE production by normal B cells (Pène et al., 1988). CD23 has also been shown to increase IgE production by B cells derived from atopic individuals (Sarfati et al., 1984).

Proteolysis of the FcεRII receptor gives rise to soluble fragments which all bind to IgE and appear to have a role in the regulation of IgE synthesis (Conrad et al., 1991). Soluble fragments can stimulate the growth and differentiation of the precursors of plasma cells, T cells and basophils.

### 7. Functions of IgE

IgE appears to be paradoxical in its role since through the same type of mechanism, namely immediate hypersensitivity, it has a protective role in parasite infections (Hagan, 1993; Capron and Capron, 1994) and is primarily responsible for the pathogenesis of allergic diseases resulting from exposure to innocuous environmental substances in atopic patients (Platts-Mills, 1993).

### 7.1. Protective role of IgE

Helminth parasites have a unique role in inducing IgE synthesis, in both an antigen-specific and also antigen non-specific manner (Ogilvie, 1964; Jarret and Bazin, 1974; Dessaint et al., 1975; Turner et al., 1979; Zakroff et al., 1989).

The production of specific IgE to helminth parasites has been linked to host - protection (Hagan et al., 1991). However the benefit of this to the host remains unclear. Capron and Dessaint (1975) proposed that the high level of non-specific IgE resulting from potentiation by parasitic helminths may serve to favour the survival of the parasite. However the presence of excess polyvalent IgE could also reduce the risk of anaphylaxis induced by parasite-specific antigens and so protect the host from the potentially lethal consequences of hyperreactivity to parasite antigens (Moqbel and Pritchard, 1990). This host protective role of parasite-specific IgE could be dominant (Pritchard, 1993).

On the other hand, IgE can also mediate antibody-dependent cell-mediated cytotoxicity reactions against helminth parasites. It has been demonstrated that IgE antibodies, in the form of immune complexes with antigen, were both sufficient and necessary to mediate macrophage and eosinophil-dependent killing of helminths such as schistosomula (Capron et al., 1977; Capron et al., 1981).

However, recently administration of anti-IgE antisera into parasitised animals was not able to decrease the capacity of the immune system to kill the parasites (Amiri et al., 1994).

## 7.2. Mechanisms of type 1 hypersensitivity

The classic Gell and Coombs classification of hypersensitivity disorders (Coombs and Gell, 1975) is applicable to discussions of cutaneous hypersensitivity disorders and remains the immunopathologic scheme used by most authors.

Type 1 hypersensitivity results from an IgE response to an allergen. Subsequently, IgE binds to the high affinity receptor of mast cells and basophils via its constant Cε3 region. On re-exposure to the same allergen, the specific cross linking of 2 molecules of IgE on sensitised mast cells or basophils by the allergen leads to degranulation with the release of inflammatory mediators resulting in allergic disease (Halliwell and Gorman, 1989).

When mast cell bound IgE is aggregated by antigen or anti-IgE, high affinity receptors are rapidly immobilised, possibly by interaction with the cytoskeleton (Mao et al., 1991). The early signal resulting in exocytosis that follows the aggregation of FcεRI is the activation of tyrosine kinase (Eiseman and Bolen, 1992). A second event which follows the aggregation of FcεRI is the activation of G-proteins (Sagi-Eisenberg, 1993). Both tyrosine kinase and G-protein activator can lead to the activation of phospholipase-C (Sagi-Eisenberg, 1993; Jouvin et al., 1994). The activation of phospholipase-C in turn leads to the release of $Ca^{2+}$ from intracellular stores and to the activation of protein kinase C (Dekker and Parker, 1994). Exocytosis is the last stage in the secretory pathway and involves fusion of the granule membranes with those of the plasma membrane. Calcium plays an important role in exocytosis in mast cells which may be facilitated by the calcium binding protein calmodulin. Indeed, calmodulin may play a role in the regulation of intracellular enzyme and cytoskeletal proteins (Chok and Shmaudershok, 1992). G-proteins are involved in the redistribution of actin filaments (Norman et al., 1994).

Activation of mast cells leads to the release of a variety of preformed (histamine, heparin, serotonin) and newly synthesised (prostaglandins, leukotrienes) inflammatory mediators. The synthesised mediators include the important arachidonic acid-derived mediators. Arachidonic acid is activated by membrane derived phospholipase and is then acted upon by either the cycloxygenase or the lipoxygenase pathway. The former leads to the generation of prostaglandins and the latter to the leukotrienes. In addition, multiple multifunctional lymphokines and cytokines are also released as a result of IgE-mediated mast cell activation. Those released include Il-1, Il-3, Il-4, Il-6 and tumour necrosis factor $\alpha$ (Burd et al., 1989; Plaut et al., 1989).

The type I reaction was initially considered to be a short-lived acute event, not necessarily leading to prolonged inflammation. However this concept has undergone revision based upon the recognition that the LPR, which can occur in a variety of target organs including skin, lung, nose and eyes, can contribute substantially to the clinical symptomatology. The LPR is also believed to be IgE dependent (Dolovitch et al., 1973).

### 7.3. IgE on antigen presenting cells

The existence of the high affinity receptors on Langerhans' cells supports a role for this cell in the pathogenesis of atopic dermatitis (AD) involving IgE-mediated antigen presentation to autologous T cells. Many observations suggest that IgE-bearing Langerhans' cells may initiate atopic dermatitis lesions and possibly contribute to the amplification of the IgE response towards environmental allergens in man and dogs (Reitamo et al., 1986; Adinoff et al., 1988; Mudde et al., 1990; Olivry et al., 1996). A recent study has demonstrated that the effectiveness of allergen presentation by monocyte-enriched peripheral blood mononuclear cells (PBMCS) was amplified by the presence of allergen-specific IgE bound to these cells (Maurer et al., 1995).

### 8. Heterogeneity of IgE

There is differential glycosylation of IgE molecules and it has been shown that IgE from ND myeloma contains much more carbohydrate than that from another myeloma (PS). This could be responsible for the heterogeneity of the IgE molecule in its ability to interact with histamine releasing factors (HRF) (MacDonald et al., 1987). MacDonald et al. (1991) have designated the IgE which binds to HRF and which is only present in some atopic patients, as 'IgE+' (MacDonald, 1987).

Lyczack et al. (1996) demonstrated the existence of 4 human IgE isoforms which include the classic secreted form and three novel isoforms with altered carboxyl terminals. These isoforms are predicted to be secreted proteins. They may have some unique properties as circulating, cytophilic immunoglobulins and may differ in their ability to carry out IgE-mediated functions through binding to their different receptors. One of those isoforms is secreted as half-molecules (heavy-light chain).

The possible heterogeneity of canine IgE has been proposed recently by Peng et al. (1997) who showed that canine IgE appeared to be composed of two subpopulations, differing in physicochemical characteristics.

## 9. Production of IgE

### 9.1. Sites of production of IgE

9.1.1. Man

The distribution of IgE producing cells in man was studied using fluorescent antibody techniques (Tada and Ishizaka, 1970). IgE producing plasma cells were found to be numerous in lymphoid tissues such as tonsils, adenoid, bronchial and peritoneal lymph nodes but to be low in the spleen and subcutaneous lymph nodes. IgE plasma cells were also detected in the respiratory and gastrointestinal mucosa suggesting that IgE antibodies may be formed locally in those organs in response to antigenic stimuli. It was suggested that the distribution of IgE plasma cells could differ depending upon the method of immunisation and/or the animal species employed. In certain situations they may be found predominantly in the spleen and lymph nodes (Ishizaka and Ishizaka, 1975).

Ishizaka and Ishizaka (1975) suggested that the preferential distribution of IgE plasma cells in the respiratory and gastrointestinal tracts in the primate might be due to frequent exposure of the lymphoid tissues to small amounts of environmental antigens which may be favourable for IgE synthesis.

Since mast cells are able to express CD40-L (see induction of IgE synthesis) and can also produce Il-4, these cells could increase the production of allergen-specific IgE by B cells in tissues from individuals previously sensitised to the allergen (Scharenberg and Kinet, 1994).

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### 9.1.2. Dogs

In the dog, IgE-producing cells have been reported to be prominent in few tissues, namely, the mucosa of the small intestine and the mediastinal and mesenteric lymph nodes (Halliwell, 1975a). However in an experimental situation Gebhard et al. (1995) reported the production of IgE-producing canine X murine heterohybrids using popliteal lymph node cells from dogs subsequent to the injection of the filarial nematode *Brugia pahangi* into the left rear paw.

### 9.2. Cells involved in the production of IgE

T helper cells which are usually characterised by expression of CD4 (CD4+), can be subdivided into Th1, Th2 and Th0 populations based on their profile of cytokine production (Romagnani, 1994).

- One subclass, Th1, which produces the cytokines interferon-$\gamma$ (IFN-$\gamma$), tumor necrosis factor-$\beta$ (TNF-$\beta$) and Il-2 among others (but not Il-4 and Il-5), is important for cell-mediated immunity, delayed hypersensitivity reactions and in the generation of protective immune responses against intracellular pathogens. INF-$\gamma$ induces switching to IgG2a in mouse B cells (Collins and Dunnick, 1992). It has been shown recently that IFN-$\gamma$ is predominant in chronic skin lesions of patients with atopic dermatitis (AD) and therefore Th1 cells may also play a role in the maintenance of the late and chronic phase of AD (Thepen et al., 1996; Werfel et al., 1996).

- The second subtype, Th2, produces Il-4, Il-5, Il-6, Il-10 and Il-13 which promote most of the humoral immune responses, including IgE, and is responsible for inducing most of the immunoglobulin class switching (Mosmann and Coffman, 1989) and for the initiation of the cutaneous inflammation in patients with AD.

- Th0 cells represent a heterogeneous population of partially differentiated effector cells comprising multiple discrete subsets that can secrete both Th1 and Th2-type cytokines (Romagnani, 1994).

Strong evidence suggests that Th1 and Th2 cells do not derive from distinct lineages, but rather develop from the same Th-cell precursor dependent upon a number of influences such as the route of antigen entry, the physical form of antigen, the type of adjuvant and the dose of antigen. The precursors are also influenced by genetic factors acting at the level of antigen presentation. The early presence of Il-4 is the most potent stimulus for Th2 differentiation, whereas Il-12 and IFN-$\gamma$ favour Th1 development (Seder and Paul, 1994).

The mechanisms responsible for the early production as well as the source of Il-4 remain unclear. Naive Th cells are able to produce small amounts of Il-4 from their initial activation and are the principal source of Il-4 production after priming. However other Il-4 sources (CD4+NK11+ cells) can also contribute to the development of Th2 responses (Seder and Paul, 1994; Constant and Bottomly, 1997). Moreover a recent study (Machado et al., 1996) demonstrated an IgE-independent release of mediators of the allergic response by mast cells and basophils challenged with allergens, including house dust mite (HDM), bee venom and parasite secretions. Therefore these authors suggested that these cells might be the initial source of Il-4 production (Machado et al., 1996). The inducing effect of Il-4 is dominant to those of the other cytokines. Recently it has been shown that Il-6 derived from antigen-presenting cells is also able to stimulate naive Th cells to become effector Th-2 cells by inducing the initial production of Il-4 in CD4+ T cells (Rincon et al., 1997).

## 9.3. Regulation of the production of IgE

IgE antibody production is normally highly regulated, which results in minimal concentrations of IgE in body fluids.

### 9.3.1. Role of the antigen in the production of IgE

Allergens are proteins or protein-bound substances which bind reagins and promote reaginic antibody responses. Any definition of an allergen must cover its two distinctive functions. The allergenicity of an allergen is its ability to elicit a local or a systemic reaction in an already allergic individual whereas the immunogenicity of an allergen is its property to stimulate the production of reaginic antibodies (Strejan et al., 1973).

Certain individuals are genetically more prone to make IgE responses than others and are therefore more likely to develop atopic disease (see atopy in man and in the dog). On the other hand, the route of entry, dosage, frequency of exposure and molecular characteristics of an antigen are also important in determining whether the antigen will be clinically important as an allergen in the population at large.

The fundamental property of immunogenicity is dependent upon the structure of the antigen (Sela, 1969; Chestnut and Grey, 1985).

The use of adjuvant can also influence the production of IgE. Aluminium hydroxide (alum) and *Bordetella pertussis* (*B. pertussis*) are known to enhance IgE

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synthesis (Jarret and Stewart, 1972) while complete Freund's adjuvant (CFA) is reported to suppress IgE but enhance IgG production (Hirashima et al., 1981).

### 9.3.2. Induction and regulation of IgE synthesis

### 9.3.2.1. Induction

Immunological reactions are not only dependent on the recognition of antigen but also on the activation of the appropriate effector system.

### 9.3.2.1.1. First step

The first step in the allergic reaction, the activation of Th-2 cells, involves a sensitisation phase, commencing with the initial exposure to the antigen bound to MHC class II molecules (Norman, 1993). Allergen-specific Th-2 cells are then activated.

### 9.3.2.1.2. Second step

Two signals are then required for the induction of human IgE synthesis by B cells (Vercelli et al., 1989).
- The first signal delivered to B cells is derived from activated Th2 cells and consists of the cytokine Il-4 (Parronchi et al., 1991).
- The second signal can be supplied by a number of stimuli. Often, it involves a physical interaction between the B cell and another cell. It can be provided by Th-2 cells by linking their surface MHC class II molecules with CD4 antigen on the B-lymphocyte surface. The linkage of the CD40 antigen, a molecule found on the B cell itself, with its ligand (CD40-L) can also provide the second signal (Parronchi et al., 1991).

These two signals activate germline ε transcription and promote further B cell differentiation, class switching and the eventual secretion of allergen-specific IgE from plasma cells.

### 9.3.2.1.3. Isotype switching and Ig synthesis

Expression of IgE is controlled both by heavy chain switching and by selection of IgE-committed cells for survival and differentiation.

The variable region of Ig is encoded by multiple germ-line elements which are assembled into complete V(D)J variable regions during B cell differentiation. The heavy chain class switching is the expression by a B lymphocyte of different heavy chain isotypes sharing the same VDJ region. This phenomen allows a single B cell clone to produce antibodies that retain variable region specificity in association with a different CH region gene, and with a different effector function (Vercelli and Geha, 1992). This is

thought to result from the recombination event that juxtaposes a downstream CH gene to the expressed V(D)J gene.

### 9.3.2.2. Regulation

It is clear that Il-4 is particularly important in regulating B cell growth. Its ability to regulate immunoglobulin isotype expression in murine B cells by the induction of germ-line transcription of the C$\epsilon$ gene, has been clearly shown, making it a substrate for the recombinase (Rothman et al., 1988). It is able to promote the isotype switch of B cells to IgE production and can act directly on B cells. However B cells require an additional activation signal. This signal induces a switch recombinase able to cause switching to any active CH gene.

It is noteworthy that Il-4 as well as other soluble factors, are not only released by T cells but also by mast cells and basophils upon cross linkage of the IgE (Burd et al., 1989; Plaut et al., 1989, Bradding et al., 1992; Brunner et al., 1993) and also by an antibody independent mechanism (Machado et al., 1996) (see cells involved in the production of IgE).

More recently, Gauchat et al. (1993) showed that mast cells and basophils can also express CD40-L. Thus through the production of Il-4, mast cells and basophils are able to induce an IgE synthesis by B cells (Gauchat et al., 1993). This suggests that these cells may play a key role in allergy not only by producing inflammatory mediators but also by directly regulating IgE production independently from T cells (Gauchat et al., 1993). However, in a recent study, mast cells were not found to be a source of Il-4 in the chronic lesional skin of patients with AD (Thepen et al., 1996).

Stimulation of human B cells with Il-4 and Epstein-Barr virus and with anti-CD40 and Il-4 have been shown to induce T cell-independent IgE synthesis (Thyphronitis et al., 1989). The second signal can therefore be provided by infection with EBV (Thyphronitis et al., 1989), monoclonal antibodies directed against CD40 (Rousset et al., 1991), and activated CD4+ cells (Gascan et al., 1991a &b). However the nature of the second signal required for IgE induction is unclear in the EBV system (Geha, 1992).

Il-13 has been found to induce isotype switching to IgE and IgG4 in humans. Il-13 acts much like Il-4 and it has been suggested that these two cytokines may share some components on their receptors, because they compete for the same high-affinity receptors (Callard et al., 1996).

The finding that IgE can be a major regulator of mouse mast cell and basophil FcεRI expression *in vivo* identifies a potentially important mechanism for enhancing the expression of effector cell function in IgE-dependent allergic reactions or immunologic responses to parasites (Lantz et al, 1997).

The low affinity IgE receptor (FcεRII or CD23) also has a role in the regulation of IgE. The soluble fragments resulting from the proteolysis of this receptor can either promote or inhibit IgE synthesis (Sarfati et al., 1992).

Hydrocortisone has been reported to provide the second signal and increase IgE levels *in vivo*, in combination with human Il-4. However, its role in the induction of IgE synthesis is still not clear. The sequential IgE isotype switching in human B cells induced by hydrocortisone leads to increased polyclonal IgE production but no increase in allergen-specific IgE (Bohle et al., 1995; Nüsslein et al., 1994).

### 9.3.2.3. Regulation of IgE synthesis by cytokines other than Il-4 and Il-13

A number of cytokines are able to modulate Il-4-dependent IgE synthesis. Il-5 and Il-6 are able to upregulate the IgE response induced by Il-4. IL-3, IL-9 and TNF-$\alpha$ are also able to enhance the IgE response. IFN-$\gamma$, INF-$\alpha$, transforming growth factor (TGF)-$\beta$, IL-8, Il-10 and Il-12 inhibit Il-4-dependent IgE synthesis (deVries, 1994; Pene et al., 1988). The mechanism of the modulation of IgE synthesis by these cytokines is still poorly understood (deVries, 1994).

Thus, in summary, extracellular signals and nuclear events shift the balance between Th1 and Th2 cell development in response to a specific allergen, and exert a profound influence on IgE synthesis and hence the development of atopic disease.

### 9.3.2.4. The "allergic breakthrough" concept

The concept of "allergic breakthrough" first introduced by Katz (1978) hypothesises that IgE antibody production is maintained at a low level following sensitisation because of the existence of normal suppressive or dampening mechanisms that exist specifically to limit the quantity of IgE antibodies produced during any particular response. It was postulated that the bypassing of this dampening mechanism by any one of a number of perturbations such as parasite or viral infestations, can result in "allergic breakthrough" if, at that point in time when the threshold of the damping mechanism is lowered, the individual becomes sensitised to one or more allergenic substances. Possible perturbation may include respiratory viral infections, endoparasites and hormonal fluctuations. In consequence, sensitisation could occur quite

coincidentally at the time when the damping mechanism has been lowered to a level insufficient to control the IgE antibody synthesis. The sensitisation might be either at the first allergen exposure or upon reexposure.

## I.2. Reaginic antibody in other species

### 1. Characteristics of reaginic antibody in other species

Binaghi and Benacerraf (1964) first described the production of reaginic antibodies in rats by immunisation with antigen plus *B. pertussis* vaccine. Later, IgE was characterised in many other species including the guinea pig (Ovary et al., 1976), monkey (Ishizaka and Ishizaka, 1968b), dog (Halliwell, 1973), mouse (Lehrer, 1976), rabbit (Zvaifler and Becker, 1966), rat (Isersky et al, 1974), cow (Neilsen and Wilkie, 1979; Gershwin, 1981), horse (Suter and Fey, 1981), pig (Roe et al 1993 ) and sheep (Yilmaz et al., 1993).

The concentration of reaginic antibody in the serum is always low, a fact that clearly distinguishes it from the other immunoglobulins. However, the discovery of IgE myelomas in the rat (Bazin et al., 1974) and the mouse (Böttsher and Hammerling, 1978) have facilitated the ready purification of IgE, whereas in other species, the lack of IgE myelomas and the low level of serum IgE make the purification of IgE more difficult. Therefore in these species, IgE has been purified by fractionation of sera from allergic, parasitised and/or immunised animals. To date, IgE ε chains have been purified and characterised only in the dog (Peng et al., 1993a) and the sheep (Yilmaz et al., 1993).

Reaginic antibodies in animals share many physicochemical and immunochemical properties to their human counterpart. They are characterised by a relatively fast electrophoretic mobility, a sedimentation coefficient of approximately 8 S and a molecular weight of between 183 and 187 KDa in the rat (Bazin et al., 1974; Bennich et al., 1978), 184 and 200 KDa in the mouse (Böttscher and Hämmerling, 1978; Liu et al., 1980) and of 200 KDa in horse (Suter and Fey, 1983). The molecular weight of the ε chain was found to be 68 KDa in the rat (Bazin et al., 1974) and 75 KDa in the mouse (Bose et al., 1984). Using PK or PCA tests, it has been shown that reaginic antibody was eluted following chromatography on Sephadex G200 in a position similar to that of man (Roe et al., 1993; Yilmaz et al., 1993).

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Rabbit reaginic antibody has been shown to be heat stable (Lindqvist, 1968). Skin sensitising activity was still detected in serum after being heated at 56°C for 32 hr (Lindqvist, 1968).

While human, canine and feline IgE were demonstrated to bind to protein A, neither bovine (Gershwin and Dygert, 1983), mouse (Ey et al., 1978) nor rat IgE (Medgyesi et al., 1978) show affinity for this protein.

The half life of serum IgE in the rat has been estimated to be 12 hr (Tada, 1975).

## 2. Cross-reactivity between IgE of different species

It has been shown by reversed cutaneous anaphylaxis (RCA) as well as by other methods (ELISA, RIA, gel diffusion, western blot and histamine release by mast cells and basophils) that human IgE cross reacts with monkey (Ishizaka and Ishizaka, 1968b), rat (Conrad et al., 1983; Kanyeresi et al., 1971; Perelmuter and Khera, 1970), bovine (Gershwin, 1981; Nielsen, 1977), ovine (Yilmaz et al., 1994), equine (Suter and Fey, 1983) and canine IgE (Halliwell et al., 1972). Feline reaginic antibody has also been shown to cross react with canine IgE (DeBoer et al., 1993).

A recent study has demonstrated that polyclonal anti-mouse IgE and anti-human IgE were able to induce RCA in dogs while monoclonal anti-human, anti-rat and anti-mouse IgG were not (Peng et al., 1996). Therefore, the IgE structure appears to be relatively well conserved between species. However, of all the five isotypes of immunoglobulin, the sequence of the IgE C region is the least well conserved across species (Patel et al., 1995).

## 3. Reaginic antibody in cats

Several studies have already shown that cat serum contains a protein with IgE-like properties (Walton et al., 1968; Powell et al., 1980; DeBoer et al., 1993; Baldwin, 1993; Foster, 1995; Foster et al., 1995). Walton et al. (1968) demonstrated the existence of reaginic antibody with whole cow's milk in a cat with dermatitis and enteritis by PK testing. In another study, reaginic antibodies to the ear mite *Otodectes cynotis* were demonstrated and are believed to be responsible for the extreme pruritus that is associated with some cases of parasitic otitis externa (Powell et al., 1980).

More recently, DeBoer et al. (1993) showed that cats have a reaginic antibody that is antigenically cross-reactive with canine IgE and has an apparent molecular weight similar to that of IgE in other species (DeBoer et al., 1993; Alaba, 1997).

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Foster et al. (1995) demonstrated that the passive cutaneous anaphylaxis (PCA) reactivity was eluted following chromatography using Sephadex G200 in a position similar to that of other species, and that feline reaginic antibodies are bound by Protein A.

Since the isolation and purification of IgE antibody by conventional methods is very difficult, Foster (1995) attempted to isolate and determine feline Cε gene sequences by the use of molecular biology techniques with the aim of preparing an IgE peptide and raising a specific antiserum. The CH3 domain was found to have sequences that were very similar to those of the other species. The whole of the CH3 sequence was cloned and sequenced, however the antisera raised against the resulting peptide recognised epitopes only in the peptide and not in the native molecule. Therefore the expressed protein appears to differ antigenically from the synthetic peptide (Foster, 1995).

## II. ATOPY IN MAN AND IN THE DOG

### 1. INTRODUCTION

### 1.1. Atopic dermatitis

### 1.1.1. Man

The term atopy, derived from a Greek word which means "out of place" or "strangeness", was first introduced by Coca and Cooke in 1923 to describe three familial diseases of man that appeared to have many features in common, namely atopic dermatitis, allergic asthma and hay fever. Although the pathogenesis of atopy was unknown, the conditions were thought to be inherited, in a dominant mode.

In 1933, atopic dermatitis (AD), considered one manifestation of atopic hypersensitivity, was defined by Wise and Sulzberger. These authors cited the cardinal features of atopy, which included an atopic family history, antecedent infantile eczema, localisation in the flexural areas, grey-brown discoloration of the skin, absence of vesicles, vasomotor instability, many immediate positive reactions to scratch or intradermal tests, and the presence of reagins in the serum.

In 1935, Hill and Sulzberger further emphasised the importance of food and airborne sensitivities in the development of AD.

Since then, human AD has traditionally been defined as an inflammatory skin disorder characterised by severe pruritus, a chronically relapsing course and a distinctive

clinical morphology and distribution. This inflammatory skin disorder is frequently associated with elevated serum IgE levels and a personal or family history of AD, allergic rhinitis and/or asthma (Leung et al., 1987; Champion and Parish, 1992; Bos et al., 1994).

## 1.1.2. Dogs

In the dog, the first case of canine atopy was reported in 1941 by Wittich. This condition is now recognised as one of the more common causes of pruritic skin disease in this species (Halliwell and Schwartzman, 1971).

Atopy is defined in dog as an inherited predisposition to develop reaginic antibodies to environmental allergens resulting in allergic disease (Halliwell and Gorman, 1989). The skin is believed to be the major target organ.

Canine atopy, canine atopic disease, canine atopic dermatitis, and canine allergic inhalant dermatitis have all been proposed as names for this disease in the dog (Halliwell and Gorman, 1989). This may reflect the uncertainty regarding the exact pathogenesis of this disease in the canine species.

## 1.2. Asthma

## 1.2.1. Man

Asthma (Greek for "panting") is a complex disease of the lower airways. Bronchial asthma is characterised by a history of episodic wheezing, by evidence of airflow obstruction that is reversible, either spontaneously or following bronchodilator therapy, and by pathological evidence of inflammatory changes in the bronchial mucosa (Arm and Lee, 1992). The inflammation of the airways is a characteristic feature of asthma and the obstruction of the airway lumen by smooth muscle constriction and mucus plugging may be the consequence of the inflammatory cascade (Arm and Lee, 1992). These pathophysiologic findings are believed to be caused by the interaction of genetic susceptibility and adverse environmental factors (Hoover and Platts-Mills, 1995).

Allergic and non-allergic asthma have both been described in man. Allergic asthma is characterised by a type 1 hypersensitivity reaction to inhaled allergen followed by a late-phase reaction, with inflammation and persistent bronchial hyperresponsiveness (Busse and Reed, 1993). It occurs most frequently in young individuals. A family history and elevated serum IgE are common features of the

disease. Although the cause of asthma in many patients can be attributed to allergen sensitisation, there are a number of individuals with asthma who have no evidence of hypersensitivity to common inhaled allergens. These "non allergic" patients have as much evidence of inflammation in the lungs, but the cause remains an enigma in many of the cases (Hoover and Platts-Mills, 1995).

### 1.2.2. Dogs

Bronchoconstriction has been induced in dogs infected with *T. canis* following nebulisation with ascaris antigen (Booth et al., 1970). Nebulisation of atopic dogs with pollen allergens to which they are sensitised, also induced bronchoconstriction (Patterson and Sparks, 1962). However, spontaneous allergic asthma appears to be exceedingly rare in the dog (Halliwell and Gorman, 1989), with only one convincing case reported in the literature (Chamberlain and Baker, 1974). This may be a reflection of the relative paucity of mast cells in the pulmonary tracts in the dog (Halliwell and Gorman, 1989).

## 2. EPIDEMIOLOGY

In the dog, reports of the incidence of atopy vary from 3.31 to 85% of the cases referred to dermatologic clinics (Halliwell and Schwartzman, 1971; Austin, 1976). According to Griffin (1993a) atopic disease occurs in some 10% to 15% of the canine population. These considerable differences probably reflect not only geographic differences but also practice bias and lack of defined criteria. The disease appears to begin between 6 months and 3 years of age in 75% of atopic dogs and onset after 7 years of age is very rare unless the dog has moved to a new environment (Griffin, 1993a). Although studies are conflicting, most report a slight female sex predisposition (Halliwell and Gorman, 1989; Griffin, 1993a).

## 3. ETIOLOGY AND PATHOGENESIS OF ATOPY

### 3.1. Studies of the immunoglobulins involved

### 3.1.1. Total immunoglobulin levels

3.1.1.1. Man

Serum levels of total IgG, IgA and IgM are generally in the normal range in humans with AD, although elevation of IgM and IgG, and even decreased IgG have been

recorded (Buckley et al., 1968; Ohman and Johansson, 1974). However, IgA deficiency appears to occur more frequently in atopic than in normal individuals (Kaufmann and Hobbs, 1970). Elevated total IgG4 has been reported in patients with atopic dermatitis (Gondo et al., 1987; Shehade et al., 1988) and a link between IgE and IgG4 production has been suggested (Shehade et al., 1988). However the biological and pathological significance of this elevated subclass remains unclear.

Total serum IgE is elevated in over 80% of patients with AD (Gurevitch et al., 1973; Stone et al., 1973; Adkinson and Platts-Mills, 1988). However normal serum levels of IgE are seen in some patients with AD who do not have coexistent allergic respiratory disease (Ohman and Johansson, 1974; Jones et al., 1975). Serum IgE levels often correlate with the extent and severity of the disease (Stone et al., 1973), although IgE concentrations may remain elevated through a prolonged lag period during remission (Gunnar et al., 1970). IgE can also be elevated in other skin disorders (Stone et al., 1973; O'Loughlin et al., 1977). Moreover, AD has been described in four children suffering from agammaglobulinemia (Peterson et al., 1962). These observations raised the question of whether IgE is simply an incidental feature of AD, secondary to defective control mechanisms governing many other characteristics of the atopic diathesis.

However, there is no doubt that IgE has a role in atopy in some allergic patients. A consistent finding is that mononuclear leukocytes spontaneously produce increased quantities of IgE *in vitro* (Saxon et al., 1980; Sampson and Buckley, 1981).

### 3.1.1.2. Dogs

Hill et al. (1995) measured the total concentration of IgE, IgA and IgG in the serum of normal, atopic and parasitised dogs and found no significant difference in total IgE level between all 3 groups. However, in both atopic and parasitised dogs, the level of IgA was shown to be lower whereas the level of IgG was higher than in the control group. On the other hand, Hall and Campbell (1993) and Mueller (1993) failed to demonstrate significant differences in the concentration of total serum IgA in atopic and normal dogs. However, the level of allergen-specific IgA at the epidermal surface of the skin appeared to be increased in atopic dogs in a similar manner to that of IgE (Mueller, 1993). The fact that total serum IgE levels do not appear to be a good predictor of atopy in dogs was also confirmed by Esch and Grier (1997).

### 3.1.2. Allergen-specific immunoglobulins involved

3.1.2.1. Man

3.1.2.1.1. Allergen-specific IgE

The level of allergen-specific IgE in the serum of atopic patients is variable and dependant on the age, the degree and duration of the most recent allergen exposure, the degree and duration of exposure to cross-reactive allergens, and whether or not immunotherapy has been employed (Gleich et al., 1982; Somville et al., 1989).

While specific IgE may be difficult to detect in infants because of the very low IgE concentration present, the levels are typically highest in school-age children and young adults. In contrast sera from children with atopic dermatitis usually have very high total IgE concentrations which may produce non-specific binding and lead to false positive serological results (Sampson and Albergo, 1984). Therefore the radioallergosorbent test (RAST) for allergen-specific IgE is reported to be unreliable in atopic dermatitis (Sampson and Albergo, 1984).

The level of allergen-specific IgE has been shown to be influenced by the pollen exposure and to peak approximately 4 weeks after a seasonal pollen exposure before gradually falling (Somville et al., 1989).

3.1.2.1.2. Allergen-specific IgG

Specific IgG antibodies to some allergens were shown to be higher in atopic than in non-atopic individuals (Chapman and Platts-Mills, 1978). However at higher exposure levels of the antigen, the concentration of allergen-specific IgG is not different between allergic and non-allergic patients (Aalberse et al., 1993).

Levels of allergen-specific IgG4 were also found to be higher in atopic patients than in normal controls (Shakib et al., 1977; Merrett et al., 1984). This is not surprising since both IgG4 and IgE synthesis were shown to be IL-4 dependent and to require the presence of Th2 cells (Ishizaka et al., 1990; Spiegelberg, 1991a&b). Nevertheless most healthy non-allergic individuals produce specific IgG4 antibodies when exposed to relatively high doses of allergens (Chapman and Platts-Mills, 1978; Platts-Mills et al., 1978). Therefore measurable titres of allergen-specific IgG were reported not to be useful in the establishment of a diagnosis of human atopy (Aalberse et al., 1983; van der Zee and Aalberse, 1991).

However, statistically, patients with food allergy or with atopic eczema were found to have higher levels of allergen-specific IgG4 than non-allergic controls (Aalberse et al., 1993).

High levels of IgG4 antibodies are frequently reported in patients undergoing immunotherapy. However without immunotherapy, IgG antibodies are not restricted to the IgG4 sub-isotype. Rather, in many cases, IgG1 antibodies are predominant, particularly in the early response to chronic exposure to allergen (Aalberse et al., 1993).

### 3.1.2.2. Dogs

#### 3.1.2.2.1. Allergen-specific IgE

Although allergen-specific IgE antibodies are usually associated with atopic disease in dogs (Halliwell and Gorman, 1989; Halliwell, 1990), normal dogs can also exhibit allergen-specific IgE responses (Lian and Halliwell, 1998). Also one report claimed that the levels of allergen-specific IgE were not elevated in atopic dogs (Codner and Lessard, 1993). This will be discussed in detail in the diagnosis section (see allergy testing).

#### 3.1.2.2.2. Allergen-specific IgG

Willemse et al. (1985a) looked at the incidence of allergen-specific IgGd antibodies in the serum of atopic dogs and found an 89% incidence in dogs with positive intradermal skin tests (IDSTs) (55/60) whereas of the dogs with negative IDSTs 11/20 (55%) had detectable IgGd antibody. They suggested that the IgGd subclass may play a role in the pathophysiology of atopy in the dog. Also Prélaud and Sainte-Laudy (1989) found high levels of allergen-specific IgG in the serum of atopic dogs.

Day et al. (1996) compared the level of allergen-specific IgG of four different subclasses in normal and atopic dogs. Whereas the concentration of *Dermatophagoides farinae* (DF) and *Dermatophagoides pteronyssinus* (DP)-specific IgG4 were shown to be higher than other subclasses in the serum of atopic dogs, lower levels of DP-specific IgG1, IgG2 and IgG3 were detected. The IgG subclass response differed from one allergen to another and the authors claimed that the results suggest a degree of IgG subclass restriction in the humoral immune response in canine atopy which may be dependent upon the nature of the allergen.

## 3.2. Genetic factors

### 3.2.1. Man

There is clear evidence that both the atopic condition in general, and also specific allergic disorders are under genetic control (Zamel et al., 1996). However, environmental factors are also highly relevant (Tada, 1975).

While there are certainly major genes which control atopy in general, there are also likely to be disease-specific genes which determine whether an atopic individual develops asthma, rhinitis or conjunctivitis. Therefore, if, as is likely, more than one gene predisposes to the syndrome of atopy, then significant numbers of individuals will carry two or more of these genes (Cookson, 1994).

Segregation analysis of atopic individuals as measured by serum IgE levels supports a recessive mode of inheritance, although further studies are required to ascertain whether this is the case when disease symptoms are used as the relevant phenotype (Meyers et al., 1994). Because many of the components that are required for an allergic response are found on several cells, atopy could theoretically result from an abnormal regulation of this process at several control points. Indeed, some of the products of the first "generation" of genes implicated in predisposition to atopy have clearly defined roles (Casolaro et al., 1996). The important role in the pathophysiology of atopy played by the MHC class II molecules, proinflammatory cytokines, high-affinity receptor for IgE, T cell receptors, adhesion molecules, chemokines and their receptors, and histamine releasing factors, makes the genes encoding for those molecules prime candidates for atopy genes, and as such they are receiving close attention in studies on the genetics of atopy (Casolaro et al., 1996). The HLA class II genes (locus for antigen-specific IgE synthesis) (Marsh et al, 1982; Casolaro et al., 1996), the chromosome 11q13 (locus for atopy) (Sandford et al., 1993; Cookson, 1994) and the chromosome 5q (locus for total serum IgE concentration) (Meyers et al., 1994; Marsh et al., 1994&1995) have all been shown to be involved in atopic diseases.

### 3.2.2. Dogs

Strong breed predilection and evidence of familial involvement in some dogs suggest a genetic basis for the disease, but the mechanism of inheritance is still unknown (Reedy and Miller, 1989). A study done by Vriesendorp et al. (1975) on the relationship

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between dog leukocyte-antigens (DLA) and atopy produced inconclusive results. However the DLA haplotype 9,4 was thought to offer protection against the disease.

In a first limited breeding study, Schwartzman et al. (1971) showed that all of the offspring from two atopic parents developed evidence of atopic disease by the age of 2.

More recently de Weck, Zunic and collaborators. (1995&1997&1998) have established colonies of high IgE and low IgE responder beagle dogs. The results suggest that the high allergen-specific IgE response observed in some dogs following experimental immunisation is inherited as a single dominant trait. On this base de Weck revived the hypothesis that atopy is based on an autosomal dominant gene with reduced penetrance. He claimed that the reduced penetrance could be explained by the need to be exposed to sensitising allergens early in life and by the fact that sensitisation by inhalation, which this worker proposes as the most likely route, is subjected to some additional environmental control. However full details of these studies have yet to be published.

Breed predispositions are evident and, depending on the geographic location, certain striking breed tendencies clearly emerge (Halliwell and Schwartzman, 1971; Scott , 1981; Schick and Fadok, 1986; Carlotti and Costargent, 1994; Saridomichelakis and Koutinas, 1996).

### 3.3. Route of allergen penetration

In dogs, the route of access of aeroallergens remains uncertain. It was always assumed to be via the respiratory tract, but it is unclear how allergens are transported from the respiratory tract to the skin via the circulation in order to cross-link IgE on mast cells (Halliwell and Gorman, 1989).

Although there is evidence in favour of the respiratory route (Butler et al., 1983), there are also other lines of evidence that argue against this route (Willemse et al., 1984; Helton-Rhodes et al., 1987). On the other hand, the localisation of the lesions in atopic dogs is similar to that of the lesions seen in allergic contact dermatitis and suggests that the percutaneous penetration of aeroallergens plays a significant role in the dog as in man (Halliwell and Gorman, 1989).

The existence of IgE positive epidermal Langerhans' cells in dogs with AD is an important finding which has added more evidence in favour of an epidermal route of allergen penetration (Olivry et al., 1996). The number of epidermal and dermal dendritic cells is higher in atopic dogs and there is an apparent correlation between the level of

expression of IgE by dendritic cells and the level of total IgE in the serum of these dogs (Olivry et al., 1996). In addition, the skin of dogs with AD was shown to be infiltrated by CD4+ and CD8+ T cells (Olivry et al., 1997a; Sinke et al., 1997). Eosinophils and proteins derived from these cells were also found in the epidermis of the lesional skin of atopic dogs (Frank and McEntee, 1995; Olivry et al., 1997a).

### 3.4. Immunopathogenesis

### 3.4.1. Role of IgE in antigen capture and production of inflammation

It has become clear over the years that there are many other factors involved in the pathogenesis of atopy than merely IgE. However the precise role of each of the many abnormalities that have been identified in atopic patients in the proposed pathogenesis of the disease remains controversial. Multiple allergic and nonallergic factors are involved, which produce variable clinical signs, confusing test results, and unpredictable responses to therapy.

The central importance of the type I hypersensitivity reaction has already been discussed in the previous section.

A number of the cutaneous factors related to the pathogenesis such as the participation of T cells directly or indirectly, and the role of IgE and Langerhans' cells in antigen presentation have also been discussed in previous sections.

Additional abnormalities have been identified in atopic patients although their exact role in the pathogenesis of the disease remains unclear. Amongst these are the possible existence of a heterogeneity of IgE and the existence of mutants of the FcεRI on mast cells (Shirakawa et al., 1994).

### 3.4.2. Mast cells

3.4.2.1. Introduction

Although mast cells are clearly critical in the pathogenesis of disorders of IgE-dependent immediate hypersensitivity, these cells are not simply troublemakers devoid of any redeeming biologic value. It is clear that they also participate in the late-phase reactions associated with allergy not only by the liberation of newly formed mediators but also by the release of cytokines, such as Il-4 and TNF-α (Schwartz and Huff, 1993).

Mast cell differentiation and proliferation is predominantly regulated by 2 separate mechanisms. The first is dependent on a fibroblast-derived growth factor, termed stem cell factor (Costa et al., 1996), and the second is dependent on T-cell-

derived cytokines such as Il-3 (Mitsui et al., 1993), Il-4 (Schmitt et al., 1987) and Il-10 (Thompson-Snipes, 1991).

Multiple transcription factors, such as AP-1 and the nuclear factor of activated T cells, are induced in the cytoplasm in activated cells in response to extracellular signals. They shuttled to the nucleus where they exert positive or negative control over gene expression. The transcription factors have an important role in the induction of cytokines in mast cells (Nechushtan and Razin, 1998).

Protein kinases C have been implicated in the regulation of mast cell growth and in the expression of AP-1 component in response to FcεRI aggregation (Razin et al., 1994).

### 3.4.2.2. Dogs

The number of mast cells, the histamine content of mast cells and the total histamine content of lesional skin in atopic dogs, are all increased as compared to normal skin (Nimmo Wilkie et al., 1990; DeMora et al., 1996). The correlation between mast cell numbers and cutaneous histamine concentration in atopic dogs was found to be very good (r = 0.84) (Nimmo Wilkie et al., 1990). However, in the dog as in man, some data suggest that mast cells play a minor role in AD (see section 3.4.4., Olivry et al., 1997a).

### 3.4.2.2.1. Mediator releasability

DeMora et al. (1996) have studied the releasability of histamine from canine skin mast cells following stimulation by two different secretagogues in normal and atopic dogs. Calcium ionophore, that activates mast cells by a mechanism independent from antigen-antibody binding, elicited a similar response from mast cell populations in both atopic and normal dogs while concanavalin A (con-A), which mimics antigen induced release, was able to induce a significant increase of histamine secretion from mast cells from atopic dogs than from nonatopic dogs. However the processes involved and the importance of these differences in the pathogenesis of canine atopy are not known.

### 3.4.3. Role of cell mediated immunity in canine atopy

### 3.4.3.1. Nature of the cutaneous inflammatory infiltrate

Olivry et al. (1997a) have shown similar patterns of inflammation in the lesions of canine and human AD. The epidermal infiltrate of lesional atopic canine skin was shown to be characterised by Langerhans' cells, T-cells expressing the αβ– or γδ -T cell receptors, occasional intact or degranulated eosinophils, and rare neutrophils. The dermis

was infiltrated by mast cells, dermal dendritic cells, memory helper T cells, and small numbers of neutrophils and eosinophils.

Similar differences between the neutrophil infiltrate found in spontaneous lesional atopic skin and in induced cutaneous LPR reported in man were seen in dogs (Olivry et al., 1997a). Indeed in the spontaneous lesional atopic skin, neutrophils were found to constitute only a small percentage of the total inflammatory cells (Olivry et al., 1997a), whereas they were the predominant cell type in the dermis of induced cutaneous LPR (Becker et al., 1988; Thomsen et al., 1993).

3.4.3.2. Studies on cell-mediated immune function

Cell-mediated immune function in atopic dogs has been studied and some abnormalities identified (Nimmo Wilkie, 1990, Nimmo Wilkie et al., 1991&1992). These workers demonstrated that the ability of histamine to induce suppression in peripheral blood mononuclear cells was significantly decreased in atopic dogs as compared to normal dogs. However the total number of histamine type 1 or type 2 receptors was not different for cells from normal or atopic dogs. On the other hand, they did not investigate the total numbers of cells that bear these receptors which might still be decreased in atopic dogs. A reduction of con-A-induced suppression by atopic canine peripheral blood mononuclear cells was obtained only at supraoptimal doses of con-A while it was not significantly different from the control group at suboptimal and optimal doses.

Nimmo Wilkie and collaborators studied immunologic parameters in normal dogs, dogs with atopic disease and dogs with induced type 1 hypersensitivity to ascaris antigen (Nimmo Wilkie, 1990). Atopic dogs were shown to have alterations of histamine-modulated function, increased cutaneous response to intradermally injected mitogens and decreased con-A induced suppression. The alterations of histamine-modulated function detected in atopic dogs were also found in dogs with induced type 1 hypersensitivity and anaphylaxis following intravenous administration of ascaris antigen. However the increased response to intradermally injected mitogens and the decrease in con-A-induced suppression reported in atopic dogs were not shown in dogs with experimentally-induced anaphylaxis (Nimmo Wilkie, 1990). Therefore the authors concluded that some of the altered cell-mediated immune functions observed in dogs with AD result from type 1 hypersensitivity (Nimmo Wilkie et al., 1990). Dogs with atopic dermatitis were also shown to have a significantly reduced response to the contact allergen dinitrochlorobenzene but paradoxically to have significantly increased response

to intradermally injected mitogen PHA and con-A and no change in lymphocyte blastogenesis (Nimmo Wilkie et al., 1991).

### 3.4.4. Basophils

Basophils share similarities with mast cells . Some of the mediators are common to both cells, as are most of the preformed granule-associated mediators. However neutral proteases are only found in very small concentrations (Schwartz and Huff, 1993). Basophils also release the newly-generated mediators derived from arachidonic acid with the exception of $PGD_2$.

Like mast cells, they can induce IgE-dependent but also IgE-independent release of Il-4 (Machado et al., 1996) and could therefore be the initial source of Il-4-production (see Immunoglobulin E). Basophils can also generate and secrete high levels of Il-13. Human basophils, like human mast cells , can also produce TNF-$\alpha$ (Schwartz and Huff, 1993).

### 3.4.5. Histamine releasing factors

Basophils are responsive to an unique set of cytokines that have been termed histamine releasing factors (HRFs) (MacDonald, 1996). HRFs are produced by a variety of cells including T and B cells, mononuclear cells, alveolar macrophages and platelets. HRFs can induce histamine release from basophils either by an IgE cell surface-dependent or an IgE-independent mechanism (MacDonald et al., 1993). They only affect basophils sensitised with IgE from atopic individuals, termed IgE+ (MacDonald, 1987).

HRFs have been detected in human respiratory secretions, late skin blister fluids in atopic patients and bronchoalveolar lavage fluids (MacDonald et al., 1993). Patients with atopic dermatitis and food hypersensitivity were found to have higher rates of spontaneous histamine release from basophils and which was believed to result from HRF production (Sampson et al., 1989).

The gene encoding an IgE-dependent HRF produced by lymphocytes from atopic children and produced in biological fluids of allergic patients has recently been cloned and prepared in recombinant form by MacDonald and collaborators (1995). Polyclonal antibodies recognised and removed the biological activity of both the recombinant and native HRF (MacDonald et al., 1995).

### 3.4.6. Keratinocytes

Keratinocytes not only function as mechanical barriers but play also important roles in mediating cutaneous immune responses and inflammation by their production of a wide variety of cytokines and by direct cell-cell contact via adhesion receptors (Takashima and Bergtresser, 1996). Keratinocytes, Langerhans' cells and dendritic epidermal T cells (γδ T cells) can communicate with each other by their production of cytokines. Keratinocytes and Langerhans' cells can promote the development, modulate the phenotype, and regulate the immune function of dendritic epidermal T cells. Conversely, dendritic epidermal T cells regulate the function of both keratinocytes and Langerhans' cells (Takashima and Bergtresser, 1996).

Interestingly, Malaviya et al. (1996) demonstrated in a very recent study that keratinocytes from normal people were able to produce histamine and were likely to be the chief cell type responsible for the histamine found in the epidermis, although Langerhans' cells and melanocytes may also contribute. Moreover, when keratinocytes were stimulated by ultraviolet B light, the quantity of histamine release was comparable to that released by mast cells . It was also shown that the release of significant amounts of histamine into the extracellular environment after irradiation was not specifically associated with cell injury or cell death (Malaviya et al., 1996). The results suggest that such release of keratinocyte-derived histamine may play an important role in mediating the inflammatory response of the skin. Ultraviolet light injury may therefore contribute to the inflammation in some atopic individuals.

### 3.4.7. Eosinophils

The recruitment of eosinophils from the blood into the tissues involve their adhesion to endothelial cells through adhesion molecules and their migration to the site which is controlled by three main cytokines: Il-3, Il-5 and granulocyte/macrophage-colony stimulating factor (GM-CSF) (Desreumaux and Capron, 1996). Il-4 alone or in combination with IFN-γ has been recently shown to induce the production of an eosinophil chemotactic protein (eotaxin) by dermal fibroblasts (Mochizuki et al., 1998).

3.4.7.1. Mediators produced and their effects

Following activation resulting from the presence of IgE or IgA and/or cytokines that interact with specific receptors on their membrane, eosinophils release their granules which contain four distinct cationic proteins.

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These proteins have major protective effects in host defences against helminth parasites but also contribute to tissue damage and to acute and chronic manifestations of inflammation in allergic diseases. Membrane receptor expression, oxidative metabolism and the release of granule proteins are all controlled by Il-3, Il-5, GM-CSF (Desreumaux and Capron, 1996) and HRFs (MacDonald, 1996). Membrane expression of VCAM-1 on endothelial cells, which is predominantly involved in the migration of eosinophils in the respiratory mucosa, is upregulated by Il-4 and Il-13 (Bochner et al., 1995).

### 3.4.7.2. Cytokines produced and their effects

Eosinophils can contribute to the pathogenesis of allergic disorders not only by releasing tissue damaging basic protein but also by producing specific lipid mediators and various cytokines. Lipid mediators include leukotriene C4, platelet activating factor and lipoxin factor. Cytokines produced by eosinophils can have autocrine growth factor activities such as Il-3, Il-5 and GM-CSF. They also have a potential role in the inflammatory response and the development of fibrosis in the case of Il-8, TNF-$\alpha$ and TGF-$\alpha$. They may be involved in the regulation of the immune system, in the case of Il-4, Il-2 and IFN-$\gamma$ (Desreumaux and Capron, 1996). Eosinophils may also promote IgE synthesis and potentially be a source of early Il-4 (Nonaka et al., 1995). However, little is known about the precise role of eosinophil-derived cytokines in human allergic diseases and it is still not clear if mediators located in the granules are activated upon release, or are inactivated and degraded (Desreumaux and Capron, 1996). Very recently eosinophils were shown to synthetise biologically active Il-12 (Crewe et al., 1998). Heinz they may be involved in the activation of Th0 and Th1 type cells seen in the late phase of AD (Crewe et al., 1998).

### 3.4.8. Late-phase reactions

#### 3.4.8.1. Man

##### 3.4.8.1.1. General introduction

It has long been appreciated that exposure of an allergic individual to an antigen produces an immediate response, often followed by a LPR. In man this late reaction occurs within 6 to 24 hours of allergen challenge, peaks after 4 to 12 hours, and is characterised clinically by oedema and erythema of the skin and by increased resistance to air flow in the nose and lung (Atkins et al., 1988).

However, in contrast, the spontaneous human AD lesions are not characterised by an immediate reaction and differ from the LPR by the lack of persistent neutrophilic infiltration, activation and degranulation (Ott et al., 1994).

### 3.4.8.1.2. Cells involved in the LPR

Histopathological examination of LPRs reveal a mixed cellular infiltrate of predominantly mononuclear cells but also of eosinophils, basophils, and neutrophils. The size of the LPR appears to be correlated with the number of activated eosinophils. Activated T cells are also present and there is a strong correlation between the number of CD4+ T cells and the number of activated eosinophils (Frew and Kay, 1988).

### 3.4.8.1.3. Mediators and cytokines involved in the late-phase reaction

Leukotrienes and cyclooxygenase products, such as $PGD_2$, are more likely to be active during the LPR than the early-phase reaction (Bousquet and Michel, 1993).

Cytokines, such as IFN-$\gamma$, Il-1, Il-3, Il-4, Il-5 and GM-CSF are likely to have a role in the generation of LPR (Frew and Kay, 1988; Kay et al., 1991). HRFs which activate all basophils and eosinophils from allergic donors and which are found in the LPR fluids, are believed to play a major role in perpetuating the LPR and the chronic inflammation, in allergic asthma (MacDonald, 1996).

### 3.4.8.1.4. Mechanisms of the late-phase reaction

The exact mechanisms of the erythematous inflammation seen in LPR are not completely understood. Mast cells appear to be the trigger cell of the reaction as the LPR rarely appears without an immediate reaction. Antigen cross-linking of mast cell bound IgE is ultimately responsible for the involvement of eosinophils, neutrophils, basophils and lymphocytes. Lymphocytes may play a key role in the generation of the late-phase inflammation by their production of cytokines (Frew et al., 1991). Neutrophils represent the first cell type seen in the inflammatory infiltrate following the immediate reaction. The eosinophil appears to be critical to the development of the LPR by the production of cytokines and eosinophil cationic proteins (particularly the major basic protein) which may play a role in enhancing basophil and mast cell activation (Charlesworth et al., 1989). Basophils may be the source of late-phase histamine release and thus be responsible for the persistent release of histamine during continuous antigen challenge to the skin.

3.4.8.2. Dogs

The existence of the LPR in dogs was first demonstrated by Becker et al. (1988). The LPR in the dog is similar to the LPR in humans (Becker et al., 1988; Thomsen et al., 1993). Following intradermal injection of antigen and/or histamine in experimentally sensitised dogs, neutrophils and eosinophils were the first cells seen in the dermis. They were followed by mononuclear cells. $LTB_4$ was found to be increased prior the infiltration by neutrophils. However, the influx of basophils was not significant (Becker et al., 1988). Nevertheless, as in human AD, neutrophils were reported to be rare in spontaneous lesional skin (Olivry et al., 1997a).

However, the decrease of clinical symptoms shown in some atopic dogs treated with misoprostol, a $PGE_1$ analogue, is in favour of a clinical relevance of allergen induced LPR (Olivry et al., 1997b).

### 3.4.9. Disregulation of the fatty acid metabolism

Therapeutic fatty acid supplementation and absorption studies in atopic and normal dogs have recently been undertaken. In one study, levels of plasma essential fatty acid (EFA) were measured in normal humans and normal dogs. They were then compared to the levels in subjects with allergic skin disease. The study concluded that normal humans and dogs have slightly lower concentrations of plasma linoleic acid (LA) and slightly higher concentrations of dihomo-gamma-linoleic acid (DGLA) and arachidonic acid (AA) than do subjects with allergic skin disease. A slight increase in DGLA and a marked increase in AA after evening primrose oil supplementation was seen in dogs but not in humans. These results point to a possible enzyme defect in humans with atopic dermatitis but argue against such a defect in dogs (Horrobbin et al., 1990).

In another study, no significant difference was found in serum n-6 fatty acids between normal, atopic, and seborrhoeic dogs (White, 1990). In a further report there was no difference in resting triglyceride levels between atopic dogs and normal dogs; however triglyceride levels were significantly lower in atopic dogs than in normal dogs after feeding corn oil (van den Broek and Simpson, 1990).

All these studies tend to imply that atopic dogs have either impaired fat absorption or altered triglyceride metabolism.

### 3.4.10. Importance of food sensitivity in atopic disease

The importance of food sensitivity in atopic disease will be discussed in the section on food hypersensitivity.

### 4. IMMUNOGLOBULIN G IN ATOPIC DISEASE

In most species, antibodies of one or more IgG subclasses are cytotropic and can bind to mast cells or basophils. These IgG antibodies are heat stable and were called "short term sensitising IgG" according to their sensitisation time which is maximal after 2 hours and persists only for a few hours (Parish, 1970).

The evidence of the involvement of IgG4 in some anaphylactic reactions and allergic diseases was first proposed by Parish in 1974. Since then, several authors have demonstrated that anti-IgG4 antibodies are able to cause histamine release from human basophils (Vijay and Perelmuter, 1977; Fagan et al., 1982).

However, allergen-specific IgG has also been shown to block the binding of allergen to cell-bound IgE (Lichtenstein et al., 1968), and IgG4 has been shown to significantly increase in patients undergoing hyposensitisation (Devey et al., 1976). Therefore, the role of IgG4 in atopic disease appears to be paradoxical and a protective role as well as a potentially harmful role have been attributed to it (van der Zee and Aalberse, 1987).

The heterogeneity of IgG4 antibodies was suggested as an explanation for this paradox by Stanworth (1987). The author postulated that the majority of IgG4 myeloma proteins which he found were unable to bind to human mast cells, represent protective antibodies and the 25% that do seem to bind were effector types.

### 5. DIAGNOSIS

### 5.1. Introduction

Atopy should be diagnosed on the basis of the history and the clinical findings. Allergy tests should only be used to confirm the diagnosis and to identify the specific allergens involved to enable hyposensitisation therapy. Both, *in vitro* and *in vivo* tests are available and which one is better remains controversial. Both have advantages and disadvantages.

## 5.2. Allergy testing

### 5.2.1. *In vivo* testing

#### 5.2.1.1. Man

##### 5.2.1.1.1. General introduction

Skin tests have represented the primary diagnostic tool in human allergy since their introduction. The intracutaneous test developed by Mentoux in 1908 was applied to immediate hypersensitivity by Schloss (1912). Some years later Lewis and Grant (1924) described the prick test. These methods have been used for decades without major modifications, but in the recent years they have been refined, and a number of standardised devices have been developed for prick testing (Pepys, 1975).

##### 5.2.1.1.2. Interpretation of *in vivo* tests

*In vivo* allergy testing is generally not used in people with atopic dermatitis because it is not helpful. Whereas negative tests are very reliable, most of the positive results are false-positives, lacking clinical correlation (Sampson and Albergo, 1984). However, skin tests can provide useful confirmatory evidence for a diagnosis of allergic asthma that has been made on clinical grounds.

The occurrence of a positive skin test does not necessarily imply that the patient is clinically allergic to that allergen. Many have shown that ST can be positive in nonallergic individuals (Bousquet and Michel, 1993). These positive results are usually attributed to the presence of impurities, contaminants or non-specific mast cell secretagogues in the allergen extract. However studies have shown that such reactions were not all irritant reactions since, in some patients, positive PK tests were obtained using their serum (Roane et al, 1968). It has also been proposed that the presence of positive skin tests in non allergic subjects may predict the onset of allergic symptoms since these reactions seems principally to occur in subjects who are first-degree relatives of atopic patients (Hagy and Settipane, 1976).

#### 5.2.1.2. Dogs

##### 5.2.1.2.1. General introduction

In dogs IDSTs are used rather than prick tests. In the UK the use of the IDST in the dog was first described by Baker (1971). The test is reported to be a useful diagnostic aid if performed properly with good allergenic extracts and if interpreted with caution (Halliwell and Gorman, 1989).

IDSTs can detect allergen- specific anaphylactic antibodies bound to mast cells, which belong predominantly, if not exclusively, to the IgE isotype. The degranulation of mast cells and the release of inflammatory mediators produce an erythematous wheal. The factors influencing the skin sensitivity to an antigen include the amount of allergen injected, the degree of sensitisation and releasability to the mediators, as well as the reactivity of the skin to the mediators released from the mast cells. A positive control such as histamine should be included (Halliwell and Gorman, 1989). The negative control is the diluent of the allergenic extract. The skin test is read after 15 to 20 minutes. The reactions are graded either by using an arbitrary scale rating +1 to +4 where +4 approximates the histamine wheal size and a +1 is a wheal that is just discernibly greater than the size of the negative control. The result should also take into account the degree of erythema and the elevation above the surface. Alternatively, one can measure the diameter of the wheals and a positive test is defined as >50% of the sum of the diameter of the positive and the negative controls (Halliwell and Gorman, 1989). Although it seems easy to perform such tests, adequate interpretation requires trained individuals who are aware of all factors that might modify the results.

The simplicity, rapidity of performance, low cost and high sensitivity explain the popularity of the IDST in atopy.

5.2.1.2.2. Causes of false-positive and false-negative results

There are a number of factors which can lead to either false-positive or false-negative results. Discussion of this is beyond the scope of this chapter.

5.2.1.2.3. Interpretation of *in vivo* tests

It is important to emphasise that a positive IDST is merely an indicator that the patient has skin-sensitising antibody, mast cells that degranulate on antigen exposure, and target tissue that responds to the released mediators. It does not necessarily mean that the patient has clinical allergy to the allergen(s) injected and results have to be interpreted in the light of the patient's history and exposure to allergen (Halliwell and Gorman, 1989; Scott et al., 1995a). On the other hand, the IDST does not appear to be 100% sensitive and a negative test does not always mean that the dog is not sensitised to this allergen (DeBoer, 1997).

### 5.2.2. *In vitro* testing

5.2.2.1. Man

5.2.2.1.1. General introduction

An immediate consequence of the discovery of IgE antibody in man was the development of assays to measure total and allergen-specific IgE. A radioimmunoassay (RIA) was first developed in 1967 and adapted to demonstrate the antibody activity of IgE to particular allergens (Wide et al., 1967). This sandwich RIA was called the radioallergosorbent test (RAST) (Wide et al., 1967). More recent methods for the detection of IgE antibody include the enzyme-linked immunosorbent assay (ELISA) (Donohue et al., 1989) and AlaSTAT or liquid phase assay (El Shami and Alaba, 1989).

5.2.2.1.2. Current methods

The principle of the ELISA and RAST is identical and both detect the same antibody. Both tests use allergens bound to a solid phase and the only difference is that RAST uses radiolabelled antibody whereas ELISA uses an antibody coupled to a suitable enzyme.

The liquid phase assay measures IgE antibody activity to allergens in the liquid phase. One of the advantages is that non-specific IgE binding to a solid phase can be virtually eliminated. Another advantage claimed by proponents over the solid-phase assays is that no potentially dominant epitope is masked in the coupling procedure, because the different coupling agents can react with different parts of the molecule. Also, the test is usually performed with undiluted serum. However, several studies in which AlaSTAT and RAST were evaluated in man showed that both correlated well with each other and had good sensitivity and specificity (McSharry et al., 1993).

5.2.2.2. Dogs

RAST was first described in the dog by Halliwell and Kunkle in 1978. More recently the development of ELISA has been reported (Kleinbeck et al., 1989). Those are the two most commonly used in vitro tests that detect relative levels of allergen-specific IgE in serum (Halliwell, 1994).

The principle of the test is the same as it is in man. Results are compared to a standard curve established by the laboratory. The standard is usually a serum or a pool of sera which are known to contain a high level of allergen-specific IgE. Results are expressed in OD or reported in relative antibody units (RAU) by comparison to the standard.

In *vitro tests* have now become available through commercial laboratories in both the USA and Europe and have been the subject of several studies, with variable results (Sousa and Norton, 1990; Miller et al., 1992; Bond et al., 1994; Plant, 1994; Bunde et al., 1997).

The replacement of anti-canine IgE by the use of the human FcɛRIα receptor to detect allergen-specific IgE by ELISA has been introduced recently (McCall et al., 1997). A study performed on a group of artificially sensitised flea allergic dogs showed an excellent correlation between the skin tests results to flea saliva and the biotinylated FcɛRIα based assay. Another study has been recently performed on normal dogs, atopic dogs and dogs with naturally occurring dermatitis (McCall et al., 1997). Results were found to be encouraging with a sensitivity of the assay of 83.3% as compared to the IDST.

VARL Liquid Gold allergen-specific IgE test (liquid-phase) was reported to have a good concordance with western blots in two blinded studies which used the latter as a standard (Griffin, 1993a).

*In vitro* tests have the great advantage that they are not influenced by antihistamines and probably not by low doses of corticosteroids, although this has yet to be proved. However levels of birch pollen-specific IgE were shown to be decreased in asthmatic individuals following treatment with intranasal glucorticoids (Pulleritis et al., 1997). Levels of allergen-specific IgE were also reported to double in some dogs after discontinuation of corticosteroids and therefore some authors recommended that they are withdrawn before *in vitro* testing (Griffin, 1989). A recent study using the FcɛRIα-based test Allercept® showed that although many dogs may be tested successfully while undergoing treatment with corticosteroids, other may be immunosuppressed sufficiently to affect IgE serum levels (McCall, 1998). *In vitro* tests can, of course, also be used on dogs with inflamed, lichenified skin, which may be inappropriate for the IDST (Halliwell and Gorman, 1989).

### 5.2.3. Correlation between *in vivo* and *in vitro* testing

5.2.3.1. Man

Comparisons between the titration of specific IgE by IDST and by immunoassay in man depend upon the quality and standardisation of allergens used in both tests. The worst correlations are obtained with HD, mold, and unstandardised dander extracts,

whereas the correlation ranges between 70 and 80% with extracts such as pollens, mites, or standardised dander extracts (Bousquet et al., 1988).

There is a generally good correlation between a strongly positive IDST and a positive *in vitro* test, and also between a negative IDST and a negative *in vitro* test in atopic individuals (Bousquet et al., 1988). In > 80% of cases where discordant results were obtained positive IDSTs were found in the absence of detectable allergen-specific IgE. The discrepancies observed in some studies could be due to the heterogeneity of the atopic population since the level of total IgE was shown to influence the relationship between skin test and RAST (Eriksson, 1989).

### 5.2.3.2. Dogs

Many studies have compared results of *in vitro* and *in vivo* tests in the dog and have shown varying correlations which are dependent upon the allergen employed. In general the correlation appears to be best in the case of pollens, and worst for HD (Halliwell and Kunkle, 1978; Willemse et al., 1985b; Kleinbeck et al., 1989; Codner and Lessard, 1993; Miller et al., 1993a; Bond et al., 1994; Plant, 1994; Bunde et al., 1997; Lian and Halliwell, 1998). Interestingly, in one study, discordant results usually consisted of a negative skin test associated with positive or strongly positive ELISA (Kleinbeck et al;, 1989). In another study, ELISA was found to be of limited value due to low specificity and a high incidence of false positives (Codner and Lessard, 1993). It has been suggested that positive results seen in clinically normal dogs with negative IDSTs may result from the high level of total IgE present in dog serum (Griffin et al., 1990).

The results of hyposensitisation based upon skin tests and *in vitro* tests have been compared and results appear to be broadly similar (Willemse et al., 1984; Sousa and Norton, 1990; Griffin and Rosenkrantz, 1991; Miller et al., 1993a). However Griffin and Rosenkrantz (1991) showed that only 22% of dogs treated with hyposensitisation based on the results of ELISA in animals with negative skin tests showed improvement.

Nevertheless, it is important to ask whether *in vitro* tests should correlate with skin testing. Since skin tests have always been considered as the "gold standard", when the *in vitro* test and skin testing do not correlate it has always been assumed that *in vitro* tests must be wrong (Halliwell, 1994). The IDST can also be prone to error. The percentage of concordance between skin testing and *in vitro* tests can be considerably decreased when dogs with weak IDST reactions and questionable clinical histories are included (Esch and Grier, 1997). The possible heterogeneity of IgE antibody may also be

an explanation for the poor correlation often observed between *in vivo* and *in vitro* tests. Therefore so called false-positive reactions in *in vitro* tests may be due in some cases to the fact that non-pathogenic IgE is being detected (Jackson et al., 1996; Lian and Halliwell, 1998).

### 5.3. Conclusion

In conclusion, there is clearly a place for both tests in the diagnosis of atopic disease and the practice of both tests on the same animal can help the dermatologist to learn more about allergic disease.

It must be emphasised once more that a positive allergy test is an indication that the animal has allergen-specific IgE but is not of itself a diagnosis, and that the use of these tests must be regarded as an adjunct in confirming the diagnosis when supported by appropriate clinical signs and after elimination of other possible diagnoses.

If it is clear that there is room for technological improvement of diagnostic tests it is also clear that there is more to allergy than the mere presence of allergen-specific IgE (DeBoer, 1996).

## III. ADVERSE REACTION TO FOODS IN MAN

### 1. INTRODUCTION

Adverse food reaction is a generic term that includes any reaction following the ingestion of a food or a food additive. It can be secondary to a food allergic reaction or food intolerance. An examination of the host immune response at the level of the intestinal mucosa is prerequisite since the intestinal mucosa is the site where food antigens first interact with cells of the immune system.

Under the ideal situation, ingestion of food in normal individual results in the development of immunologic tolerance to food antigens. However, in addition, food antigen-specific antibodies are formed, which play a role in blocking penetration of food antigens across the gastrointestinal barrier and in rapidly clearing minute quantities of food antigens that gain systemic access. Failure to develop oral tolerance in genetically predisposed individuals may result in food hypersensitivity that may be responsible for a variety of clinical illnesses. IgE-mediated disorders account for the majority of well-characterised food allergic reactions and have been studied most extensively. However other mechanisms are believed to be involved in some food allergic disorders.

## 2. CONTROL OF ANTIGEN ABSORPTION

Food antigens, obtained in part from heterologous animal species and from vegetable sources, are capable of eliciting immune responses. Therefore the individual must digest and absorb these substances without developing an adverse immune response whilst at the same time being able to recognise and respond to infectious organisms gaining access via the same route.

### 2.1. Nonspecific-regulation of antigen uptake

Digestive enzymes, gastric acidity, mucous glycoproteins, and peristalsis are important factors that both protect the host from infectious agents and direct the degradation of foods.

Glycoproteins in the mucous covering of the gastrointestinal surface provide an important nonspecific defence mechanism against parasitic, bacterial and viral attachment to the epithelium. However the mucous glycoprotein responses are not entirely non-specific and a relationship between immunological reactions and glycoprotein synthesis and release has been suggested (Lake et al., 1980; Miller et al, 1979).

The epithelial layer of the gastrointestinal tract is the final barrier to antigen passage. Its permeability is discussed in a following section.

Immaturity of these mechanisms in infants reduces the efficiency of the infant mucosal barrier (Hyman et al., 1985). Basal acid output, proteolytic activity in the small intestine, mucin composition and microvillus mucous membranes have all been shown to be immature and less functional in the young animal (Shub et al., 1983).

### 2.2. Specific regulation of antigen uptake

The humoral immune system of the gastrointestinal tract is capable of specific responses to intact infectious organisms and to solubilised antigens. The "gut-associated lymphoid tissue" (GALT) is composed of four distinct lymphoid compartments. These are the Peyer's patches, lymphocytes and plasma cells scattered throughout the lamina propria, intraepithelial lymphocytes, and mesenteric lymph nodes. Macrophages and dendritic cells located in Peyer's patches transport macromolecules from the lumen to resident precursor B and T cells.

IgA-producing cells predominate in the intestinal mucosa. IgM B cell precursors are the next most common (6-18%), followed by IgG-producing cells (3-4%) and,

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finally, by IgE and IgD isotypes (<1%) (Cornell et al., 1971; Owen and Jones, 1974; Baklien and Brandtzaeg, 1975).

The function of IgA is to trap undesirable molecules such as dietary and microbial antigens and infectious agents and block their access to the absorbing surface of the gastrointestinal tract by preventing binding to the surface cells (Walker et al., 1975).

The newborn lacks IgA in exocrine secretions (Selner et al., 1968). The relatively low concentration of IgA in the intestine in young infants together with the enormous quantity of ingested proteins, contributes to the large amount of food antigen confronting the immature GALT. These antigens may stimulate excessive production of IgE antibodies in genetically predisposed infants (Taylor et al., 1973; Soothill et al., 1976).

## 2.3. Antigen uptake, gastrointestinal tract permeability, development of oral tolerance and normal systemic immune response to food antigen

Oral tolerance is the phenomena whereby prior exposure to an antigen by the enteric route induces a specific immunological unresponsiveness on subsequent systemic exposure to the same antigen (Sanderson and Walker, 1993).

The integrity of the intestinal barrier is important in preventing inappropriate and uncontrolled antigen transport. However, under normal conditions, a small fraction of the potential antigenic load bypasses this barrier and will be absorbed. This fraction is crucial for antigen-specific immune response (Walker, 1988). There are 2 different modes of absorption of food molecules across the intestinal mucosa. In order to reduce their immunogenicity, a major part of the proteins (90%) are degraded before absorption as smaller non-immunogenic peptide fragments. These molecules penetrate through the enterocyte membrane (Fordtran et al., 1965). A minor part of the protein is however absorbed intact or at least in an immunologic form and leads to the induction of antigen-specific immune responses (Sampson, 1991). The absorption of intact food antigens, which are thought to permeate aqueous channels between adjacent enterocytes (Smulders and Wright, 1971) occurs as a natural consequence of the design of the defence systems. This mechanism generates a systemic hyporesponsivness, or oral tolerance (Sanderson and Walker, 1993).

The means by which tolerance develops is not well understood. Three principal immunological mechanisms have been implicated: clonal deletion, clonal anergy and antigen-driven suppression (Strobel and Mowat, 1998). Lymphoid cells appear to be

important in the development of oral tolerance since irradiated mice lose the ability to develop oral tolerance (Bruce et al., 1987). Murine studies have shown that CD8+ cell activation along with antigen presenting cells in the reticuloendothelial system may play a key role in the development of oral tolerance (Sampson, 1991). However Garside et al. (1995) were able to induce oral tolerance in mice depleted of CD8+ cells. Therefore these cells appear not to be prerequisites for the induction of oral tolerance and CD4+ rather than CD8+ T cells may be implicated (Garside et al., 1995).

The presence of detectable serum IgG, IgM, and IgA antibodies to food antigens is a common phenomenon in normal individuals, at least in low levels, and thus everyone has some detectable antibody to various food proteins (Sampson, 1991). Food-specific IgE can also be detected in the sera of normal children (Burgin-Wolff et al., 1980). High levels of food specific non-IgE antibodies found in some patients seem to be secondary to increased gastrointestinal permeability associated with a variety of inflammatory disorders (Sampson, 1991).

Also, in a relatively few individuals who are genetically predisposed, a breakdown or a failure in the development of oral tolerance may result in a hypersensitivity response to the ingested antigen. This may occur if the gut permeability is altered and one or both modes of absorption is affected (Isolauri et al., 1990). In the newborn, the gut barrier is incompletely developed and the antigen may not be transformed into a tolerogenic form (Strobel and Ferguson, 1984). This may explain the peak of prevalence of food allergy in young children. Increased intestinal permeability has also been demonstrated in patients with atopic eczema (see section on food induced eczema/atopy). However, it seems that the inability to induce tolerance is not merely a result of the immaturity of the digestive system or antigen-handling capacity of the neonatal gut, but is more likely to be due to a regulatory imbalance that can be partially restored with adult spleen cells (Peng et al., 1989).

## 3. CLASSIFICATION OF ADVERSE REACTIONS TO FOOD ANTIGENS

As mentioned earlier, adverse reaction to food is a general term for any clinically abnormal response attributed to the ingestion of a food or food additive. Such reactions can be divided in two broad categories; those which are immune-mediated (food hypersensitivity) and those without an immunological basis (food intolerance). The distinction between these different categories is prerequisite for a good understanding of adverse food reactions.

## 4. FOOD HYPERSENSITIVITY

### 4.1. Prevalence

The true prevalence of adverse food reactions is controversial. Many authorities suggest that it is less than 1% of the population (Sampson, 1993a). However most studies suggest that true allergy, at least in young children, may be more prevalent that the medical society thought (U.S. Department of Agriculture, 1983; Bock, 1987). Food hypersensitivity may affect 3 to 8% of the population with a peak prevalence at about one year of age (Bock, 1987; Host and Halken, 1990; Sampson, 1992). As approximately 70 to 80% of infants outgrow their food hypersensitivity, by the end of the first decade of life the prevalence drops, and then appears to remain at about 1% of the population (Sampson, 1992).

### 4.2. Etiopathogenesis

Although type I, IgE mediated reactions, are most frequently implicated, food allergic reactions may also involve type II, type III and type IV hypersensitivities. More than one hypersensitivity reaction may be involved (Sampson, 1991).

Reactions to food antigens can be immediate and occur within minutes after exposure or can be delayed, requiring repeated antigen exposure and occurring hours or days after exposure.

In some situations, although immunological mechanisms are believed to be involved, the precise pathogenesis is unknown (Sampson, 1993a).

#### 4.2.1. IgE-mediated reactions

The majority of immediate reactions to food are reported to be due to IgE-mediated hypersensitivity (Sampson, 1991) and appear to result from the production of food-specific IgE in predisposed individuals. Interaction between food antigen and specific IgE leads to a pathological process mediated by mast cells and basophils. Involvement of food-specific IgE has been demonstrated by the use of PK tests (Prausnitz and Küstner, 1921; Bock et al., 1978). Following oral challenge of the recipient with food antigens, to which the donor was allergic, cutaneous reactions similar to those presented by the patient were induced (Ratner and Gruehls, 1934). Mast cell degranulation was also reported following *in vitro* and *in vivo* passive sensitisation of intestinal mucosal mast cells (Gray et al., 1940; Selbekk et al., 1978). Local

degranulation of intestinal mast cells has been demonstrated to increase the permeability and the passage of food antigen through the gastrointestinal barrier into the body where it may be distributed to other target organs (Brostoff et al., 1979).

The liberation of a variety of cytokines by activated mast cells and basophils may also induce an IgE-mediated late-phase response (see chapters I and II).

Moreover, repeated ingestion of food allergen stimulates mononuclear cells to secrete HRF (Sampson et al., 1989). This phenomena has been associated with increased bronchial hypersensitivity in patients with asthma (Alam et al., 1987) and increased cutaneous irritability in children with atopic dermatitis (Sampson et al., 1989) (see chapter II). Individuals with atopic dermatitis and food allergy sometimes show spontaneous basophil histamine release (May and Remigio, 1982; Sampson et al., 1989). The level of histamine release *in vitro* has been demonstrated to return to normal levels over a 4-6 month period when the patient is not fed the offending food allergen and therefore appears to depend on continued ingestion of the allergen (Sampson et al., 1989).

### 4.2.2. Non IgE-mediated food hypersensitivity

Although type II , antigen-antibody - dependent cytotoxic reactions, type III, antigen-antibody complex-mediated hypersensitivity and type IV, cell-mediated hypersensitivity (Paganelli et al., 1984; Paganelli et al., 1987; Sampson, 1993a) have been reported to be implicated in patients with food hypersensitivity, there is little support for any significant role.

### 4.3. Diagnosis

The lack of definitive laboratory tests to confirm food allergy and implicate the responsible allergens is a major cause of concern. Adverse reactions may result from a variety of known and unknown immunologic responses, may occur in minutes to days following ingestion and may involve a wide variety of objective and subjective complaints as well as the development of symptoms which may be variable, intermittent or cumulative (Sampson, 1988).

History, diet diary, performance of elimination diets, open challenges, single-blind challenges and double-blind placebo-controlled food challenges (DBPCFC's) may all be utilised as diagnostic approaches in the establishment of a diagnosis of food allergy. However, the DBPCFC is the only test which can control for psychogenic

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factors and patient and physician bias and thus is the only way to accurately diagnose food hypersensitivity (Sampson, 1988). Therefore the DBPCFC represents the gold standard for diagnosing adverse reactions to food but does not necessarily implicate hypersensitivity.

Non IgE-mediated gastrointestinal disorders generally require laboratory or endoscopic studies to arrive at the correct diagnosis whereas when an IgE-mediated disorder is suspected, selective IDST or radioallergosorbent tests (RASTs) can be very helpful in the establishment of the diagnosis.

There is no evidence of any diagnostic value for food-specific IgG or IgG4 antibody levels, food immune complexes or lymphocyte activation tests.

### 4.3.1. *In vivo* tests

Prick skin tests are frequently used to screen patients with suspected IgE-mediated food allergies. The positive predictive accuracy of skin tests was reported to vary from 17% to 78% for the 6 major food antigens (egg, milk, peanut, fish, soy and wheat), whereas the negative predictive value was reported to vary from 87% to 100% (Sampson, 1984&1988). Therefore the prick skin test may be considered an excellent means of excluding IgE-mediated food allergies, but is only suggestive of the presence of clinically relevant food allergies. However there are some exceptions to this general statement. IgE-mediated sensitivity to fruits and vegetables is frequently not detected, probably because of the lability of these commercially prepared allergens (Ortoloni et al., 1989). On the other hand, children less than 2 years old may have food hypersensitivity in the absence of positive skin test presumably because of lack of skin reactivity (Menardo et al., 1985).

### 4.3.2. *In vitro* tests

It is important to remember that a local immune response in the gastrointestinal tract is not necessarily reflected in the systemic circulation, and findings based on the analysis of blood samples are not necessarily relevant for local immunity. Also, the ingested food may not be the relevant antigen, with the true offending allergen possibly appearing only during digestion.

*In vitro* tests include ELISA and RAST, basophil histamine release assay, intestinal mast cell histamine release and intragastral provocation under endoscopy (Selbekk, 1985; Nolte et al., 1989; Sampson, 1993b)

4.3.2.1. ELISA and RAST

The diagnostic value of *in vitro* tests reported in the literature is very variable and dependent on the criteria used to make the diagnosis, the population investigated and the food antigen evaluated (Ownby, 1991). Because processing proteins does influence their potential allergenicity, *in vitro* measurement of food-specific IgE antibodies raises certain issues. Commercially available food extracts are often unreliable, especially some allergens such as of vegetable origin which are labile and may lack allergenicity. Conversely, vegetable allergens may be very rich in lectins, which can bind IgE and reduce the specificity of the assay (Barnett et al., 1987).

Although RASTs and ELISAs are considered slightly less sensitive than skin tests, prick skin tests and RASTs were found to have similar sensitivity and specificity in a study which was comparing RAST with DBPCFC (Sampson, 1988). In another recent study in which the skin prick test and specific IgE for milk, egg and nut were compared with the oral challenge test, the prick test was found to be the most sensitive technique whereas RAST had the highest specificity (Roger et al., 1994). ELISA appears to be useful for predicting the probability that a food allergic patient may later develop immunological tolerance with resolution of clinical signs and low levels of antigen-specific IgE are thus useful prognostic indicators (Sampson, 1993b).

# IV. ALLERGIC SKIN DISEASES IN CATS

Allergic skin diseases comprise up to 30% of cases of skin disease in cats (Chalmers and Medleau, 1989; Carlotti, 1992). The most common causes are flea and other insect bite hypersensitivities, atopy and food hypersensitivity. Some patients are presented with more than one type. The reported incidence varies in different studies (O'Dair et al., 1996, Prost, 1996a). In a report of 88 cats which showed clinical signs compatible with allergic skin disease, a diagnosis of flea allergy was made in 45.5% of cases, atopy in 73% of cases and food allergy in 23% of cases (Prost, 1996a). However, O'Dair et al. (1996) reported that of 20 cats suspected of allergic skin disease, 10 were atopic (50%), 14 were flea allergic (70%) whereas none were diagnosed with food allergy.

Allergic skin diseases occur most commonly in young cats although cats from all ages can be affected.

The clinical presentation of the different allergic diseases can be indistinguishable. Whereas some symptoms are similar to those in the dog, the cat is not

a small dog and has its own clinical entities such as the eosinophilic granuloma complex, miliary dermatitis, self-induced alopecia and facial pruritus. Some cats can manifest various combinations of those 4 patterns (Prost, 1996a&b; Halliwell, 1997). In a recently published report of 88 confirmed cases of allergic skin disease, 24 cases were presented with self-induced alopecia, 20 were presented with eosinophilic granuloma complex (eosinophilic plaque, eosinophilic ulcer and/or linear granuloma), 13 cats had miliary dermatitis and 12 cats had facial dermatitis (Prost, 1996a). Various combinations of 2 of the 4 patterns were seen in 24 of the 88 cats (Prost, 1996a). Also, cats appear to suffer from a true allergic asthma (Moise and Spaulding, 1981; Halliwell and Gorman, 1989; Corcoran et al., 1995; Prost, 1996a&b; Halliwell, 1997) and can be presented with both cutaneous and respiratory signs resulting from exposure to the same allergen(s) (Halliwell and Gorman, 1989; Halliwell, 1997).

Although much progress has been made in the past decade in the field of allergic skin diseases in man and in dogs, little is still known about these diseases in cats despite a number of recent studies.

## 1. FELINE ATOPY

### 1.1. Introduction

Atopy was first described in the cat in 1982 by Reedy who reported positive skin tests in 15 of 20 cats presented with miliary dermatitis, extensive alopecia and/or manifestations of the eosinophilic granuloma complex. Eleven out of these 15 cats showed a good response to hyposensitisation. Currently there is increasing interest in feline atopy but reports are still few in comparison to those of canine atopy.

Although recent studies have shown the existence of an IgE-like antibody in cat serum (Walton et al., 1968; Powell et al., 1980; DeBoer et al., 1993; Baldwin, 1993; Foster, 1995; Foster et al., 1995), this immunoglobulin has not yet been fully characterised. Also, while anecdotal reports of familial involvement in feline atopy suggest a genetic component (Scott, 1987), an inherited predisposition has not yet been fully demonstrated (Halliwell and Gorman, 1989). Thus one may still argue whether the term atopy should be used in cats.

## 1.2. Pathogenesis

The pathogenesis of feline atopy is still poorly understood. No study has been performed on the production and the regulation of feline IgE. The clinical relevance of this antibody is also unknown.

On the other hand atopic cats tend to respond well to immunotherapy based on the detection of allergens involved by either IDST (Reedy, 1982; Bettenay, 1996; Prost, 1992) or *in vitro* tests (Halliwell, 1997). This suggests a role for reaginic antibody in the pathogenesis of feline atopy.

Recently, the level of allergen-specific IgG in atopic cats has been studied. Foster et al. (1997) demonstrated that the level of IgG antibody against some, but not all environmental allergens, was significantly higher in the serum from cats with confirmed allergic disease than in the serum from normal cats, cats with non-dermatologic disease and cats with undiagnosed pruritis. They suggested that allergic cats may therefore develop a Th2 lymphocyte-directed immune response characterised by the production of both allergen-specific IgG and IgE (Foster et al., 1997). Which of the three IgG subclasses is involved in this response was not addressed, and the importance of this isotype in the pathogenesis of feline atopy is speculative.

Cells found in skin biopsies from allergic cats vary according to the clinical lesion sampled. In non-inflammatory skin, lymphocytes and mast cells are prominent whereas in inflammatory skin, eosinophils are usually the predominant inflammatory cell (Scott et al., 1995b).

Roosje et al. (1995) have demonstrated that lesional skin of atopic cats with miliary dermatitis contains a predominantly $CD4^+$ infiltrate. A recent study has investigated MHC class $II^+$ and $CD1a^+$ cells in the skin of normal cats and in lesional skin of atopic cats (Roosje et al., 1997). The authors showed that the number of MHC class $II^+$/$CD1a^+$ cells were increased in the epidermis and the dermis of the lesional skin. The marked increase of MHC class $II^+$ dendritic cells seen in the epidermis is consistent with the upregulation of MHC class II after cytokine-mediated Langerhans' cells activation. These cells could have an active role in the immunopathogenesis of skin lesions in atopic cats.

Mast cells have been studied in normal cats and cats suffering from skin diseases. They were found in the epidermis of cats with eosinophilic granuloma complex whereas they were not detected in the epidermis of normal cats (Scott, 1990) or of cats suffering from non-dermatologic diseases (Foster, 1994). A study performed on cats with miliary

"eczema" showed a higher number of mast cells in the dermis of these cats than in normal cats (Scott, 1980). Dermal mast cells were also shown to be more numerous in some cats suffering from pemphigus foliaceus (Greek, 1993). On the other hand, feline mast cells vary in number with the site of the skin biopsy (Foster, 1994).

A recent study (O'Dair et al., 1996) has evaluated intestinal permeability ratios to lactulose and mannitol in 14 cats which were diagnosed as atopic and/or allergic to fleas but not allergic to food. An elevated ratio suggestive of increased intestinal permeability was found in only one cat. This cat also showed clinical signs of concurrent gastrointestinal disease.

The immunopathogenesis of feline asthma is still not clear. It has been shown to share some features with human asthma. One is an eosinophilic inflammation of central airways, a bronchoconstriction that is reversible with β-adrenergic agonists, and histologic changes in airways including epithelial erosion, globlet-cell and submucosal-gland hyperplasia and hypertrophy, and increased smooth-muscle mass (Howard and Ryan, 1982; Moise et al., 1989; McKiernan and Johnson, 1993). A model of feline asthma has been induced in ascaris naive cats by repeated immune stimulation with ascaris antigen. The histological appearance of the airway inflammation was similar to that noted in both feline and human asthma (Padrid et al., 1995). This data suggests that immune sensitisation alone could be a salient factor in the histologic changes that are observed in the asthmatic cat. On the other hand, asthmatic cats can exhibit positive skin tests and *in vitro* tests, can show a good response to hyposensitisation (Halliwell, 1997) or to the removal of the allergen (Corcoran et al., 1995).

## 1.3. Incidence, breed and sex predisposition, and age of onset

Reports of the incidence of feline atopy vary considerably. Chalmers and Medleau (1989) reported that atopy accounted for less than 1% of the cats presented for investigation of presumed allergic dermatitis at Purdue University and for 15% at the New York state College of Veterinary Medicine. However, European studies suggest a higher incidence. In the UK, 50% of cats with allergic skin disease had positive skin reactions to aeroallergens (O'Dair et al., 1996), whereas in a study in France, 73% of presumed allergic cats gave positive skin tests (Prost, 1996a). These differences probably reflect the lack of defined criteria for feline atopy and also practice bias.

No breed and sex predisposition has been reported (Prost, 1992). Age of onset ranges from 6 months to 7 years (Reedy, 1982; Scott et al., 1986; Halliwell and Gorman;

1989; Prost, 1996b). Most cases seem to commence in the young cat between the age of 6 months and three years (Scott et al., 1995b; Halliwell and Gorman; 1989; Prost, 1996a&b). In one study performed on 16 atopic cats with miliary dermatitis, 75% started to show clinical signs between 6 months and 2 years of age (Scott et al., 1986). Prost (1996b) reported an age of onset ranging from one to three years of age in 65% of cases, with 15% commencing their disease at less than one year of age.

## 1.4. Clinical signs

### 1.4.1. Cutaneous signs

As noted in the dog, pruritus is always present (Carlotti, 1992; Prost, 1992; Scott and Miller, 1993). However this is not always obvious to the owner because grooming is part of the normal behaviour of cats and also because they are secretive and can spend hours hidden, grooming or traumatising themselves. Also, pruritus is not always obvious to the practitioner when the cat is presented for a non-inflammatory alopecia without any other clinical signs. It can be seasonal or non seasonal (Scott, 1987; Carlotti and Prost, 1988). In a report of 16 cats with symptoms consistent with atopy, 11 had seasonal disease, three showed non seasonal symptoms and 2 progressed from seasonal to non seasonal (Scott, 1987). Carlotti and Prost (1988) reported non seasonal pruritus in 6 cats out of 7.

The clinical signs attributed to atopy are extremely variable. The most common signs are self-induced alopecia (also called extensive alopecia or symmetrical alopecia), miliary dermatitis, eosinophilic granuloma complex and facial and neck pruritus (Reedy, 1992; Scott, 1984&1987; Moriello, 1994; Prost, 1996a&b; Halliwell, 1997). Some cats can manifest various combinations of those 4 patterns (Carlotti and Prost, 1988; Prost, 1992; Halliwell, 1997). In a study of cats with symptoms compatible with atopy, 8 cases were presented with extensive alopecia, 11 were presented with eosinophilic granuloma complex (eosinophilic plaque, eosinophilic ulcer and/or linear granuloma), 5 cats had miliary dermatitis, 2 cats had facial pruritus and one cat had seborrhea (Prost, 1992). An association of the eosinophilic granuloma complex with one of the other syndromes was seen in three cases whereas one cat with extensive alopecia showed also some lesions of miliary dermatitis (Prost, 1992). Recurrent swelling of the chin or lower lip (eosinophilic or collagenolytic granuloma) may also be seen (Scott et al., 1995b). Cats may exhibit seborrhea as the only symptom (Prost, 1992) or foot/nail chewing (Moriello, 1994). One

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case of feline atopy was reported in association with a sterile eosinophilic folliculitis and showed an excellent response to hyposensitisation (Scott and Shanley, 1989).

Both lesions and pruritus may be localised or generalised. Primary skin lesions may or may not be present and many cats start to scratch or to lick at apparently normal skin (Carlotti, 1992). The typical chronic "eczematous " lesions (excoriation and lichenification) seen in man and the dog with AD are not reported in atopic cats. It is also very uncommon for cats to develop secondary staphylococcal infection, however secondary pyoderma may occur in some atopic cats (Scott et al., 1995b).

Atopic cats can manifest recurrent ceruminous otitis externa (Carlotti and Prost, 1988; Prost, 1992; Moriello, 1994; Halliwell, 1997).

Lymphadenopathy may be seen in some cats, especially in chronic cases that have miliary dermatitis, excoriations or eosinophilic plaques (Bettenay, 1991; Moriello, 1994; Scott et al., 1995b). Because of the excessive grooming in some animals, there may be a history of vomiting hair balls (Bettenay, 1991), or constipation (Moriello, 1994).

### 1.4.2. Respiratory signs

Cats with allergic respiratory disease can show a chronic cough suggestive of allergic bronchitis, respiratory difficulty accompanied by an expiratory wheeze compatible with asthma, rhinitis and/or conjunctivitis (Halliwell and Gorman, 1989). Carlotti and Prost (1988) reported sneezing in 5 cats and chronic conjunctivitis in one of 10 atopic cats which had cutaneous lesions.

In a recent study of 29 cats exhibiting feline asthma syndrome without cutaneous signs, the most common presentations were recurrent bouts of coughing (26 cats) and dyspnea (21 cats) while sneezing was seen in 10 cats and nasal discharge in 6 cats (Corcoran et al., 1995).

### 1.5. Diagnosis

The extreme variability of the clinical signs and the fact that the associated reaction patterns can have many other causes necessitates the consideration of a lengthy list of differential diagnoses. Therefore a careful history and clinical evaluation should first identify other possible causes of the skin or respiratory diseases under investigation (Halliwell and Gorman, 1989). In addition, atopic cats may have other concurrent allergic diseases (Foster and O'Dair, 1993; O'Dair et al., 1996; Prost, 1996a, Halliwell,

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1997). Flea allergy was reported to be associated with atopy in 3 out of 9 cats cases (33% of cases) (Foster and O'Dair, 1993), 33 out of 66 cats (50% of cases) (Prost, 1996a), and 7 out of 10 cats (70% of cases) (O'Dair et al., 1996). Although it is much less common, food hypersensitivity may also be seen with atopy (Halliwell, 1997).

Therefore, the common differential diagnoses include other hypersensitivities, ectoparasitism (cheyletiellosis, otodectic and feline notoedric acariasis), dermatophyte infection and psychogenic alopecia (Scott et al., 1995b). Other less frequent diseases to be considered in the differential diagnosis include bacterial folliculitis, telogen effluvium, idiopathic symmetrical alopecia, mycosis fungoides and mastocytosis (Bettenay, 1991; Thoday, 1990; Carlotti, 1992). The term idiopathic symmetrical alopecia is also used to describe the previously named feline endocrine alopecia which has been shown in some cats to be the result of low thyroid function (Thoday, 1990).

Although haematology and histopathology can help in the diagnosis of the pathological entities, they cannot be used to establish a diagnosis of feline atopy (Foster and O'Dair, 1993). The presence of eosinophilia is very variable, and of little diagnostic significance (Scott, 1987; Foster and O'Dair, 1993; O'Dair et al., 1996).

## 1.5.1. Allergy testing

After careful and complete elimination of other diagnoses, confirmation of the diagnosis and the identification of the allergens involved may be made with the IDST and/or *in vitro* tests.

### 1.5.1.1. IDSTs

Until recently, the IDST has been the only test available for allergy testing in the cat. It is assumed, as in the dog, that it is the gold standard and the diagnosis of atopy has thus been based on IDST results and response to hyposensitisation (Reedy, 1982; Scott, 1984; McDougal, 1986; Prost, 1992).

Allergen concentrations used in dogs were shown to be appropriate for use in cats (Bevier, 1990).

Withdrawal times for corticosteroids and antihistamines recommended are the same as those established for the dog (Halliwell and Gorman, 1989; Carlotti, 1992). Megestrol acetate has also been shown to suppress the pituitary/adrenal axis for at least as long as do corticosteroids (Chastain et al., 1981). It could therefore be expected to suppress skin reactivity in the IDST, although this has yet to be critically addressed.

Skin tests are technically more difficult than in dogs since cat skin is thinner and has also denser collagen bundles (Carlotti, 1992). Also, weal reactions in cats tend to be subtle, flat, with minimal erythema and difficult to interpret (Reedy, 1982; Prost 1996b). Restraint for skin testing in cats, with or without preceding anaesthesia, were shown in a study to induce significant increases in plasma cortisol, corticotropin, and $\alpha$-melanocyte-stimulating hormone concentrations (Willemse et al., 1993). The authors suggested that this cortisol response may explain the typical weak response to the IDST observed in cats (Willemse et al., 1993). However, some atopic cats can be as reactive as any dog.

Nonetheless sedation or anaesthesia with low doses of either xylazine, medetomidine, ketamine, ketamine-diazepam, glycopyrrolate, tiletamine- diazepam, or a general gas anaesthetic are recommended (Mueller et al., 1991; Carlotti, 1992; O'Dair and Foster, 1995; Scott et al., 1995b; Prost, 1996b).

The technique of injection is the same as described for the dog but great care must be taken to ensure that the injection is truly intradermal (Halliwell and Gorman, 1989). The reading and scoring of the reaction are also critical. In order to help the visualisation of the reaction, the IDST should be read in the dark with an indirect light source. Sometimes, feline skin test reactions develop and fade rapidly, within 10 minutes. Therefore some authors observe the site of injection continuously over 20 minutes (Carlotti, 1992), whereas others recommend reading the reaction between 5 and 15 minutes (Bettenay, 1991) or after 15 to 20 minutes (Prost, 1996b). A recent study demonstrated that degranulation following *in vivo* allergen challenge occurred after 5 minutes in 20 % of mast cells and, after 10 to 15 minutes in nearly two-thirds of them (Bevier and Dunston, 1996). In addition to immediate reactions, delayed reactions may also occur. Their significance is unknown (Halliwell and Gorman, 1989).

Non-seasonal allergens appear to be the most commonly involved in feline atopy. Of 66 cats with positive IDSTs, 38 cats (57.6%) were positive to non seasonal allergens and 25 cats (38.5%) were positive to seasonal and non seasonal allergens while only three cats were reactive to pollens alone (Prost, 1996a). In another report, non seasonal and seasonal allergens gave positive reactions in 77% and 23% of the cats respectively (Foster and O'Dair, 1993). Several studies have shown that positive reactions occur most frequently to HDM antigens. DF has been reported to be involved in 80% (Prost, 1996a) and 100% (Carlotti and Prost, 1988) of the cases, whereas DP was involved in 10% (Carlotti and Prost, 1988) and 46% of the cases (Prost, 1996a). Foster and O'Dair (1993) reported positive reactions to house dust and *Dermatophagoides* spp in 77% of cats.

Human dander, dog dander and moulds were reported to be positive in 22.5%, 17.7% and 8% respectively (Prost, 1996a).

Some allergens, such as house dust (HD), firebush and flea, were shown to induce positive skin reactions at low dilutions in normal cats. It was proposed that those reactions may be not always false-positive irritant reactions but rather true positive reactions and that a possible interpretation could be the dissociation of positive skin test reactivity and clinical sensitivity (Bevier, 1990).

### 1.5.1.2. *In vitro* tests

Recently, ELISAs for the establishment of the diagnosis of atopic disease in cats have become available in the USA and in Europe. One of them uses for the detection of allergen-specific IgE an antiserum that was raised against a chimaeric feline/murine IgE antibody. These commercial tests have not been adequately evaluated. The only report which compared the results of this assay to skin testing, assuming the latter as the gold standard in the diagnosis of feline atopy, showed a very poor correlation between the two tests (Foster and O'Dair, 1993). The specificity and sensitivity of the ELISA were reported as 57% and 11% respectively with positive and negative predictive values of 10% and 40% (Foster and O'Dair, 1993). However, in a recent study in which 81 cats received hyposensitisation based on results of ELISA using a polyclonal antiserum, the response was shown to be broadly similar to that obtained when hyposensitisation was based upon skin testing (Prost, 1992; Halliwell, 1997). Although these studies were not placebo controlled, the results raised the same question in the cat as has been asked in the dog: namely "should results of *in vitro* tests correlate with those of skin testing?".

## 1.6. Treatment

The optimal treatment of feline atopy varies with the severity of the disease and its duration. Avoidance is the ideal form of therapy (Prost, 1993), but unfortunately is not easily undertaken. Corticosteroids, antihistamines and essential fatty acids, used alone or in combination as symptomatic therapy, can be very useful (Carlotti, 1992). Hyposensitisation is often effective and could represent the most appropriate long-term control method for a cat which has non seasonal atopic disease (Reedy, 1982, McDougal, 1986; Scott et al., 1986; Carlotti and Prost, 1988; Prost, 1992; O'Dair and Foster, 1995; Bettenay, 1996; Halliwell, 1997). Topical therapy is uncommonly used in cats but can however be very effective (Scott et al., 1995b).

## 1.6.1. Symptomatic therapy

Corticosteroids are well tolerated by cats and represent the treatment of choice for controlling clinical signs. They are widely used with good results, in the early stage of the disease. Oral prednisolone (2.2 mg/kg/day for 5 to 10 days and then every other day) and injectable repositol methylprednisolone acetate (5.5 mg/kg or 20 mg/cat SC or IM at intervals of not less than 8 weeks and preferably every 12 weeks) or triamcinolone acetonide (5 mg/cat SQ) can all be used (Scott et al., 1995b). However after a few years, response to the therapy can decrease and side effects may occur. Thus in these cases another form of therapy should be tried.

The antihistamine most commonly recommended is chlorpheniramine hydrochloride (2-4 mg/cat b.i.d.). It was reported to be very effective in atopic cats with improvement in 73% of cases (Miller and Scott, 1990). Alternatively clemastine (0.67 mg/cat b.i.d.) can also be used and was shown to give good results in 50% of cases (Miller and Scott, 1994).

Fatty acid preparations containing n-3/n-6 have also been reported to be of help and seem to give a good response in 50 to 75% of cats with inflammatory skin disease (Harvey , 1991; Harvey, 1993a&b; Miller et al., 1993b). When given with either corticosteroids and/or antihistamines, they appear to have synergistic effects. The cats which improved when treated with EFA also showed abnormalities of their serum fatty acid before treatment (Harvey, 1991).

## 1.6.2. Hyposensitisation

### 1.6.2.1. Introduction and mechanisms of action

The concept that protective or blocking antibodies are induced by hyposensitisation has long been favoured since it was reported that sera from hyposensitised patients could block the PK reaction (Cooke et al., 1935). However, although allergen-specific IgG antibodies increase in hyposensitised patients, there are few studies that have shown a convincingly direct relationship between allergen-specific IgG levels and clinical efficacy (Aalberse et al., 1983; Djurup, 1985).

A rise in allergen-specific IgE is often seen early in the course of the therapy and despite this rise in the first year, patients often report considerable therapeutic benefits. IgE antibody then falls gradually over several years (Irons et al., 1977; Foucard and Johansson, 1978). The induction of autologous anti-IgE antibody, particularly antiidiotype antibodies, could be of potential benefit (Wheeler et al., 1990). Autologous

antigen/antibody complexes were demonstrated under experimental conditions to inhibit the specific response directed against the antigen contained in the complex (Saint-Rémy, 1994). Injection of such complexes have been reported to be of particular benefit in patients with AD (Leroy et al., 1993).

A potential aim of hyposensitisation is the reversal of the activity of allergen-specific T-cell activity from Th2>Th1 to Th1>Th2. The current view is that allergen-specific immunotherapy is believed to switch the allergen-specific immune response toward the production of a higher Th1 cytokine profile (Durham et al., 1991). It appears that immunotherapy leads to an upregulation of Th1-type cytokines rather than to a downregulation of Th2-type cytokines. However decreased production of Il-4 and Il-5 by Th2-like cells have been demonstrated in recent studies involving long term administration of immunotherapy (Briner et al., 1993; Secrist et al., 1993).

Recently, a study has shown a long-lived reduction in the induction of CD23 on peripheral B cells by grass-pollen-extract, in patients given allergen-specific immunotherapy. This was associated with reduced symptoms. No such reduction in CD23 was observed in patients treated symptomatically without allergen-specific immunotherapy (Jung et al., 1995).

Basophils of patients hyposensitised often show a reduced histamine release in response to allergen challenge *in vitro* (Swineford, 1972). A reduced level of IgE receptors on the cells has also been proposed as a mechanism in hyposensitisation (Bonifazi et al., 1991).

1.6.2.2. Assessment of hyposensitisation in cats

Either aqueous or alum-precipitated allergens have been used with similar results. The protocol used is the same as for the dog.

Hyposensitisation in cats was first described by Reedy (1982) who used alum-precipitated allergens to hyposensitise 15 cats who showed positive reactions on IDST. A favourable response was seen in 11 cats (73%). Favourable results were also reported in 69% of cases by McDougal (1986), 100% of cases by Scott et al. (1986), 67% of cases by Carlotti and Prost (1988) and 73% of cases by Prost (1992). However some studies have also showed less favourable results (O'Dair and Foster, 1995; Bettenay, 1996) with efficacy rates of 45% and 50% respectively. Most of the reports on hyposensitisation were based upon follow-up periods of 6 to 10 months and the lower success rate reported by Bettenay (1996) may be due to the longer study period which involved a 12 month to three year follow-up. Indeed their results at 12 months were comparable to those in

previous publications (McDougal, 1986; Scott et al., 1986; Carlotti and Prost, 1988; Prost, 1992). This is supported by the data from O'Dair and Foster (1995) who found a success rate of only 45% in a 2 year study involving 12 cats. On the other hand none of them were placebo-controlled and thus, the apparent efficacy may be overstated. Interestingly, O'Dair and Foster (1995) reported no success with advanced lesions of eosinophilic granuloma complex. A recent study showed that the different dermatologic conditions exhibit differences in their response to hyposensitisation (Halliwell, 1997) (see below). Therefore the interpretation of the success rate of some studies is difficult as all are based on numbers of cats with different reaction patterns.

Comparisons of the reported response rates to immunotherapy are difficult. Firstly, there are no standardised regimens. There is variation in protocols regarding frequency, the dose of antigen and the recommended duration of therapy. In addition, the allergens used come from different sources, and there are no universally accepted protocols for allergen standardisation. The definition of a good response can vary from one author to another. The patients may be on a variety of concurrent symptomatic medications and the clinical assessment procedures may vary from study to study. Despite these problems, hyposensitisation is widely recommended and most writers report a good clinical response, with adverse reactions being rare.

The efficacy of hyposensitisation based upon *in vitro* test results has been assessed for the first time very recently in a survey of 81 cases (Halliwell, 1997). Response to hyposensitisation and a diet change and to hyposensitisation alone was studied for each of the specific signs of atopy. The follow-up ranged from 4 to 37 months (mean of 18.5 months). Results were broadly similar for both groups (hyposensitisation and a diet change and to hyposensitisation alone). An improvement of at least 50% was noted in 75.3% of all cases to a combination of hyposensitisation and dietary change. Forty eight percent of all cases showed an improvement of 75% to 100% and 12.3% of them, an improvement of 100%. The improvement of the individual dermatological conditions ranged from 93.6% in cases of linear granuloma to 60% in cases with self-induced alopecia. A 62.6 % improvement was reported in cases of otitis externa. Cases with respiratory signs only showed 86.1% improvement (Halliwell, 1997).

## 2. FOOD HYPERSENSITIVITY

The term food hypersensitivity is used inappropriately in veterinary medicine to describe any adverse reactions to ingested food. However, as mentioned previously, some adverse reactions can be the result of intolerance rather than allergy.

### 2.1.Etiopathogenesis

As in the dog, the pathogenesis of food hypersensitivity in cats has not been established. Type I but also type III and type IV hypersensitivities are believed to be involved (Scott, 1980; Kunkle, 1984; Halliwell and Gorman, 1989).

Walton et al. (1968) studied a cat suffering from both gastrointestinal and dermatological signs due to allergy to cows milk. Positive PK tests were obtained and the PK reactivity was destroyed by heating the serum at 56°C for 30 minutes.

Dietary antigens most commonly incriminated in food allergy in cats are fish, dairy products and beef (Walton, 1967; Dakin, 1988; White and Sequoia, 1989; Guaguère, 1993). Other foods reported to be involved are pork, chicken, rabbit, horse, lamb and mutton, eggs, clam juice and cod liver oil (Walton, 1967; Stogdale et al., 1982; White and Sequoia, 1989; Carlotti et al., 1990; Guaguère, 1993; Rosser, 1993; Reedy, 1994; Scott et al., 1995b). Some cats react to all commercial foods, but are asymptomatic when fed any home prepared diet. This would appear to implicate some commonly used food additive (White and Sequoia, 1989).

There is no age, breed or sex predilection. The age of onset varies from three months to 13 years with a mean age of 4 to 5 years (Walton, 1967; White and Sequoia, 1989; Carlotti et al., 1990; Guaguère, 1993; Rosser, 1993; Denis and Paradis, 1994). It has been reported that in 70% of cases, the cat was fed with the offending diet for 2 years or longer (Scott, 1980). Recently Reedy (1994) reported a case of a cat with diagnosed food allergy that was maintained on a diet of lamb but became sensitive to lamb two years later.

### 2.2. Incidence

Although food hypersensitivity represents the third most common hypersensitivity in the cat after flea allergy dermatitis and atopy, its incidence is rare and it has been reported to comprise up to only 6% of all feline dermatologic cases (Walton, 1967; Carlotti et al., 1990; Denis and Paradis , 1994; O'Dair et al., 1996). The diagnosis was made at two Universities in the USA and one in Canada, only 2 to 4 times per year

(White and Sequoia, 1989; Rosser, 1993; Denis and Paradis , 1994). The incidence was similar in a French referral practice which reported an average of three cases per year (Guaguère, 1993). Reports of the prevalence of food allergy vary from 0% (O'Dair et al., 1996), 4% (Foster and O'Dair, 1993) to 23% (Prost, 1996a) of allergic skin diseases. Up to 15% of cats with miliary dermatitis were reported to have food hypersensitivity (Scott, 1987).

## 2.3. Clinical signs

Cats suffering from food allergy can show dermatologic and/or gastrointestinal signs, and respiratory and neurologic signs are rare (Wills, 1992). The disease is usually nonseasonal.

Pruritus is always present and can be localised or generalised (White and Sequoia, 1989; Carlotti et al., 1990; Guaguère, 1993; Rosser, 1993). Clinical signs vary widely and can be indistinguishable from other cutaneous hypersensitivities. They may include focal or symmetrical alopecia, facial and neck pruritus, miliary dermatitis, excessive hair grooming, eosinophilic granuloma complex and otitis externa. The anterior one-third of the body has been reported to be the most common region involved (White and Sequoia, 1989; Guaguère, 1993; Rosser, 1993). Angiodema, urticaria and conjunctivitis have also been reported (Halliwell and Gorman, 1989; Rosser, 1993).

Gastrointestinal signs were reported in 10 to 15% of the cases (Scott et al., 1995b). They can occur with or without skin lesions and may include vomiting, profuse watery diarrhea and inflammatory bowel disease such as lymphocytic-plasmacytic colitis (Walton et al., 1968; Stogdale et al., 1982; Nelson et al., 1984; Dakin, 1988; Dennis et al., 1992; Guaguère, 1993; Denis and Paradis, 1994).

## 2.4. Diagnosis

In the cat, as in man and the dog, the definitive diagnosis is based on a favourable response to an "elimination" diet followed by a provocative test (Walton, 1967). The animal is fed with foods to which it has either not been exposed previously or to which it has been exposed infrequently. Some clinicians attempt this by feeding the cat with a commercial hypoallergenic diet (Medleau et al., 1986; Dakin, 1988; White and Sequoia, 1989; Guaguère, 1993; Roudebush and McKeever, 1993). However in one study, an improvement was seen in only 79% of cats with confirmed food hypersensitivity using a commercial hypoallergenic diet (Roudebush and McKeever, 1993). Because of these

findings homemade diets are usually recommended as the initial test diet (White and Sequoia, 1989; Carlotti et al., 1990; Roudebush and McKeever, 1993; Reedy, 1994).

While most of the cats usually improve within three weeks of instituting the hypoallergenic diet (Scott, 1980; Stogdale et al., 1982; Kunkle, 1983; White and Sequoia, 1989; Carlotti et al., 1990), some cats improve only after 8 to 13 weeks (Carlotti et al., 1990; Guaguère, 1993; Rosser, 1993; Denis and Paradis, 1994). Indeed it was reported in a recent study that three cats of 17 cats suffering from food allergy responded to the restriction diet only after 8 weeks (Guaguère, 1993), whereas in 2 other studies, both performed on 13 cats, one cat improved after 9 weeks and one improved after only 13 weeks (Rosser, 1993; Denis and Paradis, 1994). Therefore, the restriction diet should be maintained for at least 10 weeks in order to ensure a response in all food allergic cats.

Although most of the cats relapse within 8 days of a challenge with their previous diet (Medleau et al., 1986; Dakin, 1988; Guaguère, 1993; Reedy, 1994), some of them may take longer (Rosser, 1993). Indeed, Rosser (1993) showed that symptoms could reappear at any time from 15-30 minutes to 10 days. Thus to confirm the diagnosis, cats should be fed their previous diet until a relapse is noticed, or for at least 10 to 14 days.

## 2.5. Clinical management

The clinical management is the avoidance of the offending food. It is also important to bear in mind that the cat can become sensitised to a component of its new diet (Reedy, 1994).

However, in some cases avoidance may be difficult, especially when the cat is outdoors and can find other sources of food. In such cases, the use of corticosteroid therapy can be of benefit to the cat. In cases of extreme pruritus, the administration of oral corticosteroids can also be justified during the first few weeks of the diet trial. Although food allergy in cats has been reported by some authors to be refractory to corticosteroids (Scott, 1980; Carlotti et al., 1990), these drugs were shown to have reasonable efficacy in the management of pruritus in 2 studies that incorporated rechallenge to confirm the diagnosis. A good response was shown in approximately 35% of cases in one study (Guaguère, 1993) and in 64% of cases in the other (Rosser, 1993).

# AIMS OF THE STUDIES

Details of the current state of knowledge regarding IgE mediated hypersensitivities in man and the dog and cat have been reviewed. There is a paucity of information regarding the latter species. The aims of this study, therefore, were:

* To purify feline IgE, to assess further its physicochemical characteristics, and to raise polyclonal antisera against the isotype.

* To assay the levels of IgE specific for *Dermatophagoides farinae* in the sera of atopic and normal cats, both household and laboratory reared.

* To induce IgE in normal cats using low dose antigenic challenge in aluminium hydroxide, and to compare the induced antibody with the spontaneous antibody of hypersensitive cats.

* To assess the effects of infection with *Toxocara cati* and of immunoprophylaxis with the standard battery of feline viral vaccines on the immune response of the IgE and other isotypes to an orally administered antigen.

# CHAPTER 2.

# PRODUCTION AND CHARACTERISATION OF POLYCLONAL ANTISERA AGAINST FELINE IgE

## 2.1. INTRODUCTION

IgE antibodies in animals share many physicochemical properties with IgE antibody of man. They are characterised by a relatively fast electrophoretic mobility, a sedimentation coefficient of approximately 8 S, a molecular weight of approximately 200 KDa, the capacity to fix to tissue following injection for > 3 weeks and a high sensitivity to heat and reducing agents.

Several studies have already shown that cat serum contains a protein with IgE-like properties (Walton et al., 1968; Powell et al., 1980; Baldwin et al., 1993; DeBoer et al., 1993; Foster, 1995; Foster et al., 1995). DeBoer et al. (1993) showed that cats have a reaginic antibody that is antigenically cross-reactive with canine IgE and has an apparent molecular weight similar to that of IgE from other species. Foster et al. (1995) demonstrated that the passive cutaneous anaphylaxis (PCA) reactivity was eluted following chromatography using Sephadex G200 in a position similar to that of other species, and that feline reaginic antibodies are bound by protein A.

In man (Johansson and Bennich, 1967) as in the rat and the mouse (Bazin et al., 1974; Böttscher and Hämmerling, 1978) myelomas have permitted the ready purification of IgE for the subsequent production of monoclonal antibodies. However, in the cat, the lack of myelomas secreting IgE, the presumably low level of serum IgE and the small amount of serum obtainable make the isolation of IgE very difficult.

The further characterisation of feline IgE and the development of specific antisera are prerequisites for the elucidation of the role of IgE and of immediate type hypersensitivity in a number of common feline diseases. The goal of this study was thus to produce polyclonal antisera against the immunoglobulin to permit quantification of total and allergen-specific feline IgE in diseases which may involve immediate-type hypersensitivity.

## 2.2. MATERIALS AND METHODS

### 2.2.1. Experimental animals

Normal cats and cats infected with *Toxocara cati* (*T. cati*) were obtained from a colony of experimental cats at the University of Liège (Belgium).

Normal adult (ex-breeder) parasite free cats for Prausnitz-Küstner (PK) tests and reversed cutaneous anaphylaxis (RCA) were obtained from Hill Grove Family Farm Ltd (Oxford, England).

New Zealand white rabbits were obtained from Interfauna UK limited (Cambridge, England).

All experimental protocols were in accordance with the requirements of the Animal Care and Ethics Committee and/or the UK Home Office as appropriate.

### 2.2.2. Infection of cats with T. cati

Three 10 month old cats (#5,#6,#7) and three 12 to 16 week old kittens (#1,#2,#3) were found to be naturally infected with *T. cati*. All were secreting ova in their faeces.

Three additional normal 10 week old kittens (#4,#8,#9) were experimentally infected with *T. cati*. Eggs were isolated from faeces of naturally infected cats. They were washed 3 times in 1% sodium chloride and centrifuged at 1500 rpm for 5 minutes. After discarding the final rinse, eggs were resuspended in 0.5% formalin (Sigma, Poole, UK) and kept, under oxygen, in a dark room, at 27°C. Infective 2nd stage larvae were obtained after 3 weeks. The cats were dosed with 10 embryonated ova daily for 3 days. Development of a patent infection was indicated by the appearance of the ova in the faeces within 60 to 75 days.

### 2.2.3. Ascaris antigens

Purified antigen from *Ascaris suum* (Asc-1) was purchased from Greer Laboratories (Lenoir, North Carolina).

Adult *T. cati* were obtained post mortem from naturally infected cats and antigens from body fluid were partially purified by gel filtration on Sephadex G100 (Pharmacia, Uppsala, Sweden) to remove the low molecular weight histamine releasing fractions. Two adult *T. cati* were washed with PBS, and ground with a mortar and pestle.

The worm mixture was incubated at 4°C for 48 hr. The pellet was discarded and the supernatant was centrifuged at 49000x g for 1 hr in order to remove the fine particles. The resultant solution was passed through a Sephadex G100 (Pharmacia) column and material from the first peak (the major protein peak) was concentrated to 1 mg/ml.

### 2.2.4. Preparation of dinitrophenylated antigens

Asc-1 and bovine serum albumin (BSA) (Sigma) were dinitrophenylated according to the method of Eisen (1964).

### 2.2.5. Induction of feline anti-DNP and anti-Ascaris antibodies

Cats were immunised with DNP-Asc in aluminium hydroxide (Imject Alum®; Pierce, Rockford, Illinois, U.S.A.) at a final ratio of alum to immunogen-alum mixture 1:4 (1 volume of alum for 3 volumes of immunogen fraction) in order to induce anti-DNP reaginic antibody as described for the dog (Schultz & Halliwell, 1985). Each cat received two biweekly injections of 10 µg of DNP-Asc subcutaneously over the lateral thorax at two sites on either side of the body.

### 2.2.6. Performance of intradermal skin tests (IDSTs)

Ten days after the last immunisation, cats were anaesthetised with 1 mg/kg xylazine (Virbaxyl®, Virbac limited, Cambridge, UK) and 10 mg/kg ketamine hydrochloride (Vetalar®, Parke-Davis, Pontypool, Gwent, UK) intramuscularly. The lateral chest was clipped and 0.05 ml of a solution of 10 µg/ml of DNP-BSA was injected intradermally. Phosphate buffered saline (PBS) and 1/100,000 w/v solution of histamine phosphate (ARTU Biologicals N.V., Lelystad, Holland) were used as negative and positive controls respectively. Reactions were scored on a +1 to +4 scale, incorporating both diameter and elevation above the surface. A +1 reaction was just discernibly greater than the PBS weal, and a +4 reaction approximated the size of the histamine weal.

### 2.2.7. Fractionation of sera

### 2.2.7.1. Preparation of IgE rich fractions (Fr-R)

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*T. cati* infected cats were bled by cardiac puncture ten days after the last immunisation with DNP-Asc. Sera were harvested by centrifugation after clot retraction and were stored at -30°C.

The 3-in-series 4x60 cm column set of Sephacryl S-200 (Pharmacia) was firstly carefully calibrated by filtration of normal cat serum equilibrated with 0.75M PBS, pH 7.0, containing 0.05% tween 20 (Sigma) to prevent aggregate formation.

Sera from bleedings of 4 infected cats (sera #1, #2, #7 and #8) were fractionated by the same three-step procedure that was employed to prepare IgE rich fractions of canine sera (Halliwell, 1973). A volume of 30 to 40 ml of each serum was precipitated at 33.33% saturation with ammonium sulphate and after centrifugation, further ammonium sulphate was added to the supernatant to bring the degree of saturation up to 45%. The resultant precipitate was resuspended in 0.15 M sodium phosphate buffered pH 7.2 (PBS) to the original volume and dialysed against a 10 fold volume of the same buffer for 48 hr at 4°C with 3 buffer changes, to remove the ammonium sulphate.

The resultant fraction that precipitated between 33.33% and 45% saturation with ammonium sulphate was then dialysed against 0.05 M phosphate buffer, pH 8.0, and applied to a column of diethylaminoethyl cellulose (DEAE; Whatman, Maidstone, Kent, England) equilibrated with the same buffer.

The excluded protein was concentrated and then applied to a 3-in-series 4x60 cm column set of Sephacryl S-200 (Pharmacia) and eluted using PBS. The protein eluting immediately prior to the 7S peak and the first one third of that peak was pooled and concentrated using centripet -10 concentrators (Amicon, Gloucester, UK) as fraction R (Fr-R) (fig. 2.1). The second third of the peak was pooled and concentrated as fraction R+G (Fr-R+G) (fig. 2.1).

Columns were attached to a LKB uvicord UV monitor, 2211 Superrac fraction collector and chromatogram recorder (Pharmacia).

## 2.2.7.2. Preparation of IgG fractions

Feline sera were fractionated using a 2 step procedure. Following precipitation with 33.33% saturated $(NH_4)_2SO_4$ (SASS) and centrifugation, the supernatant was discarded and the protein pellet was resuspended in PBS, pH 7.2 to the starting volume. A second precipitation with 33.33% SASS was carried on and the resuspended pellet was dialysed against a 10 fold volume of PBS at 4°C for 72h, with 3 changes.
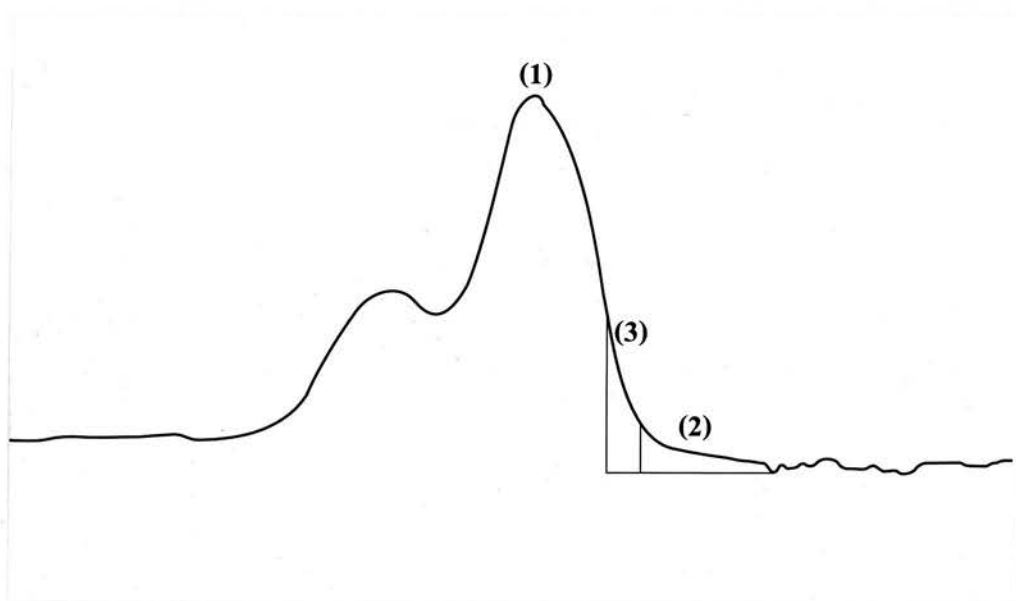
Fig. 2.1. Elution profile of the 7S peak (1), Fr-R (2) and Fr-R+G (3) from the 3-in-series column set of Sephacryl S-200.

The protein was then dialysed against 0.01M phosphate buffer at 4°C overnight and applied to a column of DEAE (DE52; Whatman, Maidstone, UK) equilibrated with the same buffer. The excluded protein was collected and concentrated. The molarity of the phosphate buffer was then increased to 0.025 and to 0.035M and the respective eluted proteins were concentrated.

### 2.2.7.3. Preparation of IgG (Fab) 2

The IgG fraction excluded from DEAE using 0.01M phosphate, pH 8 was adjusted to a concentration of 20 mg/ml and dialysed against a 0.1M sodium acetate buffer, pH 4.5, for 3 hr. Then the pH was decreased to pH 4.5 using glacial acetic acid (Sigma). Two mg of pepsin were added to 100 mg of protein and the solution was incubated at 37°C for 24 hr. After centrifugation, the precipitate was discarded. The resultant solution was dialysed against PBS in order to raise the pH to 7.4 before filtration through a column of Sephadex G-100 (Pharmacia) (Hudson and Hay, 1980). Five protein peaks eluted from the column. The peak containing the $(Fab)_2$ fraction (peak 2) was collected, concentrated and coupled to CNBr activated Sepharose 4B.

### 2.2.8. Preparation of antisera

### 2.2.8.1. Preparation of polyclonal anti-feline IgE

All fractions were sterilised by filtration through a 0.45μm nitro-cellulose filter (BDH, Luterworth, UK) prior to their injection into the rabbits.

### 2.2.8.1.1. Antisera against fraction-R

Three New Zealand white rabbits were immunised with 3 biweekly injections of 100 to 200 μg of Fr-R in 2 ml of complete Freund's adjuvant (Sigma, Poole, UK) subcutaneously at 4 sites (table 2.2).

The resultant antisera were passed over an immunoabsorbent column of Sepharose 4B (Sigma) to which was coupled serum from a parasite free cat that had been heated to 56°C for 4 hr. This column removed any activity against all immunoglobulins other than IgE and also against other heat stable serum proteins (Vaerman and Heremans, 1968). One to 2 ml of antisera were loaded on the immunoabsorbent column

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**Table 2.1. Protocols for experiments in chapter 2.**

**Manipulations of cats**

Infection with T. cati (table 2.4)
Immunisation with DNP-Asc
Intradermal skin tests (table 2.5)

**Assessment of cat sera**

PK tests (table 2.5)

**Fractionation of cat sera**

IgE rich  fraction:          IgG fractions:          IgG (Fab)$_2$

- *SASS (33.33% followed*      - *SASS (33.33%)*
  *by 45% or 55%)*            - *DEAE (0.01M,*
- *DEAE (0.05M)*                *0.025M and 0.035M)*
- *Filtration on  S-200: Fr-R*

**Production of antisera**

Anti-feline IgE              Anti-feline IgG          Anti-feline light chain
(table 2.2)                  (table 2.3)

- *Fr-R*
- *IgE eluted from putative*
  *anti-IgE column*
- *Gel band cut from SDS-page*

**Assessment of antisera**

Reversed cutaneous anaphylaxis (table 2.2)
Neutralisation of PK reactivity (fig. 2.4)
Immunoelectrophoresis (fig. 2.5)

**Table 2.2. RCA titres of polyclonal anti-feline IgE raised against IgE preparations**

| Rabbit | Fraction of IgE used for immunisation | Protein concentration of the immunising antigen | Bleeding | RCA titre |
|--------|---------------------------------------|------------------------------------------------|----------|-----------|
| R194 | Fr-R from parasitised cat 8 immunised with DNP-Asc | 0.2 mg | #5 | 800 |
|  |  |  | #7 | 400 |
|  |  |  | #10 | 200 |
| R294 | Fr-R from sera of parasitised cats 1 and 2 immunised with DNP-Asc | 0.2 mg | #4 | 3200 |
|  |  |  | #6 | 1600 |
|  |  |  | #8 | 800 |
|  |  |  | #11 | 400 |
| R394 | Fr-R from parasitised cat 7 immunised with DNP-Asc | 0.2 mg | #4 | 3200 |
|  |  |  | #5 | 800 |
|  |  |  | #8 | 400 |
| R794 | IgE preparation eluted from the putative anti-IgE column (after multiple passages of serum #6) | 0.5 mg | #3 | 400 |
| R894 | IgE preparation eluted from the putative anti-IgE column (after multiple passages of whole cat sera) | 0.3 mg |  | Negative |
| R994 | IgE preparation eluted from the putative anti-IgE column (after multiple passages of whole cat sera) | 0.5 mg | #4 | 400 |
| R1094 | IgE preparation eluted from the putative anti-IgE column (after multiple passages of whole cat sera) | 0.4 mg | #2 | 1600 |
|  |  |  | #3 | 800 |
|  |  |  | #4 | 400 |
| R195 | Fr-R from pool of stray* cat sera | 1 mg | #2 | 400 |
| R295 | Fr-R from pool of stray* cat sera | 1 mg | #2 | 200 |
| R395 | Gel band cut from SDS-PAGE |  |  | Negative |
| R196 | Fr-R from pool of stray* cat sera | 1 mg |  | Negative |
| R197 | Fr-R (55% SASS**) from pool of sera from parasitised cats immunised with HSA | 1.5 mg |  | Negative |
| R297 | Fr-R (55% SASS**) from pool of sera from parasitised cats immunised with HSA | 1 mg |  | Negative |

*: Stray cats from a cat home in Liège (Belgium).
**: IgE rich fraction resulting from a 33% SASS precipitation followed by a 55% SASS precipitation instead of 45%.

of heated normal feline serum equilibrated with PBS and the flow through (FT) collected. Bound proteins were eluted using 0.1M glycine HCl, pH 2.8 and discarded. This process was repeated several times until no protein could be eluted by glycine HCl. The last FT collected was then concentrated to the starting volume.

### 2.2.8.1.2. Other anti-IgE sera

The antiserum which had the best apparent anti-IgE activity as assessed by reversed cutaneous anaphylaxis (RCA) (R294-7) was then used to prepare an immunoabsorbent column. A 45% ammonium sulphate precipitate of the antiserum was dissolved in PBS and then coupled to Sepharose 4B. Two ml of sera from parasitised cats were applied to the immunoabsorbent column equilibrated with PBS. The bound protein was eluted using 0.1M glycine HCl, pH 2.8. The eluate was collected in 1M tris-HCL, pH 8 to avoid prolonged exposure to low pH, and the resulting eluate was concentrated. The putative IgE from sera of parasitised cats that was eluted from the column was then used to immunise 4 further rabbits using the same procedure as above (table 2.5).

Aliquots of 5 ml of sera from cats with patent infections with *T. cati* that had been immunised orally with HSA were then applied to the Sephacryl S-200 columns (see chapter 6). The first half of the 7S peak (Fr-R and Fr-R+G) was collected, concentrated and pooled. Ammonium sulphate was added to the pool to give a final concentration of 33.33%. The supernatant was kept and precipitated a second time at 55% saturation. The pellet obtained was resuspended with and dialysed against 0.15M PBS, pH 7.2 at 4°C for 24 hr with 3 changes. Two New Zealand white rabbits (R197 and R297) were immunised with the resultant solution in CFA as described above.

One rabbit was also immunised with a protein band cut from a SDS-PAGE. This protein had a MW similar to the canine IgE heavy chain (see chapter 5).

### 2.2.8.2. Preparation of polyclonal anti-feline IgG

All fractions were sterilised by filtration through a 0.45μm nitro-cellulose filter (BDH) prior to injection into rabbits.

Anti-feline IgG was prepared by immunising 3 New Zealand White rabbits with 3 biweekly injections of 0.5 ml of 1 mg aliquots of purified IgG fractions (table 2.3). The first immunisation was given in 2 ml of CFA whereas other 2 were given in 2 ml of incomplete Freund's adjuvant (IFA). Rabbits were bled every 2 weeks by venepuncture.

Resulting antisera were assessed by IEP.

**Table 2.3. Polyclonal antisera raised against feline IgG preparations**

| Rabbit | Fraction of IgG used for immunisation | Immunoabsorbent column to which the antiserum was applied |
|---|---|---|
| R494 | 0.01M | (Fab)$_2$ column |
| R594 | 0.025M | 0.01M column |
| R694 | 0.035M | 0.01M column |

2.2.8.3. Preparation of anti-feline light chain serum

Anti-feline light chain serum was prepared by adsorption and elution of two ml of anti-feline IgG R4#94 from an immunoabsorbent column of Sepharose 4B coupled to feline (Fab)$_2$.

2.2.9. Induction of reversed cutaneous anaphylaxis (RCA)

Antisera were assessed for activity against IgE by their ability to induce reversed cutaneous anaphylaxis (RCA) on intradermal injection in healthy parasite-free cats. Cats were anaesthetised with 10 mg/kg ketamine hydrochloride intramuscularly. The hair of the lateral chest area was clipped and 0.05 ml of each dilution was injected intradermally. Antisera were tested at 1:10 and 1:100 and then at doubling dilutions. Normal rabbit serum (1/10, 1/100 and 1/1000) and PBS were included as negative controls and histamine phosphate 1/100,000 w/v as a positive control.

Immediate skin reactions were recorded 15 to 20 minutes after the injection as above for IDST.

2.2.10. Homologous PK tests

Homologous PK tests were performed to assess the DNP specific IgE and the Asc specific IgE in the sera from 9 infected cats. Healthy parasite-free cats were anaesthetised with 10 mg/kg ketamine hydrochloride intramuscularly. The hair of the lateral chest area was clipped. One tenth ml of ten fold dilutions in PBS (1 -1/10,000) of each serum, sterilised beforehand by filtration through a 0.45µm nitro-cellulose filter (BDH), was injected intradermally. Injection sites were challenged 48 hr later by intradermal injection of 0.05 ml of a 10 µg/ml solution of DNP-BSA , 100 µg/ml of Asc-

1 and 100 µg/ml of *T. cati* antigen respectively. The antigens were injected into unsensitised sites as negative controls with histamine phosphate 1/100,000 w/v as a positive control. Skin reactions were recorded 15 to 20 minutes after the injection as above.

### 2.2.11. Neutralisation of PK reactivity

Antisera were also assessed for activity against IgE by their ability to remove PK reactivity from feline sera. PK tests were performed as above with sera that gave positive reactions before and after 2 and 4 passages through the anti-IgE immunoabsorbent column (R294). Two ml of the same serum was also assessed by PK test before and after 4 passages through a column of Sepharose 4B coupled to normal rabbit serum as a control.

### 2.2.12. Immunoelectrophoresis (IEP) on agar gel

IEP was performed using 1.5% agar in 0.03 M sodium barbital buffer pH 8.6. Electrophoresis was carried out for 60 min at 50 mA in 0.03 M sodium barbital buffer pH 8.6. After application of antisera, the slides were incubated at room temperature for 48 hr in a humidity chamber. Precipitates were washed and dried before being stained with amido black (Sigma).

## 2.3. RESULTS

### 2.3.1. Parasitological findings

All cats developed a patent infection with *T. cati* with ova appearing in the faeces within 60 to 75 days. Adult *T. cati* were found in the duodenum of cats #1 - #4 at autopsy 4 weeks to 12 months later. No *T. cati* were recovered in the other cats. However *Dypilidium caninum* (*D. caninum*) was found in the large intestine of cats #4 - #7 (table 2.4).

**Table 2.4. Parasitological findings in 9 cats naturally\* or experimentally\*\* infected with *T. cati*.**

| Cat | Parasitological findings | |
| --- | --- | --- |
| | Ova in faeces | At autopsy |
| #1* | yes | *T. cati* |
| #2* | yes | *T. cati* |
| #3* | yes | *T. cati* |
| #4** | yes | *T. cati* and *D. caninum* |
| #5* | yes | *D. caninum* |
| #6* | yes | *D. caninum* |
| #7* | yes | *D. caninum* |
| #8** | yes | None |
| #9** | yes | None |

### 2.3.2. IDSTs

All cats had a positive IDST to DNP-BSA with 4 cats (cats 1, 3, 6 and 7) showing a +3 reaction; 4 cats (cats 2, 4, 8 and 9), a +2 reaction; and one cat (cat 5), only a +1 reaction (table 2.5).

**Table 2.5. DNP-specific IDST and PK test results in 9 cats immunised with DNP-Asc.**

| Cat | IDST reactivity* | PK reactivity** |
| --- | --- | --- |
| #1 | +3 | 1/100 |
| #2 | +2 | 1 |
| #3 | +3 | 1/10 |
| #4 | +2 | 1/10 |
| #5 | +1 | 1/10 |
| #6 | +3 | 1/100 |
| #7 | +3 | 1/10 |
| #8 | +2 | 1/100 |
| #9 | +2 | 1/10 |

*: Strength of skin test to $10^{-2}$ mg/ml DNP/BSA.

\* \*: Highest dilution (10 fold) to which a positive test resulted using $10^{-2}$ mg/ml DNP-BSA.

### 2.3.3. Homologous PK tests

Ten-fold dilutions of sera from immunised cats were assessed for PK reactivity. Positive results were recorded with dilutions out to 1/100 (3 cats), 1/10 (5 cats), with one cat reacting only with undiluted serum when challenged by DNP-BSA (table 2.5).

Serum from only one cat (cat #4) gave a positive reaction to *T. cati* antigen (with a PK reactivity of 1/64), and no sera showed PK reactivity against Asc-1.

### 2.3.4. RCA tests

The 3 antisera raised against Fr-R of the sera from cats immunised with DNP-Asc (R194-R394) and 3 out of the 4 antisera raised against the putative IgE fraction eluted from the putative anti-IgE column (R794-R994 and R1094) were able to induce RCA at a dilution of >1/100 (table 2.2). Although 2 antisera (R294 and R394) were able to induce a weal on intradermal injection at 1/3200 dilution, the largest wheal at that dilution was obtained with R294. Reactions were not seen with normal rabbit serum. Of the 6 last antisera produced, four were always negative on RCA, and a further two (R195 and R295) only induced positive reactions in the first two bleedings.

### 2.3.5. Neutralisation of PK reactivity of reactive sera

PK reactivity to DNP and *T. cati* respectively in sera #6 and #4 decreased progressively following each passage through the putative anti-IgE immunoabsorbent column (R294) (fig. 2.2). Although the diminution in PK reactivity was evident in both cases, it was most significant in case of serum #6 (fig. 2.2). In contrast, passage of the sera through a column of normal rabbit serum coupled to cyanogen bromide activated Sepharose 4B produced only a small decrease of PK reactivity (fig. 2.2). Heating at 56°C for 4 hr removed all PK reactivity (fig. 2.2) The eluate from the R294 anti-IgE immunoabsorbent column following application of serum from cat #4 was assessed for PK reactivity to DNP. A +4 result was obtained following injection of 0.1 ml of a 375 μg/ml preparation of pooled eluates.

### 2.3.6. IEP on agar gel

### 2.3.6.1. Specificity of the putative anti-feline IgE.

The absorbed antisera which had the best apparent anti-IgE activity were assayed for their capacity to precipitate with reaginic antibodies on IEP against a range of both heated and unheated feline sera.

Rabbit antiserum R294 from the 9th bleeding and R1094 from the 5th bleeding were able to precipitate with a protein which had γ-1 electrophoretic mobility in the 9 sera from infected cats and in the serum from a parasite free cat. This protein was not

□ Serum #6
□ Serum #6 after 2 passages through the putative anti-IgE column
⊞ Serum #6 after 4 passages through the putative anti-IgE column
□ Serum #6 after 4 passages through a normal rabbit serum column
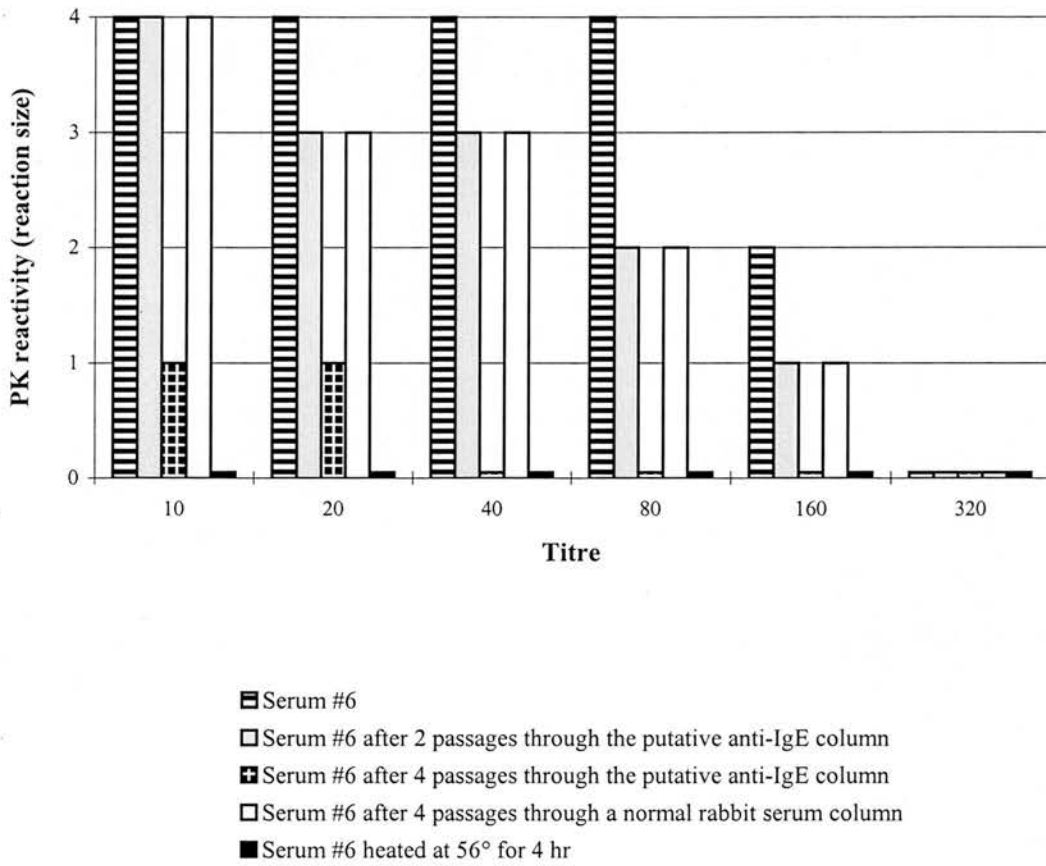■ Serum #6 heated at 56° for 4 hr

**Fig. 2.2. Effect on the PK titre to DNP of the passage of feline reagin-containing serum #6 through a putative anti-IgE immunoabsorbent column (R294)**

detectable in heated sera (fig. 2.3). The same antisera failed to detect purified feline IgG. This protein was also no longer detectable after 4 passages of the serum through the anti-IgE immunoabsorbent column.

2.3.6.2. Immunoelectrophoretic analysis of the eluate from the putative anti-IgE column.

Rabbit anti-feline light chain serum was tested for its ability to precipitate with the reaginic antibody eluted from the column R294-7 coupled to cyanogen bromide activated Sepharose 4B.

Rabbit antiserum against feline light chains detected a single protein which had γ-1 electrophoretic mobility in the putative IgE preparation eluted from the column R294-7 (fig. 2.3). However R294 was not able to precipitate with this protein, and equally the protein was not detected by anti-feline IgG (Fc-specific).

## 2.4. DISCUSSION

Foster et al. (1995) have demonstrated that reaginic activity in cat sera eluted between the IgM and IgG peaks upon Sephadex G 200, as has been shown in other species (Fireman et al., 1963; Zvaifler and Becker, 1966; Patterson et al., 1969; Hammer et al., 1971; Isersky et al, 1974; Halliwell, 1975b; Lehrer, 1976; Ovary et al., 1976; Suter and Fey, 1981; Roe et al., 1993; Yilmaz et al., 1993). Nevertheless, contrary to the dog (Peng et al., 1991&1997), feline immunoglobulins fail to bind to protein G (Reis et al., 1986; Lew et al., 1991; Foster et al., 1995) which therefore cannot be used to remove contaminating IgG from an IgE preparation. In the present study, feline reaginic antibodies were isolated from cat sera by salt precipitation followed by anion-exchange chromatography on DEAE and by Sephacryl 200 gel filtration to produce fraction-R. This fraction was shown to contain reaginic antibody by its ability to produce antisera, following immunisation of rabbits, which had activity against feline IgE as judged by neutralisation of PK reactivity and induction of RCA.

On IEP on agar gel, antisera R294 and R1094 were able to precipitate with a heat labile immunoglobulin which had γ-1 electrophoretic mobility. The protein eluted from the anti-IgE column was shown to be immunoglobulin by its detection by a rabbit anti-feline light chain serum, but it was not detected by the anti-IgE sera R294 and R1094. Neither was it detected by Fc-specific anti-feline IgG. It has been shown that canine IgE
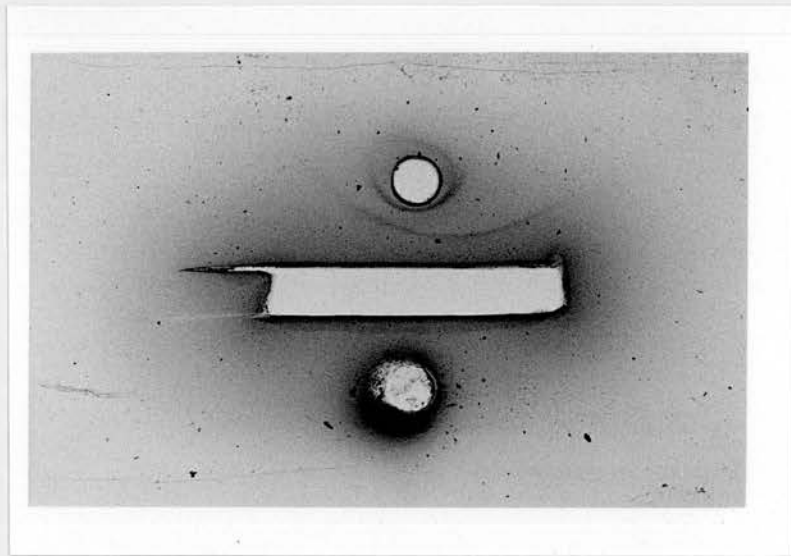
(a) Top well: cat serum #4.
    Bottom well: cat serum #4 heated at 56° for 4 hr.
    Trough: antiserum R294.
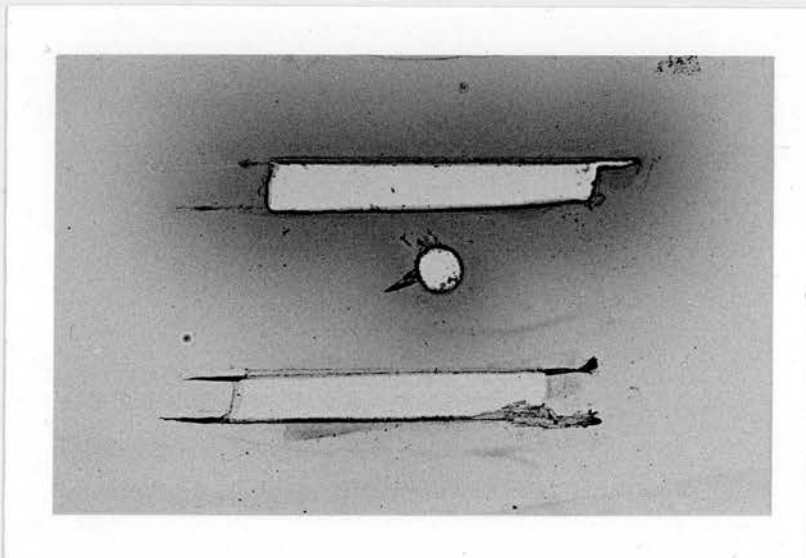


(b) Top trough: antiserum R294.
    Bottom trough: anti-feline light chain immunoglobulin.
    Well: eluate from R294 immunoabsorbent column.

**Fig. 2.3. (a and b) Antibody activity of putative anti-feline IgE serum (R294) as revealed by immunoelectrophoresis.**

is sensitive to acid treatment (Halliwell, 1975b) and the same is likely to hold true for the feline isotype. Since 0.1M glycine-HCl pH 2,7 was used to eluate the solution from the putative anti-IgE affinity column, the Fc portion of the reaginic antibody may have been denatured although it was immediately neutralised with 1M Tris-HCl to pH 8.0. This could explain why R294 failed to precipitate with the immunoglobulin in the putative IgE eluted from the anti-IgE column. It could also explain why the antisera produced against this eluate were weaker in terms of RCA activity. Therefore it may have been of benefit to eluate the proteins bound to the column using a neutral buffer such as immunopure gentle antigen/antibody elution buffer (Pierce and Warriner, UK Ltd). However, it was surprising that the last 6 antisera produced, although also raised against Fr-R from parasitised cats or stray cats, were either negative, or were able to induce RCA only in the early bleedings and rapidly became negative in later bleedings. The fractions were mixed with CFA as was the immunogen in the case of the previous antisera but the protein concentration injected was higher, although similar to the concentration recommended for the production of polyclonal antisera (Harlow and Lane, 1988). Nevertheless, it is possible that the fractions contained more contaminant proteins and that the IgE antibodies were present in too small concentrations. The first bleeding (2 weeks after immunisation) of the antisera was often able to induce a strong RCA which rapidly decreased in the subsequent bleedings. This may be the result of the presence of IgM antibody in high concentrations.

Although many researchers recommend that CFA should only be used for the first injection in antisera production for humane reasons, it was used in these studies on three occasions with full permission of the Home Office. This was done because previous experience in our laboratory has shown that the induction of antisera against IgE of animal origin, which is likely to be present in very small quantities, is exceedingly difficult using any other protocol. Painless granulomatous swellings which did not ulcerate, were the only adverse effects observed, and the rabbits were not affected systematically.

The PK test has proved to be a sensitive method to detect the presence of reaginic antibody (Prausnitz and Küstner, 1921). The sensitivity of the test has been shown to be 0.2 ng/ml in human sera and 2-3 ng/ml in mouse sera (Ishizaka, 1988). The optimal period after sensitisation for the detection of IgE using PK or PCA tests is 24 to 48 hr while for IgG, this period is 1 to 6 hr. Moreover, the sensitisation induced by IgG persists only for a short time of 1 to 2 days while the IgE binds to mast cells for about 3

weeks (Ovary, 1986). Those characteristics as well as the heat sensitivity permit distinction between IgE and IgG homocytotropic antibodies. In cats as in other species, PCA tests have also been used extensively to establish the presence of allergen specific IgE (Walton et al., 1968; Powell et al., 1980; Baldwin, 1993; Foster et al., 1995).

The life cycle of *T. cati* is complex and may include a variety of developmental stages and several hosts. Cats can become infected by 3 different routes, namely the ingestion of embryonated eggs, the transmammary passage of larvae from the queen and the ingestion of larvae in paratenic hosts (rodents, birds or insects) (Swerczek et al., 1971). The mode of infection of the cat determines the relative proportion of larvae that migrate via the tracheal versus the somatic route. When infection occurs by the ingestion of embryonated eggs, second-stage larvae (L2) hatch in the stomach and migrate through the liver and the lungs to the small intestine, where they mature into the adult stage (Hendrix and Blagburn, 1983; Swerczek et al., 1971). The migration is accomplished by larvae in the second-stage and the third-stage does not develop until larvae have returned to the digestive tract. Third-stage larvae develop in the stomach wall while the fourth stage is found in the stomach contents, the intestinal wall and intestinal contents. The patent period is about 50 days (Glickman and Schantz, 1981). In this mode of infection, some of the larvae that reach the lung also penetrate the pulmonary vein to undergo somatic migration and encyst in an arrested state in the tissues of the cat where they can survive for some years.

Therefore, in order to induce a somatic migration, kittens were infected orally with embryonated eggs. Eggs were found in the faeces 63 days after oral infection in two cats (cats #7 and #8), and 75 days in one cat (cat #4). This is in accord with the patent period reported by Glickman and Schantz (1981). Six months later, 2 of the cats (cats #7 and #8) were found to be negative whereas the third cat (cat #4) remained positive for more than 12 months. The 2 cats which became negative presumably had some larvae encysted in their tissues stimulating specific IgE against *T. cati*. However Bowman and Lynn (1995) reported that in the cat there is not the age related protection seen in dogs against *T. canis*, and this is why mature ascarids are not uncommon in cats. On the other hand, faeces were assessed for *T. cati* eggs before the experimental infection when the kittens were 7 weeks old. Although no eggs were found in their faeces, 7 weeks may not have been long enough to obtain a patent infection and the kittens may still have already been infected by the transmammary route before experimental infection. In contrast to infection by ingestion of embryonated eggs, in the latter mode of infection, larvae

acquired by the kitten do not undergo migration and develop in the stomach wall and stomach contents (Hendrix and Blagburn, 1983). If so, the 2 cats which became negative could have cleared their adult worm burden and may not have had any encysted larvae in their tissues. It is not impossible that the mother of the kittens had encysted larvae which were reactivated during her pregnancy. However this is unlikely since they were wormed before mating. In a recent survey performed in Ireland, *T. canis* were still found in faeces from 3.6% of pet dogs (4 dogs out of 112) which were afforded high level of care (O'Sullivan, 1997).

The dosage of 10 embryonated eggs for 3 days was suggested by the Department of Parasitology in Liège, Belgium in order to avoid the development of an immune response that could have led to the clearing of the larvae.

Sera from all 9 of the cats were able to give a positive PK test to DNP at 1/10 dilution with 3 sera reacting out to 1/100. However, results of PK tests when challenged by Asc-1 or *T. cati* antigen were surprising in that only one serum gave a positive test when challenged with *T. cati* antigen despite the fact that all the cats had previous patent infections with *T. cati*. It is also difficult to understand why the immunisation of the cats with DNP-Asc-1 failed to induce detectable Asc-1-specific IgE. It has been shown by Strejan et al (1973) that Asc-1 allergen is strongly immunogenic and when coupled to DNP, was far more effective in inducing DNP-specific IgE than when coupled to other proteins (Strejan and Marsh, 1971). A lower dose of Asc-1 could have been more effective since Strejan et al (1973) found that Asc-1 was more immunogenic at a dose of 1 μg than at 10 μg.

Also, in some systems, the potentiation of reagins by helminth antigens seems to require active infection and is usually transient and ceases when most of the parasites have been expelled (Orr et al., 1971). In this study, the IgE response to DNP does not appear to be related to the potentiation by helminth infection but may still have resulted from immunisation of the cats with DNP coupled to Asc-1. It is also possible that the differences seen in the IgE response could reflect differing genetic backgrounds (Tada, 1975).

Antigenic cross-reactivity between stages of *Toxocara* and other ascarid species has been described (Page et al., 1991). Rabbits, rats and mice infected with *T. canis* gave a heterogencous response to the antigens of different stages and the different hosts recognised different and cross-reacting antigens (Kennedy and Qureshi, 1986). Also, human infection with *A. suum* may be associated with cross-reacting antibodies to

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antigens from *A. suum* and *N. brasiliensis* (McWilliams et al., 1987; Kennedy et al, 1987). However, in one study, Foster (1995) used crude *A. suum* and *T. canis* antigens in PCA tests using sera from cats infected with *T. canis,* with negative results. The author hypothesised that the antigens responsible for PCA activity may have been present in different quantities at different stages of the parasite life cycle (Foster, 1995). Cox (1993) has shown that the various immune responses are stage specific and that immunity elicited by larval stages is effective against subsequent invading larvae giving rise to the situation in which the host harbours a population of adult worms but is immune to reinfestation. In the case of the cats infected by ingestion of embryonated eggs, the cats with negative PK activity to antigens of the adult worm may still have had some IgE activity against larval stages of *T. cati.*

The production of specific IgE has been linked with host-protection against helminth infection. Hagan et al (1991) showed that resistance to reinfection with *Schistosoma haematobium* in man is associated with high levels of parasite-specific IgE antibodies with specificity for adult worm and schistosomulum membrane antigens. Baldwin et al (1993) showed a strong correlation between the production of filarial-specific IgE and the death of adult *Brugia pahangi* in cats. The majority of cats with high levels of circulating microfilaria or live adults in the lymphatics did not produce significant levels of IgE. However helminths are also able to elicit extremely high levels of blocking IgG4 antibodies (Allen and Maizels, 1996). If so, such a level of specific IgG4 may have interfered with the IgE binding to the mast cells.

The reasons for the failure of most of the cats to develop a detectable IgE response to the parasite antigens studied using the PK test are thus obscure. However, since the detection of specific-IgE was not performed by enzyme-linked immunoabsorbent assay we should not exclude the possibility of the existence of another Asc-1 or *T. cati*-specific IgE isoform which was either less efficient in binding to mast cells or in inducing degranulation. It is also quite possible that antibodies of other isotypes were produced.

In conclusion, the claim of the specificity of the antisera for feline IgE is supported by a number of results. Firstly, the DNP-specific PK reactivity was removed by passage through the putative anti-IgE immunoabsorbent column. Secondly, PK reactivity was recovered in the eluate. Thirdly, this eluate contained immunoglobulin which was not IgG. Furthermore, the antisera were able to induce RCA in high dilutions and finally, they were able to detect a heat labile protein in the sera of parasitised cats

which had an electrophoretic mobility compatible with that known for this isotype in other species.

# CHAPTER 3.

# INDUCTION OF ANTIGEN-SPECIFIC IgE
# IN NORMAL CATS

## 3.1. INTRODUCTION

Although enzyme-linked immunosorbant assays (ELISAs) are marketed for the detection of allergen-specific feline IgE, details supporting the specificity of these antisera have not yet been published in the scientific literature. In a study of one of these assays, Foster and O'Dair (1993) found that the correlation with intradermal skin tests was very poor. Both the specificity and the sensitivity of the ELISA as compared with the intradermal skin test results were low with a positive and negative predictive value of 0.1 and 0.4 respectively.

The previous chapter has documented the production of polyclonal antisera specific for feline IgE. It was important therefore to ensure that the antisera could detect IgE antibodies in the sera of cats in which allergen specific IgE was induced experimentally under controlled conditions. As house dust mites are the most common allergens involved in atopic disease in dogs and cats in Europe (Foster and O'Dair, 1993; Carlotti and Costargent, 1994; Sture et al., 1995) *Dermatophagoides farinae* (DF) was chosen as the antigen. The kinetics of IgE and IgG responses specific for DF were followed during the 8 weeks of the study. In addition, IgE antibodies specific for the dinitrophenyl group (DNP) were induced in another group of cats (see chapter 2). In all cases, IgE was assayed by both homologous Prausnitz-Küstner (PK) tests and by ELISA for comparison.

## 3.2. MATERIALS AND METHODS

### 3.2.1. Animals

Nine cats, either naturally or experimentally infected with *Toxocara cati* (*T. cati*) were described in the previous chapter.

Fifteen healthy adult cats were obtained from Hill Grove Family Farm Ltd, of which 10 were used for immunisation with DF and 5 as recipients for PK tests.

### 3.2.2. Allergen extracts

DF extract (1/100 w/v) was purchased from Greer Laboratories.

### 3.2.3. Dinitrophenylation of antigen

Cat serum albumin (CSA, Sigma) and *Ascaris suum* antigen (Asc-1, Greer) were dinitrophenylated by mixing with a solution of 2 % dinitrofluorobenzene (DNFB) in ethanol (Hudson and Hay, 1980). Dinitrophenylated protein was separated from unbound DNFB by filtration through a Sephadex G-25 column (Pharmacia).

### 3.2.4. Immunisation of cats

1. The 9 cats, naturally or experimentally infected with *T. cati*, were given two subcutaneous injections at 2 week intervals of 10 μg of DNP-Asc in aluminium hydroxide (Imject™ Alum®, Pierce), and were the same cats as described in the previous chapter. Serum was harvested from blood taken prior to immunisation and 2 weeks after the second immunisation.

2. Ten cats from a parasite free colony were given 3 subcutaneous injections of 50 μg of DF in aluminium hydroxide at weeks 0, 2 and 5. Five cats (U49, B85, 46L, D09 and 794), in addition, were given at the same time, 50 μg of Asc-1 mixed with the DF antigen in the same adjuvant. All cats were bled prior to immunisation and at weekly intervals thereafter for 7 weeks.

### 3.2.5. Performance of intradermal skin tests (IDSTs)

IDSTs were performed weekly as before (chapter 2). Two fold dilutions of a 1/1000 w/v dilution of DF was injected intradermally.

### 3.2.6. PK tests

PK tests were performed as before (chapter 2). Briefly, 0.1 ml of doubling dilutions of sera were injected intradermally. Injection sites were challenged 48 hr later by intradermal injection of 0.05 ml of a 1/1000 w/v dilution of DF. For standardisation of responses between PK tests in recipient cats, a reference serum from a cat which had a strong positive skin test to a high dilution of DF was used in each test.

## 3.2.7. Antisera

The antiserum R294 (chapter 2) was passed through an affinity chromatography column containing DF coupled to cyanogen bromide activated Sepharose 4B (Pharmacia) to remove possible naturally occurring antibodies with activity against DF.

Rabbit anti-feline IgG, heavy chain (HC) specific, was purchased from Nordic Immunology (Tilburg, the Netherlands).

Monoclonal alkaline phosphatase-conjugated mouse anti-rabbit immunoglobulin was purchased from Sigma.

## 3.2.8. ELISA

### 3.2.8.1. Assay optimisation

Preliminary assays were performed to determine optimal dilutions of reagents and sera. The concentrations of coating antigen and conjugated antibody were evaluated by use of checkerboard titrations.

### 3.2.8.2. Detection of DF-specific IgE

DF-specific IgE in feline sera from sensitised cats was assessed by indirect ELISA. Polystyrene microtitre plates (Immulon 4®, Dynatech Laboratories, Billingshurst, UK) were coated overnight at 4°C with 100 µl/well of 1/5000 W/V dilution of DF extract in 0.1M sodium carbonate buffer, pH 9.6 (coating buffer). Plates were washed three times with washing buffer which was 0.05% tween 20 (Sigma) in 0.015M PBS, pH 7.2 and blocked overnight at 4°C with 0.5% human serum albumin (HSA) in washing buffer (150 µl/well). After further washing, 50 µl of each sample diluted in ELISA buffer (0.05% tween 20, 0.1% of HSA in 0.015M PBS, pH 7.2) was added to duplicate wells. After incubation at 37°C for 2 hr and further washing, 50 µl of the polyclonal anti-feline IgE diluted in ELISA buffer (1/50) was added to each well. After incubation for 2 hr at 37°C, the plates were washed and 50 µl of the alkaline phosphatase-conjugated second antibody diluted in ELISA buffer (1/2000) was added to each well and plates were incubated for a further hour at 37°C. After a last wash, 50 µl of alkaline phosphatase substrate, namely 5 mg of PNP substrate tablets (Sigma) in 5.5 ml of $MgCl_2$ in diethanolamine buffer, pH 9.8, was added to each well. Plates were

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incubated at 37°C for 30 minutes and the optical density (OD) at 405 nm read using an automated microtitre plate reader after subtraction of the mean blank well values.

### 3.2.8.3. Detection of DF-specific IgG

DF-specific IgG was assayed by indirect ELISA as performed for allergen-specific IgE. Incubation times with the serum samples were reduced from 2 hr to 1.5 hr for the serum samples and from 2hr to 1 hr for both the polyclonal rabbit anti-feline IgG and the alkaline phosphatase conjugated monoclonal mouse anti-rabbit immunoglobulin. Rabbit anti-feline IgG was used at 1/5000 and conjugated antibody at 1/2000.

### 3.2.8.4. Detection of DNP-specific IgE and IgG

The same method as above was used to assess the DNP-specific antibody except that plates were coated with 10 μg/ml of a solution of DNP-CSA for the measurement of DNP-specific IgE and 1 μg/ml in the case of DNP-specific IgG.

### 3.2.8.5. Standardisation of results

A serum containing a high level of allergen-specific IgE or IgG antibody was selected as the standard and assigned a value of 1000 relative antibody unit (RAU) value when undiluted. Doubling dilutions of the standard were included in each plate, and the results were recorded in RAU by reference to the standard curve. Preimmunisation sera were used as negative control sera in each plate.

### 3.2.8.6. Assessment of serum-samples

Serum samples were initially assessed at a dilution of 1/2 in ELISA buffer for the detection of DNP-specific IgE and DF-specific IgE in preimmunisation bleedings and 1/5 in postimmunisation bleedings. For the detection of DNP-specific IgG and DF-specific IgG, preimmunisation and postimmunisation bleedings were initially assessed at a dilution of 1/10 and 1/100 respectively. The assay was then repeated in lower or higher dilutions as appropriate. A serum sample whose resultant OD fell below the end point of the standard curve (EPSC) was deemed to be negative. For statistical analyses, these sera were given a value half way between the RAU of the EPSC and zero.

### 3.2.8.7. Determination of the standard curve

A programmable ELISA reader indicated the % error for each point on the standard curve. Since these errors tended to be higher at the lower and higher end of the curve, these points were deleted, leaving a minimum of 3 or 4 points to the standard curve for the detection of DNP and DF-specific IgE, and 4 to 5 points for the detection of allergen-specific IgG. The standard curve was generated by plotting the OD against log10 reference serum concentration.

Using linear regression analysis, the correlation coefficient of each standard curve, R, is defined through

$$R^2 = \frac{(n\Sigma\, XY - \Sigma X\, \Sigma Y)^2}{(n\, \Sigma X^2 - (\Sigma X)^2)(N\Sigma Y^2 - (\Sigma Y)^2)}$$

X represents concentration whereas Y denotes optical density

When $R^2 = 1$, all data points lie along the line the best fit, whereas when $R^2$ is very small, data points are highly scattered.

### 3.2.8.8. Validation of the assay

Allergen-specific IgE and IgG were measured in sera before and after heating to 56°C for 4 hr. In the case of both DNP and DF-specific antibody, IgE was no longer detected after heating, whereas IgG levels were substantially unaffected (figs 3.1 (a and b) and 3.2 (a and b)). This supports the isotype specificity of the anti-IgE antiserum.

### 3.2.9. Statistical analysis

Correlation of IgE and IgG were calculated using a Pearson's rank coefficient correlation. The results were considered statistically significantly different if $p < 0.05$.
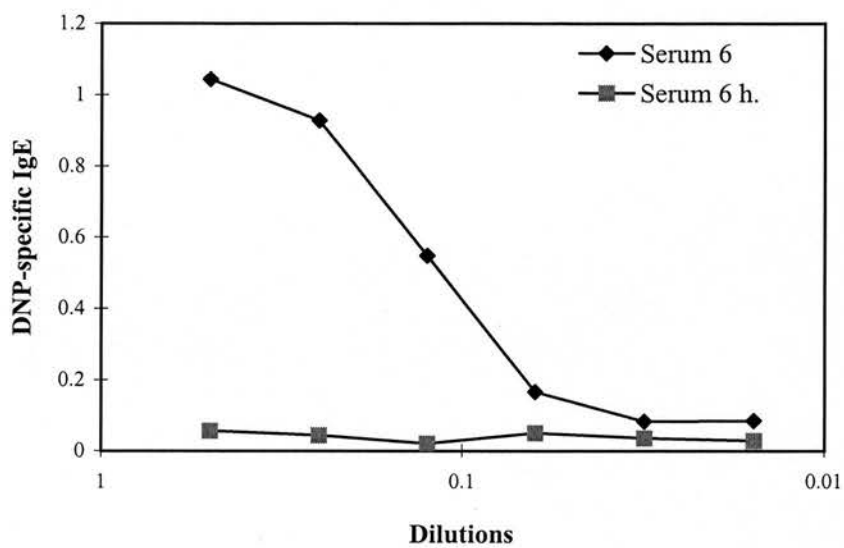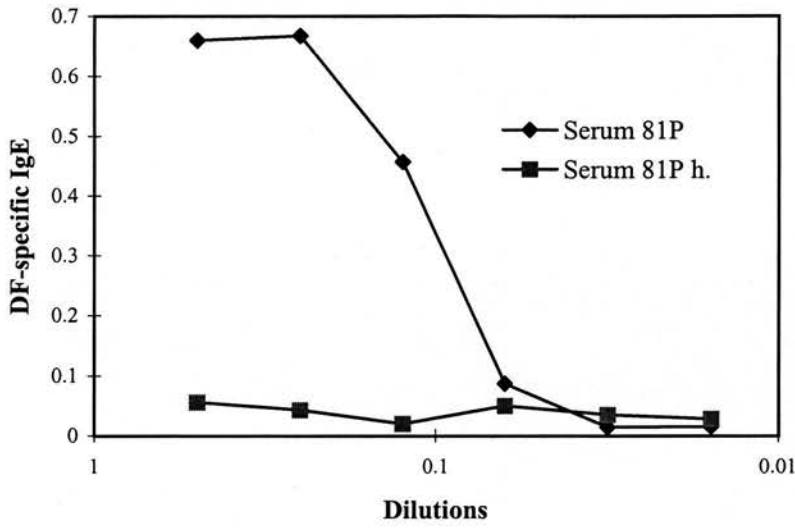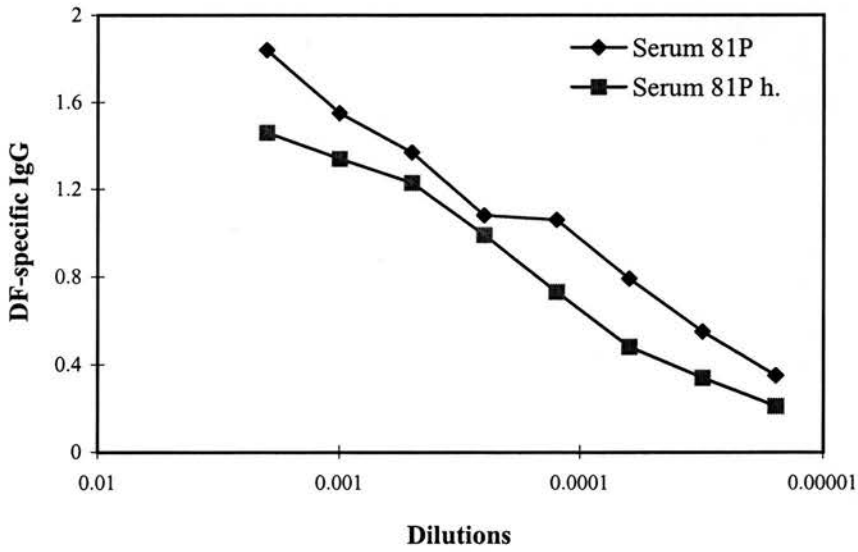
(a)
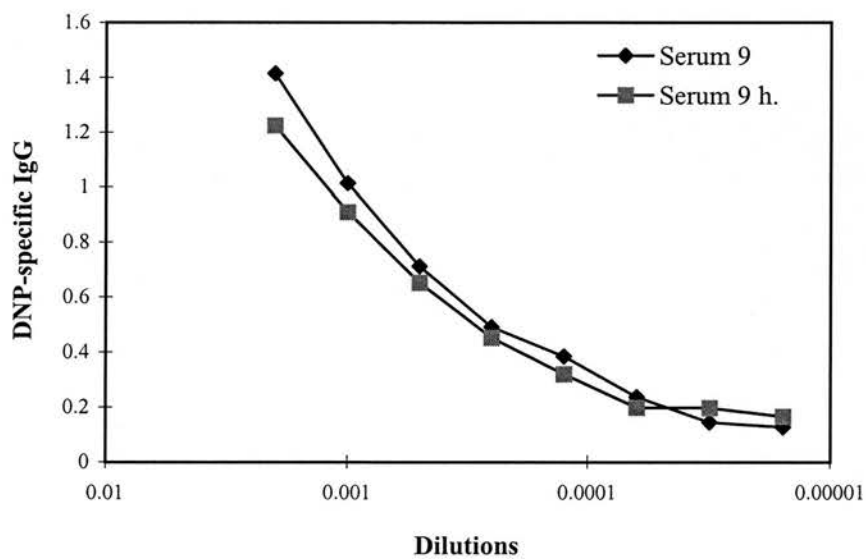


(b)

**Fig. 3.1. DNP-specific IgE (a) and IgG (b) in cat sera before and after heating at 56 °C for 4 hr (h = sera after heating). IgE and IgG levels reported as absorbance at 405 nm.**

(a)



(b)

**Fig. 3.2. DF-specific IgE (a) and IgG (b) in cat sera before and after heating at 56 °C for 4 hr (h = sera after heating). IgE and IgG levels reported as absorbance at 405 nm.**

### 3.3. RESULTS

3.3.1. DNP-specific response

No DNP-specific antibody was detected in the sera of cats prior to their immunisation with DNP-Asc. Six out of the 9 cats developed an IgE response against DNP as assessed by ELISA (fig. 3.3). However all the cats were positive to the IDST and all showed positive PK tests. Serum from 8 of the cats gave positive PK tests at 1/10 with 3 sera reacting out to 1/100. One cat had PK reactivity only to undiluted serum. All cats developed a DNP-specific IgG response (fig. 3.3).

3.3.2. DF-specific response

3.3.2.1. IDSTs

The 10 cats immunised with DF were skin tested weekly commencing immediately prior to the first injection. Assessment of the level of serum DF-specific IgE by ELISA and of the highest dilution of DF antigen to induce a positive skin test following intradermal injection revealed some striking anomalies and little evident correlation. Firstly, DF-specific IgE was in general, detectable in sera prior to the time at which the cat showed positive skin tests. Indeed, 4 cats failed to develop positive skin tests during the 8 weeks of the experiment despite developing some of the highest levels of serum allergen-specific IgE. In general, strongly positive skin tests to high dilutions of antigen were always accompanied by high levels of detectable allergen-specific IgE. However the reverse was not the case. The individual responses are noted in fig. 3.4 (a-j)).

3.3.2.2. ELISA

OD values slightly greater than the blanks were detected in some of the preimmunisation sera. These values were unchanged in sera after passage through an immunoabsorbent column of DF coupled to Sepharose 4B (data not shown) which implied non-specific binding. These OD values were therefore subtracted from the results in each case before expression in RAU following reference to the standard curve.

All cats developed both an IgG and an IgE response to DF following immunisation (fig. 3.5 (a and b)). The kinetics appeared different, in that a detectable IgE response appeared earlier, and had fallen by week 5 prior to the third immunisation.
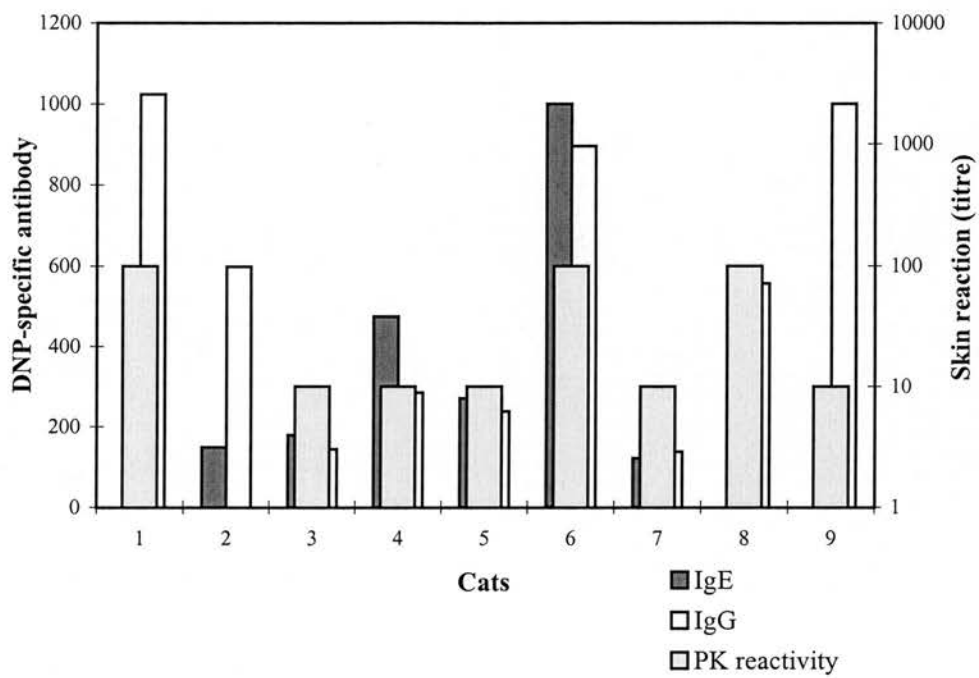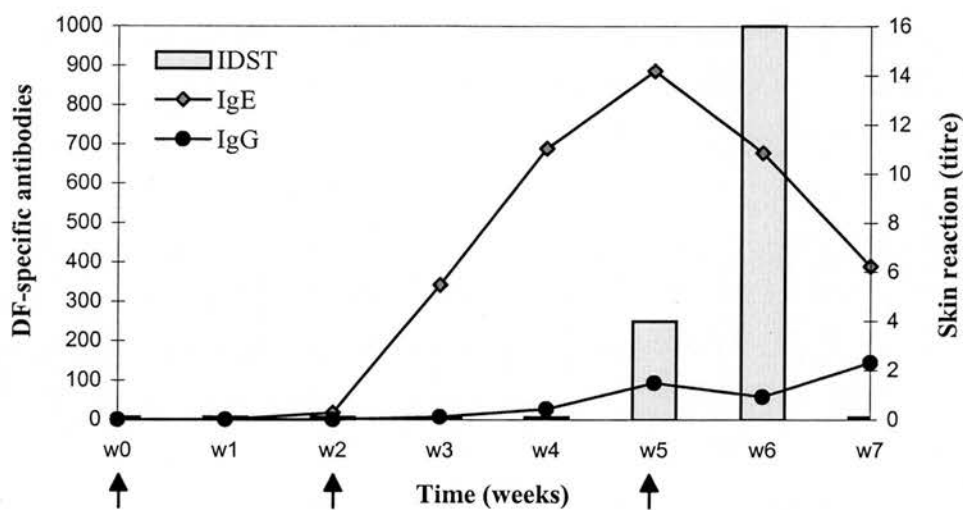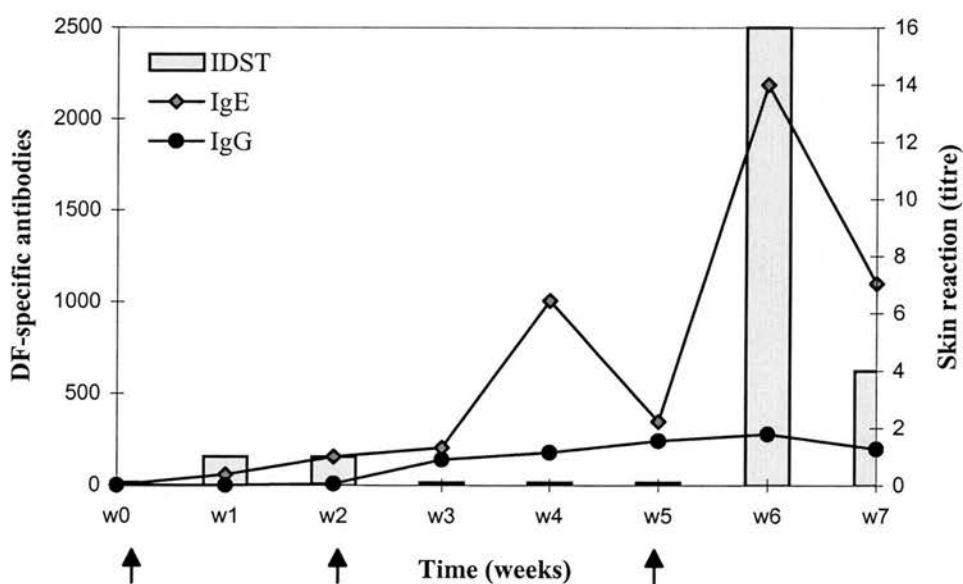
Fig. 3.3. DNP-specific IgE and IgG responses, and PK reactivity to DNP
in 9 cats immunised with DNP-Asc. Antibody level expressed in RAU.
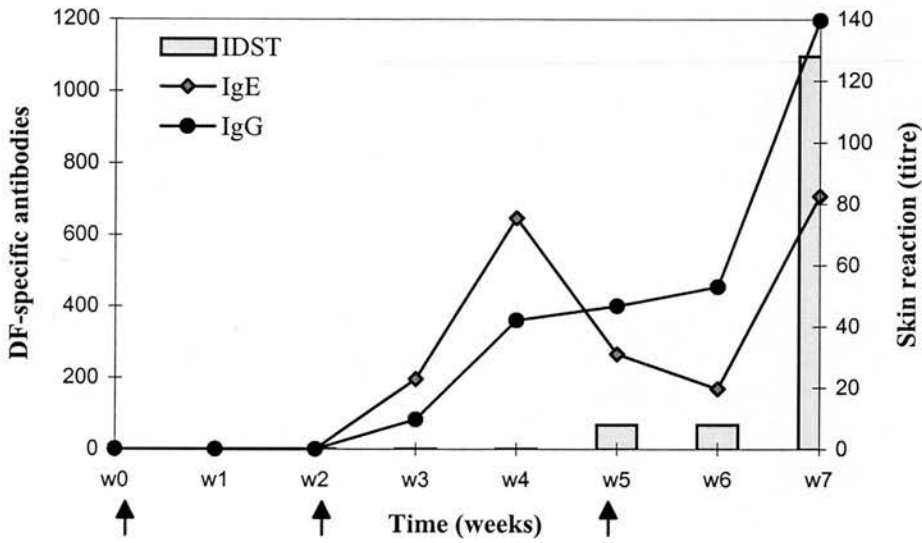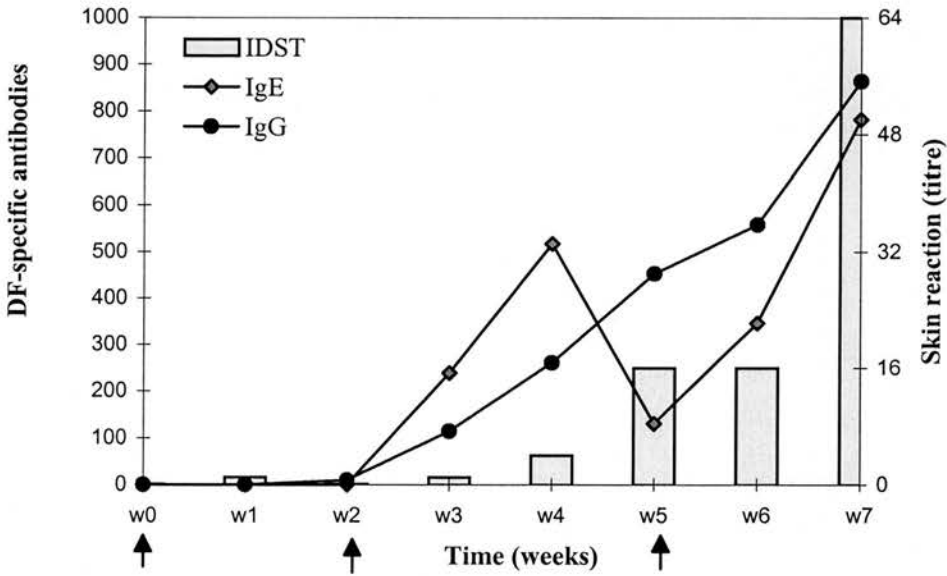
**Fig. 3.4. The relationship between the DF-specific IgE and IgG (in RAU) , and the highest dilution of antigen to gave positive IDST (skin reaction titre) in 10 cats immunised with DF.**

**▲ = immunisation. IgG results are divided by 1000.**
**(a) cat A60 and (b) cat U49**

(c) Cat A71



(d) Cat B85

Fig. 3.4. (continued) The relationship between the DF-specific IgE and IgG (in RAU) , and the highest dilution of antigen to gave positive IDST (skin reaction titre) in 10 cats immunised with DF.

▲ = immunisation.  IgG results are divided by 1000.

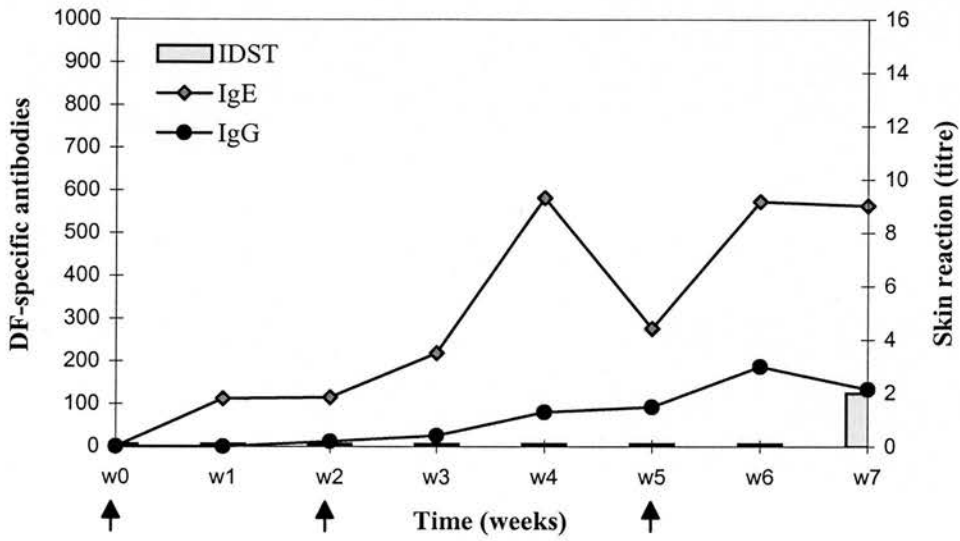(c) cat A71 and (d) cat B85
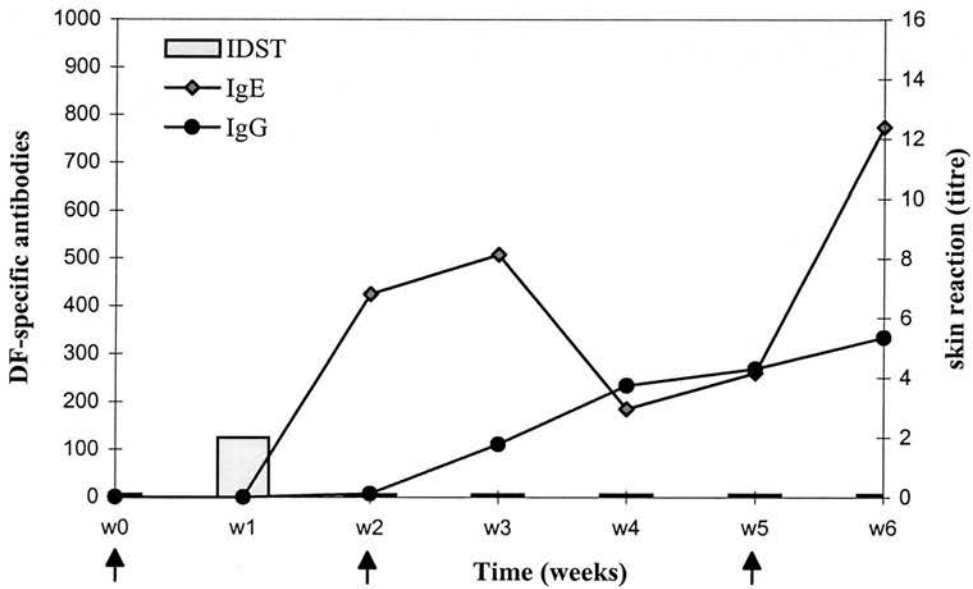
(e) Cat 46L



(f) Cat D09

Fig. 3.4. (continued) The relationship between the DF-specific IgE and IgG (in RAU) , and the highest dilution of antigen to gave positive IDST (skin reaction titre) in 10 cats immunised with DF.
⬆ = immunisation.  IgG results are divided by 1000.
(e) cat 46L and (f) cat D09

(g) Cat 60T



(h) Cat 81P

**Fig. 3.4. (continued) The relationship between the DF-specific IgE and IgG (in RAU) , and the highest dilution of antigen to gave positive IDST (skin reaction titre) in 10 cats immunised with DF.**
**▲ = immunisation. IgG results are divided by 1000.**
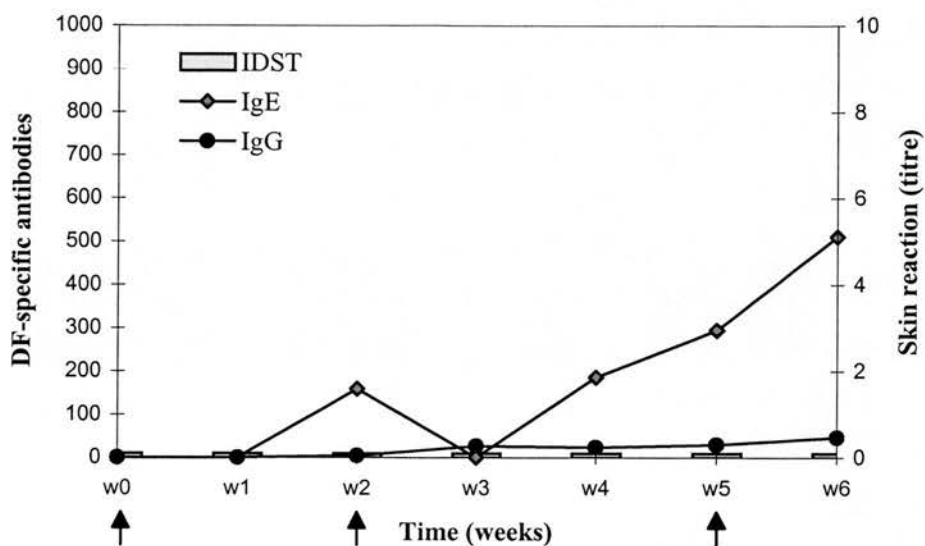**(g) cat 60T and (h) cat 81P**
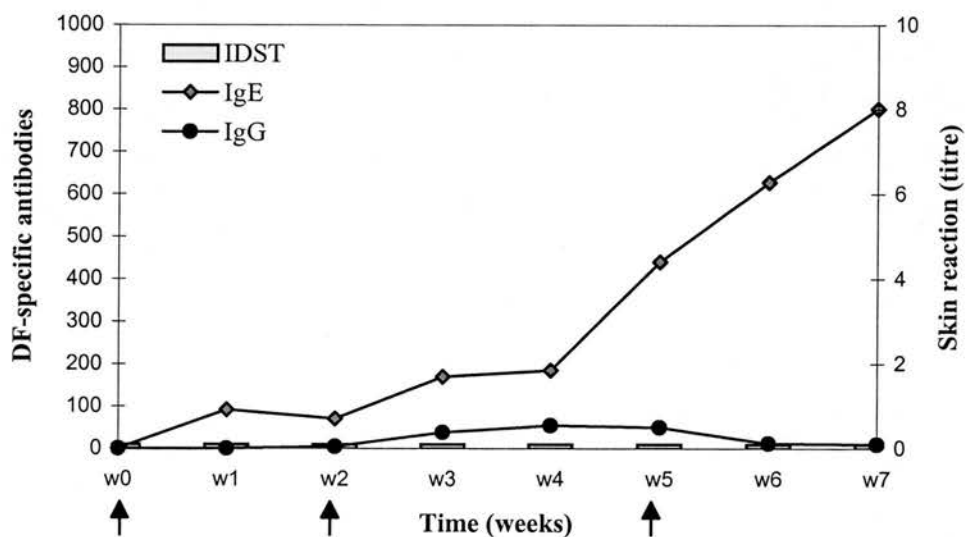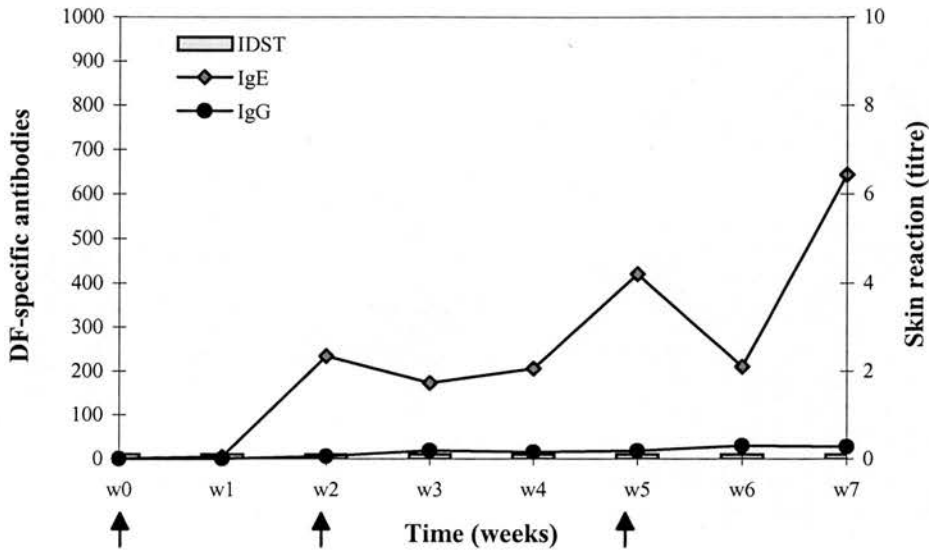
(i) Cat 794
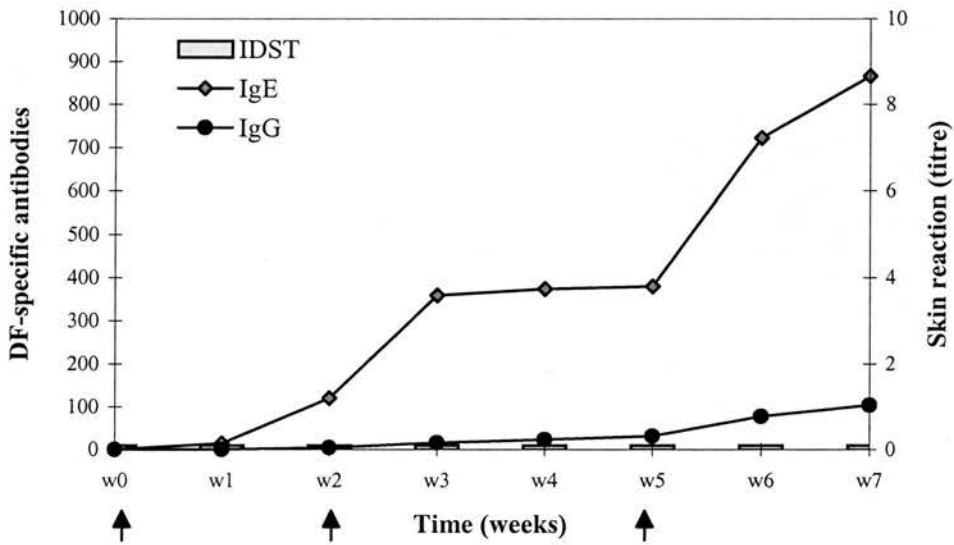


(j) Cat 735

**Fig. 3.4. (continued) The relationship between the DF-specific IgE and IgG (in RAU) , and the highest dilution of antigen to gave positive IDST (skin reaction titre) in 10 cats immunised with DF**
**↑ = immunisation.  IgG results are divided by 1000.**
**(i) cat 794 and (j) cat 735**

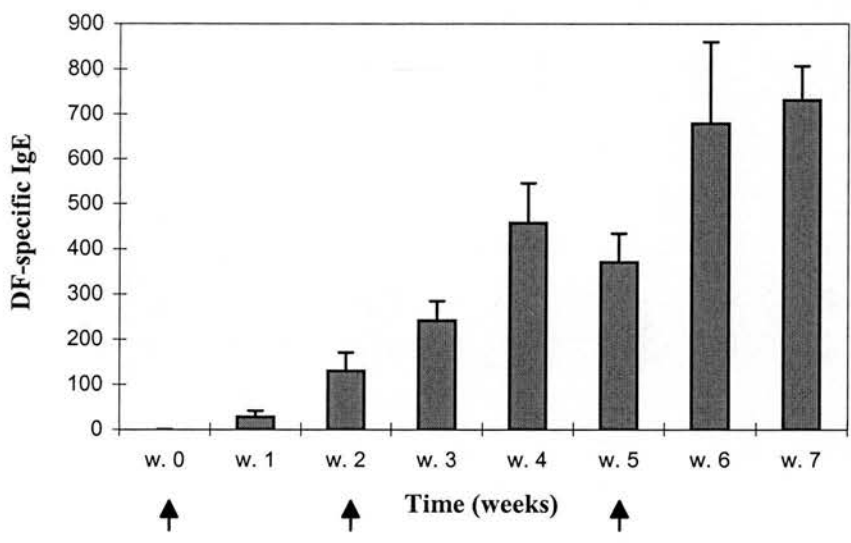(a)
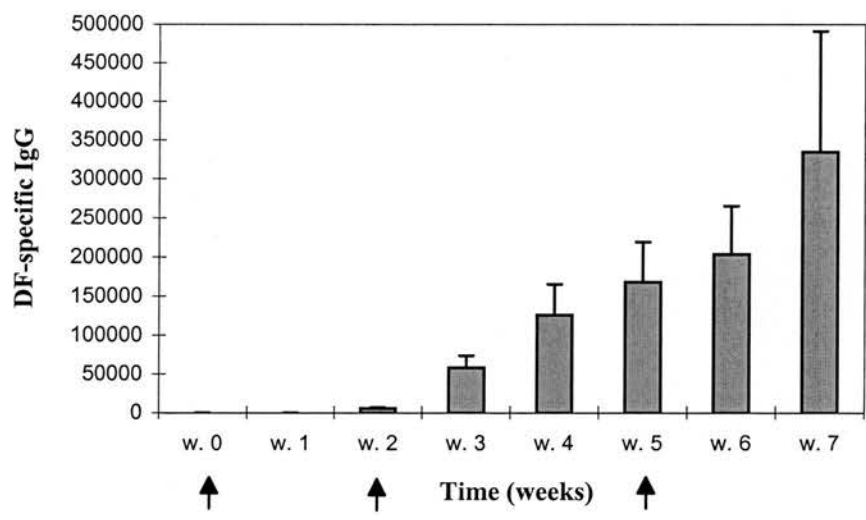


(b)

Fig. 3.5. Mean and standard error of the DF-specific IgE (a) and DF-specific
IgG (b) responses in 10 cats immunised with DF. Antibody expressed in RAU.
↑ = immunisation.

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In contrast, a detectable IgG antibody response commenced later and rose gradually through the 8 weeks (fig. 3.5 (a and b)). Assessment of both isotypes in all serum samples in each cat revealed no significant correlation between them (Pearson's rank correlation coefficient). However, when all the ELISA values for DF were considered together, there was a significant correlation between Log DF-specific IgE and IgG antibodies at weeks 2, 4 and 5 (r=0.499, 0.396 and -0.504 respectively, p<0.05, fig. 3.6) although some samples had very high IgG and low IgE levels and vice versa (fig. 3.4 (a-j)). The levels of both isotypes were not correlated at weeks 3, 6 and 7 (r=0.120, 0.010 and 0.026 respectively, p>0.05, fig. 3.6). The correlation between Log DF-specific IgE and IgG antibodies was also significant when all the data were considered together over the 8 week period (weeks 0 to 7, 10 cats, r=0.790, p<0.01, fig.3.7). The 5 cats immunised with DF alone (A71, 60T, 81P, A60 and 735) in alum produced responses that did not differ noticeably from those immunised with both DF and Asc-1 in alum (U49, B85, 46L, D09 and 794).

### 3.3.2.3. PK tests

Positive PK tests were only obtained using sera from cats with positive IDSTs and the IDST reactivity tended to parallel the PK titre. Thus the strongest PK test results were in general obtained with postimmunisation sera from cats which had strong positive skin tests to high dilutions of DF (up to 1/320). On the other hand sera from cats which had positive IDSTs to low dilutions of DF, gave either negative or only weakly positive PK reactions. Furthermore postimmunisation sera from the 4 cats which failed to develop positive skin tests but had high levels of serum allergen-specific IgE as assessed by ELISA showed no PK reactivity. On the other hand, allergen-specific IgE was detected by ELISA in cats which had PK reactivity as well as in cats which had no such reactivity. In both situations the IgE was heat labile and was not detected by the ELISA after the serum was heated at 56°C for 4 hr. Heating the sera also completely abolished the PK reactivity.

## 3.4. DISCUSSION

One of the classical characteristic properties of reaginic antibody is its sensitivity to heating (Coca and Grove, 1925). Ishizaka et al. (1967) observed that reaginic antibody to ragweed failed to precipitate with monospecific antiserum to IgE after exposure to
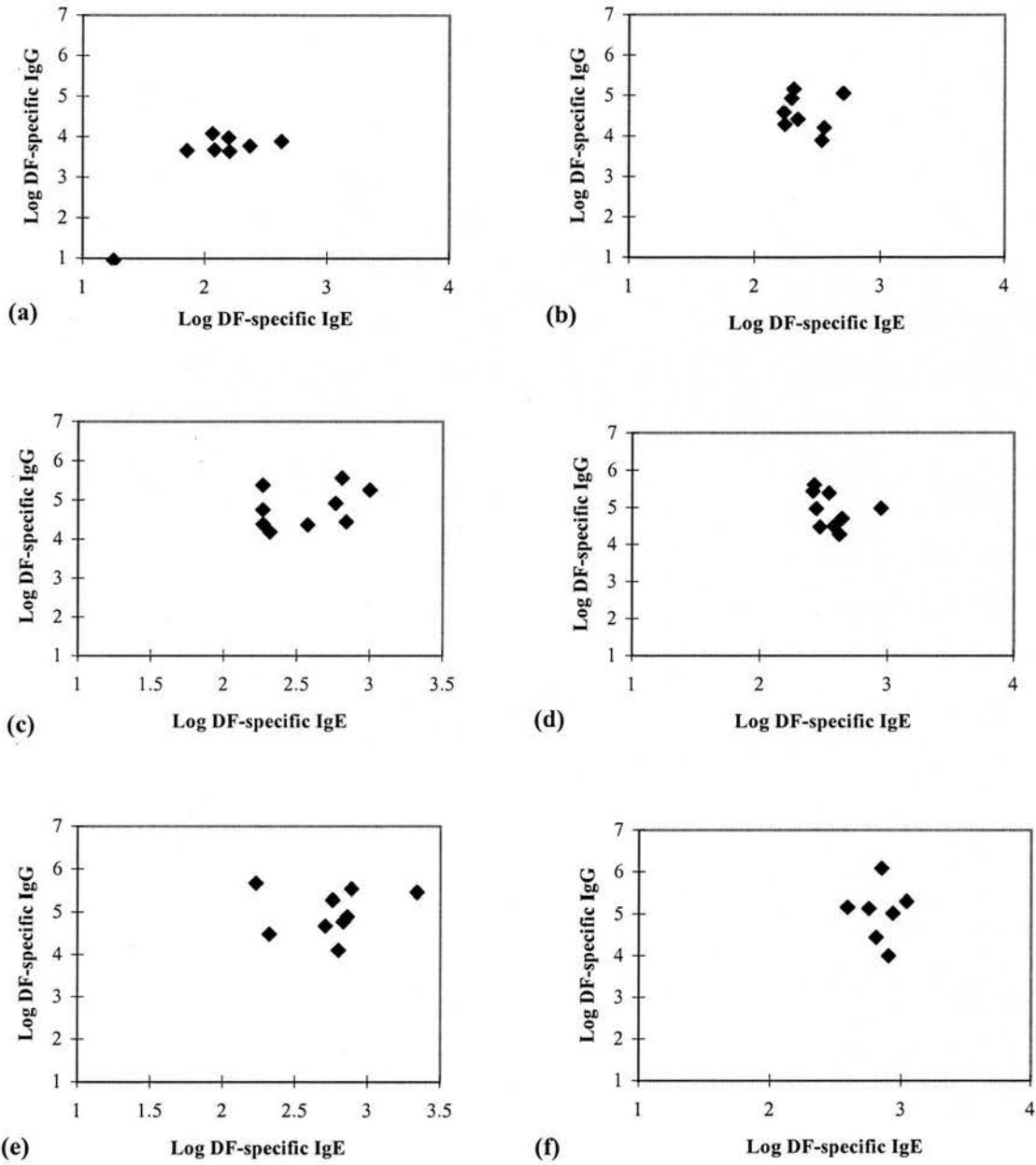
**Fig. 3.6. DF-specific IgE and IgG in RAU in sera from cats immunised with DF at week 2 (a), week 3 (b), week 4 (c), week 5 (d), week 6 (e) and week 7 (f).**

The levels of the two isotypes are significantly correlated at week 2 ((a) r =0.499, p < 0.05), week 4 ((c) r =0.396, p < 0.05) and week 5 ((d) r =-0.504, p < 0.05). The correlation was not significant at week 3 ((b) r =0.120, p > 0.05), week 6 ((e) r = 0.010, p > 0.05) and week 7 ((f) r =0.026, p > 0.05).
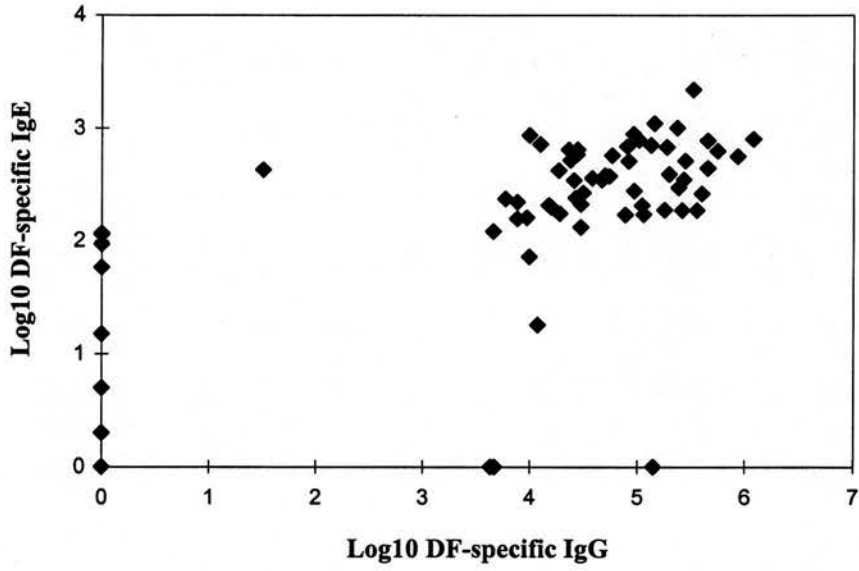
Fig. 3.7. DF-specific IgE and IgG in RAU in sera from cats immunised with DF. The levels of the two isotypes are significantly correlated (r = 0.790, p < 0.01).

56°C for 2 to 4 hr. Schwartzman and Rockey (1967) demonstrated that canine reaginic antibody is comparable to human reaginic antibody in many characteristics including heat sensitivity. Powell et al. (1980) demonstrated the heat sensitivity of reaginic antibody in cats. Such treatment does not inactivate other classes of antibody and thus allows the differentiation between IgE and other isotypes. In the present study, the complete abolition of detectable allergen-specific IgE in postimmunisation sera heated to 56°C for 4 hr demonstrated the specificity of the rabbit anti-feline IgE. It was noteworthy that in the case of the ELISA for DF-specific IgE, above background OD values were obtained in preimmunisation sera. The fact that the OD values were unchanged after passage through a DF immunoabsorbent column, and that the rabbit anti-feline IgE had also been passed over such a column indicated non-specific binding. It has been shown in dogs that the high background levels of IgE antibody, associated with parasitism, may interfere with the ELISA and lead to a higher incidence of false positive results. In one study, Griffin et al. (1990) demonstrated that different types of antigens were affected differently by the serum IgE concentration. It is important therefore, that true controls are identified in the case of each allergen system to obviate false positive results. Although the cats used in the DF study were parasite free at the time of the experiment, the possibility of previous parasitism cannot be excluded.

Only duplicates of a single dilution were performed in the ELISA in the present study. Bullock and Walls (1977) found that reactivity curves showed an extremely steep slope at the end point. The antibody affinities of various sera may differ, leading to many possible dose-response curves (de Savigny and Voller, 1980). In this respect, testing a whole range of serum dilutions would have been more accurate. However the amount of antiserum available was too low to allow such procedure.

In the *T. cati* infected cats, immunisation with DNP-Asc produced a DNP-specific IgE response (assessed either by ELISA or PK tests) in all cases. Surprisingly, ELISA results did not correlate with PK test results. Indeed, the antiserum was not able to detect any DNP-specific IgE in the sera of 3 cats although all of these cats had good PK reactivity. The PK test is considered a sensitive test with an estimated minimum concentration of human IgE required for sensitising human skin for a positive reaction of 0.2 ng/ml (Ishizaka and Ishizaka, 1975). Therefore this finding could be related to the lower sensitivity of the ELISA in comparison with the PK test. However, since the PK tests were performed almost 2 years before the ELISA tests and samples had been thawed and refrozen a number of times, the IgE may have been denatured in those cats

by freezing and thawing or storage conditions. On the other hand, this is unlikely since in dogs, as in man, IgE appears to be remarkably stable in serum stored at room temperature for short durations and limited freeze/thaw cycles are unlikely to substantially alter quantitative results (Bazaral and Hamburger, 1972; Halliwell, 1975; Hill and DeBoer, 1994). Another explanation could be that the high level of DNP-specific IgG in cats 1 and 9 could have interfered with the binding of allergen-specific IgE to the antigen in the ELISA. However cat 6 showed a high level of IgE and a good PK reactivity in spite of a high level of IgG. Also the cats that showed positive PK tests and no detectable IgE on ELISA may have had higher levels of DNP-specific immunoglobulins other than IgG and IgE. It has been shown that dogs sensitised to ragweed not only develop ragweed-specific IgE and IgG level but also ragweed-specific IgA, IgM and IgD-like antibody (Peng et al, 1991; Yang et al., 1995). The reasons for this discrepancy are thus obscure.

Injections of Asc-1 together with DF in 5 parasite free cats failed to stimulate a higher DF-specific IgE than in cats given DF alone. It is well-known that helminths are particularly adept at stimulating IgE synthesis. Helminth infection has the ability to potentiate IgE responses to unrelated antigens and therefore to disproportionately elevate serum levels of total non-parasite-specific IgE (Ogilvie, 1964; Jarrett and Bazin, 1974; Turner et al., 1979; Zakroff et al., 1989). Halliwell (1973) showed that subcutaneous injections in dogs of *A. suum* antigen alone or coupled to haptens was able not only to stimulate the production of reagin to haptens to which the antigen was coupled but also to any protein with which it was simultaneously administered. However the parasite status of the dogs was not known, although in those days *T. canis* infection in dogs was almost universal. The apparent disparity in the ability of ascaris antigens to stimulate allergen-specific IgE production in the 2 experiments may thus merely relate to the parasite status.

In the DF immunised cats, the IgE response commenced early, being detectable 2 weeks after immunisation whereas IgG was essentially undetectable until week 3. Furthermore, the IgE level tended to fall off after the second immunisation in 5 cats whereas the IgG antibody continued to rise. IgE thus appears to be an early antibody in the cat, as has been shown in the rat (Tada, 1975). The decrease in DF-specific IgE after 5 weeks, as contrasted with the IgG response, could, of course merely be reflective of the shorter half-life of the former isotype, rather than an actual lowering of the production relative to IgG. The IgE antibody level depends not only on the dose of the antigen and

the adjuvant used but also on the interaction of genetic background and environmental factors (Levine et Vaz, 1970; Vaz et al., 1971). It has been demonstrated that the IgE response to an allergen in mice depends on the strain immunised (Levine et Vaz, 1970). The marked variations of the antibody response seen in these cats are most probably the result of the genetic differences between them as it was suggested for the outbred dogs in the study of Schultz and Halliwell (1985).

The cats immunised with DF showed no clinical signs of allergy in spite of the strongly positive IDST developed by some of them. It is possible that this resulted from the limited exposure to DF in the laboratory conditions. However the subcutaneous route used for antigen administration, both for the induction of IgE and for antigen challenge, is different to the natural disease in which the percutaneous route is believed to be responsible for clinical signs (Halliwell, 1990; Olivry et al., 1996). On the other hand it is noteworthy that no species in which IgE was experimentally induced, including man, have shown any clinical signs of allergic disease (Marsh et al., 1972). The only study that came close to inducing spontaneous disease involved a study of basenji-greyhound crosses who developed not only bronchospasm, but also dermatitis upon repeated nebulisation with Ascaris antigen (Butler et al., 1983). However these dogs were either natively allergic to *A. suum* or had become sensitised by repeated aerosol challenge with a purified *Ascaris* antigen. This emphasises the fact that it is not merely the presence of IgE that distinguishes atopic individuals from normals. It has been suggested that this might be because the reaginic response can be accompanied by high levels of blocking antibodies (Marsh et al., 1972) but another reason could be the heterogeneity of the reaginic antibody response as discussed below.

In the present study, circulating levels of DF-specific IgE did not correlate with tissue-bound IgE as assessed by IDSTs. In particular, 4 cats with high levels of IgE never developed a positive IDST. Moreover, it was noteworthy that there was a marked lack of correlation between the level of IgE as assessed by ELISA and by PK tests. Also, positive PK tests were never obtained using sera from cats which were IDST negative, although such sera had high levels of IgE as assessed by ELISA. On the other hand, sera from the cats which were only IDST positive to a low dilution of DF showed only a weak PK reactivity whereas sera from cats which had a positive IDST to high dilutions of DF showed much greater reactivity. It is, of course, possible that the cats which developed a high level of DF-specific IgE without a positive IDST may have developed a high level of blocking IgG. However, the level of IgG measured in these cats was not

higher than in the cats with a positive IDST to a high dilution of DF that showed strong PK reactivity. Therefore the DF system appears to be different than the DNP system, in that sera from all the 9 cats immunised with DNP showed PK reactivity.

It could also be hypothesised that the negative IDSTs in the face of the positive ELISAs could be due to a delay in circulating IgE fixing to mast cells. Such a phenomena is believed to occur clinically in some cases of canine atopic disease in which the positive in vitro tests develop weeks or months before a positive IDST. However this possibility could be excluded as the sera from these cats failed to give positive PK tests.

The most rational explanation for this finding would be the existence of heterogeneity of IgE in cats. Theoretically, a number of possibilities exist by which subclasses of IgE could differ. Firstly, the IgE antibody could be heterogeneous in terms of its ability to respond to histamine releasing factors (HRF), which are cytokines derived from macrophages, lymphocytes, mononuclear cells, platelets and vascular endothelial cells. It has been hypothesised that it is not the production of IgE but, rather the ability of the IgE to interact with the HRF which differentiates atopics from non atopic individuals (Lichtenstein, 1988). The basis of this heterogeneity is not yet understood but has been speculated to be due to differential glycosylation of the IgE molecule (MacDonald, 1996). Thus the type of IgE produced by the cats experimentally immunised with DF, in contrast to that produced by atopic cats, may not be reactive with HRF. In cats which become positive to the IDST, this first IgE antibody response (IgE-) could have been followed by the production (switching) to the second type of IgE antibody (IgE+). The cats which stayed negative to the IDST would never have developed the second IgE isotype and such animals would have positive ELISA results without positive IDST or PK reactivity. However in man, HRF's appear to be involved only in the late phase reaction resulting from the activation of basophils, and the reaction is seen in only a subpopulation of allergic individuals (MacDonald, 1996). Therefore it is unlikely that, in the present study, the interaction of IgE with HRF would explain why some of the cats in which DF-specific IgE was experimentally induced did not develop an immediate response to the IDST.

Secondly it is possible is that the IgE antibody experimentally induced in the normal cats may be less effective at inducing degranulation, as is the case with IgG homocytotropic antibody which requires larger amounts of antigen to elicit positive IDSTs or passive transfer tests. In this regard it has been shown in man that anti-IgG4

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appears to be less effective than anti-IgE in eliciting basophil histamine release and that the amount of anti-IgG4 required to induce a response is very high (Valent and Bettelheim, 1992). It could also be that the IgE experimentally induced in the normal cats may have a low affinity for mast cell receptors and therefore not be able to interact with them in order to sensitise mast cells. In a recent study, Lyczack et al. (1996) demonstrated the existence of 4 human IgE isoforms They suggested that this could be an additional possible explanation for the functional heterogeneity of IgE and that IgE may differ in its primary protein structure as well as glycosylation. Each isotope may have some unique properties as circulating, cytophilic immunoglobulins, and may differ in their ability to carry out IgE-mediated functions through binding to their different receptors. One of those isoforms is secreted as half-molecules (heavy-light chain) and therefore could be less able to arm mast cells and basophils for antigen-triggered release and may indeed inhibit such release (Lyczack et al., 1996). Interestingly, it has been also shown that the relative levels of mRNAs encoding various isoforms appears to be altered in allergic and parasitic diseases (Diaz-Sanchez et al., 1995). These findings suggest that the existence of more than one type of IgE in cats is not unexpected and that the IgE antibody experimentally induced in normal cats may be different from the IgE produced in allergic cats. Moreover, it is not impossible that the cats which developed a positive IDST were genetically predetermined to be atopic.

Findings of recent studies in dogs are also strongly suggestive of a heterogeneity of canine IgE. Lian and Halliwell (1998) skin tested and blood sampled both normal and atopic dogs for the detection of allergen-specific IgE and found positive IDSTs and ELISAs in most of the normal dogs.

Jackson et al. (1996) studied leucocyte histamine release in atopic dogs, normal dogs and artificially sensitised dogs and found comparable results to those found in that study, in that histamine release induced by antigen or anti-IgE was generally found only in atopic dogs.

Using a monoclonal and polyclonal antibodies specific for canine IgE, Peng et al. (1993b) found that the ELISA values measured by the monoclonal antibody correlated less well with PCA titres when compared to the ELISA values measured by the polyclonal antibody. They were able to isolate 2 different isotypes of canine IgE (IgE1 and IgE2) which differed from each other by their charge, their affinity for protein A and their antigenicity (Peng et al., 1997). Carbonhydrate moieties appeared not to be involved in the differences between the 2 types of IgE. In spite of these differences, both

exhibited typical IgE characteristics, such as heat sensitivity, identical molecular weight and capacity to induce 48-hr PK tests.

Since PK test results can be influenced by the recipient (Augustin, 1978), the PK tests in these studies were performed with a positive serum included in each assay as a control. This serum was used a total of 9 times on 5 recipients with an identical titre (1/320) on every occasion although the reaction size varied from one cat to another. This supports the validity of the test as used in this study. The use of Evans blue and the PCA test could have been more sensitive and resulted in higher observed titres, but such an approach limits the repeated use of the cats. Interestingly PK tests have been found to have limitations in dogs as the titre of a given serum may vary widely when assessed in different recipient animals (Peng et al., 1993a).

In conclusion, the present study showed that normal cats developed a reaginic antibody response to both a hapten (DNP) and an environmental allergen (DF) which was readily detected by use of the polyclonal anti-IgE in an ELISA. There was a significant overall correlation between allergen-specific IgE and IgG responses. However, the level of allergen-specific IgE was not correlated with either skin test or PK reactivity, whereas the positive PK reactivity was generally correlated with positive skin test reactivity. That results are consistent with the existence of a heterogeneity of IgE antibodies in cats.

# CHAPTER 4.

# MEASUREMENT OF DF-SPECIFIC IgE IN THE SERA FROM SPONTANEOUSLY ALLERGIC CATS

## 4.1. INTRODUCTION

Feline atopy was first described in 1982 by Reedy who reported positive skin tests and a good response to immunotherapy in cats presented with miliary dermatitis, self-induced alopecia and/or eosinophilic granuloma complex (Reedy, 1982).

IgE immunoglobulin has been shown to be involved in the pathogenesis of atopy although its clinical relevance remains unknown. Because the intradermal skin test (IDST) has been the only test available for allergy testing in the cat, the diagnosis of atopy has been based on this test and response to hyposensitisation for many years (Reedy, 1982; Scott, 1984; Prost, 1992). Recently, ELISA for feline IgE has become commercially available in the USA and in Europe. However these tests have not been adequately evaluated. The only study which compared the results of ELISA to the IDST, which was assumed to be the gold standard in the diagnosis of feline atopy, reported a very poor correlation between the two. The authors concluded that the ELISA used was unreliable in establishing a diagnosis of feline atopic disease (Foster and O'Dair, 1993). Nevertheless, in a recent study in which 81 cats received hyposensitisation based on the results of radioallergosorbent tests (RAST), the response was shown to be broadly similar to that obtained when hyposensitisation was based upon the results of skin testing (Halliwell, 1997).

In the previous chapter (chapter 3), it was demonstrated that allergen-specific IgE could be induced in normal cats by immunisation with *Dermatophagoides farinae* (DF) in aluminium hydroxide. The allergen-specific IgE response was shown not to be correlated with the skin reactivity, which raised the question of a possible heterogeneity of IgE in this species. It was therefore of interest to know if cats suffering from atopy had higher levels of DF-specific IgE than normal cats. The level of DF-specific IgG was also assayed.

## 4.2. MATERIALS AND METHODS

### 4.2.1. Cat sera

Cats were presented to the Royal (Dick) School of Veterinary Studies at the University of Edinburgh and to the Faculté de Medecine Vétérinaire at the Université de Liège in Belgium. Three groups of cats were represented in the study:

*Atopic cats.* The 10 cats comprising this group had a mean of age of 6.5 years (range 3 years to 12 years). Six of the cases were spayed females and the 4 males were castrated. The diagnosis of atopic disease was made by the combination of clinical signs, absence of any other dermatological disease that could account for their disease, and the presence of at least one positive reaction to the IDSTs using a panel of 57 allergens. A hypoallergenic food trial and strict flea control were performed in each case to exclude those diseases. One cat had also a concomitant adverse reaction to food, and responded partially to a hypoallergenic diet.

*Control group 1.* This group comprised 15 normal healthy household cats that were presented to the University of Liege for routine examination. The average age of the group was 4.5 years (range 9 months to 13 years). The group was composed of 7 females (2 spayed) and 8 males of whom 6 were castrated. All were free of skin disease and ectoparasites.

*Control group 2.* This group comprised 11 normal healthy laboratory reared cats that were obtained from Hill Grove Family Farm Ltd. The mean age was 9 years (range 4 to 12 years). All cats were entire females. The cats had been raised under laboratory conditions and presumably exposed at best to only low levels of DF.

### 4.2.2. Allergen extracts

DF extract (1/100 w/v) was purchased from Greer Laboratories.

Fifty three additional allergens were selected for intradermal skin tests of which 42 were obtained from Greer laboratories whereas 12 were obtained from ARTU (Artuverin Allergens; ARTU Biologicals, Lelystad, The Nederlands) (table 4.1). Allergens were used at appropriate dilutions which were believed to be non-irritant for the dog (August, 1982) and therefore non-irritant for the cat (Bevier, 1990).

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**Table 4.1. Allergens and concentration used for the IDST**

| Number | Allergens | Units | Concentration | Source |
|---|---|---|---|---|
| | **Mites/Dusts** | | | |
| 1 | *Dermatophagoides pteronyssinus* | w/v | 1:10000 | Greer |
| 2 | *Acaris siro* | NE | 100 | ARTU |
| 3 | *Dermatophagoides farinae* | w/v | 1:10000 | Greer |
| 4 | *Tyrophagus putresceantiae* | NE | 100 | ARTU |
| 5 | House dust | PNU | 1000 | Greer |
| | **Epithelial group** | | | |
| 6 | Human epithelium | mg | 10 | ARTU |
| 7 | Cat epithelium | PNU | 500 | Greer |
| 8 | Horse dander | PNU | 500 | Greer |
| 9 | Sheep epithelium | PNU | 500 | Greer |
| 10 | Mixed feathers | mg | 100 | ARTU |
| 11 | Cotton linters | w/v | 1:1000 | Greer |
| | **Insects** | | | |
| 12 | American/German cockroach | PNU | 500 | Greer |
| 13 | Flea | w/v | 1000 | Greer |
| | **Trees** | | | |
| 14 | White ash | PNU | 1000 | Greer |
| 15 | Ash | NE | 1000 | ARTU |
| 16 | Tag alder | PNU | 1000 | Greer |
| 17 | Alder | NE | 1000 | ARTU |
| 18 | American beech | PNU | 1000 | Greer |
| 19 | Beech | NE | 1000 | ARTU |
| 20 | White oak | PNU | 1000 | Greer |
| 21 | White birch | PNU | 1000 | Greer |
| 22 | Birch | NE | 1000 | ARTU |
| 23 | American elm | PNU | 1000 | Greer |
| 24 | Elm | NE | 1000 | ARTU |
| 25 | Box elder | PNU | 1000 | Greer |
| 26 | Elder | NE | 1000 | ARTU |
| 27 | Plane | NE | 1000 | ARTU |
| 28 | White pine | PNU | 1000 | Greer |
| 29 | Hawthorn | PNU | 1000 | Greer |
| | **Weeds** | | | |
| 30 | Yellow dock | | 1000 | Greer |
| 31 | Jerusalem oak | | 1000 | Greer |
| 32 | Lambs quater | | 1000 | Greer |
| 33 | Commun mugwort | | 1000 | Greer |
| 34 | Nettle | | 1000 | Greer |
| 35 | English plantain | | 1000 | Greer |
| 36 | *Brassica spp* | | 1000 | Greer |
| 37 | *Chrysanthemum spp* | | 1000 | Greer |
| 38 | Red clover | | 1000 | Greer |
| 39 | Heather | | 1000 | ARTU |
| 40 | Kentucky blue | | 1000 | Greer |
| 41 | Meadow fescue | | 1000 | Greer |
| 42 | Orchard/Cocksfoot | | 1000 | Greer |
| 43 | Perrenial rye | | 1000 | Greer |
| 44 | Sweet vernal | | 1000 | Greer |
| 45 | Timothy | | 1000 | Greer |
| 46 | Velvet/Yorkshire fog | | 1000 | Greer |
| 47 | Red top/Bent | | 1000 | Greer |
| 48 | Couch | | 1000 | Greer |
| | **Moulds/Smuts** | | | |
| 49 | *Aspergillus mix* | | 1000 | Greer |
| 50 | *Alternaria tenius* | | 1000 | Greer |
| 51 | *Botryttis cinerea* | | 1000 | Greer |
| 52 | *Penicillium mix* | | 1000 | Greer |
| 53 | Barley smut | | 1000 | Greer |
| 54 | Oat smut | | 1000 | Greer |

### 4.2.3. Antisera

The production of polyclonal rabbit anti-feline IgE used in this study was described in chapter 2. The antiserum was passed through an affinity chromatography column containing DF coupled to cyanogen bromide activated Sepharose 4B (Pharmacia) to remove possible naturally occurring antibodies with activity against DF.

Goat anti-feline IgG, Fc specific, alkaline phosphatase-conjugated, was obtained from Bethyl Laboratories Inc. (Montgomery, Alabama, USA).

### 4.2.4. IDSTs

Prior to the IDST, all antiinflammatory drug therapy was discontinued for at least 4 weeks in the case of oral and topical medications or at least 8 weeks in the case of injectable glucocorticoids or progestagen therapy (Barbet and Halliwell, 1989; Halliwell and Gorman, 1989).

IDSTs were performed as in the previous chapters.

### 4.2.5. In vitro enzyme-linked immunoabsorbent assays (ELISAs)

4.2.5.1. Detection of DF-specific IgE

Assay conditions were optimised in preliminary experiments (chapter 3). Results were expressed in RAU by reference to the standard which was included in each plate. Serum samples were initially assessed at a dilution of 1/5 and repeated at higher or lower dilutions if the results did not fall in the sensitive part of the curve. Plates were read from 30 minutes to 45 minutes.

4.2.5.2. Detection of DF-specific IgG

Serum samples were assayed for DF-specific IgG by direct ELISA. They were initially assessed at a dilution of 1/100. Incubation time with the serum samples was reduced from 2 hr to 1.5 hr and, from 2 hr to 1 hr with the alkaline phosphatase-conjugated goat anti-feline IgG. Polyclonal anti-feline IgG was used at 1/100. Plates were read from 30 minutes to 45 minutes.

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4.2.5.3. Statistical analysis

Nonparametric Mann Whitney tests were used to perform the statistical analysis and to compare the IgE and IgG levels in the 3 groups of sera. Differences were considered significant at p<0.05.

Correlations between IgE and IgG were calculated using Pearson's rank coefficient correlation. The results were considered statistically significantly different at p<0.01.

## 4.3. RESULTS

### 4.3.1. IDST results

All the 10 cats showed skin test reactivity to at least one of the house dust mite (HDM) and/or house dust (HD) antigens (table 4.2). The most common allergens implicated by the IDST were DF and HD which both gave positive reactions in 8 cats (table 4.2). Six cats were positive to *Dermatophagoides pteronyssinus* (DP) and four cats were also positive to flea antigen (table 4.2). Other allergens to which positive reactions were seen were *Tyrophagus putresceantiae* (n=2), mixed moth (n=1), cockroach (n=1), white ash (n=1), birch (n=1), american elm (n=1), *Brassica spp* (n=1), *Chrysanthemum spp* (n=2), red clover (n=2), Heather (n=1), velvet/Yorkshire fog (n=1), red top/bent (n=1), and couch (n=1).

**Table 4.2. Levels of DF-specific IgE and IgG, and IDST results for DF, DP and HD antigens in the 10 atopic cats**

| Atopic cats | IDST | | | DF-specific IgE (RAU) | DF-specific IgG (RAU) |
|---|---|---|---|---|---|
| | DF | DP | HD | | |
| 166324 | +3 | +4 | +3 | 953 | 295793 |
| 165767 | neg. | neg. | +3 | 576 | 15259 |
| 166349 | +3 | neg. | +2 | 282 | 1722 |
| 166064 | neg. | +1 | +1 | 657 | 31170 |
| 161640 | +3 | neg. | neg. | 97 | 14456 |
| 165841 | +2 | +1 | +2 | 447 | 1317 |
| 166735 | +3 | neg. | +2 | 680 | 5543 |
| 8317 | +4 | neg. | +3 | 421 | 26031 |
| 640 | +2 | +2 | neg. | 327 | 17644 |
| Noirfa | +3 | +3 | +2 | 468 | 21177 |

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### 4.3.2. ELISA results

Although the median level of antibody to DF in the atopic cats of both isotypes was higher than that of control group 1 (457.5 vs 330 RAU for IgE and 16451 vs 9860 RAU for IgG) in neither case was the difference significant (table 4.3, fig. 4.1(a) and 4.1(b). However, in the control group 2 (laboratory reared cats) the median levels (37 RAU for IgE and 343 RAU for IgG) were significantly lower than both the atopic cats and the cats in control group 1 (p<0.001). The levels of DF-specific IgE and IgG were significantly correlated (r = 0.629, p<0.01) (fig. 4.2).

**Table 4.3. Median and range of levels of DF-specific IgE and DF-specific IgG in RAU in the atopic, normal household (control group 1) and laboratory raired cats (control group 2).**

|  | Atopic cats n = 10 | Control group 1 n=15 | Control group 2 n=11 |
|---|---|---|---|
| DF-specific IgE |  |  |  |
| Median | 457 | 330 | 37 |
| Range | 97-953 | 108-478 | 31-87 |
| DF-specific IgG |  |  |  |
| Median | 16451 | 9860 | 343 |
| Range | 1317-295493 | 78-38539 | 78-8367 |

### 4.4. DISCUSSION

Only one cat out of the 10 cats which showed positive IDST results had an adverse reaction to food. This is in accord with the other reports on food allergy in cats in which the incidence is reported to be rare (Walton, 1967; White and Sequoia, 1989; Carlotti et al., 1990; Foster and O'Dair, 1993; Rosser, 1993; Denis and Paradis, 1994; O'Dair et al., 1996).

DF is believed to be the most common allergen implicated in feline atopy. Positive reactions to DF have been reported in 77 to 100% of atopic cats (Carlotti and Prost, 1988; Foster and O'Dair, 1993; Prost, 1996a). The results of the IDST in the present study were thus in agreement with other reports, with 80% of the cats showing positive reactions to DF.
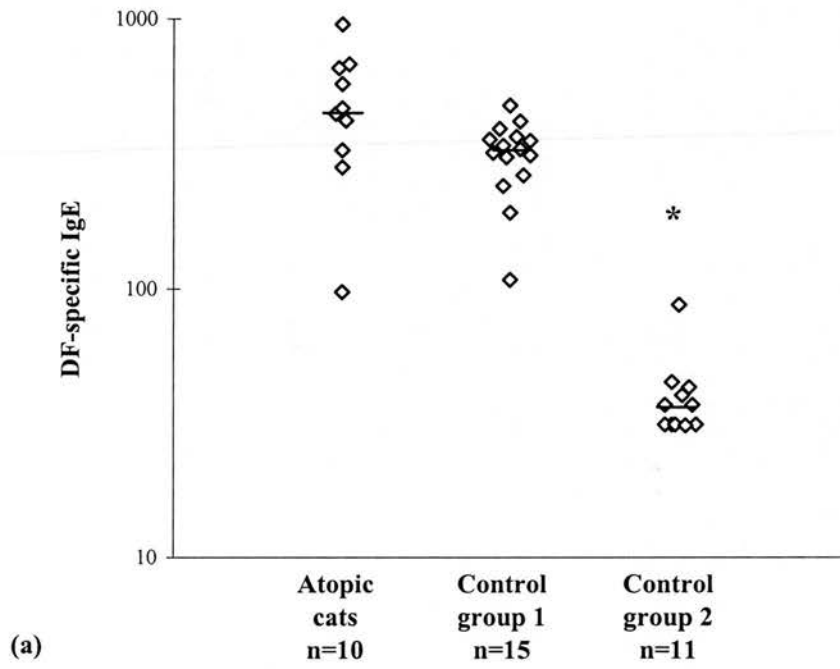
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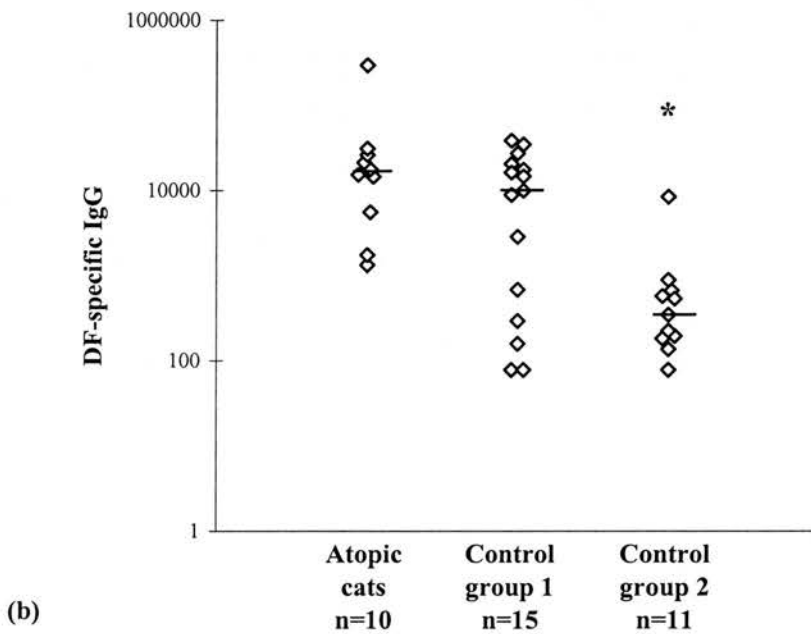
**Fig. 4.1. Levels of DF-specific IgE (a) and IgG (b) in RAU in serum from atopic, normal pet (group 1) and laboratory reared cats (control group 2).**
— Bar represents the median for each group.

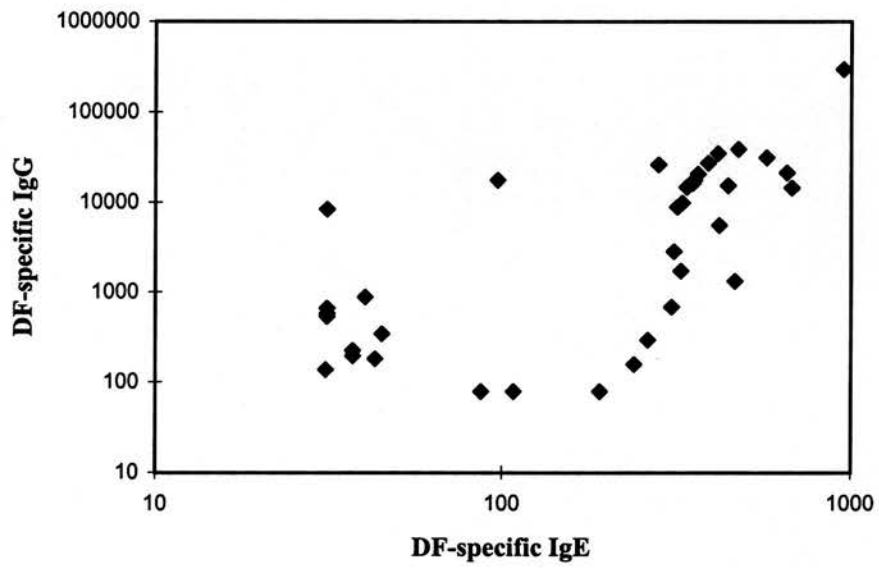* = significantly lower than the atopic group and control group 1 (p<0.001).

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Fig. 4.2. Df-specific IgE and IgG in RAU in sera from atopic and normal cats.
The levels of the two isotypes are significantly correlated (r=0.617, p<0.01).

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In human allergic patients, a good correlation has been reported between a strongly positive IDST and a positive *in vitro* test and also between a negative prick-puncture test and a negative *in vitro* test (Bousquet and Michel, 1993). In dogs, the correlation depends upon the allergen employed and appears to be good for ragweed and poor for HD (Halliwell and Kunkle, 1978; Willemse et al, 1985b; Lian and Halliwell, 1998). Contrary to man, in the dog discordant results were reported to usually consist of negative skin tests associated with positive ELISA results (Kleinbeck et al., 1989; Codner and Lessard, 1993).

The ELISA has only been introduced recently for confirmation of the diagnosis of atopic disease in cats (Foster and O'Dair, 1993). The antiserum used in the latter assay was raised against a chimaeric feline/murine IgE antibody and there is no published data supporting its specificity. A comparison of this test with results of IDSTs, assuming the latter as the gold standard, showed a poor correlation between the two and it was concluded that ELISA was unreliable in establishing a diagnosis of feline atopy. In the present study also the IDST did not always correlate well with the *in vitro* test. Of the 10 atopic cats, 2 cats were IDST negative to DF but had high levels of DF-specific IgE as assessed by ELISA whereas one had a very low level of DF-specific IgE but showed a strong skin test reactivity to DF. Although DF antigen at 1/10,000 dilution is believed to be non-irritant, it is possible that it was close to an irritant concentration in one cat and therefore the result could have represented a true false positive reaction in this case.

All the normal pet cats had detectable levels of DF-specific IgE, and high levels were found in 2 of them. However the median level of DF-specific IgE in sera from these cats was not significantly different from the median of that in the atopic cats. But it should be emphasised that the normal pet cats were not skin tested and some of the cats may have had a positive IDST to this antigen, as was recently reported in normal dogs (Lian and Halliwell, 1998). Although none of the cats showed otitis externa, some of them may have been asymptomatic carriers of *Otodectes cynotis*. Therefore, in such a case the positive ELISA tests seen in normal pet cats could have been the result of antigenic cross-reactivity between DF and *Otodectes*. Indeed cross-reactivity between HDM and *Sarcoptes* has been reported in man using both skin tests and RAST (Falk and Bolle, 1980). Because *Sarcoptes* and *Otodectes* are antigenically very close, it would not be surprising that HDM would also cross-react with *Otodectes* (Saridomichelakis et al., 1997). It was noteworthy that levels of both DF-specific IgE and IgG in the laboratory

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reared cats were significantly lower than that in both the other groups. Also, the rabbits which produced the antisera were shown to have antitibodies that reacted with DF. Therefore those rabbits may have been infected with *Psoroptes* and contain *Psoroptes*-specific IgE antibodies in their sera. For this reason, the antisera were passed through an immunoaffinity column coupled to DF prior to be used in ELISA.

The fact that not only is DF the most frequently implicated allergen in feline atopy, but also that DF-specific IgE is found in normal cats is of great interest. It has been suggested in man that the powerful allergic immune response commonly provoked by Dermatophagoides pteronyssinus (DP) could result from its ability to cleave the low affinity IgE Fc receptor (CD23) from the surface of IgE-secreting B cells (Hewitt et al., 1995). This could ablate an important feedback inhibitory mechanism that normally limits IgE synthesis. Furthermore, fragments of CD23 released by HDM have been reported to promote IgE production (Sutton and Gould, 1993).

The level of specific IgG to some aeroallergens has been shown to be higher in cats with either atopy, food and/or flea allergy than in normal cats or cats with pruritic skin disease but negative IDSTs (Foster et al., 1997). However this was not so in the case of HDM-specific IgG levels which were not significantly greater in allergic cats as compared to normal cats (Foster et al., 1997). In both the clinical and the experimental studies (chapter 3) the levels of DF-specific IgE and IgG were significantly correlated. This is not surprising since both isotypes are under the control of a Th-2 type response.

In conclusion, this antiserum also detected allergen-specific IgE in atopic cats and in normal pet cats kept under normal household conditions, but significantly lower levels were found in laboratory reared cats. In both the experimental and clinical studies there was a significant overall correlation between allergen-specific IgE and IgG responses. However, the level of allergen-specific IgE was not correlated with the skin test results. This is also consistent with the results found in normal cats in which DF-specific IgE was induced experimentally and supports the possible existence of heterogeneity of IgE antibodies in cats.

# CHAPTER 5.

# PHYSICOCHEMICAL STUDIES ON FELINE IgE AND CROSS-REACTIVITY OF FELINE IgE WITH CANINE AND HUMAN IgE

## 5.1. INTRODUCTION

Although a number of recent studies have been performed on IgE in cats, it has not been possible to purify this immunoglobulin by conventional chromatographic techniques. Recently Foster (1995) has attempted to isolate and determine feline Cε gene sequences using a nested polymerase chain reaction technique with primers based on the established sequences for sheep, rat, mouse and human homologues. He was able to establish a partial sequence for the feline Cε gene. However the antisera raised against the synthetic peptide recognised epitopes only in the peptide and not in the native molecule.

Semi-purified feline IgE has been examined by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and the MW of putative feline IgE was reported to be approximately 80 kDa (DeBoer et al., 1993). However the amino acid sequence of this molecule has never been established.

Cross-reactivity has been demonstrated between feline IgE and canine IgE using a monoclonal anti-canine IgE (DeBoer et al., 1993). However the existence of a cross-reactivity has never been shown between human and feline IgE.

The aims of this study were to assess by SDS-PAGE different fractions of partially purified IgE using conventional chromatographic techniques, and to assess the antigenic cross-reactivity of feline reaginic antibody with canine IgE and human IgE using polyclonal anti-human and anti- canine IgE of different origins.

## 5.2. MATERIALS AND METHODS

### 5.2.1. Physicochemical studies on feline IgE

#### 5.2.1.1. Experimental animals

Stray kittens and adult cats were provided by the Dog and Cat Home (Société Protectrice des Animaux, SPA) of Vervier, Belgium.

One New Zealand white rabbit was obtained from Interfauna UK limited.

All experimental protocols were in accordance with the requirements of the Animal Care and Ethics Committee and/or the UK Home Office as appropriate.

#### 5.2.1.2. Sera and IgE antibodies

The stray cats were anaesthetised with 20 μg/kg medetomidine hydrochloride (Domitor®, Smithkline Beecham, Louvain-La-Neuve, Belgium) and 10 mg/kg ketamine hydrochloride (Imalgène 1000®, Rhône Mérieux, Brussels, Belgium) and bled by cardiac puncture. Blood was centrifuged and approximately 45 ml (kittens) to 90 ml (adults) of sera were collected from each cat. They were then euthanised with 5 ml of pentobarbital sodium (Natriumpentobarbital 20%®, Kela, Hoosgraten, Belgium) and their gastrointestinal (GI) tract opened to check for the presence of adult *Toxocara cati* (*T. Cati*).

Sera from cats parasitised with *T. cati* were those from the cats immunised with DNP-Asc (see chapter 2).

Sera with a high level of *Dermatophagoides farinae* (DF)-specific IgE were the sera from the cats B85 and A71 experimentally sensitised to DF (bleeding 8), which showed a strong intradermal skin test (IDST) to this antigen (see chapter 3).

Partially purified canine IgE was kindly provided by Prof. R.E.W. Halliwell.

Monoclonal mouse IgE was purchased from Sigma (Sigma).

#### 5.2.1.3. Antisera

Rabbit anti-feline IgE used were R294 and R1094 (chapter 2).

Rabbit anti-feline IgG, heavy chain (HC)-specific, was purchased from Nordic Immunology.

5.2.1.4. Immunoaffinity chromatography columns

DF antigen (Greer laboratories, Lenoir, North Carolina, USA) and polyclonal antisera against feline IgE (R294 and R1094) and against feline IgG were conjugated to cyanogen bromide activated Sepharose 4B (see appendix).

A protein A column (HiTrap affinity columns) was purchased from Pharmacia.

5.2.1.5. Preparation of feline IgE rich fractions

Different approaches were used to obtain feline IgE rich fractions in order to assess these samples for their IgE content by SDS-PAGE.

(a) Sixty ml of a pool of sera from stray cats and cats parasitised with *T. cati* were precipitated with ammonium sulphate. The protein that precipitated between 33% and 45% saturation was equilibrated with 0.05M phosphate buffer, pH 8.0, and passed over a column of DEAE equilibrated with the same buffer (see chapter 2). The excluded protein was collected, concentrated and then passed over a 3-in-series column set of Sephacryl S-200 in PBS. Fr-R and Fr-R+G were collected and concentrated to a protein concentration of 1 mg/ml for Fr-R and 3.2 mg/ml for Fr-R+G (see chapter 2). These fractions were assessed by SDS-PAGE.

(b) Fraction R+G obtained above (a) was passed through the putative anti-feline IgE (R294 or R1094) affinity column equilibrated with PBS. The protein bound to the column was eluted with 0.1M glycine HCl buffer, pH 2.8. The eluate was neutralised immediately with 1M Tris-HCl buffer, pH 8 and concentrated. It was then assessed by SDS-PAGE.

(c) Five replicates of two ml each of serum from parasitised cats were passed through the putative anti-feline IgE affinity column and the eluates were collected, pooled together and concentrated to a protein concentration of 750 µg/ml. The resulting fraction was assessed by SDS-PAGE.

(d) Three ml aliquots of sera from cats which had a high level of DF-specific IgE were passed 3 times through an immunoaffinity column of Sepharose 4B coupled to DF. A total of 36 ml of sera were applied on the columns. Bound material was eluted and neutralised as above. In order to remove contaminating IgG, the IgE-containing fraction was repeatedly passed down to through an immunoaffinity column of anti-feline IgG, which was heavy chain specific, equilibrated with PBS, until the eluate peak was

negligible (4 times). The fall-through (FT) was concentrated to 2 mg/ml and assessed by SDS-PAGE.

(e) Sera from 2 stray cats were fractionated by the 3 step procedure (a). The resulting Fr-R (1 mg/ml) was cross-adsorbed by successive passages through an immunoaffinity column of anti-feline IgG and the FT concentrated to a final protein concentration of 2.5 mg/ml. One ml of this solution was dialysed in PBS overnight at 4°C and filtered before application to the protein A column (HiTrap affinity columns, Pharmacia, Biotech) equilibrated with 0.1M sodium phosphate buffer, pH 8.0. Material bound to the column was eluted with 0.1M glycine HCl buffer, pH 2.8 and neutralised immediately with 1M Tris-HCl buffer, pH 8.0. FT (400 µg/ml) and eluate (300 µg/ml) were both assessed by SDS-PAGE. The FT peak was chosen to perform the terminal amino-acid sequencing.

5.2.1.6. Assessment of the different fractions by SDS-PAGE

IgE containing fractions were analysed by SDS-PAGE using a 10% polyacrylamide gel run under reducing conditions as described by Laemmli (1970).

Gels were cast and run using the mini-Protean system (82 x 73 x 0.75 mm) or the large format gel (20 x 20 cm) (Bio-Rad Lab, Ltd., Hemel Hempstead, UK). To form the mini-gel, 3.5 ml of 10% acrylamide resolving gel containing 0.33% N, N - methylenebisacrylamide (acrylamide bis) and 0.5% sodium dodecylsulphate (SDS) in 0.375M Tris-HCl buffer pH 8.8 were polymerised by the addition of 50 µl of a freshly prepared 10% ammonium persulphate solution and 10 µl of N, N, N, N' - tetramethylethylene diamine (Temed, BDH, Poole, UK). The resolving gel was poured into each gel sandwich and carefully overlaid with distilled water (dH$_2$0). After complete polymerisation, the water was removed. The 4% acrylamide stacking gel containing 0.1% acrylamide bis, 0.25% SDS in 0.125M Tris-HCl buffer, pH 6.8, a 10% ammonium persulphate solution and Temed was added and allowed to polymerise with a 10-well comb in place.

Samples were diluted 1:1 in SDS-PAGE sample buffer containing 10% glycerol, 2% SDS, 5% 2-β-mercaptoethanol and 0.1% bromophenol blue in 62mM Tris-HCl, pH 6.8. Samples were then heated in a boiling water bath for at least 2 minutes before immediate loading onto a gel. Twenty µl of each preparation were loaded and run for approximately 55 minutes at room temperature at a constant 200 V. Power was supplied

by a Pharmacia Power Supply EPS 500/400 power pack. Running buffer, 192mM glycine, 25mM Tris and 1% SDS, pH 8.3, was freshly prepared before each run.

Gels were stained for 30 minutes with 0.25% Coomassie blue solution (Coomassie brilliant blue, R-250, Biorad Laboratories Ltd, Hemel Hempstead, Herts) in 45% methanol, 7.5% alcohol, 45% $H_2O$ and destained in successive changes of 25% methanol, 7.5% acetic acid, 67.5% $H_2O$.

When large format gels were used, they were cast and run following the same method as used for the mini-gel. However the stacking gel (40 ml) and the resolving gel (20 ml) were degassed under vacuum prior to the addition of the ammonium persulphate solution and Temed.

Large format gels were cast using 15-lane combs and loaded with 80 to 100 μl of sample. Gels were run for approximately 4 hours at room temperature at 50mA constant current. Power was supplied by a Pharmacia Power Supply EPS 500/400 power pack.

Large format gels were stained in 0.25% Coomassie blue solution as the mini gel and destained overnight in 25% methanol:7.5% acetic acid:75.5% $H_2O$.

In addition to the high molecular weight markers usually employed (Bio-Rad Lab, Ltd., Hemel Hempstead, UK), partially purified canine IgE and/or purified mouse monoclonal IgE were used as positive controls and feline IgG, as negative control molecular weight markers.

## 5.2.1.7. Preparation of a gel band in order to immunise a rabbit

An IgE preparation resulting from the elution of serum #6 (see chapter 2) after passages through the anti-IgE affinity column was applied to SDS-PAGE on a large-format gel. Gel was polymerised with a comb composed of one large and 2 small wells in place. Four hundred and fifty μl corresponding to 450 μg of the feline IgE sample were diluted in the sample buffer 1:1 and loaded on the large well without being heated. A sample of 50 μl of canine IgE and the molecular weight standard were loaded on the small wells. After electrophoresis, the gel was washed in several changes of deionized water and transferred to 0.3M $CuCl_2$ for 10 minutes. The relevant band of the gel corresponding to the molecular weight (MW) of the IgE heavy chain was excised using a scalpel, and destained by incubation in 0.25M EDTA, 0.25M Tris, pH 9.0 with 3 changes of 5 minutes each. The gel was pestled in 0.5 ml of PBS and stored at -20°C prior to immunisation. In order to obtain the 3 fractions necessary for the immunisation of the rabbit, the procedure was performed 3 times. Each sample was mixed with

Freund's complete adjuvant (CFA) prior to injection as for the production of the other antisera (see chapter 2). This antiserum was assessed for its activity against feline IgE by reversed cutaneous anaphylaxis (RCA) as described in chapter 2.

5.2.1.8. Immunoblot for terminal amino-acid sequencing of suspected feline IgE

In order to perform an amino (N)-terminal amino-acid sequencing, Tris-Tricine SDS-PAGE (Schägger gel) was made (Schägger and von Jagow, 1987). Gels were cast and run using the mini-Protean system as described above. To form the mini-gel, 10 ml of Tris-Tricine gel mixture containing 10% acrylamide bis and 0.1% SDS in 1M Tris buffer pH 8.45 were polymerised by the addition of 50 μl of a freshly prepared 10% ammonium persulphate solution and 10 μl of Temed. Three and a half ml of the resolving gel was poured into each gel sandwich and carefully overlaid with dH$_2$0. After complete polymerisation, the water was removed. The 4% acrylamide stacking gel containing 0.1% acrylamide bis, 0.25% SDS in 0.125M Tris-HCl buffer, pH 6.8, a 10% ammonium persulphate solution and Temed was added and allowed to polymerise with a 10-well comb in place.

Samples (feline IgE rich fraction prepared as in (e) and partially purified canine IgE) were diluted 3:2 in SDS-PAGE sample buffer and then heated, as described above. Five μl of high molecular weight markers (Bio-Rad Lab), 45 μl of feline IgE and 25 μl of canine IgE preparations were loaded and run for approximately 1 hour 20 minutes at room temperature at a constant 110 V. Running buffers used were in the upper tank 100mM Tris buffer, 100mM Tricine and 0.1% SDS and the lower tank 1M Tris buffer, pH 8.9.

The gel was transblotted onto "Immobilon P" soaked in 10 mM (3-(cyclohexylamino)-1-propanesulfonic acid) (CAPS) in 10% methanol using a semi-dry blotter.

Briefly, 6 sheets of Whatman chromatography paper number 3 (Whatman, Maidstone, UK) and a sheet of PVDF membrane with 0.2 mm pore size (Immobilon P, Millipore Corporation, Bedford, Massachussetts, USA) were trimmed to the appropriate size. Three sheets of chromatography paper were soaked in CAPS buffer and placed on the anode graphite plate. The immobilon P membrane was soaked in methanol and in dH$_2$0 and then placed on top of the 3 layers of paper. The gel was placed carefully on the immobilon P membrane and the sandwich was completed by the addition of 3 further sheets of paper soaked in CAPS buffer. The transfer cell was completed by placing the

cathode plate on the top and the constant current of 80 mA was applied from a Bio-Rad power pack (model 160/1.6) for 1 hour 30 minutes.

The blot was immediately stained for 10 seconds with 0.25% Coomassie blue solution as for the gel and destained in methanol:acetic acid:$H_2O$ 5:1:5.

The band on the resulting blot that corresponded to the MW of IgE heavy chain was cut and sent to Mr Pat Baker at the Microchemical Facilities of Brabaham Institute in Cambridge for a terminal amino acid sequencing.

## 5.2.2. Assessment of antigenic cross-reactivity of feline IgE with canine and human IgE

### 5.2.2.1. Experimental animals

Recipients were normal adult parasite free cats (ex-breeder) obtained from Hill Grove Family Farm Ltd.

### 5.2.2.2. Sera

Sera from cats parasitised with *T. cati* were the sera from the cats #6 and #4 which were immunised with DNP-Asc (see chapter 2).

Sera B85 and 735, bleeding 8 were from the cats experimentally sensitised to DF, which showed a strong IDST and/or developed a good IgE response to DF antigen (see chapter 3).

### 5.2.2.3. Antisera

Polyclonal rabbit, goat and sheep anti-human IgE, HC-specific were purchased from Nordic Immunology.

Twelve polyclonal antisera, 3 from goats and 9 from rabbits specific for canine IgE, were previously produced by Prof. R.E.W. Halliwell in his laboratory. One goat and 2 rabbit antisera were purified antibodies.

### 5.2.2.4. Immunoaffinity chromatography columns

Normal rabbit serum, polyclonal goat anti-canine IgE and rabbit anti-human IgE were conjugated to cyanogen bromide activated Sepharose 4B.

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5.2.2.5. Induction of RCA

RCA was performed as before (see chapter 2). Antisera were tested at dilutions of 1:100 and 1:1000. The antisera which gave a strong reactions at 1/1000 dilution were assessed at doubling dilutions from 1/100. A rabbit anti-feline IgE (R294 or R1094) known for its ability to induce strong RCA was included as a positive control.

5.2.2.6. Immunoabsorption of feline sera rich in IgE by goat anti-canine IgE

Sera from 2 cats (#4 and #6) were fractionated by the 3 step procedure as described above (a). The resulting Fr-R+G (1 mg/ml) was adsorbed by successive passages through an immunoaffinity column of anti-canine IgE column. The proteins bound were eluted, collected and the pH immediately neutralised as described above. The eluate was concentrated up to a protein concentration of 620 µg/ml and assessed by SDS-PAGE as described above.

5.2.2.7. Immunoabsorption of feline sera rich in IgE by rabbit anti-human IgE

Rabbit anti-human IgE was also assessed for activity against IgE by its ability to remove ELISA reactivity from feline serum (B85 and 735) after passages through an anti-human IgE immunoabsorbent column. ELISAs were performed as described in the clinical study on atopic cats (chapter 4). All fractions were tested at a protein concentration of 44 mg/ml, which corresponded at a serum dilution of approximately 1/2. Samples were tested in quadruplicate. Bleeding 8 of cat U49 was used as the standard.

## 5.3. RESULTS

5.3.1. Physicochemical studies on feline IgE

5.3.1.1. Fractions assessed by SDS-PAGE

(a) Fractions R and R+G

Multiple protein bands were seen in the gel (fig. 5.1). However the two major bands were the IgG heavy chain and the light chain. A pale band corresponding to the MW of canine IgE was present. This band had an apparent MW of 80 kDa. However the MW of the protein bands were only estimated by reference to the marker and the positive control and were not calculated.

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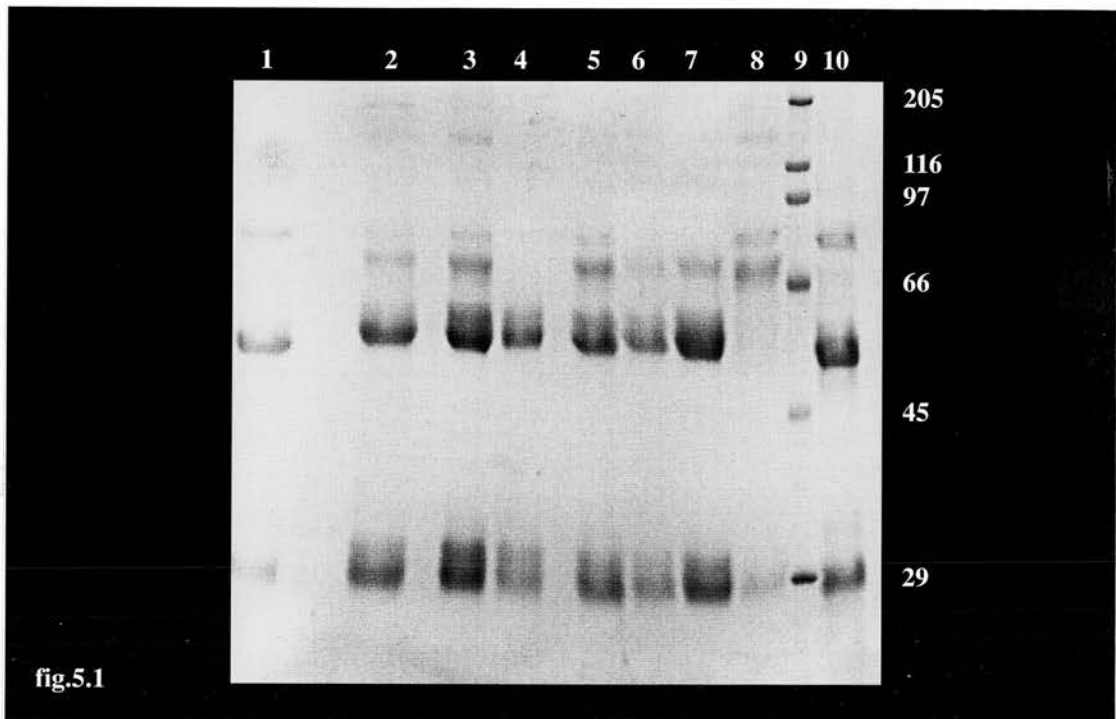
**Fig. 5.1. SDS-gel electrophoresis. Assessment of Fr-R and R+G and of the feline proteins in the fall through (FT) and eluate from the protein A affinity column**

| 1 | Canine IgE partially purified |
|---|---|
| 2 | Fr-R |
| 3 | Fr-R+G |
| 4 | Feline IgG eluted from the anti-IgG affinity column |
| 5 | FT from the anti-IgG affinity column |
| 6 | Eluate from the protein A affinity column |
| 7 | Eluate from the protein A affinity column |
| 8 | FT from the protein A affinity column |
| 9 | Molecular weight marker |
| 10 | Canine IgE partially purified |

(b and c) Eluates from the putative anti-feline IgE affinity column.

Multiple proteins were again present in the fractions eluted from the anti-feline IgE affinity column (fig. 5.2). The two major protein bands seen on the gel were also the IgG heavy chain and the light chain. In the Fr-R+G eluted from the anti-feline IgE affinity column, a minor very thin and pale band corresponded to the canine IgE heavy chain molecule with a MW of 80 kDa. In the sera of parasitised cats eluted from the putative anti-feline IgE affinity column, two close protein bands seen on the gel were of an approximate calculated MW of 83 and 87 kDa, whereas the approximate calculated MW of the canine IgE was of 79 kDa.

(d) Eluates from the DF affinity column adsorbed by successive passages through an affinity column of anti-feline IgG heavy chain.

The major protein bands corresponded to IgG heavy chain and to another protein of approximately 66 to 70 kDa (fig. 5.3). A thin band corresponded to the MW of canine IgE heavy chain and had an estimated MW of 80 kDa. Another paler, thin band of slightly higher MW was seen. Because the gel was run for longer than normal, the light chain was not visible.

(e) Eluate and FT from the protein A affinity column.

When run on the gel, the FT peak was found to contain only 3 protein bands (fig. 5.1). One band corresponded to the light chain and one had a molecular weight of approximately 70 kDa. The last band was pale but relatively large and had a similar molecular weight to the canine IgE heavy chain which was of approximately 80 kDa. None of the bands corresponded to the heavy chain of IgG.

The eluate from the protein A affinity column was composed of 3 protein bands (fig. 5.1). The major band corresponded to IgG heavy chain while another band corresponded to the light chain. The third band was minor and had an approximate molecular weight of 70 kDa.

5.3.1.2. RCA tests

The antiserum resulting from the immunisation of a rabbit with the gel bands was not able to induce RCA in a recipient cat.
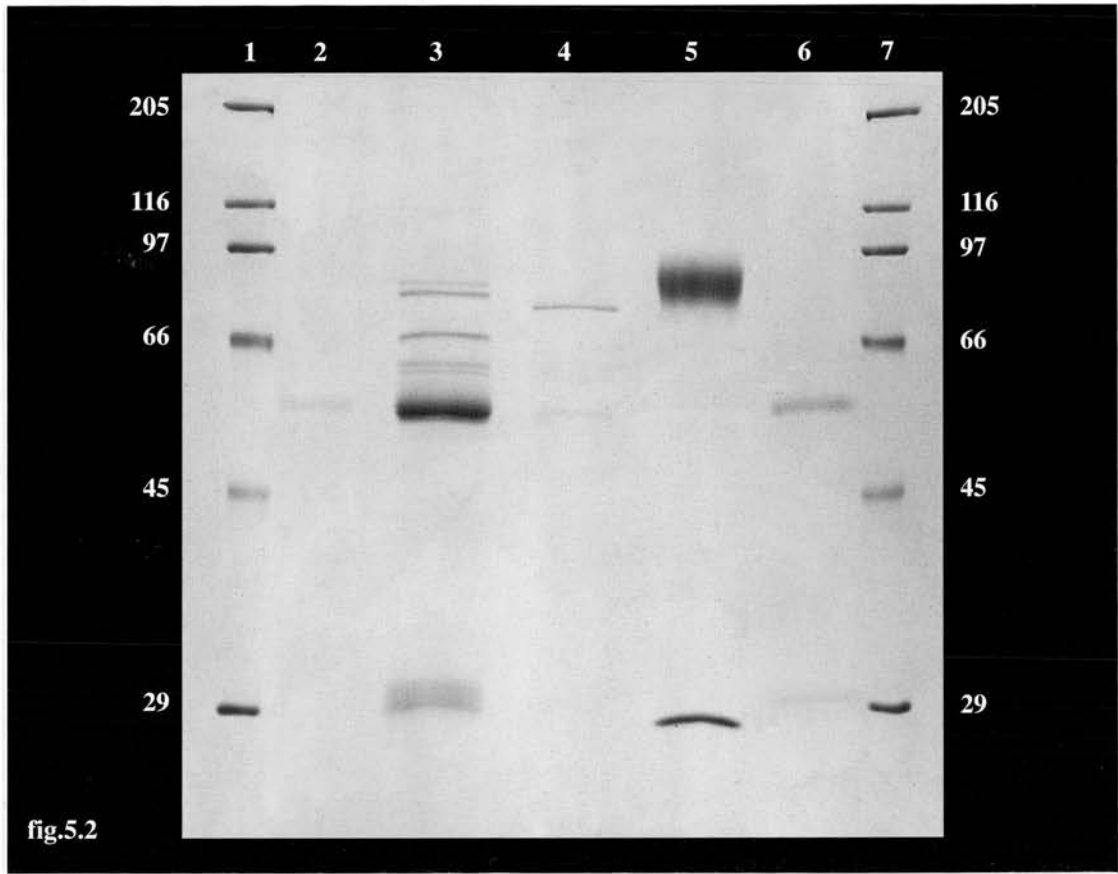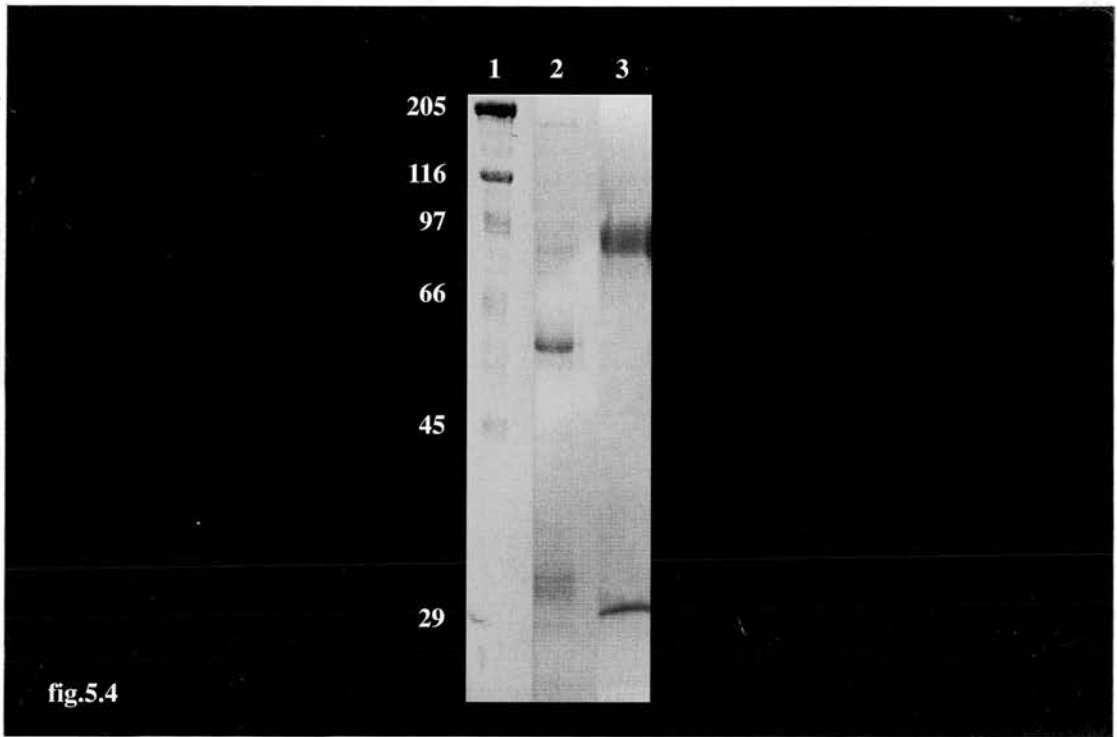
**Fig. 5.2. SDS-gel electrophoresis. Assessment of Fr-R+G eluate from the putative anti-feline IgE affinity column**

1       Molecular weight marker

2       Feline IgG

3       Eluate from the anti-feline IgE affinity column

4       Canine IgE partially purified

5       Mouse IgE

6       Feline IgG

7       Molecular weight marker

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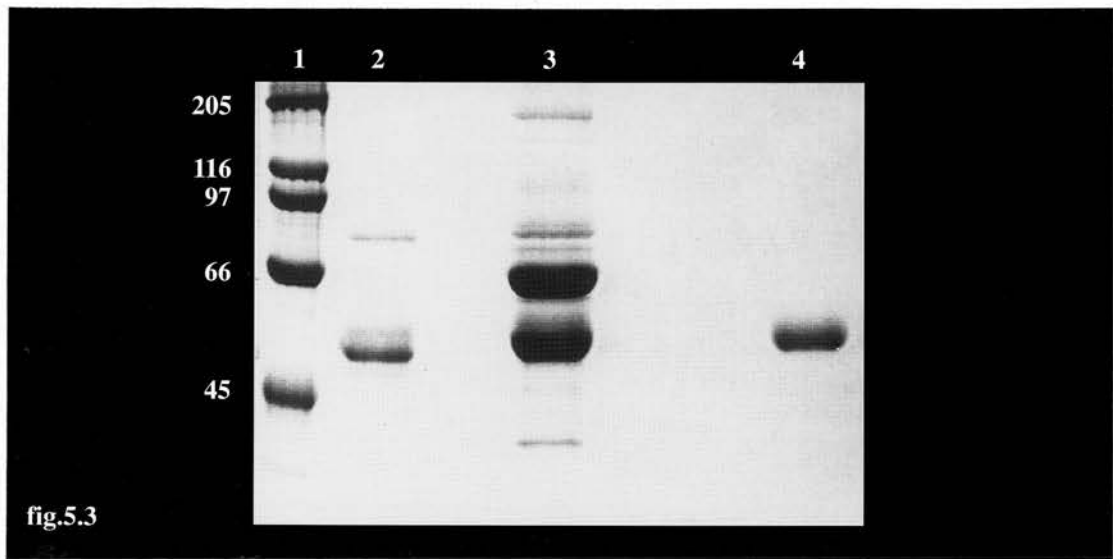
**Fig. 5.3. SDS-gel electrophoresis. Assessment of serum from DF immunised cats eluted from the HDM affinity column**

1       Molecular weight marker

2       Canine IgE partially purified

3       Serum from parasitised cat eluted from the HDM affinity column

4       Feline IgG eluted from the anti-IgG affinity column

### 5.3.1.3. Amino-terminal amino-acid sequencing

The N-terminal amino acid sequence of the protein sent for sequencing (see section 5.1.8) shared more identity with the terminal amino acid sequence of a sheep fibrinogen β-chain (50% identity) or $\alpha_2$-macroglobulin (25% identity) and was not of an antibody (table 5. 1). Only one residue (11) of this molecule was common to the human IgE heavy chain (5% identity) (table 5.1).

**Table 5.1. Terminal amino-acid sequence of the approximately 80 kDa feline protein, the human IgE heavy chain, the fibrinogen β-chain and the $\alpha_2$-macro- globulin**

|  | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Feline protein | V | L | P | L | V | E | V | P | X | L | L | D | L | P | G | V | D | D | N | P |
|  |  |  |  | E | K |  | L | G |  | D |  | V | K | A | S? | L | G | A |  |  |
| Human IgE | A | S | T | Q | S | P | S | V | F | P | L | T | R | C | C | K | N | I | P | S |
| Fibrinogen β-chain |  |  |  |  |  |  |  |  | G | Y | L | D | Y | D | E | W | D | D | N | R |
| $\alpha_2$-Macro-globulin | P | Q | Y | M | V | L | V | P | S | L | L | H | T | E | T | T | E | K | G | C |

### 5.3.2. Assessment of antigenic cross-reactivity of feline IgE with canine and human IgE

### 5.3.2.1. Induction of reversed cutaneous anaphylaxis

### 5.3.2.1.1. Rabbit and goat anti-canine IgE

Twelve polyclonal antisera were assessed for activity against IgE by their ability to induce reversed cutaneous anaphylaxis (RCA) on intradermal injection in healthy parasite-free cats

All rabbit and goat anti-canine IgE were able to induce RCA at a dilution of 1/100 (table 5.2). However 5 rabbit antisera had a RCA titre of 1000, 4 antisera (2 rabbits and 2 goats) had a RCA titre of 1600 and one antiserum (the purified goat antiserum), a RCA titre of 4000 (table 5.2). Because of the small amount of specific purified goat and rabbit antisera available, the goat antiserum GB was chosen to be coupled to the cyanogen bromide activated Sepharose 4B.

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## 5.3.1.2. Rabbit, goat and sheep anti-human IgE

Rabbit anti-human IgE was the only antiserum which was able to induce a RCA at a dilution of 1/10 and 1/100 although weak reactions only resulted. An immunoaffinity column was made with this antiserum.

**Table 5.2. RCA titres of polyclonal anti-canine and anti-human IgE**

| Anti-human IgE | RCA titre* | Anti-canine IgE | RCA titre |
|---|---|---|---|
| R | 100 | R1 | 100 |
| G | Negative | R2 | 100 |
| S | Negative | R3 | 1000 |
| | | R4 | 1000 |
| | | R5 | 1000 |
| | | R6 | 1000 |
| | | R7 | 1000 |
| | | Rp | 1600 |
| | | GG | 1600 |
| | | GB | 1600 |
| | | GpW | 4000 |
| | | RpZ | 1600 |

R = rabbit antiserum, Rp = rabbit antiserum: purified antibody
G = goat antiserum, Gp = goat antiserum: purified antibody
S = sheep antiserum
* Highest dilution of antiserum to which a weal resulted following intradermal injection

## 5.3.2.2. Immunoabsorption of feline sera rich in IgE by goat anti-canine IgE

The eluted proteins consisted of three bands on the gel (fig. 5.4). The major band corresponded to the heavy chain of IgG and the lowest band, to the light chain. The third band was very pale and had a MW which was similar to the mouse IgE heavy chain and was estimated to be of approximately 80 kDa. However the MW was not calculated.

**Fig. 5.4. SDS-gel electrophoresis. Assessement of Fr-R+G eluted from the anti-canine IgE affinity column**

1       Molecular weight marker

2       Eluate from the anti-canine IgE affinity column

3       Mouse IgE

### 5.3.2.3. Immunoabsorption of feline sera rich in IgE by rabbit anti-human IgE

Two ml of serum from 2 cats that developed a strong positive IDST to DF antigen and/or that had a high level of DF-specific IgE (B85 and 735) was assessed by ELISA before and after 4 passages through a rabbit anti-human IgE immunoabsorbent column. Two ml of these feline sera rich in DF-specific IgE were also assessed by ELISA before and after 4 passages through a column of Sepharose 4B coupled to normal rabbit serum. The later fraction was used as a control.

The levels of DF-specific IgE were significantly different before and after 4 passages through the anti-human IgE immunoaffinity column. However, no significant difference was seen between the results of the sera after the passages on the normal rabbit serum immunoaffinity column and the passages on the anti-human IgE immunoaffinity column.

## 5.4. DISCUSSION

### 5.4.1. Physicochemical studies on feline IgE

The molecular weight of human monoclonal IgE heavy chains ($\varepsilon$ chain) have been reported to vary from 69.8 kDa (Kochwa et al, 1972), 70 kDa (Peng et al., 1996), 72.3 kDa (Ishizaka, 1988), to 72.5 kDa (Bennich and Johansson, 1971). The MW of canine $\varepsilon$ chains have also been estimated to vary and to range from 73 kDa (Peng et al., 1993a), 75 kDa (Peng et al., 1996) to 77 kDa (DeBoer et al., 1993) whereas the MW of mouse IgE heavy chain has been reported to be 75 KDa (Bose et al., 1984) and of sheep and pig to be of 72 and 73 KDa respectively (Yilmaz et al., 1993; Roe et al., 1993).

In the present study a protein which had a similar MW to both canine and mouse IgE was present in almost all fractions assessed. However, although different methods were used to attempt to obtain an IgE rich fraction, resulting samples were always contaminated by other proteins. Feline IgG heavy chain, with a calculated MW of 57 kDa was found to be the major protein present in all samples, except in the FT from the protein A column. Even after 4 passages of the sample eluted from the HDM column, through the feline anti-IgG column, IgG was still present in large amounts. Another band of protein with a MW of approximately 70 kDa was visible on most of the gels. This protein was believed to be albumin.

The protein which had an apparent MW of 80 kDa was not found in the eluate from the protein A column. This could be because of the low protein content of the

fraction. However further concentration of this fraction would have led to an overloading of the gel with IgG. On the other hand, each concentration of the sample led to loss of protein and therefore of IgE molecules, presumably already present in very small quantities. The 80 kDa band was present in the FT of the protein A column, with 2 other bands. One corresponded to the immunoglobulin light chain whereas the other one had an approximate MW of 70 kDa, which could correspond to the albumin which has been reported to have a MW of 69 kDa (Spitzauer et al., 1995) or the IgM heavy chain which has a MW of 74 kDa (Grant, 1995). Nevertheless, Foster (1995) has found the MW of feline IgM heavy chain to be 85 KDa. However after elution from DEAE at 0.05M phosphate buffer pH 8.0 and gel filtration chromatography on Sephacryl S-200, the fraction would be very unlikely to contain any IgM antibodies. Therefore it was assumed that the light chain may belong to the feline protein which had a similar MW to canine IgE and could have represented the IgE molecule. This is the reason why this fraction was chosen to perform a terminal amino acid sequencing. Also, Foster et al. (1995) demonstrated that although the fraction eluted from the protein A column induced stronger positive passive cutaneous anaphylaxis (PCA) tests, the FT was also able to induce positive PCA tests. It appears therefore that in cats, as in dogs (Peng et al., 1991), a proportion of IgE does not bind to protein A. Peng et al. (1997) have suggested that this could be reflective of heterogeneity of canine IgE. The N-terminal amino acid sequencing of the band with a MW similar to the canine or mouse ε chain was not consistent with the expected IgE heavy chain. Whereas the amino acid homology between ε chain and other immunoglobulin heavy chains is low (Liu, 1986), ε chain has been shown to have a high homology between species. However while the N-terminal amino acid sequencing allows to differentiate an antibody from another protein, this method would not permit the differentiation between isotypes. The protein sequenced may still have masked the presence of a smaller amount of ε chain.

DeBoer et al. (1993) separated by SDS-PAGE feline serum proteins eluted from a monoclonal anti-canine IgE immunoaffinity matrix. They have estimated the MW of a putative feline IgE heavy chain to be of approximately 80 kDa and therefore to be slightly higher than canine IgE heavy chain. The sample was contaminated by IgG and another protein which had a MW of 71 kDa and was believed to be albumin (DeBoer et al., 1993). However in this study the band corresponding to the light chain was almost not apparent on the gel and should have been stronger in comparison to the thickness of the IgE heavy chain band. Therefore the band of the light chain seems more to

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correspond to the weaker IgG heavy chain band, and the protein band of 79.7 kDa may have been a contaminant rather than the ε chain.

In dogs, Peng et al. (1991) have reported that 100% of the IgA and IgG, 98% of the IgM and 87% of IgE binds to the protein A. The affinity of feline immunoglobulins for protein A was found to be completely different from the affinity of canine immunoglobulins (Grant, 1995). Indeed, the major fraction of cat IgM and IgA was found not to bind to protein A, and this could be the result of different subclasses of these immunoglobulins with different affinities. However, as in dogs (Peng et al., 1991), the bulk of the feline IgG subclasses have been reported to bind to protein A (Grant, 1995). In the present study, all the feline IgG was recovered in the fraction that bound to the protein A column.

Feline immunoglobulins are reported to have a very low affinity for protein G and therefore the latter is not useful to the purification of IgE in the cat (Foster et al., 1995; Grant, 1995).

In the fractions eluted from the putative anti-feline IgE column and from the HDM column, the band corresponding to the canine or mouse ε chain was a doublet. The bottom band was thicker than the top band. These findings could have resulted from the denaturation of the protein by the acidity of glycine-HCl buffer. However only a single band was present in the fraction eluted from the goat anti-canine IgE column. Because the band protein was very thin, the double band may not have been visible.

IgG was found in almost all samples, even in the fraction eluted from the putative anti-feline IgE column. However this anti-feline IgE coupled to the Sepharose 4B had been absorbed by passage through column of heated normal cat serum and proved to be specific for feline IgE in the ELISA. Thus it appears that non-specific binding of IgG occurs easily and that it is difficult to obtain an IgE fraction that is not contaminated by IgG. While the passage of such fractions through the protein A column allows depletion of the IgG, it is not a good method for purifying IgE since not all IgE binds to protein A.

The antibody raised against the gel band which had a MW similar to the canine IgE did not shown any activity against feline IgE. It is possible that the protein band injected was composed of a protein other than IgE, as for example the protein which was assessed by N-terminal amino acid sequencing. It could also be the result of the denaturation of the protein band by the mercaptoethanol contained in the SDS-PAGE sample buffer since it has been shown that IgE is sensitive to the mercaptoethanol (Rockey and Schwartzman, 1967; Ishizaka and Ishizaka, 1968a). Therefore the

antiserum raised against the putative IgE heavy chain may not have been able to recognise the native protein. It is also possible that there was not sufficient protein present to generate an immune response.

It would have been of great interest to perform a western blot analysis on these gel using the polyclonal anti-feline IgE (R294) and goat anti-canine IgE. Unfortunately the small quantity available of the samples and the reagents made the performance of such studies difficult.


5.4.2. Assessment of antigenic cross-reactivity of feline IgE with canine and human IgE

In the present study, while only a weak RCA was obtained using polyclonal rabbit anti-human IgE, goat and sheep polyclonal antibodies were not able to induce RCA. Also, the absorption of sera of cats sensitised to DF with polyclonal rabbit anti-human IgE did not result in a reduction of reaginic antibody in those sera as compared with multiple passages over a column of normal rabbit serum. However it is possible that the net amount of anti-human IgE on the column was too low to bind sufficient feline IgE to remove ELISA reactivity, which would have reduced the effective sensitivity of this approach. On the other hand, the weak positive RCA at a dilution of 1/100 seen with the rabbit anti-human IgE is unlikely to be a false positive reaction but rather reflective of a true but weak cross-reactivity between human and feline IgE, since a negative control was used in each test.

In previously reported studies IgG, IgA and IgM were all demonstrated to cross-react with the equivalent human isotype (Grant, 1995). The cross-reactivity between IgE of several species with human IgE was assessed by gel diffusion and rat mast cell binding using a sheep anti-human IgE (Nielsen, 1977). The percentage of cross-reactivity of feline and canine sera with the sheep anti-human IgE was reported to be very low (Nielsen, 1977). However, in another study, Halliwell et al. (1972) demonstrated the antigenic similarity between canine IgE and human IgE. They showed that the PK titre to ragweed was decreased by absorption of canine reaginic serum with anti-human IgE and that anti-human IgE was able to elicit RCA in the dog. More recently, Peng et al. (1993a and 1996) have directly demonstrated the cross reactivity of dog IgE with human IgE by western blot analysis using either a polyclonal or a monoclonal anti-human IgE. Moreover, canine reaginic antibody from dogs suffering from allergic disease is able to sensitise both human and equine basophils (Prélaud et al., 1993; Sainte-Laudy and Prost, 1996). It has been shown by RCA as well as by other

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methods (ELISA, RIA, gel diffusion, western blot and histamine release by mast cells and basophils) that human IgE also cross-reacts with IgE from monkey (Ishizaka and Ishizaka, 1968b), rat (Conrad et al., 1983; Kanyeresi et al., 1971; Perelmuter and Khera, 1970), bovine (Gershwin, 1981; Nielsen, 1977), ovine (Yilmaz et al., 1994) and equine (Suter and Fey, 1983).

A recent study has demonstrated that polyclonal anti-mouse IgE and anti-human IgE were able to induce RCA in dogs while monoclonal anti-human, anti-rat and anti-mouse were not (Peng et al., 1996). Therefore the IgE structure appears to be well conserved from species to species. However, of all five isotypes of immunoglobulin, the sequence of the IgE C region is the least well conserved across species (Patel et al., 1995).

Feline reaginic antibody has been demonstrated to cross react with canine IgE by RCA and by absorption on affinity column using a monoclonal anti-canine IgE (DeBoer et al., 1993). Both monoclonal and polyclonal anti-canine IgE induced a wheal reaction at a dilution of 1/1000, 15 minutes after intradermal injection. In the present study, all the polyclonal anti-canine IgE were able to induce RCA, with some of them showing titres of 1600 and even 4000 for a specific purified goat anti-canine IgE.

In conclusion, feline IgE appears to be more difficult to isolate and purify than canine IgE. One of the reasons is that cat sera are available in smaller quantities than dog sera. However, feline IgE, through its affinity for protein A and protein G, behaves differently from canine IgE and IgE from other species. The existence, in almost all samples, of a protein contaminant with an apparent MW similar to the MW of IgE from other species, makes it even more difficult to identify feline IgE. Cross-reactivity between feline IgE and canine IgE has been demonstrated by RCA. However the level of cross-reactivity between human IgE and feline IgE was obviously low in that, although a positive RCA at low dilution resulted, anti-human IgE was not able to remove IgE ELISA reactivity.

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# CHAPTER 6.

# THE EFFECTS OF ENDOPARASITISM AND OF THE ADMINISTRATION OF VIRAL VACCINES ON THE IMMUNE RESPONSE TO ORALLY ADMINISTERED ANTIGEN IN CATS: POSSIBLE IMPLICATIONS FOR THE PATHOGENESIS OF FOOD HYPERSENSITIVITY

## 6.1. INTRODUCTION

The immunopathogenesis of food hypersensitivity in cats remains unclear although reaginic antibody has been clearly shown to be involved in one cat with allergy to cows milk (Walton et al., 1968). Recently, models of food allergy have been successfully developed in dogs (Guilford and Badcoe, 1992, Ermel et al., 1997). In the first model, pups were sensitised to codfish antigen by oral and parenteral administration and showed markedly elevated food-specific IgE in their serum (Guilford and Badcoe, 1992). Another model was developed in dogs from an atopic colony. The dogs showed an IgE-response to food antigens after being immunised with a live attenuated distemper/hepatitis vaccine and injected with three different food allergens and one pollen (Ermel et al., 1997). It is reported that endoparasitic infections as well as immunoprophylaxis with live attenuated virus predispose to the development of an IgE response to antigens administered at the same time (Turner et al., 1979; Frick and Brooks, 1983).

The aims of this study were to assess whether immunoprophylaxis with the standard battery of viral vaccines and/or infection with *Toxocara cati* (*T. cati*) facilitates the induction of IgE or other antibody responses in kittens to a specific antigen administered with food. Human serum albumin (HSA) was chosen as the antigen because none of the cats would have been previously exposed to this protein.

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## 6.2. MATERIALS AND METHODS

### 6.2.1. Animals

Seventeen domestic short hair (DSH) cats (13 males and 4 females) aged approximately 4 months were obtained from Hill Grove Family Farm Ltd (table 6.1). All cats were parasite-free, and came from a colony which has been parasite free for many generations.

Twenty additional DSH male parasite free kittens, aged 7 to 10 weeks, were obtained from the Central Animal Laboratory of the University of Nijmengen, The Netherlands, for infestation with *T. cati* in Belgium (table 6.2).

After nursing, kittens were weaned onto Whiskas® chicken and beef for kittens (Waltham Centre for Pet Nutrition, Leicestershire, UK). They were fed once a day and water was available *ad libitum*.

**Table 6.1. Parasite-free cats sensitised with HSA**

| Cat number | Sex | Age (weeks) at the time of sensitisation | Immuno-prophylaxis |
|---|---|---|---|
| 13L | m | 18 | |
| 13N | m | 18 | |
| W02 | m | 18 | vaccinated |
| W05 | m | 18 | |
| W06 | m | 18 | vaccinated |
| W07 | m | 18 | vaccinated |
| W11 | m | 17 | vaccinated |
| W14 | m | 17 | |
| 88A | m | 18 | vaccinated |
| 88B | m | 18 | vaccinated |
| 30B | m | 18 | vaccinated |
| 558 | m | 18 | |
| 563 | m | 18 | |
| 13O | f | 18 | |
| 13P | f | 18 | |
| W16 | f | 17 | |
| W17 | f | 17 | |

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**Table 6.2. Cats infected with _T. Cati_ and sensitised with HSA**

| Cat number | Sex | Age (weeks) at the time of infection | Age (weeks) at the time of sensitisation | Presence of _T. cati_ eggs in faeces | Immuno-prophylaxis |
|---|---|---|---|---|---|
| 298 | m | 10 | 20 | positive | |
| 301 | m | 10 | 20 | positive | |
| 302 | m | 10 | 20 | positive | |
| 303 | m | 10 | 20 | positive | vaccinated |
| 304 | m | 10 | 20 | positive | |
| 305* | m | 10 | 20 | negative | |
| 307 | m | 8 | 18 | positive | |
| 308 | m | 8 | 18 | positive | vaccinated |
| 309 | m | 8 | 18 | positive | |
| 310 | m | 8 | 18 | positive | |
| 312 | m | 8 | 18 | positive | |
| 317 | m | 7 | 17 | positive | vaccinated |
| 318 | m | 7 | 17 | positive | vaccinated |
| 319 | m | 7 | 17 | positive | |
| 320 | m | 7 | 17 | positive | vaccinated |
| 324* | m | 7 | 17 | negative | |
| 325 | m | 7 | 17 | positive | vaccinated |
| 326* | m | 7 | 17 | negative | |
| 331 | m | 7 | 17 | positive | |
| 337 | m | 7 | 17 | positive | |

* Cats which were excluded from the study

6.2.2. Infection of cats with T. cati

The twenty kittens aged between 7 and 10 weeks were experimentally infected with _T. cati_. Infective 2nd stage larvae were provided by Dr Epe from the Institute for Parasitology der Tierarztliche Hochschule Hannover (Hannover, Germany). A single dose of approximately 250 embryonated _T. cati_ eggs suspended in 1 ml of water was administered orally to each cat. Faeces were examined for the appearance of ova 2 months later. The parasitised cats were euthanised at the end of the study with 5 ml of

sodium pentobarbital (Natriumpentobarbital 20%®) and the gastrointestinal (GI) tract opened to assess the worm burden.

### 6.2.3. Antigen employed

Fraction V of human serum albumin (HSA), 96-99% pure (Sigma), was used for oral sensitisation of the cats and for the detection by ELISA of HSA-specific IgE, IgG, IgA and IgM.

### 6.2.4. Sensitisation of cats

Each cat was dosed with 100 mg of HSA added to their food daily from days 7 to 28 inclusive (fig. 6.1).

### 6.2.5. Vaccination protocol

Seven of the parasitised cats and the same number of parasite-free cats were immunised with a live trivalent freeze-dried vaccine derived from attenuated feline calicivirus, feline herpesvirus, and feline panleucopenia virus (Katavac CHP®, Solvay Duphar B.V., Weesp, Holland) on day 7 and day 21 (tables 6.1 and 6.2, fig. 6.1).

### 6.2.6. Blood samples

Blood was obtained by jugular venepuncture at weeks 0, 2, 4 and 8 (bleedings 1 to 4) (fig. 6.1). After clot retraction, serum was harvested by centrifugation and stored at -20°C.

### 6.2.7. Antisera employed

Polyclonal rabbit anti-feline IgE was the same antibody as used in the previous studies (chapters 2 and 3).

Alkaline phosphatase-conjugated goat anti-feline IgG, IgA and IgM (heavy chain specific) were obtained from Bethyl Laboratories Inc. (Montlucon, France).

Unconjugated goat anti-feline IgM was purchased from Nordic Immunology (Tilburg, The Netherlands).

Mouse monoclonal alkaline phosphatase-conjugated anti-rabbit and anti-goat immunoglobulin were purchased from Sigma.
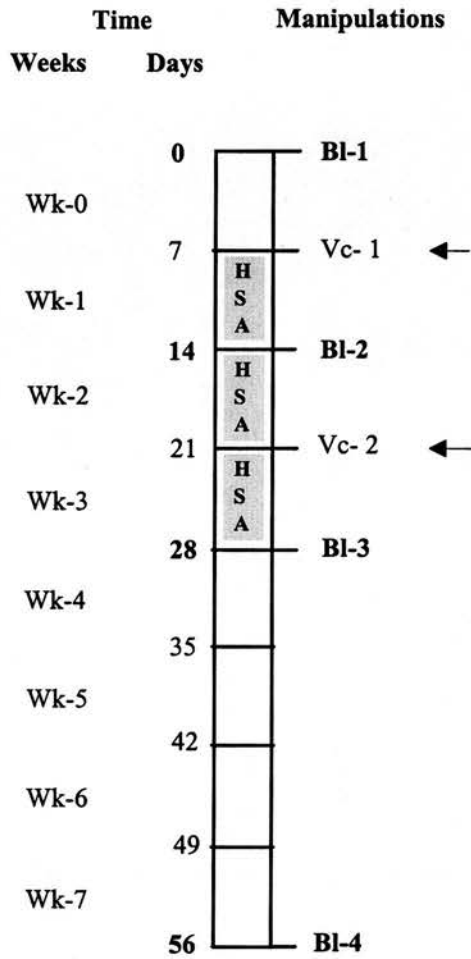
**Fig. 6.1. Protocol for experiments in chapter 6: feeding of HSA, vaccination (Vc ←) and bleeding (Bl) schedules.**

### 6.2.8. ELISA for HSA-specific antibodies

#### 6.2.8.1. Assay optimisation

Assay conditions were optimised in preliminary experiments (chapter 3). Sera with a high level of HSA antibody of each isotype were pooled and used as the standard and given an arbitrary relative antibody unit value (RAU) of 1000 when undiluted. Doubling dilutions of the reference serum were prepared in ELISA buffer (2.5% donkey serum (DS) in washing buffer) to generate a standard curve which was included in each plate (see chapter 3). Preimmunisation bleedings were used as the negative controls and were included in each assay.

#### 6.2.8.2. Procedure for the detection of HSA specific-IgE

Serum samples were assayed for HSA-specific IgE by indirect ELISA at an initial dilution in ELISA buffer of 1/2. The ELISA was performed exactly as described previously except that the plates were coated with 100 μl of 0.5% HSA and that 25% DS was used as the blocking agent. The anti-feline IgE was diluted 1/35 in ELISA buffer.

Since the level of serum HSA-specific IgE was too low to generate a standard curve with a minimum of 3 points, results for this isotype were reported in optical density (OD) units. The OD was read when the standard reached an OD of 0.56.

#### 6.2.8.3. Procedure for the detection of HSA-specific IgG, IgA and IgM

Sera were assayed for HSA-specific IgG, IgA and IgM by direct ELISA. Serum samples were initially assessed at a dilution of 1/10 in ELISA buffer. Incubation times for the test sera were reduced from 2 hr to 1.5 hr and for the conjugate from 2 hr to 1 hr. When samples were assessed with the unconjugated polyclonal anti-feline IgM, the addition of the monoclonal alkaline phosphatase-conjugated mouse anti-goat immunoglobulin was performed under the same conditions as for the detection of HSA-specific IgE.

Polyclonal alkaline phosphatase conjugated anti-feline IgG and IgA were used at 1/500 and 1/150 respectively whereas alkaline phosphatase-conjugated anti-feline IgM was used at 1/500 and 1/1000. The polyclonal unconjugated anti-feline IgM was assessed at 1/100, 1/1000 and 1/5000 dilutions.

A standard curve was established with a minimum of 4 points for the detection of IgG and IgA, and HSA-specific antibody was expressed in RAU by reference to the

standard curve. If the result did not fall in the sensitive part of the standard curve, the assay was repeated at a lower or higher dilution as appropriate. A serum sample whose OD at 1/2 fell below the end point of the standard curve (EPSC) was deemed to be negative. For the purpose of statistical analyses, these sera were assigned a value half way between the RAU of the EPSC and zero, which was 3.906.

### 6.2.9. Statistical analysis

Results were analysed using a statistical computer package (MINITAB release 11.0 Minitab Inc., State College, PA, USA). The non-parametric Mann-Whitney test, an unpaired statistical test, was used to compare the HSA-specific IgA and IgG responses between different groups of cats whereas the levels of HSA-specific IgE were analysed by analysis of variance (ANOVA). Results were considered significant at $p<0.05$.

Correlations between the levels of IgG and IgA at week 4 and 8 in the parasite-free and parasitised cats were calculated using Spearman's Rank correlation test. Data were previously transformed to log10 values. Results were considered statistically significant at $p<0.01$.

## 6.3. RESULTS

### 6.3.1. Infection with *T. cati*

*T. cati* eggs were found in the faeces of 12 of the cats 8 weeks after being orally dosed with embryonated eggs, whereas in 5 cats *T. cati* eggs were seen in the faeces only after 10 weeks. Adult *T. cati* were found in the digestive tract in all the 17 cats at the end of the study, four months after oral infection (table 6.2). In the case of three cats (305, 324 and 326) no ova were detected in the faeces and they were excluded from the study. However as the latter 3 were not necropsied, their true infective status was unknown.

### 6.3.2. Immune response to HSA

#### 6.3.2.1. HSA-specific IgE response

Positive OD were obtained in the presensitisation bleedings and were assumed to be the result of non-specific binding (see discussion). In general these data were only slightly positive however an OD>0.1 was seen in 24 cats and >0.2 in one of them (cat

13O) (table 6.3 (a and b)). There was no significant difference between the OD values found in the presensitisation bleedings in the parasitised and parasite-free cats.

Although weak, an HSA-specific IgE response was detectable in most of the parasitised cats one week after exposure to HSA (week 2) and rose to a maximum three weeks after exposure to HSA (week 4). Levels of IgE at week 8 decreased in 29 cats out of the 34 (tables 6.3 (a and b) and 6.4 (a), fig. 6.2). The lowest levels of HSA specific IgE were found in the parasite-free cats with two of them showing essentially undetectable responses (13O and W16) (table 6.3 (b), fig. 6.2).

The group of cats infected with *T. cati* had significantly higher levels of HSA-specific IgE in each bleeding than did the group of parasite-free cats ($p<0.001$). However the most significant differences between the two groups were found 3 weeks after exposure to HSA (week 4) (table 6.4 (a), fig. 6.2 and 6.3).

Although the vaccinated cats had generally higher levels of antigen-specific IgE than the non-vaccinated cats, significant differences between these two groups were only seen at weeks 2 and 4 from the parasite-free cats (table 6.4 (a), fig. 6.2 and 6.3). The parasitised cats which were not vaccinated had higher levels of HSA-specific IgE at week 8 than did the vaccinated cats although this difference was not significant (fig. 6.2 and 6.3).

### 6.3.2.2. HSA-specific IgG response

The cats developed an HSA-specific IgG response between the first and the third week following exposure to HSA (fig. 6.4 and 6.5). However, one cat from the parasitised group (cat 301) and 4 cats from the parasite-free group (cats 13L, 13N, 13O and W17) remained negative during the 2 months of the study. Four parasite-free cats (cats 563, W11, 13P and W16) had HSA-specific IgG only in the last bleeding (week 8), and only at very low levels. IgG antibody levels decreased in 20 cats out of the 34 at week 8, which was 4 weeks after conclusion of the antigenic challenge (tables 6.3 (a and b) and 6.4 (b)). Interestingly, the parasite-free cats which had undetectable or only low levels of HSA-specific IgG also showed either a weak or undetectable IgE response. However this was not the case in the parasitised cats with the exception of one cat (312) that showed a low response for all isotypes including IgE. Also all these non-responder or weakly responding cats were non-vaccinated with the exception of one cat (W11).

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**(a)**

| | IgE | | | | IgG | | | | IgA | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Wk-0 | Wk-2 | Wk-4 | Wk-8 | Wk-0 | Wk-2 | Wk-4 | Wk-8 | Wk-0 | Wk-2 | Wk-4 | Wk-8 |
| 13L | 0.172 | 0.183 | 0.571 | 0.216 | 3.906 | 3.906 | 3.906 | 3.906 | 3.906 | 3.906 | 22 | 23 |
| 13N | 0.176 | 0.127 | 0.105 | 0.404 | 3.906 | 3.906 | 3.906 | 3.906 | 3.906 | 3.906 | 3.906 | 19 |
| W02* | 0.109 | 0.277 | 0.657 | 0.475 | 3.906 | 3.906 | 455 | 596 | 3.906 | 3.906 | 937 | 1182 |
| W05 | 0.106 | 0.061 | 0.285 | 0.223 | 3.906 | 3.906 | 42 | 16 | 3.906 | 3.906 | 59 | 3.906 |
| W06* | 0.101 | 0.183 | 0.603 | 0.426 | 3.906 | 3.906 | 376 | 313 | 3.906 | 3.906 | 222 | 196 |
| W07* | 0.161 | 0.346 | 0.431 | 0.285 | 3.906 | 3.906 | 82 | 59 | 3.906 | 3.906 | 149 | 75 |
| W11* | 0.104 | 0.325 | 0.25 | 0.21 | 3.906 | 3.906 | 3.906 | 17 | 3.906 | 3.906 | 20 | 20 |
| W14 | 0.073 | 0.084 | 0.24 | 0.166 | 3.906 | 3.906 | 50 | 139 | 3.906 | 3.906 | 100 | 59 |
| 88A* | 0.098 | 0.063 | 0.152 | 0.278 | 3.906 | 3.906 | 104 | 32 | 3.906 | 3.906 | 83 | 62 |
| 88B* | 0.139 | 0.507 | 0.328 | 0.115 | 3.906 | 3.906 | 38 | 47 | 3.906 | 3.906 | 79 | 32 |
| 30B* | 0.117 | 0.413 | 0.437 | 0.316 | 3.906 | 3.906 | 27 | 35 | 3.906 | 3.906 | 31 | 19 |
| 558 | 0.16 | 0.418 | 0.46 | 0.202 | 3.906 | 3.906 | 29 | 15 | 3.906 | 3.906 | 18 | 20 |
| 563 | 0.139 | 0.192 | 0.342 | 0.275 | 3.906 | 3.906 | 3.906 | 37 | 3.906 | 3.906 | 12 | 35 |
| 13O | 0.258 | 0.24 | 0.336 | 0.273 | 3.906 | 3.906 | 3.906 | 3.906 | 3.906 | 3.906 | 3.906 | 3.906 |
| 13P | 0.101 | 0.125 | 0.152 | 0.199 | 3.906 | 3.906 | 3.906 | 32 | 3.906 | 3.906 | 3.906 | 8 |
| W16 | 0.036 | 0.126 | 0.084 | 0.137 | 3.906 | 3.906 | 3.906 | 17 | 3.906 | 3.906 | 3.906 | 11 |
| W17 | 0.082 | 0.246 | 0.283 | 0.154 | 3.906 | 3.906 | 3.906 | 3.906 | 3.906 | 3.906 | 3.906 | 8 |

**(b)**

| | IgE | | | | IgG | | | | IgA | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Wk-0 | Wk-2 | Wk-4 | Wk-8 | Wk-0 | Wk-2 | Wk-4 | Wk-8 | Wk-0 | Wk-2 | Wk-4 | Wk-8 |
| 298 | 0.173 | 0.402 | 0.828 | 0.42 | 3.906 | 3.906 | 694 | 353 | 3.906 | 3.906 | 993 | 229 |
| 301 | 0.12 | 0.284 | 0.607 | 0.162 | 3.906 | 3.906 | 3.906 | 3.906 | 3.906 | 3.906 | 3.906 | 3.906 |
| 302* | 0.074 | 0.18 | 0.662 | 0.178 | 3.906 | 3.906 | 163 | 89 | 3.906 | 3.906 | 215 | 104 |
| 303* | 0.105 | 0.301 | 0.449 | 0.286 | 3.906 | 3.906 | 36 | 213 | 3.906 | 3.906 | 32 | 17 |
| 304 | 0.128 | 0.28 | 0.636 | 0.222 | 3.906 | 3.906 | 150 | 42 | 3.906 | 3.906 | 255 | 51 |
| 307 | 0.098 | 0.498 | 0.761 | 0.606 | 3.906 | 3.906 | 1224 | 1432 | 3.906 | 3.906 | 1586 | 1973 |
| 308* | 0.073 | 0.447 | 0.702 | 0.242 | 3.906 | 3.906 | 453 | 254 | 3.906 | 3.906 | 501 | 247 |
| 309 | 0.118 | 0.341 | 0.701 | 0.304 | 3.906 | 3.906 | 345 | 190 | 3.906 | 3.906 | 355 | 198 |
| 310 | 0.124 | 0.473 | 0.703 | 0.352 | 3.906 | 3.906 | 397 | 248 | 3.906 | 3.906 | 584 | 356 |
| 312 | 0.134 | 0.216 | 0.318 | 0.056 | 3.906 | 3.906 | 52 | 33 | 3.906 | 3.906 | 114 | 60 |
| 317* | 0.126 | 0.397 | 0.658 | 0.537 | 3.906 | 3.906 | 437 | 1571 | 3.906 | 3.906 | 339 | 387 |
| 318* | 0.098 | 0.323 | 0.652 | 0.305 | 3.906 | 3.906 | 411 | 298 | 3.906 | 3.906 | 506 | 206 |
| 319 | 0.104 | 0.419 | 0.422 | 0.461 | 3.906 | 3.906 | 532 | 570 | 3.906 | 3.906 | 4497 | 591 |
| 320* | 0.124 | 0.332 | 0.69 | 0.548 | 3.906 | 3.906 | 1037 | 613 | 3.906 | 3.906 | 1173 | 1857 |
| 325* | 0.139 | 0.267 | 0.677 | 0.556 | 3.906 | 3.906 | 853 | 2560 | 3.906 | 3.906 | 5940 | 744 |
| 331 | 0.116 | 0.028 | 0.624 | 0.561 | 3.906 | 3.906 | 212 | 102 | 3.906 | 3.906 | 273 | 1860 |
| 337 | 0.034 | 0.29 | 0.574 | 0.337 | 3.906 | 3.906 | 258 | 262 | 3.906 | 3.906 | 571 | 635 |

**Table 6.3. Levels of HSA-specific IgE antibody (in OD units), and IgG and IgA antibodies (in RAU) in parasite-free cats (a) and parasitised cats (b)**
* = vaccinated cats,  Wk = week

**Table 6.4. Median and range of HSA-specific IgE (a), IgG (b) and IgA (c)**

(a) HSA-specific IgE, results in OD units

| | Week 2 | | | Week 4 | | | Week 8 | |
|---|---|---|---|---|---|---|---|---|
| | pf | p | | pf | p | | pf | p |
| Vc+NVc | 0.192* | 0.323* | Vc+NVc | 0.328** | 0.658** | Vc+NVc | 0.223* | 0.337* |
| | (0.063-0.507) | (0.18-0.397) | | (0.152-0.657) | (0.449-0.702) | | (0.115-0.475) | (0.178-0.556) |
| Vc | 0.325°° | 0.323 | Vc | 0.431°° | 0.662 | Vc | 0.285 | 0.305 |
| | (0.063-0.507) | (0.18-0.397) | | (0.152-0.657) | (0.449-0.702) | | (0.115-0.475) | (0.178-0.556) |
| NVc | 0.155°° | 0.315 | NVc | 0.284°° | 0.63 | NVc | 0.209 | 0.344 |
| | (0.061-0.418) | (0.028-0.498) | | (0.084-0.571) | (0.318-0.828) | | (0.137-0.404) | (0.056-0.606) |

(b) HSA-specific IgG, results in RAU

| | Week 4 | | | Week 8 | |
|---|---|---|---|---|---|
| | pf | p | | pf | p |
| Vc+NVc | 27** | 397** | Vc+NVc | 32** | 254** |
| | (4-455) | (4-1224) | | (4-596) | (4-2560) |
| Vc | 82° | 437 | Vc | 47° | 298 |
| | (4-455) | (36-1037) | | (17-596) | (89-2560) |
| NVc | 4° | 302 | NVc | 16° | 219 |
| | (4-50) | (4-1224) | | (4-139) | (4-1432) |

(c) HSA-specific IgA, results in RAU

| | Week 4 | | | Week 8 | |
|---|---|---|---|---|---|
| | pf | p | | pf | p |
| Vc+NVc | 22** | 501** | Vc+NVc | 20** | 247** |
| | (4-937) | (4-5940) | | (4-1182) | (4-1973) |
| Vc | 83° | 501 | Vc | 62° | 247 |
| | (20-937) | (32-5940) | | (20-1182) | (17-1857) |
| NVc | 8° | 463 | NVc | 15° | 293 |
| | (4-59) | (4-4497) | | (4-35) | (4-1973) |

Vc = vaccinated cats and NVc = non vaccinated cats.
Differences between the levels of antibodies in the parasitised and parasite free cats:
*: test significant at $p<0.05$ and **: at $p<0.001$.
Differences between the levels of antibodies in the vaccinated and non-vaccinated cats in both groups: °: test significant at $p<0.05$ and °°: at $p<0.001$.

149
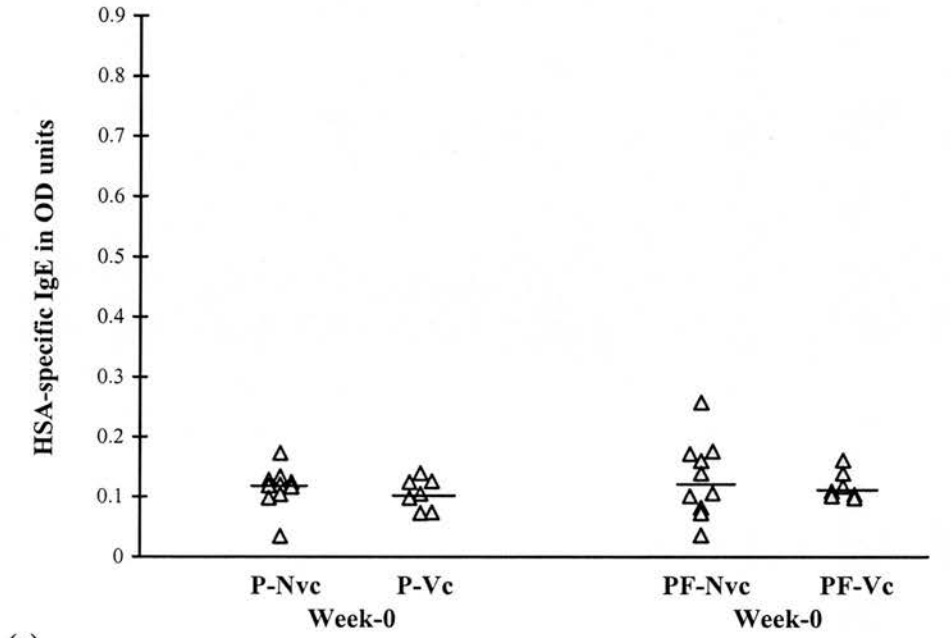
**(a)**
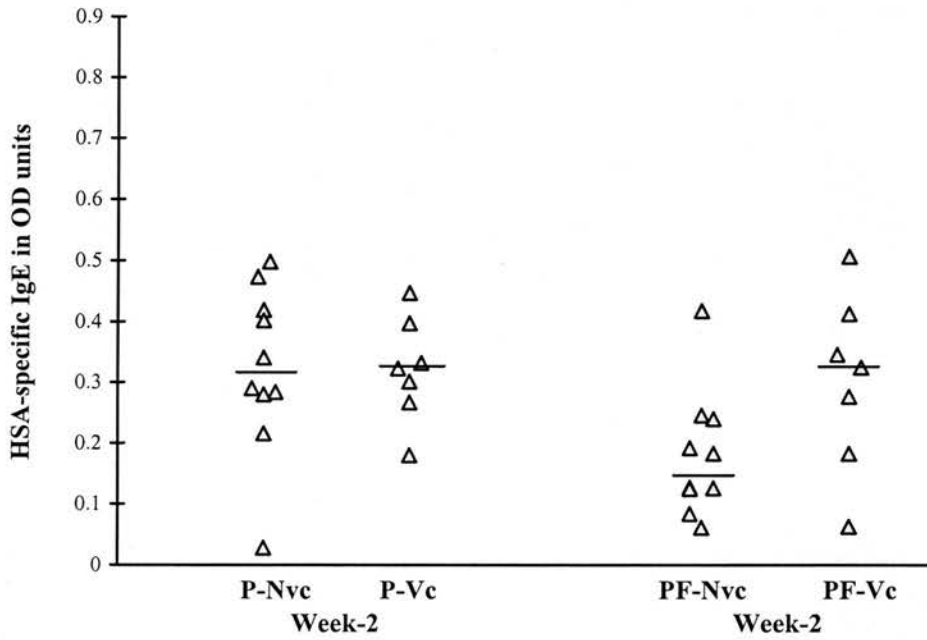


**(b)**

Fig. 6.2. Levels of HSA-specific IgE in OD units in vaccinated (Vc) and non-vaccinated (NVc) parasitised (P) and parasite-free (PF) cats at week 0 (a) and week 2 (b).
——— = median.
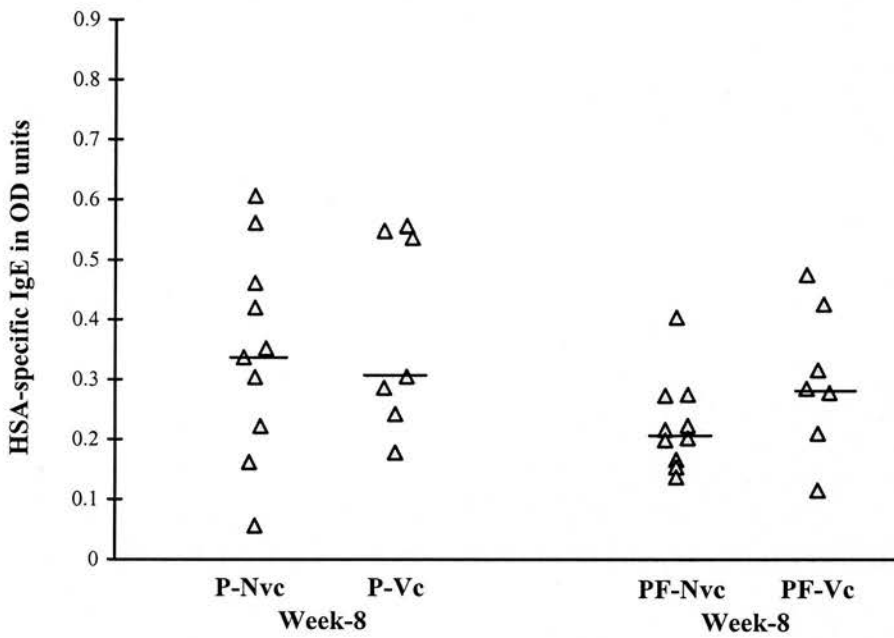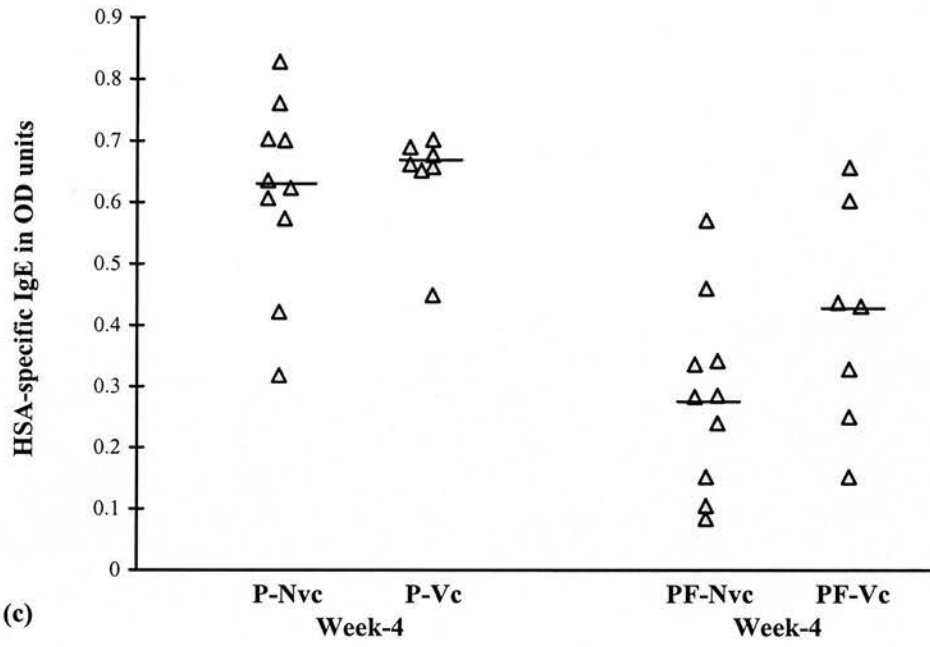
**Fig. 6.2. (continued) Levels of HSA-specific IgE in OD units in vaccinated (Vc) and non-vaccinated (NVc) parasitised (P) and parasite-free (PF) cats at week 4 (c) and week 8 (d).**

——— = median.
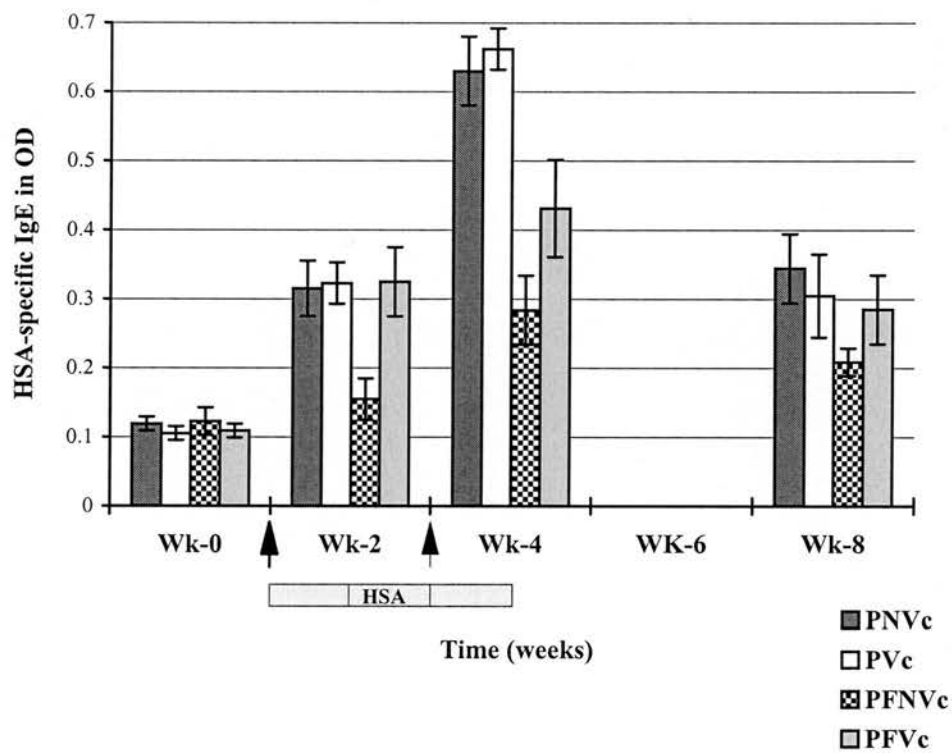
Fig. 6.3. Median and standard error of the levels of HSA-specific IgE in OD units in vaccinated (Vc) and non-vaccinated (NVc) parasitised (P) and parasite-free (PF) cats in the four bleedings.
↑ = vaccination, ⬜HSA⬜ = HSA fed.

152
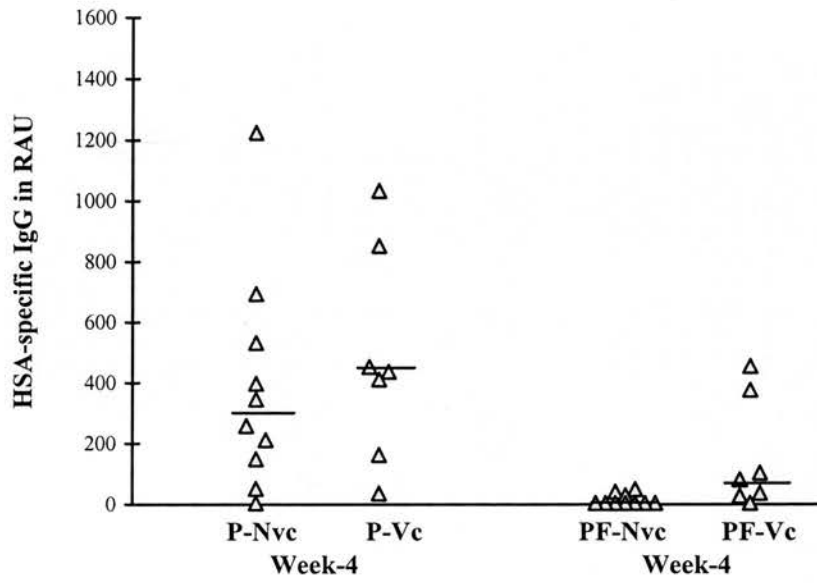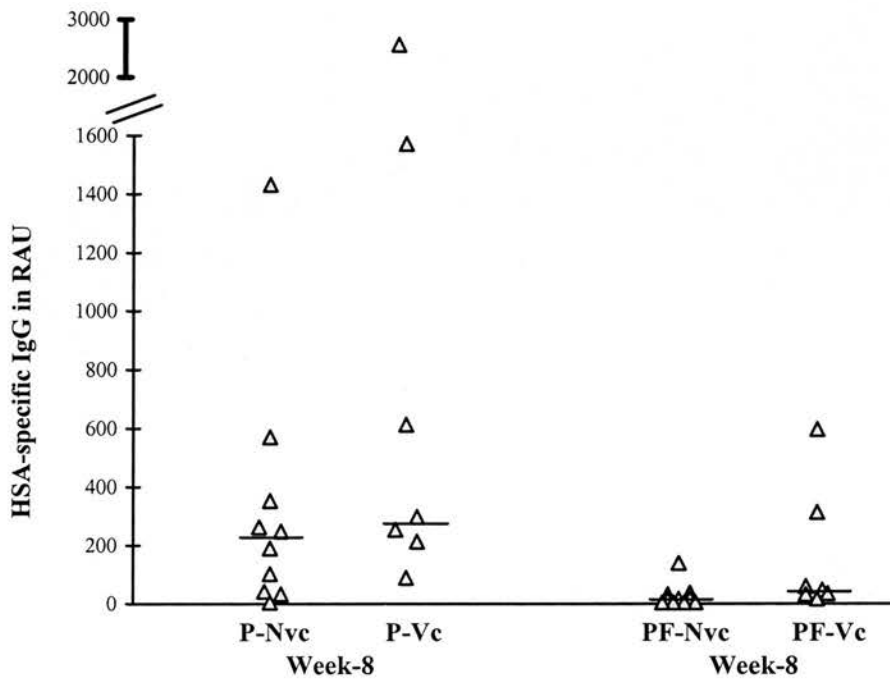
**Fig. 6.4. Levels of HSA-specific IgG in RAU in vaccinated (Vc) and non-vaccinated (NVc) parasitised (P) and parasite-free (PF) cats at week 4 (a) and week 8 (b).**

——— = median.

153
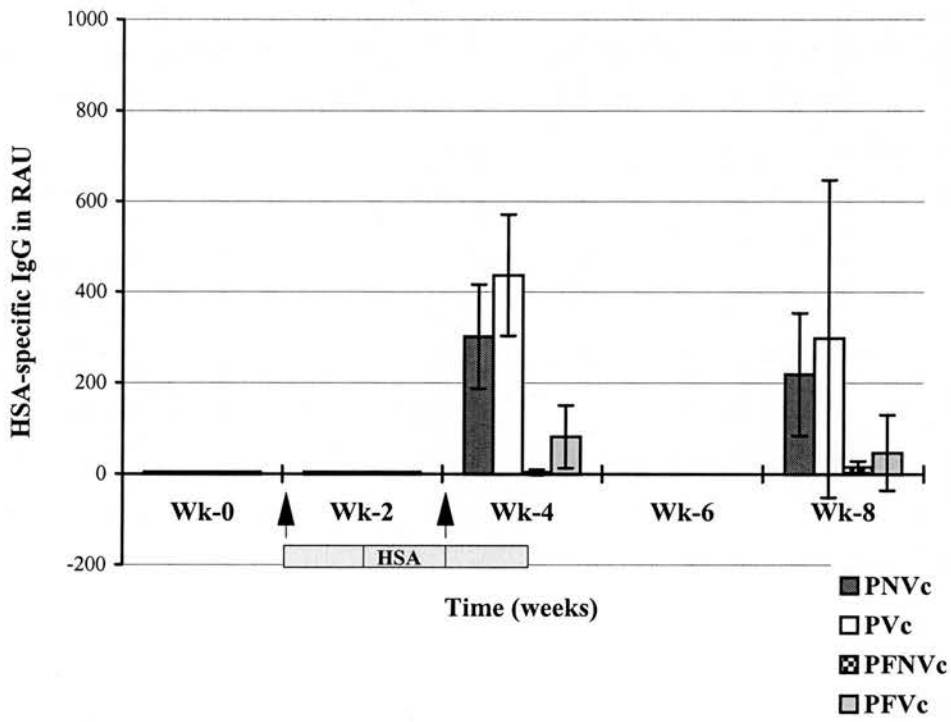
**Fig. 6.5. Median and standard error of the levels of HSA-specific IgG in RAU in vaccinated (Vc) and non-vaccinated (NVc) parasitised (P) and parasite-free (PF) cats in the four bleedings.**
↑ = vaccination, ▭HSA▭ = HSA fed.

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Cats infected with *T. cati* had significantly higher levels of antigen-specific IgG antibody than did the parasite-free cats at weeks 4 and 8 (p<0.001) (table 6.4 (b), fig. 6.4 and 6.5).

Significant differences between the level of HSA-specific IgG from the cats which were vaccinated and the cats which were not vaccinated were only seen at weeks 4 and 8 from the parasite-free cats, with the vaccinated cats showing higher levels (fig. 6.4 and 6.5).

### 6.3.2.3. HSA-specific IgA response

As was the case with HSA-specific IgG, HSA-specific IgA could only be detected from week 4 (fig. 6.6 and 6.7). One parasite-free cat (cat 13O) and one parasitised cat (cat 301) never developed an IgA response to HSA. Four parasite-free cats (cats 13N, 13P, W16 and W17) also showed only a weak IgA response in the last bleeding. The levels of allergen-specific IgA in the last bleeding declined in 21 out of the 34 cats (see tables 6.3 (a and b) and 6.4 (c)).

The group of parasite-free cats developed only a weak IgA response to HSA and differences between the levels of HSA-specific IgA in both bleedings 3 and 4 (weeks 4 and 8) from parasitised and parasite-free cats were highly significant (p<0.001) (table 6.4 (c), fig .6.6 and 6.7).

Significant differences between the levels of HSA-specific IgA in the vaccinated and non-vaccinated cats were only found at weeks 4 and 8 in the case of the parasite-free cats, with higher levels resulting in the vaccinated group (fig. 6.6 and 6.7).

### 6.3.2.4. HSA-specific IgM response

HSA-specific IgM could not be detected in the sera of any of the 17 cats of either group employing either the alkaline phosphatase-conjugated or unconjugated goat anti-feline IgM.

### 6.3.2.5. Correlation between the levels of HSA-specific IgG and IgA

The IgG and IgA responses at weeks 4 and 8, in the parasite-free and the parasitised cats were highly correlated (p<0.01) (fig. 6.8 (a and b)). When the correlations between the levels of IgG and IgA were assessed either at week 4 or week 8, they were significant in both parasite-free and parasitised cats (p<0.01) (fig. 6.9 and 6.10).

155
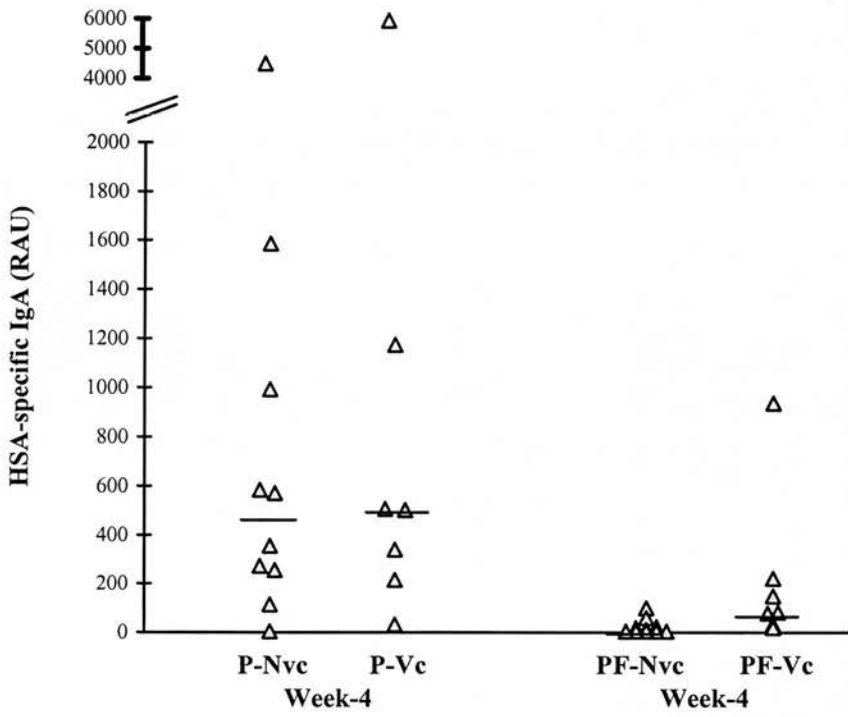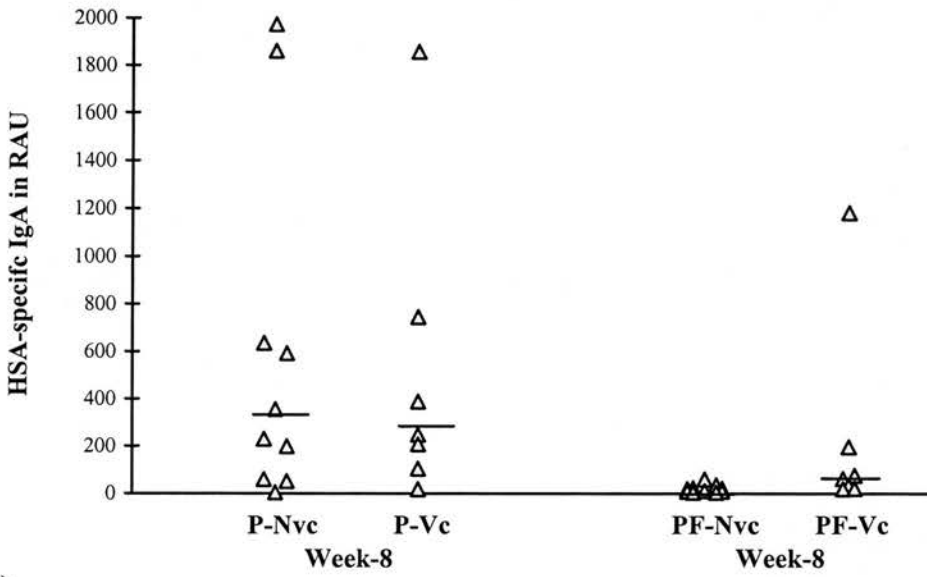
**Fig. 6.6. Levels of HSA-specific IgA in RAU in vaccinated (Vc) and non-vaccinated (NVc) parasitised (P) and parasite-free (PF) cats at week 4 (a) and week 8 (b).**

———— = median.

156
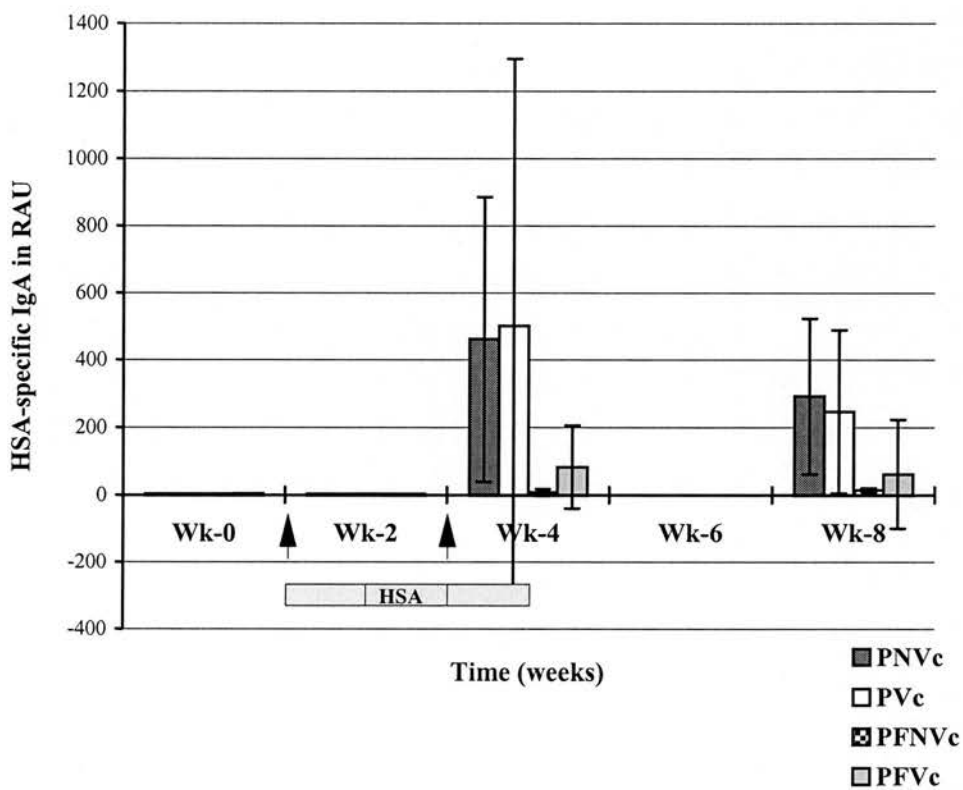
Fig. 6.7. Median and standard error of the levels of HSA-specific IgA in
RAU in vaccinated (Vc) and non-vaccinated (NVc) parasitised (P) and
parasite-free (PF) cats in the four bleedings.
↑ = vaccination, [ HSA ] = HSA fed.
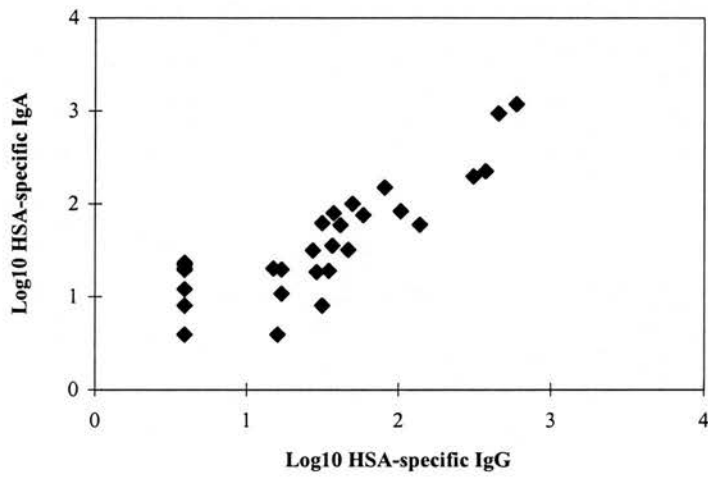
**(a)**
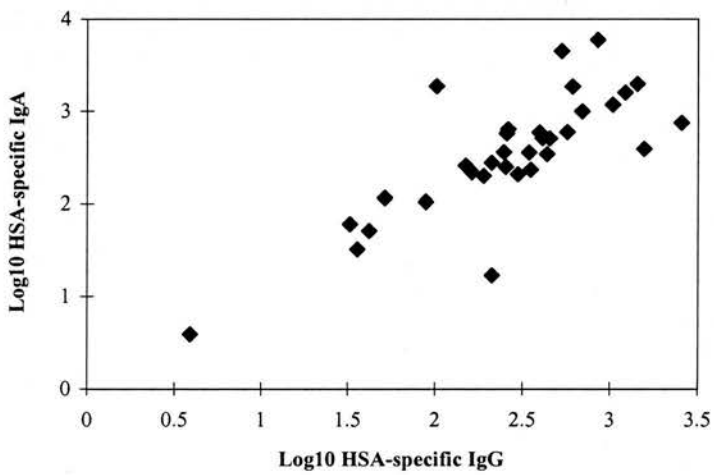


**(b)**

**Fig. 6.8. Correlations between the levels of HSA-specific IgA and IgG at week 4 and week 8 in parasite-free cats (a) and parasitised cats (b).**

**The levels of the 2 isotypes are significantly correlated ((a) r=0.869, p<0.01 and (b) r=0.830, p<0.01).**

158
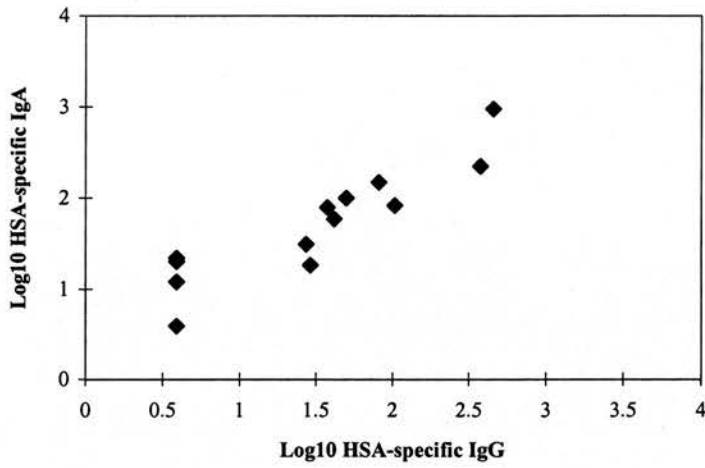
**(a)**
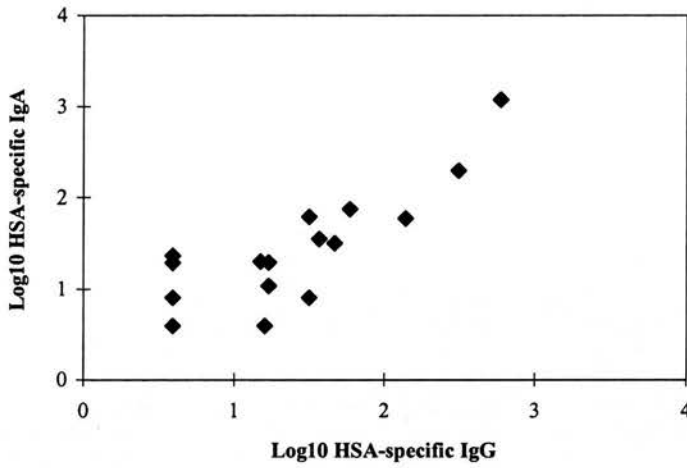


**(b)**

**Fig. 6.9. Correlations between the levels of HSA-specific IgA and IgG in parasite-free cats, at week 4 (a) and week 8 (b).**

**The levels of the 2 isotypes are significantly correlated ((a) r=0.923, p<0.01 and (b) r=0.811, p<0.01).**
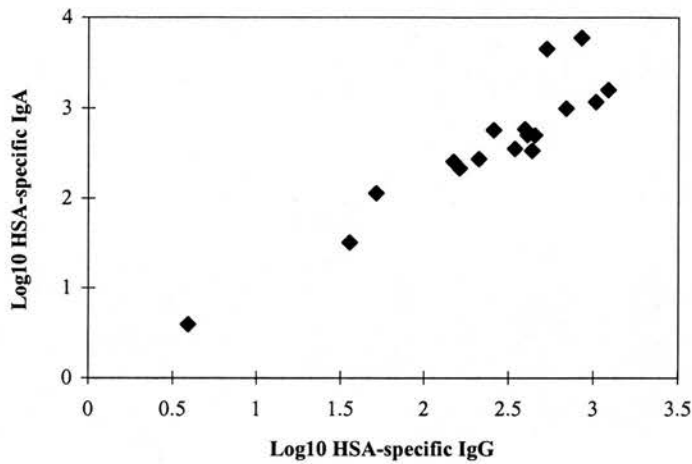
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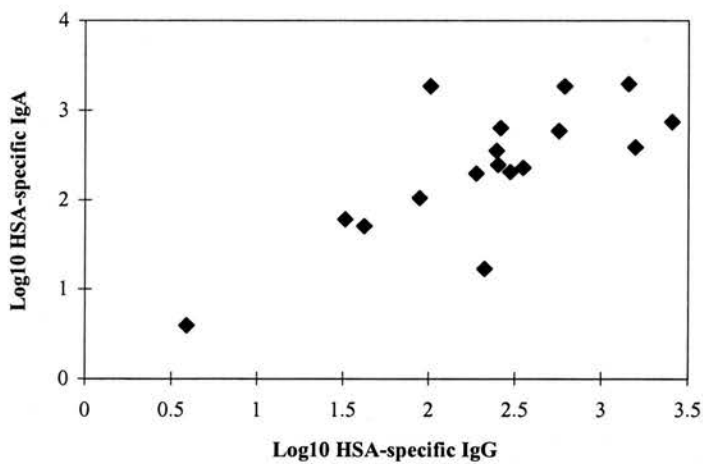


**(b)**

**Fig. 6.10. Correlations between the levels of HSA-specific IgA and IgG in parasitised cats, at week 4 (a) and week 8 (b).**

**The levels of the 2 isotypes are significantly correlated ((a) r=0.930, p<0.01 and (b) r=0.751, p<0.01).**

# 4. DISCUSSION

A single dose of approximately 250 *T. cati* embryonated eggs induced a patent infection in 17/20 cats after 8 to 10 weeks, and the infection persisted during the 2 months of the study. The three kittens which were found to be negative 10 weeks after exposure to *T. cati* eggs were excluded from the study and 3 females randomly selected from the group of parasite-free cats (881, 564 and 565) were also excluded. Two groups of 17 cats each (parasitised cats and parasite-free cats) were thus constituted.

Presensitisation bleedings (week 0) showed slightly positive OD values in the HSA-specific IgE assay. IgE cross-reactivity has been reported against albumins of different animal origins and the high frequency of asthma to animal hair/dander proteins occurring in man may be a consequence of this cross-reactivity (Spitzauer et al., 1995). Because the cats were fed a diet containing beef and chicken during the study, they may have developed some IgE directed against bovine serum albumin and/or chicken serum albumin. Such antibodies could have cross-reacted with HSA and given false-positive ELISA results. Nevertheless the slightly positive OD values seen in the sera of cats before being exposed to HSA were still present after heating at 56°C for 4 hr which precludes this explanation. Slightly positive OD values were also detected in the heated sera of the cats after exposure to HSA. However antibodies of other isotypes were not detected in the presensitisation bleedings and thus these findings could not have resulted from the detection of other isotypes by the polyclonal anti-feline IgE. Therefore these values were attributed to non-specific binding. There was no significant difference between the levels in the parasitised and parasite-free cats.

The oral administration of HSA daily for 3 weeks induced an antibody response involving IgG, IgA and IgE isotypes which was detected in the serum. Although weak, an IgE response was already detectable one week after oral exposure to HSA in the cats infected with *T. cati* and in some of the parasite-free cats, whereas the IgG and IgA responses started to develop later. However firm conclusions cannot be made as the sensitivity of the ELISA for each isotype could have differed. The IgE response was of shorter duration than that of the other isotypes. These results are in accordance with the kinetics of the IgE and IgG antibodies in the study on the response to *Dermatophagoïdes farinae* following parenteral immunisation (chapter 3) and the more rapid decline could be a reflection of the shorter half-life of IgE. In the present study, the variations in the antibody response seen in cats within each group is most probably the result of genetic

differences between them. It has been shown in the dog that the length and the intensity of the IgE response depends on the age at the time of immunisation and on the strain, and that dogs can be segregated into high and low IgE responders (de Weck, 1997; Zunic et al., 1998).

The cats did not show any dermatological or GI signs. However it is not impossible that the administration of a higher dose of HSA (rather than the 100 mg which was given daily) and/or oral challenge of the cats with HSA after their 3 weeks of sensitisation could have led to the development of clinical signs. On the other hand, these cats had no known genetic predisposition to allergy. Guilford and Badcoe (1992) were able to develop a model of food allergy in 20 puppies by oral administration of cod protein and parenteral administration of the same protein in alum. All pups developed positive IDSTs and detectable antigen-specific IgE in their serum. Oral challenge of the sensitised puppies with codfish daily for 8 to 10 days resulted in transient vomiting and pruritus in 20% and 85% of pups respectively and mild to moderate diarrhoea in the majority of them. More recently a model for food allergy has been induced in an atopic dog colony employing serial immunisations subcutaneously with three food antigens and one pollen extract in alum, and subsequent oral challenge with the food antigens (Ermel et al., 1997). The pups were also vaccinated with live attenuated distemper/hepatitis vaccine (Ermel et al., 1997). All pups responded to the antigenic challenge by producing high levels of food-specific IgE. However the overall IgE response was weak in comparison to the response of other isotypes. In general, oral sensitisation with food protein alone does not result in substantial food-specific IgE production, and animal models of food allergy have usually required alternative routes of antigen presentation, such as intravenous, intraperitoneal or subcutaneous with adjuvant. Frick et al. (1990) and Ermel et al. (1997) have reported a highly significant difference in the IgE response to food antigen in dogs immunised parenterally as compared with matched control dogs that received the food antigens in their diet. However the former in no way mimics the natural mode of sensitisation which occurs in food hypersensitivity. It was therefore noteworthy that in the present study, most of the cats developed an HSA-specific IgE response following oral administration of the antigen for 3 weeks.

Almost all the cats developed a serum IgG and IgA response to the HSA. It is reported that low levels of IgG and IgA antibodies to various food are commonly found in the serum of normal humans (Kletter et al., 1971; Johansson et al., 1984; Paganelli et al., 1984). The presence of these food antibodies results from antigenic stimulation but is

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of limited clinical relevance. Absorption of small amounts of antigen through the gut mucosa occurs under normal conditions in healthy adults (Husby et al., 1985).

Oral tolerance, a form of peripheral tolerance in which mature lymphocytes in the peripheral lymphoid tissues are rendered non functional or hyporesponsive by prior oral administration of antigen in a normal individual, has been shown to depend on the nature and the dose of the antigen, and on a number of host factors such as the genetic background, the maturity of the host, the digestive flora and the level of antigen uptake (Strobel and Mowat, 1998). The parasite-free cats showed much lower levels of food-specific antibodies than did the parasitised cats, with two of them apparently developing tolerance to the HSA. With the exception of one cat, these poorly or non-responsive cats were also unvaccinated. Therefore the presence of *T. cati* and the inoculation of the cats with a live-attenuated virus seems to have prevented the development of oral tolerance in favour of an active immunity with activation of CD4+ T cells leading to the induction of helper and memory cells. Interestingly, the cats which had a low IgE response to HSA had also low levels of other isotypes in their sera whereas the opposite was not always seen.

It was noteworthy that the apparently tolerant cats were both females. Furthermore the other two females in the study had either undetectable or very low levels of HSA-specific IgE, IgG and IgA. However because of the small number of females and because none of them were parasitised, it is not possible to say if the sex had any influence on the immune response.

Cats infected with *T. cati* gave a much stronger antigen-specific-antibody response in the case of all three isotypes. Amongst the 5 cats which did not develop an IgG response to HSA, 4 were parasite-free. One of the 2 cats which did not give an IgA response and the two cats which responded weakly or not at all in respect of IgE were also parasite-free. The marked increase of HSA-specific IgE seen in the cats infected with *T. cati* was not unexpected since it is well known that the IgE antibody response to unrelated allergens is potentiated in individuals with helminth infections. The increase in total IgE has been reported to be largely a consequence of non-specific polyclonal activation of IgE B cells (Jarret and Miller, 1982). A Th2 dominated response, exhibiting a characteristic pattern of lymphokine production (Il-4), versus a Th1 response, producing IFN-$\gamma$, may be responsible for the elevated levels of IgE seen in helminth infections (Romagnani, 1990; King et al., 1992). A preponderance of Th2 cells has been

reported in human patients with IgE antibody against various helminths, including *T. canis* (Romagnani, 1990).

There are conflicting hypotheses on the relationship between the non-specific polyclonal potentiation of IgE induced by helminth infection and allergic conditions (Moqbel and Pritchard, 1990). It has been proposed that infection with helminths predisposes to atopy (Masters and Barrett-Connor, 1985). The contrary view suggest that parasite infection prevents or modulate atopy (the "blocking theory") (Moqbel and Pritchard, 1990). Thirdly, it is possible that the atopic state can be protective against parasite infection (Moqbel and Pritchard, 1990). In the first hypothesis, the undeniable ability of most helminths to induce a specific IgE response and a mastocytosis in the infected host could undoubtedly enhance the allergic state. Also genetically predisposed atopic individuals seem to be more prone to develop atopy/asthma following the additional allergenic stimulation provided by parasite infection (Moqbel and Pritchard, 1990). The infection of cats with *T. cati* could therefore have a role in the development of food hypersensitivity in predisposed cats by the potentiation of the IgE response to orally administered antigen.

HSA-specific IgG was also found to be increased in the parasitised cats. Like the IgE response, the IgG4 response in man has been shown to depend on Th2 cells and their cytokine profile (Spiegelberg et al., 1991a), and helminth infection results in heightened parasite-specific and -non specific IgG4 antibody levels (IgG1 in the mouse) (Zakroff et al., 1989). In the present study, all subclasses of IgG were presumably detected by the ELISA and therefore the subclass specificity of the response is unknown.

Individuals with inflammatory bowel disease frequently have high levels of food-specific antibodies of the IgE and other isotypes (May et al., 1977; Johansson et al., 1984). Therefore the higher levels of food-specific antibodies seen in the parasitised cats may also have resulted from inflammation of the GI tract induced by the parasites. It could be hypothesised that the inflammation could facilitate the absorption of food antigens across the GI barrier. On the other hand, the inflammation could also facilitate antigen capture by the antigen presenting cells, which would be increased in number and situated in the mucosa close to the lumen of the GI tract. In such a case the induction of oral tolerance could be prevented (Strobel et al., 1985).

The level of total scrum IgG has also been shown to be increased in both atopic and parasitised dogs (Hill et al., 1995). However the concentration of total serum IgA was found to be lower in both groups than in healthy dogs. The authors suggested that

similar regulatory mechanisms governing immunoglobulin synthesis occurred in atopic and parasitised dogs which may lead to heightened IgG synthesis but to down-regulation of IgA production (Hill et al., 1995). IgA deficiency has been also reported to be associated with a higher incidence of atopic disease in humans (Kaufman and Hobbs, 1970; Buckley, 1975) and dogs (Campbell et al., 1991). However other studies failed to demonstrate lower IgA levels in atopic dogs (Hall and Campbell, 1993; Mueller,1993). In the present study, the level of allergen-specific IgA was shown to be significantly increased in the cats sensitised to HSA which were infected with *T. cati*. IgA producing cells have also been shown to be upregulated by Il-5 and Il-6 (McGhee et al., 1989). Therefore the increase in the levels of HSA-specific IgA found in the parasitised cats is not surprising. However the level of total IgA was not assessed in these cats.

Inoculation of the kittens with a vaccine derived from attenuated feline calicivirus, feline herpes virus and feline panleucopenia virus led to a significant increase in the levels of HSA-specific IgE in weeks 2 and 4, and in the levels of HSA-specific IgG and IgA in weeks 4 and 8 in the parasite-free cats. Vaccination with respiratory viruses has been reported to enhance the production of total and allergen-specific IgE in atopic individuals which suggests an IgE immunoregulatory role for certain viral infections, especially in atopy-prone children (Frick et al., 1979). Using the offspring of a colony of atopic dogs, Frick and Brooks (1983) showed that routine immunisation with modified live distemper virus increased the production of IgE to environmental allergens administered by injection. Pups given routine immunisation with pollen extracts had higher titres of IgE antibodies than did their control littermates which were not vaccinated until after the last pollen extract injection. However despite the marked reaginic antibody production to pollens, the dogs showed no evidence of atopy during the 14 months of study, except an occasional transitory rash on the face of one of the dogs. In addition, this study did not rule out the possibility of concomitant parasite infection.

In the model of food allergy induced in high IgE-producing atopic dogs immunised subcutaneously with food and pollen antigen extracts, the pups were vaccinated with live attenuated distemper/hepatitis vaccine (Ermel et al., 1997). The inbred colony of hypersensitive dogs developed IgE antibody to pollens and food antigens after immunisation with a live virus vaccine, followed immediately by a subcutaneous injection of food antigen extract in alum. It has been demonstrated that the stimulation of a naive immune system by a viral infection induces a response to the

challenging antigens in a somewhat non-specific manner, which will develop more specificity after further exposure (Frick and Brooks, 1983; Frick, 1986). In animals genetically predisposed to allergy and exposed to an initial early infection or viral challenge, the immune system is capable of responding to "bystander" antigens with more vigour. The CD4+/CD8+ cell ratio has been shown to rise in both atopic and non atopic humans immunised with a live attenuated measles virus vaccine (Peirsol and Frick, 1983). The ratio was higher and took longer to return to baseline in the atopic individuals than in the non atopic group. However the level of total serum IgE rose only in atopic subjects who had a high initial IgE level and not in atopic patients with a low IgE level or in non atopic patients (Peirsol and Frick, 1983).

More recently, Epstein-Barr virus in the presence of Il-4 has been demonstrated to stimulate human B cells and to induce T cell-independent IgE-synthesis (Thyphronitis et al., 1989). Viral infection (e.g. rotavirus) can also damage the gut mucosa which will become more permeable to other food allergens in the diet (Ermel et al., 1997). In the present study, the HSA-specific IgE response was only enhanced following immunoprophylaxis with live attenuated virus in parasite-free cats. It is unclear why the response in the parasitised cats was not similarly enhanced. The potentiation of the immune response induced by immunoprophylaxis was however much weaker than that resulting from endoparasitism.

Highly significant correlations were found between the levels of HSA-specific IgG and IgA. This would imply a similar mechanism in the immune responses involving these two isotypes.

Surprisingly an IgM response was never detected despite using 2 different polyclonal anti-feline IgM reagents. Small amounts of IgM antibodies to food have been shown to be present in the sera of normal individuals (Paganelli et al., 1984). In a study on the immune response to cow's milk in normal infants, IgM antibodies were only found in a few infants and were only present at low levels (Kletter et al., 1971). Low levels of IgM antibodies to cow's milk protein were also reported by Hunter et al. (1968). In contrast, Minden et al. (1966) were not able to detect any bovine serum albumin-specific IgM antibody in human sera. However in some pathological conditions such as inflammatory bowel disease, sera were found to contain high levels of IgM antibody to β-lactobglobulin (Falchuck and Isselbacher, 1976).

IgM producing cells are found in the mucosal lamina propria and secretory IgM is generated in the same way as secretory IgA (Mestecky and McGhee, 1987). Therefore,

the cats may still have had HSA-specific IgM in gastrointestinal secretions whilst failing to show a systemic response. In contrast to IgA, it is possible that the IgM produced locally may not be able to enter the circulation (Conley and Delacroix, 1987; Mestecky and McGhee, 1987).

In conclusion, the oral administration of HSA to kittens for three weeks induced antigen-specific IgE, IgG and IgA responses although the response was weak in the parasite-free cats. Administration of the trivalent freeze-dried virus vaccine significantly enhanced the production of allergen-specific IgE, IgG and IgA in parasite-free cats but had no effect on the production of allergen-specific antibodies in parasitised cats. These findings are supportive of a possible role of endoparasitism and vaccination in enhancing the IgE response to orally administered antigen, and hence possibly, in genetically susceptible individuals, in the development of food hypersensitivity.

# CHAPTER 7.

# GENERAL DISCUSSION

One aim of this work was to purify and further characterise feline IgE. The sera employed came from cats which were parasitised with *T. cati*, either naturally or experimentally. Therefore these sera were presumed to contain high levels of total IgE since levels of serum IgE were reported to be even higher in parasitised cats than in parasitised dogs (Alaba, 1997). Sera collected from several cats were pooled in order to obtain larger volumes similar to those used previously for the purification of canine IgE (Schwartzman et al., 1971; Halliwell, 1973). An attempt was made to obtain a fraction of IgE as pure as possible using the conventional methods of purification (Schwartzman et al., 1971; Peng et al., 1993a; Foster et al., 1995). However the fractions of semi-purified IgE were still found to contain other proteins in much higher concentration than the IgE antibody itself. An immunoaffinity column was made with the polyclonal anti-feline IgE and the IgE-rich fractions were passed over this column. The eluate from the anti-feline IgE column would be expected to be pure IgE. However the passage of these fractions through the anti-feline IgE affinity column resulted in non-specific binding of other proteins and the IgE antibodies in the eluate were still present in too low concentration to be characterised.

Although a protein with an apparent MW of approximately 80 KDa was present in all fractions assessed by SDS-PAGE, it was not possible to demonstrate the presence of feline IgE since a protein contaminant with a similar MW was also present and may have masked the IgE antibody. Foster (1995) also noted the presence of various proteins in the PCA positive column fractions, and attempts to further purify feline IgE were unsuccessful (Foster, 1995). On the other hand, Foster found the MW of the IgM heavy chain to be 85 KDa, which is very close to the MW expected for the IgE heavy chain. Therefore it would be even more difficult to isolate IgE on a gel where IgM is also present, as the latter was still found in some of the IgE-rich fractions partially purified by Foster (1995). However the chromatographic procedures used in the studies reported herein would be extremely unlikely to yield contamination with IgM unless the feline sera contained monomeric IgM, which has not been described.

Whereas feline IgE appears to share some antigenic determinants with the canine protein, the antigenic relationship between human and feline IgE apparently is less close.

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Indeed, while a strong cross-reactivity between canine and feline IgE was demonstrated using a polyclonal anti-canine IgE, human IgE was found to cross-react only weakly with feline IgE when assessed by RCA using 3 polyclonal anti-human IgE sera. Similar findings were noted by Foster (1995) who was not able to induce RCA by injection of monoclonal anti-human IgE. However polyclonal antibodies which recognise multiple epitopes are more likely to demonstrate cross-reactivity than are monoclonal antibodies which react only with a single epitope (Peng et al., 1996). Polyclonal anti-human IgE was shown to cross-react with canine IgE whereas monoclonal anti-human IgE did not (Becker et al., 1993).

Although it was not possible to purify and to isolate IgE on a gel, Fr-R contained sufficient IgE to immunise rabbits and induce polyclonal antisera directed against this isotype. Using affinity chromatography it was possible, as assessed by the ELISA and IEP, to remove any activity the antiserum had against other feline immunoglobulins and other heat stable proteins.

This is thus the first study that has described the production of a polyclonal antiserum to feline IgE and demonstrated the specificity of the antiserum for this isotype. Indeed, although a few reports have assessed the level of total or allergen-specific IgE in cats, none of them gave details of the production of the antisera employed or their specificity (Foster and O'Dair, 1993; Alaba, 1997).

The specificity of the antiserum is a key factor in ensuring the accuracy of an ELISA. Development of an ELISA using this polyclonal antiserum permitted a number of studies aimed at the understanding of the pathogenesis of allergic diseases in cats.

The kinetics of both the DF and HSA-specific IgE responses was found to be similar to the kinetics of IgE in other species. In general, the IgE response was shown to be earlier than the IgG and IgA responses and the length of the IgE response was shorter than that of the other isotypes. The IgE response to DF appeared to be boostable which contrasts with the findings of Zunic and collaborators (1998) in dogs. In the latter study, only puppies immunised immediately after birth were found to show such IgE responses (Zunic et al., 1998).

The studies on the DF-specific IgE levels in the three groups of cats (cats with atopy, normal household cats, and the normal laboratory reared cats in which IgE was induced experimentally) are of particular significance. DF-specific IgE was detected in the atopic cats, and in the laboratory reared cats following immunisation with DF, but also in normal household cats. Yet no clinical signs resulted in these two last groups.

Antibody was essentially absent from sera from the laboratory reared cats. In the 10 cats in which DF-specific IgE was induced experimentally, strongly positive skin tests were always correlated with positive PK tests, but neither were correlated with ELISA results. Furthermore, the development of HSA-specific IgE following oral immunisation resulted in no clinical signs.

This emphasises that it is not only the presence of IgE that distinguishes allergic individuals from normals and that the precise role of IgE in the pathogenesis of allergic diseases (atopy and food allergy) is not clear. Similar findings have been demonstrated in man and in dogs and strongly suggest the existence of heterogeneity of IgE. It seems likely that there is more than one type of IgE; with a particular subtype being responsible for allergic diseases.

As reported in both man and the dog, house dust mites (HDM) are reported to be the strongest allergens involved in the pathogenesis of atopic disease in cats. Whereas people react more frequently to *D. pteronyssinus* (DP) (Platts-Mills and Chapman, 1987), DF is more commonly involved in dogs (Prélaud 1991; Carlotti and Costargent, 1994; Sture et al., 1995; Lian and Halliwell, 1998) and cats (Carlotti and Prost, 1988; Foster and O'Dair 1993; Prost, 1996a; Halliwell, 1997). *Der p* 1, a major allergen of DP, was demonstrated to be able, through its protease activity, to disrupt the IgE network and to upregulate IgE production (Hewitt et al., 1995). It could be assumed that DF would also have such protease activity. Thus *Dermatophagoides* antigens appear to have a remarkable propensity to induce a response favouring the IgE isotype. It is not surprising therefore, that IgE to DF is found commonly in normal dogs and cats with no sign of allergic disease. The concept of heterogeneity of IgE could well account for discordance between ELISA, IDST and PK test results found in this study and in previous studies in dogs (Jackson et al., 1996; Lian and Halliwell, 1998; Peng et al., 1998). It is also of interest that ELISA using the high affinity receptor for IgE seems to detect only a small proportion of DF-specific IgE in the serum of dogs with atopic dermatitis and positive IDST to DF (McCall, 1998). A logical explanation of these findings is that some of the IgE induced by HDM may differ in its structure from the remainder and also from the IgE induced by the other allergens in having a lower affinity for FcεRI receptor. In such a case, the level of DF-specific IgE would not be expected to correlate with the presence and the severity of the clinical disease.

However, although the IgE response to HDM appears to be a unique phenomenon, it must be emphasised that IgE specific for other allergens is sometimes found in normal individuals, although generally at low levels.

This is the first report that has shown a specific IgE response to a food antigen given orally without concomitant parenteral administration. The studies also assessed the influence of the vaccination and endoparasitism on the specific IgE response. The route of antigenic challenge used was similar to that pertaining to spontaneous sensitisation that would occur in individuals with food allergy. In the models of food hypersensitivity successfully developed in dogs, puppies were sensitised not only via the oral route but also employing concomitant parenteral administration of food and non-food related antigens in aluminium hydroxide (Guilford et al., 1991; Frick et al., 1990; Guilford and Badcoe, 1992; Ermel et al., 1997).

Although weak, the majority of the parasite-free unvaccinated cats did develop an HSA-specific IgE response. However endoparasitism and vaccination were clearly shown to enhance the IgE response to the orally administered antigen. These factors may have a role in the development of food hypersensitivity and also of atopy in cats with an appropriate genetic background. This is important since a high percentage of pet cats are still infected with *T. cati* and almost all of them are vaccinated at a young age, and then revaccinated at intervals.

In conclusion, these studies have demonstrated for the first time the production and the specificity of a polyclonal anti-feline IgE. Using this antiserum, DF-specific IgE could be detected in atopic and also in normal household cats. However it was generally absent or very low in laboratory reared cats. DF-specific IgE was induced in normal laboratory reared cats following immunisation with DF in aluminium hydroxide. It was noteworthy that the levels of DF-specific IgE did not correlate with skin tests or with PK reactivity. No clinical signs resulted. These findings could not result from heterogeneity of the Fcε receptor, but can only be explained by the existence of a functional heterogeneity of IgE in cats. It was also shown that both endoparasitism and vaccination with a standard battery of viral vaccines in cats enhanced the IgE, IgA and IgG response to an orally administered antigen, and could therefore have a role in the development of food hypersensitivity in cats with an appropriate genetic background. Similarly to the cats sensitised to DF, the cats sensitised to HSA developed no clinical signs. This is also supportive of functional heterogeneity of feline IgE.

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# Veterinary immunology and immunopathology

## Production and characterisation of polyclonal antisera against feline IgE

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## Abstract

Cats, naturally or experimentally infected with *Toxocara cati* were immunised with dinitrophenylated ascaris antigen (DNP-Asc). All cats developed immediate skin reactivity to DNP coupled to bovine serum albumin (DNP–BSA) and the sera of the nine cats had a heat labile homocytotropic antibody detectable by homologous Prausnitz–Küstner (PK) tests. Reagin-rich fractions were prepared from these sera and used for the preparation of polyclonal antisera in rabbits. Resultant antisera were passed through a immunoabsorbent column of Sepharose 4B coupled to heated normal cat serum. An immunoabsorbent column prepared with the resultant antisera removed the PK reactivity from the cat sera, and the activity was recovered following acid elution. The antiserum failed to detect any recognised immunoglobulin in cat sera, but precipitated with a heat labile protein with $\gamma$-1 electrophoretic mobility in the sera of parasited cats. These findings support the contention that the antisera are specific for feline IgE. © 1998 Published by Elsevier Science B.V. All rights reserved.

*Keywords:* Immunoglobulin E (IgE); Polyclonal antisera; *Toxocara cati*; Dinitrophenylated Ascaris (DNP-Asc);
Feline

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## 1. Introduction

Cats suffer from numerous skin diseases whose pathogenesis may involve immediate-type hypersensitivity reactions but are still poorly understood. Among these are miliary dermatitis, self-induced alopecia, eosinophilic granuloma complex and pruritus of the face, neck and pinnae (Scott et al., 1995). Respiratory disease of allergic origin, so-called feline asthma, may also result from an IgE-mediated pathogenesis (Halliwell and Gorman, 1989; Corcoran et al., 1995). Unfortunately, many cases of diseases that are probably allergic in aetiology remain undiagnosed because of their corticosteroid responsiveness, and the relative lack of side effects of such drugs in the feline species. Although IgE has not been immunochemically identified in the cat, there is convincing evidence that reaginic antibody is produced in association with certain disease states.

Reaginic antibody has been characterised in many species including the dog (Halliwell et al., 1972). IgE antibodies in animals share many physicochemical properties with IgE antibody of man. They are characterised by a relatively fast electrophoretic mobility, a sedimentation coefficient of approximately 8 S, a molecular weight of approximately 200 000 Da, the capacity to fix to tissue following injection for > 3 weeks and a high sensitivity to heat and reducing agents (Halliwell et al., 1972).

Several studies have already shown that cat serum contains a protein with IgE-like properties (Walton et al., 1968; Powell et al., 1980; De Boer et al., 1993; Baldwin et al., 1993; Foster et al., 1995). Walton et al. (1968) demonstrated the existence of reaginic antibody to whole milk antigen in a cat with dermatitis and enteritis using Prausnitz–Küstner (PK) testing. In another study, reaginic antibodies to the ear mite *Otodectes cynotis* were demonstrated and are believed to be responsible for the extreme pruritus that is associated with some cases of parasitic otitis externa (Powell et al., 1980). More recently, De Boer et al. (1993) showed that cats have a reaginic antibody that is antigenically cross-reactive with canine IgE and has an apparent molecular weight similar to that of IgE from other species. Foster et al. (1995) demonstrated that the passive cutaneous anaphylaxis (PCA) reactivity was eluted following chromatography using Sephadex G200 in a position similar to that of other species, and that feline reaginic antibodies are bound by protein A. The latter author used PCA in which the antigen is injected intravenously as opposed to the PK test in which the antigen is injected intradermally.

The existence of myelomas have permitted the ready purification of IgE for the subsequent production of monoclonal antibodies in man (Johansson and Bennich, 1967), and the rat and the mouse (Bazin et al., 1974; Böttcher and Hämmerling, 1978). However, in the cat, the lack of myelomas secreting IgE, the presumably low level of serum IgE and the small amount of serum obtainable make the isolation of IgE very difficult.

The further characterisation of feline IgE and the development of specific antisera are prerequisites for the elucidation of the role of IgE and of immediate type hypersensitivity in a number of common feline diseases. The goal of this study was thus to produce polyclonal antisera against the immunoglobulin to permit quantification of total and allergen-specific feline IgE in diseases which may involve immediate-type hypersensitivity.

## 2. Materials and methods

### 2.1. Experimental animals

Infected and normal cats were obtained from a colony of experimental cats at the University of Liège (Belgium).

Normal adult (ex-breeder) parasite-free cats for PK tests and reversed cutaneous anaphylaxis (RCA) were obtained from Hill Grove Family Farm (Oxford, UK).

New Zealand white rabbits were obtained from Interfauna UK (Cambridge, UK).

All experimental protocols were in accordance with the requirements of an Animal Care and Ethics Committee and/or the UK Home Office as appropriate.

### 2.2. Infection of cats with Toxocara cati

Three 10-month old cats (#5,#6,#7) and three 12 to 16-week old kittens (#1, #2, #3) were found to be naturally infected with *T. cati*. All were secreting ova in their faeces.

Three additional normal 10-week old kittens (#4, #8, #9) were experimentally infected with *T. cati*. The eggs were isolated from the faeces of naturally infected cats. They were washed three times in 1% sodium chloride and centrifuged at 1500 rpm for 5 min. After discarding the final rinse, the eggs were resuspended in 0.5% formalin (Sigma, Poole, UK) and kept, under oxygen, in a dark room, at 27°C. Infective second stage larvae were obtained after 3 weeks. The cats were dosed with 10 embryonated ova daily for 3 days. Development of a patent infection was indicated by the appearance of the ova in the faeces within 60 to 75 days.

### 2.3. Ascaris antigens

Purified antigen from *Ascaris suum* (Asc-1) was purchased from Greer (Lenoir, NC).

Adult *T. cati* were obtained post mortem from naturally infected cats and antigens from body fluid were partially purified by gel filtration on Sephadex G100 (Pharmacia, Uppsala, Sweden) to remove the low molecular weight histamine releasing fractions. Material from the major protein peak was concentrated.

### 2.4. Preparation of dinitrophenylated antigen

Asc-1 and bovine serum albumin (BSA) (Sigma) were dinitrophenylated (DNP) according to the method of Eisen (1964).

### 2.5. Induction of feline anti-DNP and anti-Ascaris antibodies

Cats were immunised with DNP-Asc in aluminium hydroxide (Imject Alum®; Pierce, Rockford, IL, USA) in order to induce anti-DNP reaginic antibody as described for the dog (Schultz and Halliwell, 1985). Each cat received two biweekly injections of 10 μg

of DNP-Asc subcutaneously over the lateral thorax at two sites on either side of the body.

## 2.6. Performance of intradermal skin tests (IDST)

Cats were anaesthetised with 1 mg/kg xylazine (Virbaxyl®, Virbac, Cambridge, UK) and 10 mg/kg ketamine hydrochloride (Vetalar®, Parke-Davis, Pontypool, Gwent, UK) intramuscularly. The lateral chest was clipped and 0.05 ml of a solution of 10 $\mu$g/ml of DNP–BSA was injected intradermally. Phosphate buffered saline (PBS) and 1/100000 w/v solution of histamine phosphate (ARTU Biologicals, Lelystad, Netherlands) were used as negative and positive controls, respectively. Reactions were scored after 15 to 20 min using a +1 to +4 scale, incorporating both diameter and elevation above the surface. A +1 reaction was just discernibly greater than the PBS wheal, and a +4 reaction approximated the size of the histamine wheal.

## 2.7. Fractionation of sera

T. cati-infected cats were bled by jugular venipuncture. Sera were harvested by centrifugation after clot retraction and were stored at −30°C.

Sera were fractionated by the same three-step procedure that was employed to prepare IgE-rich fractions of canine sera (Halliwell et al., 1972). Briefly, serum that precipitated between 33.33 and 45% saturation with ammonium sulphate was dialysed against 0.05 M phosphate buffer, pH 8.0, and applied to a column of diethylaminoethyl cellulose (DEAE; Watman, Maidstone, Kent, UK) equilibrated with the same buffer. The excluded protein was concentrated and then applied to a three-in-series 4 × 60 cm column set of Sephacryl S-200 (Pharmacia) and eluted using PBS. The protein eluting immediately prior to the 7 S peak and the first one third of that peak was pooled and concentrated using Centripet-10 concentrators (Amicon, Gloucester, UK) as fraction R (Fr-R).

## 2.8. Preparation of polyclonal antisera to IgE

Three New Zealand white rabbits were immunised with three biweekly injections of 100 to 200 $\mu$g of Fr-R in 1 ml of Freund's complete adjuvant (Sigma) subcutaneously at four sites.

The resulting antisera were passed over an immunoabsorbent column of Sepharose 4B (Sigma) to which was coupled serum from a parasite-free cat that had been heated to 56°C for 4 h. This was repeated a number of times until no protein was eluted by glycine–HCl buffer, pH 2.7. This column removed any activity against immuno-globulins other than IgE and also against other heat stable serum proteins (Vaerman and Heremans, 1968).

The antiserum which had the best apparent anti-IgE activity as assessed by reverse cutaneous anaphylaxis (RCA) (R294-7) was then used to prepare another immunoab-sorbent column. A 45% ammonium sulphate precipitate of the antiserum was dissolved in PBS and then coupled to Sepharose 4B. Protein from sera of parasited cats that bound

to the column following the subsequent elution with glycine–HCl, was used to immunise four further rabbits using the same procedure as above.

## 2.9. Preparation of anti-feline light chain serum

Feline IgG was first prepared from a 33.3% ammonium sulphate precipitate of normal feline serum by exclusion from DEAE in 0.01 M phosphate buffer, pH 8.0. Rabbit anti-feline IgG was subsequently prepared by immunising New Zealand white rabbits with $3 \times 1$ mg aliquots biweekly in Freund's incomplete adjuvant. Anti-feline light chain serum was then prepared by adsorption and elution of the anti-feline IgG from an immunoabsorbent column of Sepharose 4B to which was coupled feline $(Fab)_2$. The latter was prepared by pepsin digestion of feline IgG at pH 4.5 for 24 h and subsequent isolation using Sephadex G-100 (Pharmacia).

## 2.10. Induction of reversed cutaneous anaphylaxis

Antisera were assessed for activity against IgE by their ability to induce RCA on intradermal injection in healthy parasite-free cats. Cats were anaesthetised with 10 mg/kg ketamine hydrochloride intramuscularly. The hair of the lateral chest area was clipped and 0.05 ml of each dilution was injected intradermally. Antisera were tested at 1:10 and 1:100 and then at doubling dilutions. Normal rabbit serum (1/10, 1/100 and 1/1000) and PBS were included as negative controls and histamine phosphate 1/100 000 w/v as a positive control.

Immediate skin reactions were recorded 15 to 20 min after the injection as above for IDST.

## 2.11. Homologous Prausnitz–Küstner tests

Homologous PK tests were performed to assess the DNP-specific IgE and the Asc-specific IgE in the sera from nine infected cats. Healthy parasite-free cats were anaesthetised with 10 mg/kg ketamine hydrochloride intramuscularly. The hair of the lateral chest area was clipped. One-tenth millilitre of 10-fold dilutions in PBS (1–1/10000) of each serum was injected intradermally. Injection sites were challenged 48 h later by intradermal injection of 0.05 ml of a 10-$\mu$g/ml solution of DNP–BSA, 100 $\mu$g/ml of Asc-1 and 100 $\mu$g/ml of *T. cati* antigen. Negative controls (PBS, DNP-BSA 10 $\mu$g/ml or Asc 100 $\mu$g/ml) and a positive control (histamine phosphate 1/100000 w/v) were injected into unsensitised sites. Skin reactions were recorded 15 to 20 min after the injection as above.

## 2.12. Neutralisation of PK reactivity of reactive sera

Antisera were also assessed for activity against IgE by their ability to remove PK reactivity from feline sera. PK tests were performed as above with sera that gave positive reactions before and after two and four passages through the anti-IgE immunoabsorbent column (R294).

## 2.13. Immunoelectrophoresis on agar gel

Immunoelectrophoresis (IEP) was performed using 1.5% agar in 0.03 M barbital buffer pH 8.6. Gels were run for 55 min at 50 mA and incubated in a humidity chamber at room temperature for 48 h (Grabar and Williams, 1953).

## 3. Results

### 3.1. Parasitological findings

All cats developed a patent infection with *T. cati* with ova appearing in the faeces within 60 to 75 days. After euthanasia, adult *T. cati* were found in the duodenum of cats #1–#4. No *T. cati* were recovered in the other cats. However *Dypilidium caninum* was found in the large intestine of cats #4–#7.

### 3.2. IDST

All cats had a positive IDST to DNP–BSA with four cats (cats 1, 3, 6 and 7) showing a +3 reaction; four cats (cats 2, 4, 8 and 9), a +2 reaction; and one cat (cat 5), only a +1 reaction.

### 3.3. Homologous PK tests

The 10-fold dilutions of sera from immunised cats were assessed for PK reactivity. Positive results were recorded with dilutions out to 1/100 (three cats),1/10 (five cats), with one cat reacting only with undiluted serum when challenged by DNP–BSA.

Serum from only one cat gave a positive reaction with *T. cati*. No sera showed PK reactivity against Asc-1.

### 3.4. RCA tests

Six of the seven antisera raised against the putative IgE rich fraction (RI94–R394, R794, R994 and R1094) were able to induce RCA at a dilution of > 1/100 (Table 1).

Table 1
RCA titres of seven polyclonal antisera raised against feline IgE preparations

| Antiserum | RCA titre * |
|-----------|-------------|
| R194 * * | 800 |
| R294 * * | 3200 |
| R394 * * | 3200 |
| R794 * * * | 400 |
| R894 * * * | negative |
| R994 * * * | 400 |
| R1094 * * * | 1600 |

* Titre using doubling dilutions of each antiserum from 1/100.

* * Antisera raised against Fr-R.

* * * Antisera raised against the protein eluted from the putative anti-IgE immunoabsorbent column.
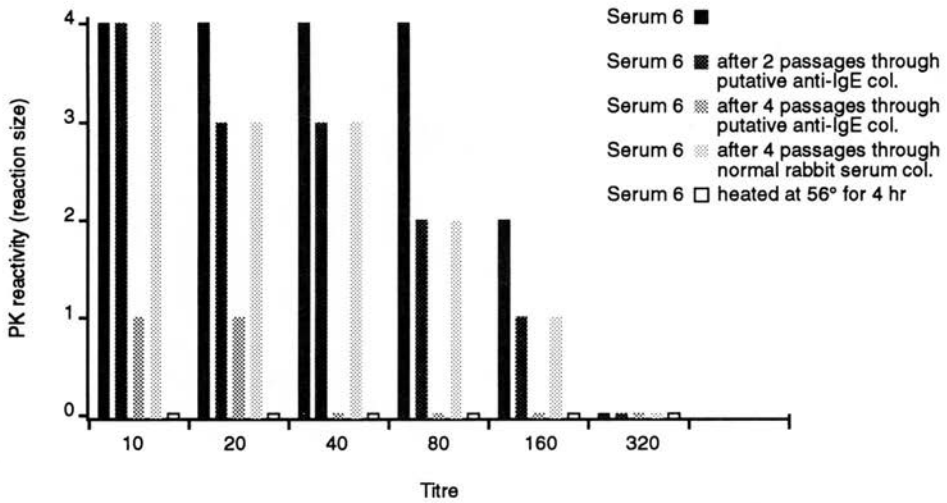
Fig. 1. Effects of the passage of feline reagin-containing serum #6 through a putative anti-IgE immunoabsorbent column on the PK titre to DNP.

Although two antisera (R294 and R394) were able to induce a wheal on intradermal injection at 1/3200 dilution, the largest wheal at that dilution was obtained with R294 which gave stronger reactions. Reactions were not seen with normal rabbit serum.

## 3.5. Neutralisation of PK reactivity of reactive sera

PK activity to DNP and *T. cati*, respectively in sera #4 and #6 decreased progressively following each passage through the putative anti-IgE immunoabsorbent column (R294). Although the diminution in PK reactivity was evident in both cases, it was most significant in case of serum #6 (Fig. 1). In contrast, passage of the sera through a column of normal rabbit serum coupled to cyanogen bromide activated Sepharose 4B produced only a small decrease of PK reactivity (Fig. 1). Heating at 56°C for 4 h removed all PK reactivity (Fig. 1). When the eluate from the immunoabsorbent column used for serum #4 was assessed for PK reactivity to DNP, a +4 result was obtained following injection of 0.1 ml of a 375-$\mu$g/ml preparation of pooled eluates.

## 3.6. Immunoelectrophoresis on agar gel

### 3.6.1. Specificity of the putative anti-feline IgE

Rabbit antiserum R294 from the ninth bleeding and R1094 from the fifth bleeding were able to precipitate with a protein which had $\gamma$-1 electrophoretic mobility in the nine sera from infected cats and in the serum from a parasite-free cat, but this protein was not detectable in heated sera (Fig. 2). The same antisera failed to detect purified feline IgG. This protein was also no longer detectable after four passages of the serum through the anti-IgE immunoabsorbent column.
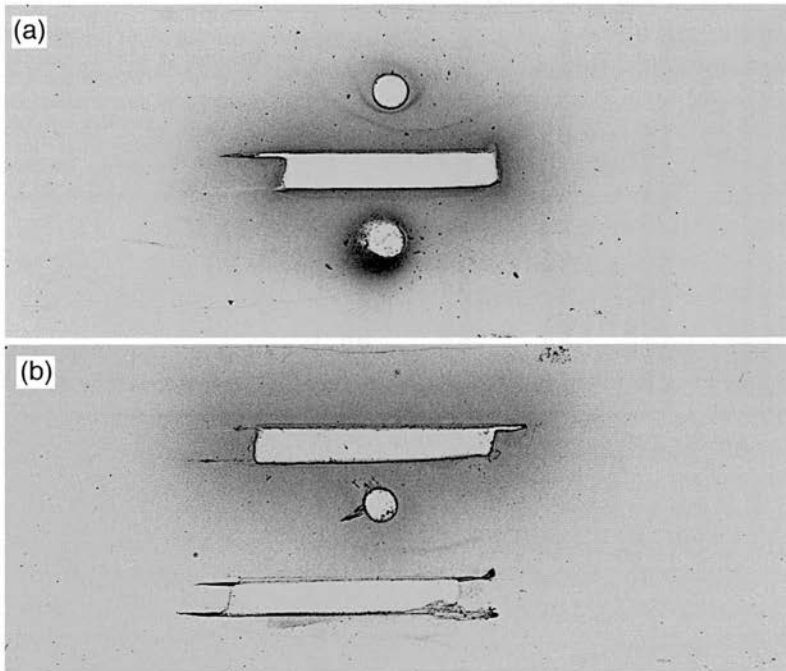
Fig. 2. Antibody activity of putative anti-feline IgE serum (R294) as revealed by immunoelectrophoresis. (a) Top well: cat serum #4. Bottom well: cat serum #4 heated at 56° for 4 h. Trough: antiserum R294. (b) Top trough: antiserum R294. Bottom trough: anti-feline light chain immunoglobulin. Well: eluate from R294 immunoabsorbant column.

### 3.6.2. Immunoelectrophoretic analysis of the eluate from the putative anti-IgE column

Rabbit antiserum against feline immunoglobulin light chain detected a single protein which had γ-1 electrophoretic mobility in the putative IgE preparation eluted from the column R294-7 (Fig. 2). However, R294 was not able to precipitate with a protein in this putative IgE, and equally the protein was not detected by anti-feline IgG (Fc-specific).

## 4. Discussion

Collectively, the data presented are persuasive evidence of the specificity of the polyclonal antiserum for feline IgE. On IEP on agar gel, antisera R294 and R1094 were able to precipitate with a heat labile immunoglobulin which had γ-1 electrophoretic mobility. The protein eluted from the anti-IgE column was shown to be an immunoglobulin by its detection by a rabbit anti-feline light chain serum, but was not detected by the anti-IgE sera R294 and R1094. Neither was it detected by Fc-specific anti-feline IgG. It has been shown that canine IgE is sensitive to acid treatment (Halliwell et al., 1975) and the same is likely to hold true for the feline isotype. Since 0.1 M glycine–HCl pH 2,7 was used to elute the solution from the putative anti-IgE affinity column, the Fc

portion of the reaginic antibody may have been denatured although it was immediately neutralised with 1 M Tris–HCl to pH 8. This could explain why R294 failed to precipitate with the immunoglobulin in the putative IgE eluted from the anti-IgE column. It could also explain why the antisera produced against this eluate were weaker in terms of RCA activity.

Although many researchers recommend that CFA should only be used for the first injection in antisera production for humane reasons, it was used in these studies on three occasions with full permission of the Home Office. This was done because previous experience in our laboratory has shown that the induction of antisera against IgE of animal origin, which is likely to be present in very small quantities, is exceedingly difficult using any other protocol. Painless granulomatous swellings which did not ulcerate, were the only adverse effects observed, and the rabbits were not affected systemically.

The PK test has proved to be a sensitive method to detect the presence of reaginic antibody (Prausnitz and Küstner, 1921). The sensitivity of the test has been shown to be 0.2 ng/ml in human sera and 2–3 ng/ml in mice sera (Ishizaka, 1988). The optimal period after sensitisation for the detection of IgE using PK or PCA tests is 24 to 48 h while for IgG, this period is 1 to 6 h. Moreover, the sensitisation induced by IgG persists only for a short time of 1 to 2 days while the IgE binds to mast cells for about 3 weeks (Ovary, 1986). Those characteristics as well as the heat sensitivity permit distinction between IgE and IgG homocytotropic antibodies. In cats, as in other species PCA tests have also been used extensively to establish the presence of allergen specific IgE (Walton et al., 1968; Powell et al., 1980; Baldwin et al., 1993; Foster et al., 1995).

Sera from eight of the nine cats immunised with DNP-Asc were able to give a positive PK test to DNP at 1/10 dilution with three sera reacting out to 1/100. However, results of PK tests when challenged by Asc-1 or *T. cati* antigen were surprising in that only one serum gave a PK reaction when challenged with *T. cati* antigen despite the fact that all had previous patent infection with *T. cati*. It is also difficult to understand why the immunisation of the cats with DNP-Asc-1 failed to induce detectable Asc-1-specific IgE. It has been shown by Strejan et al. (1973) that Asc-1 allergen was strongly immunogenic and when coupled to DNP was far more effective in inducing DNP-specific IgE than when coupled to other proteins (Strejan and Marsh, 1971). A lower dose of Asc-1 could have been more effective since Strejan et al. (1973) found that Asc-1 was more immunogenic at a dose of 1 $\mu$g than at 10 $\mu$g.

Also, in some systems, the potentiation of reagin by helminth antigens seems to require active infection and is usually transient and ceases when most of the parasites have been expelled (Orr et al., 1971). In this study, the IgE response to the DNP does not appear to be related to the potentiation by helminth infection but may still be related to the immunisation of the cats with DNP coupled to Asc-1. It is also possible that the differences seen in this study in the level of DNP-specific IgE could depend upon the genetic background (Tada, 1975).

Antigenic cross-reactivity between stages of *Toxocara* and other ascarid species has been described (Page et al., 1991). However, in one study, Foster et al. (1995) used crude *A. suum* and *T. canis* antigens in PCA tests using sera from cats infected with *T. canis*, with negative results. The author hypothesised that the antigens responsible for

PCA activity may have been present in different quantities at different stages of the parasite life cycle (Foster et al., 1995). Cox (1993) has shown that the various immune responses are stage specific. The life cycle of *T. cati* is complex and depends on the route of infection of the cat. In contrast to transmammary infection in which larvae acquired by the kitten apparently do not migrate (Swerczek et al., 1971), infection by the ingestion of embryonated eggs leads to somatic migration with accumulation of arrested larvae in the body tissues (Bowman, 1995). Thus, the cats with negative PK activity to antigens of the adult worm may still have had some IgE activity against larval stages of *T. cati*.

The reasons for the failure of most of the cats to develop a detectable IgE response to the parasite antigens studied using the PK test are thus obscure. However, since the detection of specific-IgE was not performed by enzyme-linked immunoassay we should not exclude the possibility of the existence of another Asc-1 or *T. cati*-specific IgE isoform which was either less efficient in binding to mast cells or in inducing degranulation. It is also quite possible that antibodies of other isotypes were produced.

In conclusion, the claim of the specificity of the antisera for feline IgE is supported by a number of results. Firstly, the DNP-specific PK reactivity was removed by passage through the putative anti-IgE immunoabsorbent column. Secondly, PK reactivity was recovered in the eluate. Thirdly, this eluate contained immunoglobulin which was not IgG. Also, the antisera were able to induce RCA in high dilution and finally, they were able to detect a heat labile protein in the sera of parasited cats which had an electrophoretic mobility compatible with that known for this isotype in other species.

## Acknowledgements

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# Veterinary immunology and immunopathology

## Production and characterisation of polyclonal antisera against feline IgE

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# Production and characterisation of polyclonal antisera against feline IgE

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## Abstract

Cats, naturally or experimentally infected with *Toxocara cati* were immunised with dinitrophenylated ascaris antigen (DNP-Asc). All cats developed immediate skin reactivity to DNP coupled to bovine serum albumin (DNP–BSA) and the sera of the nine cats had a heat labile homocytotropic antibody detectable by homologous Prausnitz–Küstner (PK) tests. Reagin-rich fractions were prepared from these sera and used for the preparation of polyclonal antisera in rabbits. Resultant antisera were passed through a immunoabsorbent column of Sepharose 4B coupled to heated normal cat serum. An immunoabsorbent column prepared with the resultant antisera removed the PK reactivity from the cat sera, and the activity was recovered following acid elution. The antiserum failed to detect any recognised immunoglobulin in cat sera, but precipitated with a heat labile protein with $\gamma$-1 electrophoretic mobility in the sera of parasited cats. These findings support the contention that the antisera are specific for feline IgE. © 1998 Published by Elsevier Science B.V. All rights reserved.

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## 1. Introduction

Cats suffer from numerous skin diseases whose pathogenesis may involve immediate-type hypersensitivity reactions but are still poorly understood. Among these are miliary dermatitis, self-induced alopecia, eosinophilic granuloma complex and pruritus of the face, neck and pinnae (Scott et al., 1995). Respiratory disease of allergic origin, so-called feline asthma, may also result from an IgE-mediated pathogenesis (Halliwell and Gorman, 1989; Corcoran et al., 1995). Unfortunately, many cases of diseases that are probably allergic in aetiology remain undiagnosed because of their corticosteroid responsiveness, and the relative lack of side effects of such drugs in the feline species. Although IgE has not been immunochemically identified in the cat, there is convincing evidence that reaginic antibody is produced in association with certain disease states.

Reaginic antibody has been characterised in many species including the dog (Halliwell et al., 1972). IgE antibodies in animals share many physicochemical properties with IgE antibody of man. They are characterised by a relatively fast electrophoretic mobility, a sedimentation coefficient of approximately 8 S, a molecular weight of approximately 200 000 Da, the capacity to fix to tissue following injection for $> 3$ weeks and a high sensitivity to heat and reducing agents (Halliwell et al., 1972).

Several studies have already shown that cat serum contains a protein with IgE-like properties (Walton et al., 1968; Powell et al., 1980; De Boer et al., 1993; Baldwin et al., 1993; Foster et al., 1995). Walton et al. (1968) demonstrated the existence of reaginic antibody to whole milk antigen in a cat with dermatitis and enteritis using Prausnitz–Küstner (PK) testing. In another study, reaginic antibodies to the ear mite *Otodectes cynotis* were demonstrated and are believed to be responsible for the extreme pruritus that is associated with some cases of parasitic otitis externa (Powell et al., 1980). More recently, De Boer et al. (1993) showed that cats have a reaginic antibody that is antigenically cross-reactive with canine IgE and has an apparent molecular weight similar to that of IgE from other species. Foster et al. (1995) demonstrated that the passive cutaneous anaphylaxis (PCA) reactivity was eluted following chromatography using Sephadex G200 in a position similar to that of other species, and that feline reaginic antibodies are bound by protein A. The latter author used PCA in which the antigen is injected intravenously as opposed to the PK test in which the antigen is injected intradermally.

The existence of myelomas have permitted the ready purification of IgE for the subsequent production of monoclonal antibodies in man (Johansson and Bennich, 1967), and the rat and the mouse (Bazin et al., 1974; Böttcher and Hämmerling, 1978). However, in the cat, the lack of myelomas secreting IgE, the presumably low level of serum IgE and the small amount of serum obtainable make the isolation of IgE very difficult.

The further characterisation of feline IgE and the development of specific antisera are prerequisites for the elucidation of the role of IgE and of immediate type hypersensitivity in a number of common feline diseases. The goal of this study was thus to produce polyclonal antisera against the immunoglobulin to permit quantification of total and allergen-specific feline IgE in diseases which may involve immediate-type hypersensitivity.

## 2. Materials and methods

### 2.1. Experimental animals

Infected and normal cats were obtained from a colony of experimental cats at the University of Liège (Belgium).

Normal adult (ex-breeder) parasite-free cats for PK tests and reversed cutaneous anaphylaxis (RCA) were obtained from Hill Grove Family Farm (Oxford, UK).

New Zealand white rabbits were obtained from Interfauna UK (Cambridge, UK).

All experimental protocols were in accordance with the requirements of an Animal Care and Ethics Committee and/or the UK Home Office as appropriate.

### 2.2. Infection of cats with Toxocara cati

Three 10-month old cats (#5,#6,#7) and three 12 to 16-week old kittens (#1, #2, #3) were found to be naturally infected with *T. cati*. All were secreting ova in their faeces.

Three additional normal 10-week old kittens (#4, #8, #9) were experimentally infected with *T. cati*. The eggs were isolated from the faeces of naturally infected cats. They were washed three times in 1% sodium chloride and centrifuged at 1500 rpm for 5 min. After discarding the final rinse, the eggs were resuspended in 0.5% formalin (Sigma, Poole, UK) and kept, under oxygen, in a dark room, at 27°C. Infective second stage larvae were obtained after 3 weeks. The cats were dosed with 10 embryonated ova daily for 3 days. Development of a patent infection was indicated by the appearance of the ova in the faeces within 60 to 75 days.

### 2.3. Ascaris antigens

Purified antigen from *Ascaris suum* (Asc-1) was purchased from Greer (Lenoir, NC).

Adult *T. cati* were obtained post mortem from naturally infected cats and antigens from body fluid were partially purified by gel filtration on Sephadex G100 (Pharmacia, Uppsala, Sweden) to remove the low molecular weight histamine releasing fractions. Material from the major protein peak was concentrated.

### 2.4. Preparation of dinitrophenylated antigen

Asc-1 and bovine serum albumin (BSA) (Sigma) were dinitrophenylated (DNP) according to the method of Eisen (1964).

### 2.5. Induction of feline anti-DNP and anti-Ascaris antibodies

Cats were immunised with DNP-Asc in aluminium hydroxide (Imject Alum®; Pierce, Rockford, IL, USA) in order to induce anti-DNP reaginic antibody as described for the dog (Schultz and Halliwell, 1985). Each cat received two biweekly injections of 10 μg

of DNP-Asc subcutaneously over the lateral thorax at two sites on either side of the body.

## 2.6. Performance of intradermal skin tests (IDST)

Cats were anaesthetised with 1 mg/kg xylazine (Virbaxyl®, Virbac, Cambridge, UK) and 10 mg/kg ketamine hydrochloride (Vetalar®, Parke-Davis, Pontypool, Gwent, UK) intramuscularly. The lateral chest was clipped and 0.05 ml of a solution of 10 $\mu$g/ml of DNP–BSA was injected intradermally. Phosphate buffered saline (PBS) and 1/100 000 w/v solution of histamine phosphate (ARTU Biologicals, Lelystad, Netherlands) were used as negative and positive controls, respectively. Reactions were scored after 15 to 20 min using a +1 to +4 scale, incorporating both diameter and elevation above the surface. A +1 reaction was just discernibly greater than the PBS wheal, and a +4 reaction approximated the size of the histamine wheal.

## 2.7. Fractionation of sera

T. cati-infected cats were bled by jugular venipuncture. Sera were harvested by centrifugation after clot retraction and were stored at $-30°$C.

Sera were fractionated by the same three-step procedure that was employed to prepare IgE-rich fractions of canine sera (Halliwell et al., 1972). Briefly, serum that precipitated between 33.33 and 45% saturation with ammonium sulphate was dialysed against 0.05 M phosphate buffer, pH 8.0, and applied to a column of diethylaminoethyl cellulose (DEAE; Watman, Maidstone, Kent, UK) equilibrated with the same buffer. The excluded protein was concentrated and then applied to a three-in-series 4 × 60 cm column set of Sephacryl S-200 (Pharmacia) and eluted using PBS. The protein eluting immediately prior to the 7 S peak and the first one third of that peak was pooled and concentrated using Centripet-10 concentrators (Amicon, Gloucester, UK) as fraction R (Fr-R).

## 2.8. Preparation of polyclonal antisera to IgE

Three New Zealand white rabbits were immunised with three biweekly injections of 100 to 200 $\mu$g of Fr-R in 1 ml of Freund's complete adjuvant (Sigma) subcutaneously at four sites.

The resulting antisera were passed over an immunoabsorbent column of Sepharose 4B (Sigma) to which was coupled serum from a parasite-free cat that had been heated to 56°C for 4 h. This was repeated a number of times until no protein was eluted by glycine–HCl buffer, pH 2.7. This column removed any activity against immuno-globulins other than IgE and also against other heat stable serum proteins (Vaerman and Heremans, 1968).

The antiserum which had the best apparent anti-IgE activity as assessed by reverse cutaneous anaphylaxis (RCA) (R294-7) was then used to prepare another immunoab-sorbent column. A 45% ammonium sulphate precipitate of the antiserum was dissolved in PBS and then coupled to Sepharose 4B. Protein from sera of parasited cats that bound

# Veterinary immunology and immunopathology

## Feline immunoglobulin E: induction of antigen-specific antibody in normal cats and levels in spontaneously allergic cats

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## Abstract

Sera from 10 cats with symptoms consistent with atopy, from 15 normal household cats and from 11 laboratory maintained cats were assessed for allergen-specific IgE and IgG to *Dermatophagoides farinae* (DF) by enzyme-linked immunosorbent assay (ELISA). In addition, 10 normal cats were immunised with DF and intradermal skin tests (IDST) were performed weekly. Sera from the latter were also assessed for DF-specific IgE by ELISA and using Prausnitz–Küstner (PK) tests. Although DF-specific IgE was detectable in all the atopic cats, there was no significant difference between the levels in this group and in the clinically normal household cats. However levels in both these groups were significantly higher than those in the laboratory maintained cats. Detectable DF-specific IgE was induced in all of the 10 cats, but the levels were not correlated with the development of positive IDSTs, nor with the level of IgE as assessed by PK tests. These findings are consistent with a possible heterogeneity of IgE antibody in cats. © 1998 Elsevier Science B.V. All rights reserved.

*Keywords:* Immunoglobulin E (IgE); *Dermatophagoides farinae* (DF); Enzyme-linked immunosorbent assay (ELISA); Intradermal skin test (IDST); Cat

## 1. Introduction

Allergic skin disease is a common reason for the presentation of cats to veterinarians. One important cause is believed to be feline atopy, although an inherited predisposition

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to this disease has not been proved and thus the true designation of atopy in this species remains uncertain. The existence of reaginic antibody in cats has already been shown by passive cutaneous anaphylaxis (PCA) tests and by demonstrating heat or mercapto-ethanol sensitivity (Walton et al., 1968; Powell et al., 1980; Foster et al., 1995). Further evidence was provided by DeBoer et al. (1993) who also demonstrated shared anti-genicity between canine immunoglobulin E (IgE) and the feline homologue. Cat bladder tissue was sensitised with sera from a parasitised cat and contraction noted following challenge with monoclonal anti-canine IgE antibody. The development of in vitro tests for the measurement of allergen-specific IgE is thus an important goal as an aid to the practice of feline dermatology.

Although enzyme-linked immunosorbent assays (ELISAs) are marketed for the detection of allergen-specific feline IgE, details supporting the specificity of these antisera have not yet been published in the scientific literature. In a study of one of these assays, Foster and O'Dair (1993) found that the correlation with intradermal skin tests was very poor. Both the specificity and the sensitivity of the ELISA as compared with the intradermal skin test results were low with a positive or negative predictive value of 0.1 and 0.4, respectively.

The previous paper has documented the production of polyclonal antisera that were adjudged specific for feline IgE (Gilbert and Halliwell, 1997). It was important therefore to ensure that the antisera could detect IgE antibodies in the sera of cats with symptoms consistent with atopy and in the sera of normal cats in which allergen specific IgE was induced experimentally under controlled conditions. As house dust mites are the most common allergens involved in atopic disease in dogs and cats in Europe (Foster and O'Dair, 1993; Carlotti and Costargent, 1994; Sture et al., 1995) *Dermatophagoides farinae* (DF) was chosen as the antigen. The kinetics of IgE and IgG responses specific for DF in cats experimentally immunised were followed during the 8 weeks of the study.

## 2. Materials and methods

### 2.1. Animals

#### 2.1.1. Clinical study

Serum was collected from 10 cats seen at the Royal (Dick) School of Veterinary Studies (R(D)SVS) at the University of Edinburgh and at the Faculté de Medecine Vétérinaire, Université de Liège in Belgium, in which a diagnosis of atopic disease was made by a combination of compatible clinical signs and positive intradermal skin tests (IDSTs) to one or more environmental allergens. The mean age of the cats was 6.5 years (range 3 to 12 years). Six of the cases were spayed females and the 4 males were castrated. In addition serum was collected from 15 healthy pet cats with no history of skin disease, presented to the Université de Liège for routine procedures (negative control group 1) and from 11 healthy laboratory reared cats (Hill Grove Family Farm, Oxford, UK) which had presumably never been exposed to significant amounts of DF allergens (negative control group 2). The mean ages of control group 1 and group 2 were 4.5 years (range 9 months to 13 years) and 9 years (range 4 to 12 years), respectively.

While all cats from control group 2 were entire females, control group 1 was composed of 7 females (2 spayed) and 8 males of whom 6 were castrated.

### 2.1.2. Experimental study

Ten healthy adult cats were obtained from Hill Grove Family Farm (Oxford, UK) and were used for immunisation with DF.

Five additional healthy adult cats, also from Hill Grove Family Farm, were used as recipients for Prausnitz–Küstner (PK) tests.

### 2.1.3. Antiserum production

New Zealand white rabbits, for antibody production, were obtained from Interfauna UK (Cambridge, UK).

All experimental protocols were in accordance with the requirements of the UK Home Office.

## 2.2. Allergen extracts

*Dermatophagoides farinae* (DF) extract (1/100 w/v) was purchased from Greer Laboratories (Lenoir, NC, USA).

Atopic cats were skin tested with a range of 52 additional allergens as described in a previous study on canine atopic disease (Sture et al., 1995).

## 2.3. Induction of DF-specific antibodies in normal cats

Ten parasite free cats were given 3 injections of 50 $\mu$g of DF in aluminium hydroxide (Imject Alum®; Pierce, Rockford, USA) at weeks 0, 2 and 5. Five of the 10 cats were also given, at the same time, 50 $\mu$g of *Ascaris* antigen (Asc-1; Greer Laboratories) mixed with the DF antigen in the same adjuvant. All cats were bled prior to immunisation (week 0) and at weekly intervals thereafter for 7 weeks (week 1 to week 7).

## 2.4. Performance of IDST

Cats were anaesthetised with 1 mg/kg xylazine (Virbaxyl®, Virbac, Cambridge, UK) and 10 mg/kg ketamine (Vetalar®, Parke-Davis, Pontypool, Gwent, UK). The lateral chest was clipped and 0.05 ml of 2 fold dilutions of a 1/1000 w/v dilution of DF (experimental cats) or of a 1/10000 w/v dilution of DF (clinical cases) and of appropriate dilutions of the other allergens were injected intradermally. Phosphate buffered saline (PBS) and a 1/100000 w/v solution of histamine phosphate (ARTU Biologicals, Lelystad, Netherlands) were used as negative and positive controls, respectively. Reactions were scored after 15 to 20 min on a $+1$ to $+4$ scale, incorporating both diameter and elevation above the surface. A $+1$ reaction was just discernibly greater than the PBS wheal, and a $+4$ reaction approximated the size of the histamine wheal.

## 2.5. PK tests

Healthy parasite-free cats were anaesthetised with 10 mg/kg ketamine hydrochloride intramuscularly and the hair of the lateral chest area was clipped. One-tenth ml of doubling dilutions from 1/10 of sera from the experimental cats were injected intradermally. Injection sites were challenged 48 h later by intradermal injection of 0.05 ml of a 1/1000 w/v dilution of DF. The antigen and also PBS were injected into unsensitised sites as negative controls, with a 1/100 000 w/v injection of histamine phosphate as a positive control. Skin reactions were recorded 20 min later using a +1 to +4 scale as in the IDST. For standardisation of responses between PK tests in different recipient cats, a reference serum from a cat which had a strong positive skin test to a high dilution of DF was used in each test.

## 2.6. Antisera employed

Polyclonal rabbit anti-feline IgE was prepared as described in the previous paper (Gilbert and Halliwell, 1997). The antiserum was passed through an affinity chromatography column containing DF coupled to cyanogen bromide activated Sepharose 4B (Pharmacia, Uppsala, Sweden) to remove possible naturally occurring antibodies with activity against DF.

Goat anti-feline IgG, Fc specific, alkaline phosphatase-conjugated, was obtained from Bethyl Laboratories (Montgomery, AL, US) for the measurement of DF-specific IgG in sera from clinical cases.

Rabbit anti-feline IgG, Fc specific, was purchased from Nordic Immunology (Tilburg, Netherlands) for the detection of DF-specific IgG in sera from experimental cats.

Monoclonal alkaline phosphatase-conjugated anti-rabbit immunoglobulin was purchased from Sigma (Poole, UK).

## 2.7. Enzyme-linked immunosorbent assay (ELISA)

### 2.7.1. DF-specific IgE

DF-specific IgE in feline sera was assessed by indirect ELISA. Polystyrene microtitre plates (Immulon 4®, Dynatech Laboratories, Billingshurst, UK) were coated overnight at 4°C with 100 μl/well of 1/5000 W/V dilution of DF extract in 0.1 M sodium carbonate buffer, pH 9.6 (coating buffer). Plates were washed three times with washing buffer which was 0.05% Tween 20 (Sigma) in 0.015 M PBS, pH 7.2 and blocked overnight at 4°C with 0.05% human serum albumin (HSA) (Sigma) in washing buffer (150 μl/well). After further washing, 50 μl of each sample diluted in ELISA buffer (0.05% Tween 20, 0.1% of HSA in 0.015 M PBS, pH 7.2) was added to duplicate wells. After incubation at 37°C for 2 h and further washing, 50 μl of the polyclonal anti-feline IgE diluted in ELISA buffer (1/50) was added in each well. After incubation for 2 h at 37°C, the plates were washed and 50 μl of the alkaline phosphatase-conjugated monoclonal anti-rabbit immunoglobulin diluted in ELISA buffer (1/2000) was added to each well and plates were incubated for a further hour at 37°C. After a last wash, 50 μl

of alkaline phosphatase substrate, namely 5 mg of PNP substrate tablets (Sigma) in 5.5 ml of $MgCl_2$ in diethanolamine buffer, pH 9.8, was added to each well. Plates were incubated at 37°C for 30 min and the optical density (OD) at 405 nm read using an automated microtitre plate reader after subtraction of the mean blank well values.

### 2.7.2. DF-specific IgG

In the case of the clinical study, a direct ELISA was used for the detection of DF-specific IgG, whereas in the case of the experimental study, DF-specific IgG was assayed by indirect ELISA as performed for allergen-specific IgE.

Incubation times were reduced from 2 h to 1 h 30 for the serum samples and from 2 h to 1 h for both the polyclonal rabbit anti-feline IgG and the alkaline phosphatase-conjugated goat anti-feline IgG. Incubation time for the monoclonal mouse anti-rabbit immunoglobulin was 1 h. Rabbit and goat anti-feline IgG were used at 1/5000 and 1/100, respectively. Monoclonal mouse anti-rabbit immunoglobulin was used at 1/2000.

### 2.8. Standardisation of results

A serum containing a high level of allergen-specific IgE or IgG antibody was selected as the standard and assigned a value of 1000 relative antibody units (RAU). Dilutions of the standard were included in each plate, and the results were recorded in RAU by reference to the standard curve.

### 2.9. Statistical analyses

Non-parametric Mann–Whitney test was used in the clinical study to perform the statistical analysis and to compare the IgE and IgG levels in the atopic cats and the 2 groups of negative control cats. Differences were considered significant at $P < 0.05$.

Correlation between the IgE and IgG antibody levels were calculated using a Pearson's rank coefficient correlation. The results were considered statistically significantly different if the probability ($P$) was less than 0.05.

## 3. Results

### 3.1. Validation of the assay

Allergen-specific IgE and IgG were measured in sera before and after heating to 56°C for 4 h. DF-specific IgE was no longer detected after heating, whereas IgG levels were substantially unaffected (Fig. 1a,b). This supports the isotype specificity of the anti-IgE antiserum.

### 3.2. Clinical study

### 3.2.1. IDST results

All the 10 cats showed a skin reactivity to at least one of the house dust mite (HDM) and/or house dust (HD) antigens (Table 1). The most common allergens implicated by
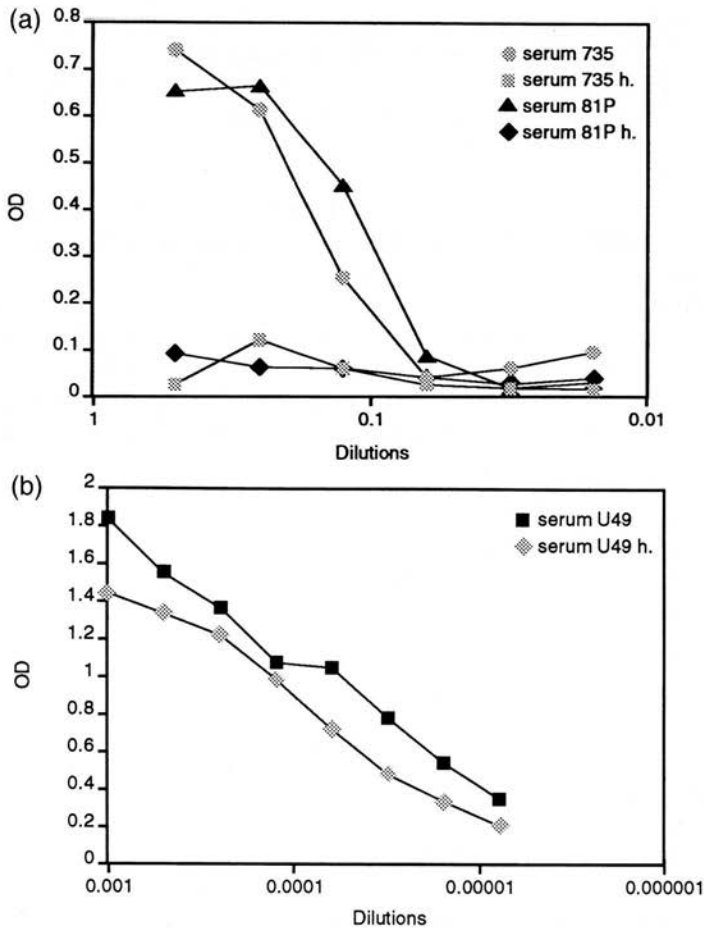
Fig. 1. DF-specific IgE (a, $n = 2$) and IgG (b) in cat sera before and after heating at 56°C for 4 h ($h$ = sera after heating). IgE and IgG levels reported as absorbance at 405 nm.

the IDST were DF and HD which both gave positive reactions in 8 cats (Table 1). Six cats were positive to *Dermatophagoides pteronyssinus* (DP) and four cats were also positive to the flea antigen (Table 1). Other allergens to which positive reactions were seen were *Tyrophagus putresceantiae* ($n = 2$), mixed moth ($n = 1$), cockroach ($n = 1$), white ash ($n = 1$), birch ($n = 1$), american elm ($n = 1$), *Brassica* spp. ($n = 1$), *Chrysanthemum* spp. ($n = 2$), red clover ($n = 2$), Heather ($n = 1$), Velvet/Yorkshire fog ($n = 1$), red top/bent ($n = 1$), and couch ($n = 1$).

### 3.2.2. ELISA results

Although the median level in the atopic cats of both isotypes was higher than that of control group 1 (457.5 vs. 330 RAU for IgE and 16451 vs. 9860 RAU for IgG) in neither case was the difference significant (Table 2, Fig. 2a and b). However, in the

Table 1
Levels of DF-specific IgE and IgG, and IDST results for DF, DP and HD antigens in the 10 atopic cats

| Atopic cats | IDST | | | DF-specific IgE (RAU) | DF-specific IgG (RAU) |
| --- | --- | --- | --- | --- | --- |
| | DF | DP | HD | | |
| 166324 | +3 | +4 | +3 | 953 | 295793 |
| 165767 | neg. | neg. | +3 | 576 | 15259 |
| 166349 | +3 | neg. | +2 | 282 | 1722 |
| 166064 | neg. | +1 | +1 | 657 | 31170 |
| 161640 | +3 | neg. | neg. | 97 | 14456 |
| 165841 | +2 | +1 | +2 | 447 | 1317 |
| 166735 | +3 | neg. | +2 | 680 | 5543 |
| 8317 | +4 | neg. | +3 | 421 | 26031 |
| 640 | +2 | +2 | neg. | 327 | 17644 |
| Noirfa | +3 | +3 | +2 | 468 | 21177 |

control group 2, the median levels (37 RAU for IgE and 343 RAU for IgG) were significantly lower than in both the atopic cats and control group 1 ($P < 0.05$). The levels of DF-specific IgE and IgG were significantly correlated ($r = 0.629$, $P < 0.01$) (Fig. 3).

## 3.3. Experimental study

### 3.3.1. ELISA results

OD values slightly greater than the blanks were detected in some of the preimmunisation sera. These values were unchanged after the sera were passed through an immunoabsorbant column of DF coupled to Sepharose 4B (data not shown) which implied non-specific binding. These OD values were therefore subtracted from the results in each case before expression in RAU following reference to the standard curve.

All cats developed both an IgG and an IgE response to DF following immunisation (Fig. 4a,b). The kinetics appeared different, in that a detectable IgE response appeared earlier, and had fallen by week 5 prior to the third immunisation. In contrast, a detectable IgG antibody response commenced later and rose gradually through the 8

Table 2
Median and range of level of DF-specific IgE and DF-specific IgG in RAU in the atopic, normal household (control group 1) and laboratory maintained cats (control group 2).

| | Atopic cats $n = 10$ | Control group 1 $n = 15$ | Control group 2 $n = 11$ |
| --- | --- | --- | --- |
| *DF-specific IgE* | | | |
| Median | 457.5 | 330 | 37 |
| Range | 97–953 | 108–478 | 31–87 |
| *DF-specific IgG* | | | |
| Median | 16451 | 9860 | 343 |
| Range | 295493–1317 | 78–38539 | 78–8367 |
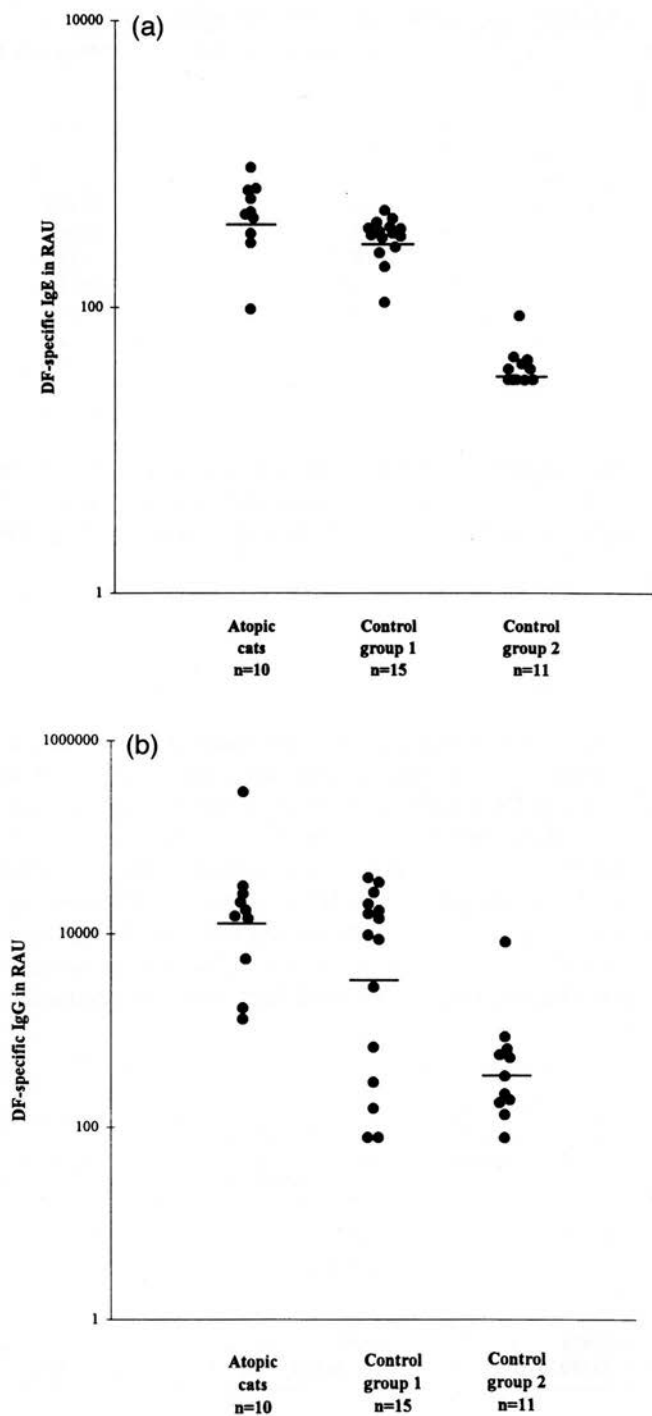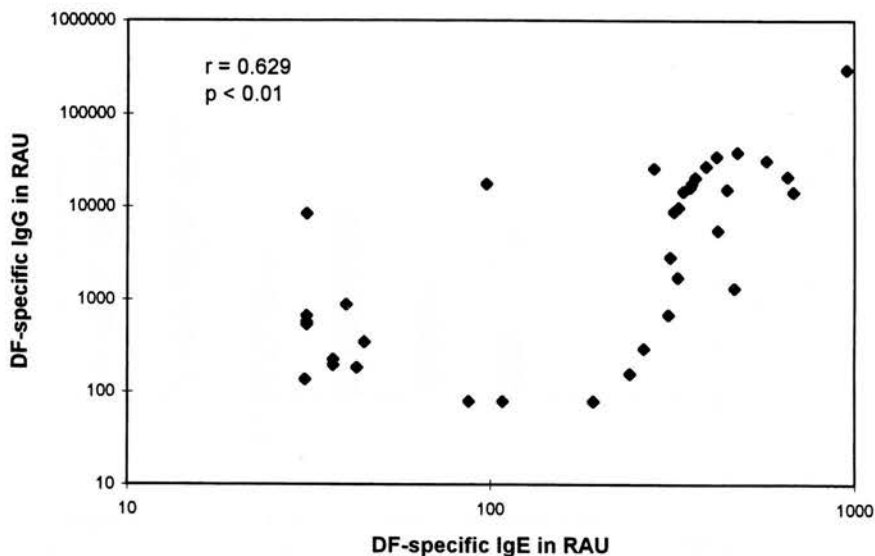
Fig. 3. DF-specific IgE and IgG in RAU in sera from atopic and normal cats. The levels of the two isotypes are significantly correlated ($r = 0.617$, $P < 0.01$).

weeks (Fig. 4a,b). Assessment of both isotypes in all serum samples in each cat did not reveal a significant correlation between them. However, when all the ELISA values for DF were considered together, there was a significant correlation between log DF-specific IgE and IgG antibodies ($r = 0.790$, $P < 0.01$), although some samples had very high IgG and low IgE levels and vice versa (Fig. 5). The 5 cats immunised with DF alone in alum produced responses that did not differ noticeably from those immunised with both DF and Asc-1 in alum.

### 3.3.2. IDST results

The 10 cats immunised with DF were skin tested weekly commencing immediately prior to the first injection. Assessment of the level of serum DF-specific IgE by ELISA and of the highest dilution of DF antigen to induce a positive skin test following intradermal injection revealed some striking anomalies and no correlation. Firstly, DF-specific IgE was, in general, detectable in sera prior to the time at which the cat showed positive skin tests. Indeed, 4 cats failed to develop positive skin tests during the 8 weeks of the experiment despite developing some of the highest levels of serum allergen-specific IgE. In general, strongly positive skin tests to high dilutions of antigen were always accompanied by high levels of detectable allergen-specific IgE. However the reverse was not the case. Representative examples of the varying responses are shown in Fig. 6a – d).

Fig. 2. The level of DF-specific IgE (a) and DF-specific IgG (b) in RAU in serum from atopic cats, normal pet cats (control group 1), and laboratory reared cats (control group 2). Bar represents the geometric mean for each group.
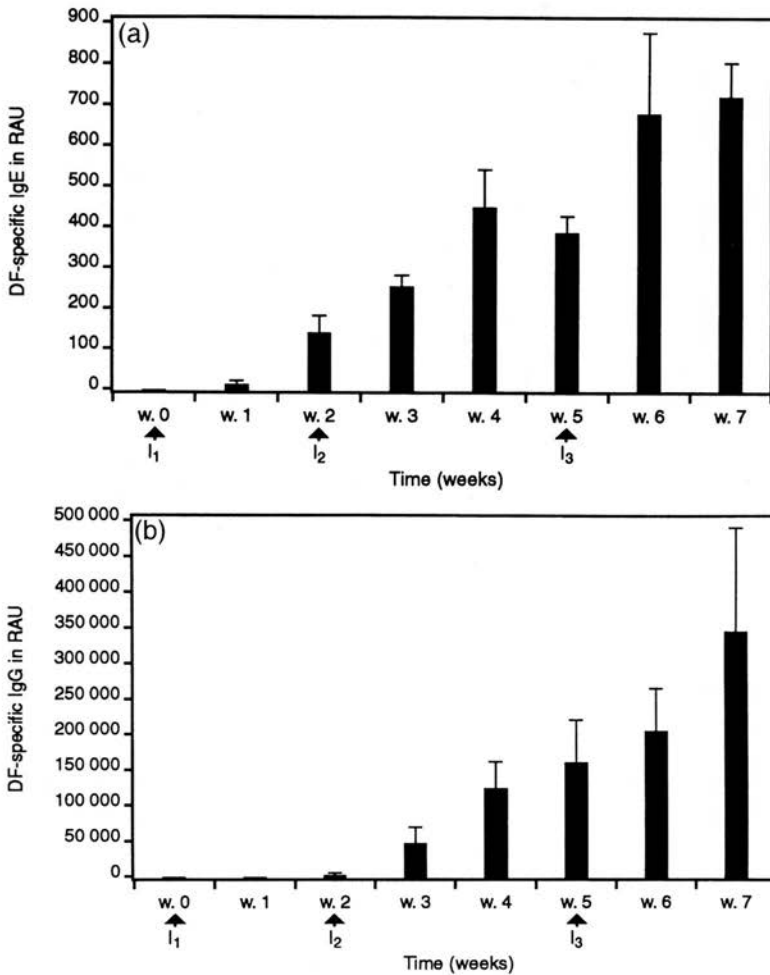
Fig. 4. Mean and standard error of the DF-specific IgE (a) and DF-specific IgG (b) responses in RAU in 10 cats ($I_1$, $I_2$, $I_3$ = immunisation dates).

### 3.3.3. PK test results

Positive PK tests were only obtained from cats with positive IDSTs and the IDST reactivity tended to parallel the PK titre. Thus the strongest PK test results were in general obtained with postimmunisation sera from cats which had strong positive skin tests to high dilutions of DF (up to 1/320). On the other hand sera from cats which had positive IDSTs only to low dilutions of DF, gave either negative or only weakly positive PK reactions. Furthermore postimmunisation sera from the 4 cats which failed to develop positive skin tests but had high levels of serum allergen-specific IgE as assessed by ELISA showed no PK reactivity. On the other hand, allergen-specific IgE was detected by ELISA in cats which had PK reactivity as well as in cats which had no such
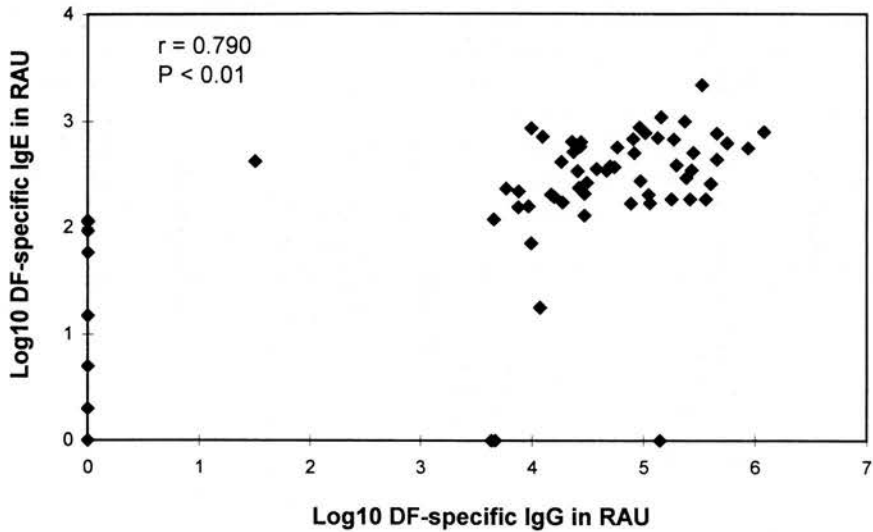
Fig. 5. DF-specific IgE and IgG in RAU in 78 blood samples from cats immunised with DF. The levels of the two isotypes are significantly correlated ($r = 0.790$, $P < 0.01$).

reactivity. In both situations the IgE was heat labile and was not detected by the ELISA after the serum was heated at 56°C for 4 h. Heating the sera also completely abolished the PK reactivity.

## 4. Discussion

One of the classical characteristic properties of reaginic antibody is its sensitivity to heating (Coca and Cooke, 1923). Ishizaka et al. (1967) observed that reaginic antibody to ragweed failed to precipitate with monospecific antiserum to IgE after exposure to 56°C for 2 to 4 h. Schwartzman and Rockey (1967) demonstrated that canine reaginic antibody is comparable to human reaginic antibody in many characteristics including heat sensitivity. Powell et al. (1980) demonstrated the heat sensitivity of reaginic antibody in cats. Such treatment does not inactivate other classes of antibody and thus allows the differentiation between IgE and other isotypes. In the present study, the complete abolition of detectable allergen-specific IgE in postimmunisation sera heated to 56°C for 4 h demonstrated the specificity of the rabbit anti-feline IgE.

*Dermatophagoides farinae* is believed to be the most common allergen implicated in feline atopy. Positive reactions to DF have been reported in 77 to 100% of the atopic cats (Carlotti and Prost, 1988; Foster and O'Dair, 1993; Prost, 1996). The results of the IDST in the present study were in agreement with other reports, with 80% of the cats showing positive reactions to DF.

The ELISA has only been introduced recently in the establishment of the diagnosis of atopic disease in cats (Foster and O'Dair, 1993). The antiserum used in the latter assay was raised against a chimaeric feline/murine IgE antibody and there is no published

data supporting its specificity. A comparison of this test with results of IDST, assuming the latter as the gold standard, showed a poor correlation between the two and it was concluded that ELISA was unreliable in establishing a diagnosis of feline atopy. In the present study the IDST also did not always correlate well with the in vitro test. Of the 10 atopic cats, 2 cats were IDST negative to DF but had a high level of DF-specific IgE as assessed by ELISA whereas one had a very low level of DF-specific IgE but showed a strong skin reactivity to DF. Although DF antigen at 1/10 000 dilution is believed to be non-irritant, it is possible that it was close to an irritant concentration in one cat and therefore could have represented a true false positive reaction in this cat. All the normal
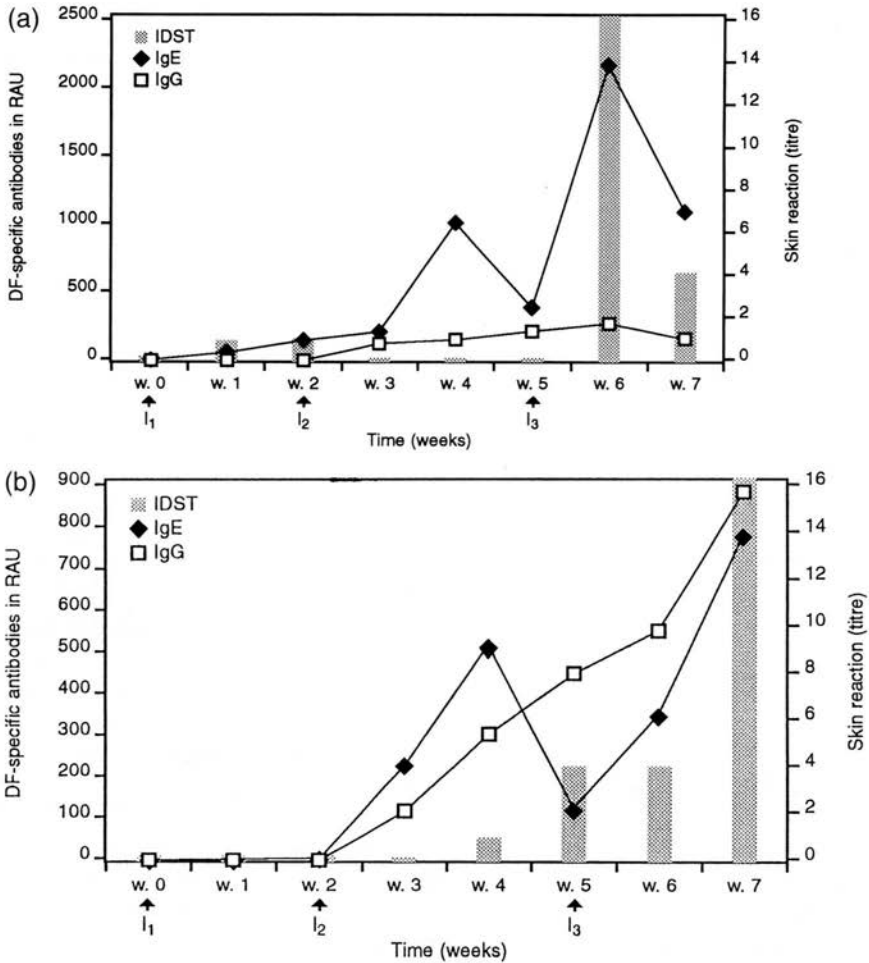


Fig. 6. The relationship between the DF-specific IgE and IgG (in RAU) and the highest dilution of antigen to give positive IDST (skin reaction titre) in 4 representative cats immunised with DF: cats U49 (a), B85 (b), A71 (c) and 735 (d) ($I_1$, $I_2$, $I_3$ = immunisation dates). IgG results are divided by 1000.
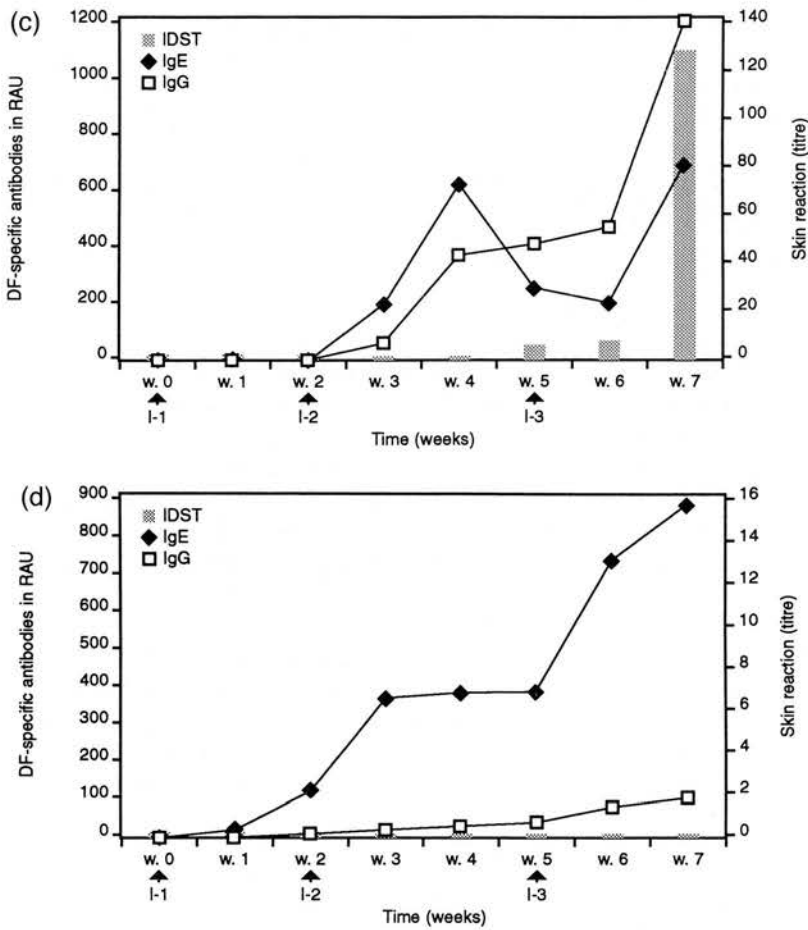
Fig. 6 (continued).

pet cats had detectable levels of DF-specific IgE, and high levels were found in 2 of them. However the median level of DF-specific IgE in sera from these cats was not significantly different from the median of that in the atopic cats. But it should also be emphasised that the normal pet cats were not skin tested and some of the cats may have had a positive IDST to this antigen, as was recently reported in normal dogs (Lian, 1997). On the other hand, it was also shown in the present study that normal cats may have allergen-specific IgE without developing skin test reactivity to this antigen. It was noteworthy that levels of both DF-specific IgE and IgG in the laboratory reared cats were significantly lower than that in both the other groups.

The fact that not only is DF-specific IgE the most frequently implicated allergen in feline atopy, but also is found in normal cats is of great interest. It has been suggested in man that the powerful allergic immune response commonly provoked by HDM could

result from its ability to cleave the low affinity IgE Fc receptor (CD23) from the surface of IgE-secreting B cells (Hewitt et al., 1995). This could ablate an important feedback inhibitory mechanism that normally limits IgE synthesis. Furthermore, fragments of CD23 released by HDM have been reported to promote IgE production (Sutton and Gould, 1993).

Injections of Asc-1 together with DF in 5 parasite free cats failed to stimulate a higher DF-specific IgE than in cats given DF alone. It is well-known that helminths are particularly adept at stimulating IgE synthesis. Helminth infection has the ability to produce an increase in non-parasite-specific IgE (Ogilvie, 1964; Zakroff et al., 1989) and furthermore to potentiate IgE responses to unrelated antigens. They also may disproportionately elevate serum levels of total non-specific IgE (Jarrett and Bazin, 1974; Turner et al., 1979). Halliwell (1972) showed that subcutaneous injections in dogs of *Ascaris suum* antigen alone or coupled to haptens was able not only to stimulate the production of reagin to haptens to which the antigen was coupled but also to any protein with which it was simultaneously administered. However the parasite status of the dogs was not known, although in those days *Toxocara canis* infection in the dogs was almost universal. The apparent disparity in the ability of ascaris antigens to stimulate allergen-specific IgE production in the 2 experiments may thus merely relate to the parasite status.

The DF-specific IgE response commenced early, being detectable 2 weeks after immunisation whereas IgG was essentially undetectable until week 3. Furthermore, the IgE level tended to fall off after the second immunisation whereas the IgG antibody continued to rise. IgE thus appears to be an early antibody in the cat, as has been shown in the rat (Tada, 1975). The decrease in DF-specific IgE after 5 weeks, as contrasted with the IgG response, could, of course merely be reflective of the shorter half-life of the former isotype, rather than an actual lowering of the production relative to IgG. The IgE antibody level depends not only on the dose of the antigen and the adjuvant used but also on the interaction of genetic background and environmental factors (Levine and Vaz, 1970; Vaz et al., 1971). It has been demonstrated that the IgE response to an allergen in mice depends on the strain immunised (Levine and Vaz, 1970). The marked variations of the antibody response seen in these cats are most probably the result of the genetic differences between them as it was suggested for the outbred dogs in the study of Schultz and Halliwell (1985).

The level of specific IgG to some aeroallergens has been shown to be higher in cats with either atopy, food and/or flea allergy than in normal cats or cats with pruritic skin disease but negative IDSTs (Foster et al., 1997). However this was not so in the case of HDM-specific IgG levels which were not significantly greater in allergic cats as compared to normal cats (Foster et al., 1997). In both the clinical and the experimental studies the levels of DF-specific IgE and IgG were significantly correlated. This is not surprising since both isotypes are under the control of a Th-2 type response.

The cats immunised with DF developed no clinical signs of allergy in spite of the strongly positive IDST developed by some of them. It is possible that this resulted from the limited exposure to DF in the laboratory conditions. However, the subcutaneous route used for antigen administration, both for the induction of IgE and for antigen challenge, is different to the natural disease in which the percutaneous route is believed

to be responsible for clinical signs (Halliwell, 1990; Olivry et al., 1996). However it is noteworthy that no species in which IgE was experimentally induced, including man, have shown any clinical signs of allergic disease (Marsh et al., 1972). This emphasises the fact that it is not merely the presence of IgE that distinguishes atopic individuals from normals. It has been suggested that this might be because the reaginic response can be accompanied by high levels of blocking antibodies (Marsh et al., 1972) but another reason could be the heterogeneity of the reaginic antibody response as discussed below. It was also noteworthy that relatively high levels of DF-specific IgE were also found in sera from some normal cats kept in normal household conditions, and presumably exposed to HDM antigen.

In the present study, circulating levels of DF-specific IgE did not correlate with tissue-bound IgE as assessed by IDSTs. In particular, 4 cats with high levels of IgE never developed a positive IDST. Moreover, it was noteworthy that there was a marked lack of correlation between the level of IgE as assessed by ELISA and by PK tests. Also, positive PK tests were never obtained using sera from cats which were IDST negative, although such sera had high levels of IgE as assessed by ELISA. On the other hand, sera from the cats which were only IDST positive to a low dilution of DF showed only a weak PK reactivity whereas sera from cats which had a positive IDST to high dilutions of DF showed much greater reactivity. It is, of course, possible that the cats which developed a high level of DF-specific IgE without a positive IDST may have developed a high level of blocking IgG. However, the level of IgG measured in these cats was not higher than in the cats with a positive IDST to a high dilution of DF who showed strong PK reactivity.

It could also be hypothesised that the negative IDST in the face of the positive ELISAs could be due to a delay in circulating IgE fixing to mast cells. Such a phenomenon is believed to occur clinically in some cases of canine atopic disease in which the positive in vitro tests develop weeks or months before a positive IDST. However this possibility could be excluded as the sera from these cats failed to give positive PK tests.

The most rational explanation for this finding would be the existence of heterogeneity of IgE in cats. Theoretically, a number of possibilities exist by which subclasses of IgE could differ. Firstly, the IgE antibody could be heterogeneous in terms of its ability to respond to histamine releasing factors (HRF), which are cytokines derived from macrophages, lymphocytes, mononuclear cells, platelets and vascular endothelial cells. It has been hypothesised that it is not the production of IgE but, rather the ability of the IgE to interact with the HRF which differentiates atopics from non atopic individuals (Lichtenstein, 1988). Thus the type of IgE produced by the cats experimentally immunised with DF and also that found in normal cats, in contrast to that produced by atopic cats, may not be reactive with HRF. In cats which become positive to the IDST, this first IgE antibody response (IgE − ) could have been followed by the production (switching) to the second type of IgE antibody (IgE + ). The cats which stayed negative to the IDST would never have developed the second IgE isotype and such animals would have positive ELISA results without positive IDST or PK reactivity. However HRF's appear to be involved only in the late phase reaction resulting from the activation of basophils. This reaction is seen in only a subpopulation of allergic individuals

(MacDonald, 1996). Therefore it is unlikely that, in the present study, the interaction of IgE with HRF would explain why some of the cats in which DF-specific IgE was experimentally induced did not develop an immediate response to the IDST.

Secondly, it is possible that the IgE antibody experimentally induced in the normal cats may be less effective at inducing degranulation, as is the case with IgG homocytotropic antibody (Valent and Bettelheim, 1992). It could also be that the IgE experimentally induced in the normal cats may have a low affinity for mast cell receptors and therefore not be able to interact with them in order to sensitise mast cells. In a recent study, Lyczak et al. (1996) demonstrated the existence of 4 human IgE isoforms. They suggested that this could be an additional possible explanation for the functional heterogeneity of IgE and that IgE may differ in its primary protein structure as well as glycosylation. Each isotope may have some unique properties as circulating, cytophilic immunoglobulins, and may differ in their ability to carry out IgE-mediated functions through binding to their different receptors (Lyczak et al., 1996). Interestingly, it has also been shown that the relative levels of mRNAs encoding various isoforms appears to be altered in allergic and parasitic diseases (Diaz-Sanchez et al., 1995). These findings suggest that the existence of more than one type of IgE in cats is not unexpected, and that the IgE antibody experimentally induced in normal cats may be different from the IgE produced in allergic cats. Moreover, it is not impossible that the cats which developed a positive IDST were genetically predetermined to be atopic.

Jackson et al. (1996) studied the leucocyte histamine release in atopic dogs, normal dogs and artificially sensitised dogs and found comparable results to those found in the present study, in that histamine release induced by antigen or anti-IgE was generally found only in atopic dogs. The same concepts could also account for the presence of IgE in normal cats without clinical disease, although, of course, the latter were not skin tested.

In conclusion, the present studies showed that normal cats developed a reaginic antibody response to an environmental allergen (DF) which was readily detected by use of the polyclonal anti-IgE in an ELISA. This antiserum also detected allergen-specific IgE in atopic cats and in normal pet cats kept under normal household conditions, but significantly lower levels were found in laboratory reared cats. In both the experimental and clinical studies there was a significant overall correlation between allergen-specific IgE and IgG responses. However, the level of allergen-specific IgE was not correlated with either skin test or PK reactivity, whereas the positive PK reactivity was generally correlated with positive skin test reactivity. These results are consistent with the existence of a heterogeneity of IgE antibodies in cats.

## Acknowledgements

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