PATHOGENETIC STUDIES
ON FELINE EOSINOPHILIC GRANULOMA COMPLEX

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CONTENTS

Abbreviations 1

Chapter 1  Introduction and aims 3

Chapter 2  Histopathological study of feline eosinophilic dermatoses 25

Chapter 3  Ultrastructural study of cutaneous lesions in feline eosinophilic granuloma complex

Chapter 4  Piecemeal degranulation (PMD) morphology in feline circulating eosinophils

Chapter 5  Characterization of biological activities of feline eosinophil granule proteins

Chapter 6  Discussion

Conclusions

Summary

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**ABBREVIATIONS** (in alphabetical order)

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cfegs</td>
<td>clusters of free eosinophil granules</td>
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<td>CLC-P</td>
<td>Charcot-Leyden crystal protein</td>
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<td>DI</td>
<td>degranulation index</td>
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<td>EAR</td>
<td>eosinophil-associated ribonuclease</td>
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<td>ECL</td>
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<td>ECP</td>
<td>eosinophil cationic protein</td>
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<td>EDN</td>
<td>eosinophil-derived neurotoxin</td>
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<td>EGC</td>
<td>eosinophilic granuloma complex</td>
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<td>EG</td>
<td>eosinophilic granuloma</td>
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<td>EP</td>
<td>eosinophilic plaque</td>
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<td>EPO</td>
<td>eosinophil peroxidase</td>
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<td>Feld I</td>
<td><em>Felis domesticus</em> allergen I</td>
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<td>GM-CSF</td>
<td>granulocyte macrophage-colony stimulating factor</td>
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<td>H &amp; E</td>
<td>haematoxylin and eosin</td>
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<td>IL</td>
<td>interleukin</td>
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<td>IU</td>
<td>indolent ulcer</td>
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<td>LAMP</td>
<td>lysosome-associated membrane protein</td>
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<td>LB</td>
<td>lipid body</td>
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<td>MBP</td>
<td>major basic protein</td>
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<td>MMP</td>
<td>matrix metalloproteinase</td>
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<td>PMD</td>
<td>piecemeal degranulation</td>
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<td>RNase</td>
<td>ribonuclease</td>
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<td>RER</td>
<td>rough endoplasmic reticulum</td>
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<td>TEM</td>
<td>transmission electron microscopy</td>
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<td>TGF</td>
<td>transforming growth factor</td>
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<td>Th2</td>
<td>T helper 2</td>
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<td>TNF</td>
<td>tumour necrosis factor</td>
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<td>VTO</td>
<td>vesiculotubular organelles</td>
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Chapter 1

Introduction and aims
Introduction

Eosinophils are among the most misunderstood cells in mammalian biology and perspectives on their multifaceted roles have undergone profound changes.

A few decades ago, eosinophils were considered to play a beneficial role both in the innate host defence against helminth parasites and in dampening allergic inflammation, by neutralising mast cell-derived inflammatory mediators (Wardlaw et al., 1995).

Then, research on human asthma induced a drastic change of eosinophils “good reputation” and their detrimental roles became object of investigation. Eosinophils were considered keyplayers of tissue damage in allergic inflammation by releasing their cytotoxic granule proteins, previously considered beneficial to kill parasites. In addition, as they were demonstrated to release a wide array of proinflammatory substances, including granule proteins, lipid mediators, cytokines and chemokines, their role in inflammation was considered to be proinflammatory rather than anti-inflammatory (Wardlaw et al., 1995).

At current writing, based on the biological rule that the ability to induce pathological changes can not represent a “raison d’être” for any existing cell type, beneficial physiological roles of eosinophils have come up again and are under investigation (Rosenberg and Domachowske, 2001). In addition, the detrimental contribution of eosinophils to asthma has been recently reassessed (Leckie et al., 2000, Adamko et al., 2003). On the other hand, there is now evidence that the role of eosinophils in host protection against parasites is probably limited to selected parasitic diseases (Behm and Ovington, 2000, Meeusen and Balic, 2000).

Despite being commonly reported (Day and Hall, 2000, Scott et al., 2001), feline eosinophil-associated disorders, including eosinophilic granuloma complex, are poorly understood. They are generally associated to immune-mediated or parasitic causes, analogous to human eosinophil-driven diseases, although specific functions and contents of the cat eosinophil are currently unknown. Nevertheless, feline and human eosinophils are likely to possess similar biology. In fact, the cat has been proven to represent an adequate experimental model for human eosinophil-driven allergic airway inflammation (Padrid et al., 1995, Padrid et al., 1996) and several analogies between feline and human
atopic dermatitis have been demonstrated (Roosje et al., 1997, Roosje et al., 1998, Roosje et al., 2002a, Roosje et al., 2002b).

Owing to the poor current knowledge of feline eosinophil-associated diseases and eosinophils, the studies included in this thesis centred on feline EGC and cat eosinophil functions and contents.

**Feline eosinophilic granuloma complex**

Feline eosinophilic granuloma complex (EGC) consists of a group of lesions, defined as eosinophilic plaque (EP), eosinophilic granuloma (EG) and indolent ulcer (IU), that affect the skin and the mucous membranes of the cat (Scott et al., 2001).

Clinical features of EP, EG and IU have been well delineated (Scott et al., 2001) and a distinctive histological appearance of each entity has been reported (Gross et al., 1992, Yager and Wilcock, 1994).

EP is described as pruritic, erosive, glistening, firm papules and plaques commonly affecting the inguinal region and the inner thighs (Scott et al., 2001). Epidermal hyperplasia with spongiosis, prominent eosinophilic exocytosis and ulceration, with perivascular to diffuse eosinophilic infiltration of the dermis are reported as common histopathological features (Gross et al., 1992, Yager and Wilcock, 1994).

EG (synonyms: collagenolytic granuloma, linear granuloma) classically occurs as variably pruritic, firm, cord-like papules and plaques linearly orientated on the caudal thigh, or as single, papular to nodular lesions located anywhere on the body, including lip margins and oral cavity (Scott et al., 2001). Sharply delineated dermal foci of amorphous to granular eosinophilic debris are reported as distinctive histopathological features of EG on haematoxylin and eosin (H & E) stained sections. Smaller foci of the same debris are also described in EG and defined as flame figures, by analogy with the microscopical aspect of flame figures in human Wells’ syndrome, i.e. dermal flame-like extensions of a brightly eosinophilic material adherent to collagen (Wells and Smith, 1979). In some cases, granulomatous reaction with epithelioid and multinucleated cells surrounds this debris (Gross et al., 1992, Yager and Wilcock, 1994).
IU (synonyms: eosinophilic ulcer, rodent ulcer, lip ulcer) refers to a glistening, non-bleeding, well-circumscribed ulcer with the aspect of an ulcerated nodule rather than a true ulcer (Foil, 1995). IU most commonly affects the upper lip, at the philtrum or opposite to the upper canine tooth (Scott et al., 2001). Reported histopathological findings of IU vary from ulcerative neutrophilic and fibrosing dermatitis, to, less commonly, ulcerative dermatitis with foci of eosinophilic debris similar to that observed in EG (Gross et al., 1992, Scott et al., 2001).

Despite being reported in veterinary dermatopathology textbooks (Gross et al., 1992, Yager and Wilcock, 1994), the specificity of histopathological findings of clinical entities of EGC is controversial, in fact, overlapping histopathological features, suggestive of more than one clinical form, have been described in individual biopsies (Rosenkrantz, 1993, von Tscharner and Bigler, 1989). This inconsistency is not surprising, taking into account that most reported data on EGC derive from retrospective evaluation of clinical cases included in studies on feline allergic skin diseases.

The fundamental question that raises from the described histological lesions of EGC concerns the histogenesis of the eosinophilic debris. It is commonly referred to as “collagen degeneration” and it has been suggested to represent a mixture of degenerate collagen, degranulated eosinophils and eosinophil granule contents (Gross et al., 1992, Yager and Wilcock, 1994).

The pathogenesis of the presumed collagen alteration in feline EGC is a subject of controversy since decades. It has been speculated that collagen fibres would degenerate as a consequence of eosinophil degranulation, being their specific target, or that they would be damaged by eosinophil products, being “innocent bystanders” (Fairley, 1991, Gross et al., 1992, Power and Ihrke, 1995, Yager and Wilcock, 1994). A primacy of collagen damage has been also hypothesised, with secondary eosinophil recruitment (Bucci, 1966).

The presence of collagen damage in feline EGC has been recently investigated. It has been reported that collagen fibres entrapped in the middle of large foci of eosinophilic debris showed no-more-frequent abnormalities, on trichrome stained sections, than collagen fibres in other dermal areas (Fernandez et al., 2000). Thus, although based on the results of light microscopic examination, it was speculated that these foci had the same ultrastructure of human flame figures, i.e. normal collagen bands surrounded by
degranulating eosinophils and free eosinophil granules (Aberer et al., 1988, Davis et al., 1998, Stern et al., 1984). However, to date, no ultrastructural studies of EGC lesions have been reported to definitely confirm a structural analogy between feline and human flame figures.

From an etiological viewpoint, EGC is currently considered a cutaneous reaction pattern incited by common underlying allergic causes, including hypersensitivity reactions to flea, environmental and food allergens (Scott et al., 2001). However, in many cases, no triggering factors are identified.

Little original research has been done on the aetiology of EGC. Recently, the contribution of a hypersensitivity reaction towards the cat allergen *Felis domesticus* allergen I (Feld I) to the pathogenesis of EGC has been studied. Despite the lack of eosinophils in the inflammatory cells 48 hours after the application of the allergen on the skin, it was concluded that autosensitisation to this allergen might play a role in the inflammation observed in EGC (Wisselink et al., 2002).

A genetic heritable “dysregulation” of eosinophil response has been suggested to predispose to the spontaneous development of EGC, based on the observation of high incidence of EG and IU, with no detectable underlying disorders, in a closed breeding colony of interrelated specific pathogen-free cats (Power and Ihrke, 1995). However, lesions were waxing and waning, with spring and summer seasonality (Power and Ihrke, 1995), and in cats from the same colony, the onset of IU coincided with flea exposure (Colombini et al., 2001).

Taking into account the overall information, plus the above-mentioned ignorance of feline eosinophils functions and contents, it is not surprising that our current understanding of EGC does not greatly differ from what reported more than 35 years ago in one of the first original descriptions of this disease complex (Bucci, 1966).

**Human eosinophils**

Information about human eosinophils has rapidly expanded over the past decades, especially since their detrimental role in asthma has been discovered. The heightened interest regarding the eosinophil is manifested by a dramatic increase in publications dealing with this cell. For example, before 1975, approximately 100 publications per year
could be identified from the National Library of Medicine database under the search term eosinophil (Gleich et al., 1993), whereas in 2003 over 1000 articles on eosinophils were published in one year. Hence, we will focus only on those aspects studied in this thesis, namely eosinophil morphology and contents, with an emphasis on morphological markers of eosinophil activation and granule contents.

The ultrastructural morphology of human eosinophils differs between resting, activated and degranulating cells and these differences reflect functional changes (Dvorak, 1994). We use the term activation to describe the transition of the eosinophil from a resting through a degranulation state, which is the end result of full activation of the eosinophil. Both resting and activated eosinophils have been described in blood and tissue, whereas degranulating eosinophils are recognised at sites of inflammation (Dvorak, 1994). However, there is some evidence that, in certain cases, eosinophils may also degranulate in blood (Karawajczyk et al., 2000). In addition, activated and degranulating eosinophils have been described under various in vitro conditions (Dvorak et al., 1991, Dvorak et al., 1992, Dvorak, 1994). Adamko et al. (2002) and Gleich (2000), among others, have recently reviewed mechanisms of eosinophil tissue recruitment, activation and degranulation.

Most information on the morphology of mature resting eosinophils derives from transmission electron microscopy (TEM) studies on circulating eosinophils from healthy donors. Mature resting eosinophils are approximately 8 µm in diameter, have an irregular surface profile with broad projections, and a bi-lobed nucleus filled with partially condensed chromatin. Rough endoplasmic reticulum (RER) and Golgi structures are minor cytoplasmic organelles. In appropriately prepared electronmicroscopic samples, scattered electron-dense glycogen particles may be observed (Dvorak and Weller, 2000). Other populations of organelles have been recognised in the eosinophil cytoplasm, namely three categories of granules, which consist of (a) specific granules, (b) primary granules and (c) small dense granules; (d) lipid bodies (LB) and (e) vesiculotubular organelles (VTO). The morphological characteristic and the contents of these organelles will be described here below.

a. Specific granules, also known as secondary granules, are ellipsoid organelles that comprise more than 95% of the cell granules and represent distinguishing eosinophils’ morphologic features (Dvorak et al., 1988). They contain two compartments, a central electron-dense crystalline core and an outer matrix, which selectively house four highly
basic proteins (Dvorak and Weller, 2000). By immunoelectronmicroscopic techniques, major basic protein (MBP) has been localised to the granule core and eosinophil cationic protein (ECP), eosinophil-derived neurotoxin (EDN) and eosinophil peroxidase (EPO) to the matrix (Egesten et al., 1986, Giembycz and Lindsay, 1999). These basic proteins, which account for the high affinity of eosinophil granules for the red acid dye eosin, were first recognised for their toxicity but they are currently known to be functionally pleiotropic.

MBP accounts for the majority of the granule protein found in eosinophils of certain animal species, as guinea pigs (Gleich et al., 1973), and was so named for that fact. It is a potent helminthotoxin (Ackerman et al., 1985) and cytotoxin (Motojima et al., 1989) and possesses bactericidal activity (Lehrer et al., 1989).

ECP is a member of the ribonuclease (RNase) A superfamily. Accordingly, it possesses ribonuclease activity, although limited (Slifman et al., 1986). In addition, it has been demonstrated to be helminthotoxic (Ackerman et al., 1985), cytotoxic (Motojima et al., 1989) and bactericidal (Lehrer et al., 1989).

EDN is another member of the RNase A superfamily that shares 67% sequence homology to ECP (Slifman et al., 1986). EDN is a more potent RNase than ECP (Slifman et al., 1986) but a poor helminthotoxic (Ackerman et al., 1985) and cytotoxic (Motojima et al., 1989). EDN and ECP are both neurotoxic (Giembycz and Lindsay, 1999) and possess antiviral activity against certain RNA viruses (Domachowske et al., 1998a, Domachowske et al., 1998b).

EPO is a heme-containing protein with helminthotoxic (Ackerman et al., 1985), cytotoxic (Motojima et al., 1989) and bactericidal activities (Borelli et al., 2003, Persson et al., 2001).

Eosinophil specific granules selectively store also hydrolytic enzymes, a wide array of preformed cytokines, including Interleukin (IL)-2, IL-4, IL-5, IL-6, tumour necrosis factor (TNF)-α and transforming growth factor (TGF)-α, and chemokines, including RANTES and eotaxin (Egesten et al., 2001).

Based on the presence of hydrolytic enzymes in specific granules and the localisation of lysosome-associated membrane protein (LAMP)-1 and 2 to their membranes, it has been
Introduction and aims

recently hypothesised that specific granules can be regarded as specialised lysosomes (Persson et al., 2002).

b. - Primary granules are roughly spherical, large, electron-dense, unicompartmental granules identified in the eosinophil cytoplasm. These granules are named primary because they develop early in eosinophil maturation. They comprise approximately 5% of the cell granules (Dvorak et al., 1988). Their specific content, localised by immunoelectron microscopy (Dvorak et al., 1988), is Charcot-Leyden crystal protein (CLC-P). This protein, unlike what previously thought (Weller et al., 1982), lacks lysophospholipase activity and is currently considered a member of the galectin superfamily (Ackerman et al., 2002). Its role in eosinophil biology remains unresolved. In primates, CLC-P forms bipyramidal crystals, which may be encountered, *in vitro* and *in vivo*, in eosinophil-related diseases (Giembycz and Lindsay, 1999).

c. - Small granules, which are another type of spherical, electron-dense, membrane-bound granules, have been also described, in small numbers, in the eosinophil cytoplasm (Dvorak and Weller, 2000). Due to their enzyme contents, they are considered cytochemically analogous to lysosomes (Dvorak and Weller, 2000). In addition, they might participate to the granule proteins transport during eosinophil degranulation (Egesten et al., 2001, Logan et al., 2003).

d. - Occasional LB, which are large, spherical, electron-dense, lipid-rich, not membrane-bound inclusions, have been identified in the eosinophil cytoplasm (Dvorak and Weller, 2000). Cyclooxygenases, lipoxygenases and phospholipase A₂, which are enzymes implicated in proinflammatory eicosanoids generation in activated eosinophils, have been localised to LB (Bandeira-Melo et al., 2002).

e. - VTO, formerly known as microgranules, are eosinophils’ specific organelles. They appear as narrow, electron-lucent, membrane-bound tubules that bend assuming the aspect of a dumbbell, a C, a reverse donut or a biconcave disc (Dvorak and Weller, 2000). Their function is still unclear. VTO, which form by endocytosis (Calafat et al., 1993), are considered to play a role in granule contents release after cell activation, in fact, they are also referred to as secretory vesicles (Egesten et al., 2001).

While the morphology of resting eosinophils is well defined, reported morphological features of activated eosinophils are greatly variable, changing with the eosinophil
population, the activating stimulus and the different techniques used to characterise the activation state (Kroegel et al., 1994, Weller, 1997).

The morphology of activated circulating eosinophils is still a subject of debate. Activated circulating eosinophils have been traditionally identified in patients with eosinophil-mediated diseases, usually accompanied by blood eosinophilia, based on their lighter density at cell separation by density-gradient centrifugation (Fukuda and Gleich, 1989).

Hypodense eosinophils, besides having augmented biological activities compared to their normodense counterparts (Owen et al., 1987, Rothenberg et al., 1987, Rothenberg et al., 1988, Rothenberg et al., 1989), have been reported to show, among others, electronmicroscopic changes suggestive of granule content release, i.e. electron-lucent granules (Caulfield et al., 1990, Peters et al., 1988), which is considered a key event following activation (see further) and probably accounts for their reduced protein contents and, in turn, reduced density (Peters et al., 1988). In addition, specific degranulation morphology has been recently reported in hypodense eosinophils (Karawajczyk et al., 2000). Nevertheless, although eosinophil hypodensity has been widely studied, a consensus mechanism for its generation has not been established yet (Fukuda and Gleich, 1989, Kroegel et al., 1994).

Morphological markers of activation in cultured eosinophils have been studied in depth and they appear to be analogous to those of tissue eosinophils. Activated cultured eosinophils often assume a motile shape (uropods) and display increased numbers of small granules, VTO, glycogen particles and LB, many of which are also enlarged (Dvorak and Weller, 2000). These morphological changes are associated to the enhanced cell activity.

Activated cultured and tissue eosinophils display striking morphological features when they release their granules content. Three different eosinophil degranulation modes are distinguishable at transmission electron microscopy (TEM) examination and consist of (a) exocytosis, (b) piecemeal degranulation (PMD) and (c) eosinophil cytolysis (ECL).

a. - Exocytosis is characterised, in case of single secretion, by the release of membrane-free granule contents in the interstitial space via fusion of granule and plasma membranes (Egesten et al., 2001). The ultimate ultrastructural result of this form of secretion is a hypogranular to agranular eosinophil with diminished or absent
cytoplasmic specific granules (Dvorak, 1994, Dvorak and Weller, 2000). Compound exocytosis, in which granules fuse inside the cell, forming large intracytoplasmic degranulation chambers that then fuse with the cell membrane, may also occur (Egesten et al., 2001). Exocytosis has been occasionally observed in vivo (Dvorak et al., 1993) and it has been described in vitro (Henderson et al., 1983, Yamazaki et al., 2001).

b. - PMD differs from regulated exocytosis in that is a vesicle-mediated secretion. Small, protein-containing, membrane-bound transporting vesicles bud off from, and gradually empty, the specific granule (Dvorak et al., 1991, Dvorak et al., 1992). However, despite protein-filled budding vesicles have been depicted in electronmicroscopic images (Dvorak et al., 1992), the contribution of granules membrane to the formation of transporting vesicles membranes is unclear (Lacy et al., 2001, Logan et al., 2002, Logan et al., 2003, Mahamudi-Azer et al., 2002, Persson et al., 2002). The result of PMD in electron-microscope images is a viable cell filled with partially to completely empty electron-lucent specific granules (Dvorak, 1994, Dvorak and Weller, 2000). PMD has been reported in vivo in various diseases, including allergic respiratory disease (Erjefält et al., 1999) and inflammatory bowel disease (Dvorak, 1980, Erjefält et al., 2001), and it has been described also in vitro (Dvorak et al., 1991, Dvorak et al., 1992). PMD permits, in a stimulus-dependent manner, the selective release of individual granule proteins (Capron et al., 1989, Torpier et al., 1988), cytokines (Bandeira-Melo et al., 2001) and chemokines (Lacy et al., 1999) from a common storage organelle.

c. - ECL is characterised, at TEM examination, by cell and nuclear membrane rupture and release of clusters of free eosinophil granules (Cfegs), intact or at different stages of dissolution, into surrounding structures (Dvorak, 1994, Persson and Erjefält, 1997). ECL, in vivo, appears to be an important mode of degranulation in a variety of diseases, including eosinophil-rich respiratory diseases (Erjefält et al., 1998, Erjefält et al., 1999, Persson and Erjefält, 1997), atopic dermatitis (Cheng et al., 1997), skin lesions of hypereosinophilic syndrome (Dvorak et al, 1990) and, as previously mentioned, Wells’ syndrome (Aberer et al., 1988, Davis et al., 1998, Stern et al., 1984). ECL may occur also in vitro (Weiler et al., 1996, Yamazaki et al., 2001).

Whether cytolytic degranulation represents a regulated active secretory process or a passive cell injury, possibly resulting from the release of cytotoxic granule proteins by PMD, is still subject of controversy (Erjefält and Persson, 2000, Logan et al., 2003, Walsh, 2001). However, the differential evoking of ECL and PMD, both in vivo (Cheng et al.,

The biological significance of the differential inducement of the above-described eosinophil degranulation pathways is currently unknown. It has been hypothesised that mechanisms that govern eosinophil degranulation pathways are partly dependent on the different kinds of stimuli to which the cell is exposed (Karawajczyk et al., 1995). It has been also conjectured that ECL would be probably more important in defence mechanisms against parasites and in eosinophilic allergic diseases, producing a rapid and entire release of granule products, whereas PMD would contribute to eosinophil-dependent immunoregulation providing a selective and long lasting release of granule contents (Erjefält and Persson, 2000).

**Feline eosinophils**

While our knowledge on human eosinophils has considerably expanded in the last decades, only ultrastructural and cytochemical aspects of feline eosinophils had been partially elucidated. Feline eosinophil ultrastructure has not been delineated with a wealth of details comparable to human eosinophil ultrastructure, however, based on two original studies (Presentey at el., 1980, Ward et al., 1972), it appears to be similar to its human counterpart (Dvorak and Weller, 2000) and to what reported in other animal species (McEwen, 1992).

Mature feline circulating eosinophils are 6 to 9 µm in diameter, have a bi-lobed nucleus with peripherally condensed chromatin and no visible nucleolus (Ward et al., 1972). The Golgi zone is not well developed and very few mitochondria, RER profiles and scattered glycogen granules are visible (Ward et al., 1972).

Two eosinophil-specific organelles have been recognised, namely, specific granules and VTO. Specific granules are round to elongated and contain a polymorphic layered core, composed of concentric rings, embedded in a less dense matrix (Presentey at el., 1980, Ward et al., 1972). Dumbbell- to ring-shaped VTO have been also observed (Presentey at el., 1980, Ward et al., 1972). Neither primary granules, nor small granules or LB have been originally reported in feline eosinophils. However, primary granules and LB have
been recently depicted in two microphotographs of feline eosinophils without giving further information (Young, 2000).

Morphological changes suggestive of an activation state have not been described in cat eosinophils and their degranulation mechanisms are not known. Nevertheless, *in vivo*, hypodense eosinophils have been separated by density-gradient centrifugation in cats (Padrid et al., 1995, Tompkins et al., 1990).

The cat eosinophil granule content is greatly unknown, the only information available, derived from cytochemical studies, is the lack of peroxidase staining (Jain et al., 1989, Presentey at el., 1980).
Chapter 1

Aims

Against the background of the poor knowledge of both feline EGC and cat eosinophil biology, the objectives of the present studies were:

1. To elucidate the histological features of EGC lesions (Chapter 2);

2. To investigate the ultrastructure of flame figures in EGC lesions (Chapter 3);

3. To investigate the ultrastructure of feline eosinophils and the presence of morphological features indicative of their activation in blood (Chapter 4);

4. To investigate the protein contents and functions of feline eosinophil granules (Chapter 5).

In the study described in chapter 2, the histopathological phenotype and the diagnostic specificity of histopathological findings of EP, EG and IU were studied on H & E stained skin specimens of cats with EGC. To elucidate the conflicting histogenesis of small- and large-sized foci of eosinophilic debris observed on H & E stained sections, in the same study, their staining properties were evaluated also on trichrome stained sections.

To investigate the ultrastructure of feline flame figures, in chapter 3, the ultrastructural morphologies of collagen fibres and degranulating eosinophils were analysed in flame figures of feline EGC and compared with what reported in Wells’ syndrome.

Next, in chapter 4, an ultrastructural study of feline peripheral eosinophils, focused on the morphology of eosinophils and eosinophil granules, was conducted in cats with different eosinophil-associated diseases and various blood eosinophil counts.

In chapter 5, a study on the cat eosinophil granule protein content was conducted. Cat granule proteins were extracted, examined for their peroxidase, RNase and bactericidal activities and the homology of their N-terminal sequence with that of similar proteins from other species was investigated.
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Chapter 1


Chapter 2

Histopathological study of feline eosinophilic dermatoses

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Histopathological study of feline eosinophilic dermatoses

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Abstract A retrospective study was conducted on skin specimens from 24 cats with eosinophilic granuloma complex. The specimens were stained with haematoxylin and eosin and Gallego's trichrome stain. In all specimens, flame figures and/or large foci of so-called 'collagen degeneration' were detected and histopathological features were not predictive of the clinical picture. Use of the term eosinophilic dermatosis was advocated in diagnostic dermatopathology. On trichrome-stained sections, normally stained collagen fibres were identified in the middle of both flame figures and large foci of 'collagen degeneration' and the debris surrounding collagen bundles showed the same tinctorial properties as eosinophil granules. Eosinophil degranulation around collagen bundles seemed to represent the major pathogenetic event in these lesions, analogous with human flame figures. The term flame figures might therefore be more accurately used to designate those foci of eosinophilic to partly basophilic debris commonly referred to as 'collagen degeneration'.

Keywords: cat, collagen, eosinophilic granuloma, eosinophils, granuloma.

INTRODUCTION

The term eosinophilic dermatoses has been proposed to describe those feline diseases included in the eosinophilic granuloma complex (EGC), Nevertheless, the term EGC is still commonly used. Eosinophilic plaque (EP), eosinophilic granuloma (EG) and indolent ulcer (IU) represent clinical entities classically included in EGC. Clinical and histopathological features distinctive of these three entities have been reported.

EP occurs as pruritic, erythematous and erosive coalescing papules and plaques. Epidermal hyperplasia with spongiosis and prominent eosinophilic exocytosis with possible formation of intra-epidermal eosinophilic vesiculo-pustules and diffuse eosinophilic infiltration of the dermis are reported as common features.

EG (synonyms: collagenolytic granuloma, linear granuloma) classically occurs as multiple nodules, variably pruritic, orientated linearly on the caudal thigh or, less common, as single nodular lesions located anywhere on the body, including the footpads, the lower lip and the oral cavity. Dermal foci of an amorphous to granular, eosinophilic to partly basophilic debris are reported as being distinctive histopathological features of EG. This debris is commonly referred to as, so-called, 'collagen degeneration', and is considered to represent a mix of degenerated collagen and degranulated eosinophils. Moreover, small foci of 'collagen degeneration', in which degenerated collagen fibres are surrounded by degranulated eosinophils, are commonly described and named flame figures. Dermal infiltrates in EG vary from predominantly eosinophilic to lymphocyte and histiocytic. In some cases epithelioid and multinucleated cells form a palisading granulomatous reaction around the eosinophilic debris. The epidermis is moderately hyperplastic and occasionally ulcerated.

IU (synonyms: rodent ulcer, eosinophilic ulcer, lip ulcer) refers to a painless, non-pruritic and non-bleeding ulcerated lesion most commonly located on the upper lip. Reported histopathological findings vary from an ulcerative dermatitis with a diffuse eosinophilic infiltrate and foci of 'collagen degeneration', although less prominent than in EG, to an ulcerative neutrophilic and fibrosing dermatitis.

In spite of the report of a distinctive histopathological appearance, pathological features consistent with those of two or more of the recognized entities of the EGC have been observed simultaneously in feline cutaneous biopsies, although never reported in an original study.

The histopathogenesis of both flame figures and large foci of 'collagen degeneration' in the feline EG remains unclear. The primacy of 'collagen degeneration' remains the subject of debate. In a recent study of feline EG specimens, it was shown that Masson's trichrome collagen staining abnormalities did not characterize collagen fibres entrapped in areas of 'collagen degeneration'. Based on this finding, it was speculated that degeneration of collagen was not involved in the pathogenesis of the lesions. However, in this study, flame figures and large foci of 'collagen degeneration' were not differentiated even though it is still not known whether the two lesions have the same histopathogenesis.

The purposes of this study were to investigate the diagnostic specificity of the histopathological findings of the three entities of EGC and to study the histological features and staining properties of feline flame figures and large foci of 'collagen degeneration'.
MATERIALS AND METHODS

A retrospective study was conducted on skin specimens from 24 cats with EGC lesions, diagnosed clinically by a veterinary dermatologist, and three normal skin specimens from one necropsied cat as the control. EGC lesions were represented by nine EP all located on the ventral abdomen, 11 EG with/without linear configuration and various cutaneous distribution, one intra-oral EG and three IU of the upper lip. One cutaneous biopsy from each case was examined. Specimens were processed in a routine fashion and stained with haematoxylin and eosin (H&E) and Gallego’s trichrome stain (IV variant).9

The H&E-stained specimens were examined in a blind fashion and the presence of flame figures and/or large foci of ‘collagen degeneration’ was recorded, as were other histopathological findings. Flame figures and/or large areas of ‘collagen degeneration’ were defined respectively as single collagen bundles surrounded by small amounts of amorphous to granular eosinophilic debris and as well-demarcated large areas of amorphous to granular, eosinophilic to partly basophilic, debris. With Gallego’s trichrome stain, collagen fibres stain blue, nuclei stain purple, and keratin, cytoplasm, muscle fibres and erythrocytes stain green. Gallego’s trichrome-stained sections were examined in a blind fashion and the presence of collagen bundles in the middle of flame figures and of large areas of ‘collagen degeneration’ was recorded. Moreover, collagen fibres were specifically scrutinized for abnormalities in size, outline and staining.

Table 1. Results of the histopathological findings on H&E-stained EGC sections

<table>
<thead>
<tr>
<th>Case</th>
<th>Clinical diagnosis</th>
<th>Histopathological findings</th>
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<tbody>
<tr>
<td>1</td>
<td>EP</td>
<td>Flame figures</td>
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<tr>
<td>2</td>
<td>EP</td>
<td>Ulcer, spongiosis, intraepidermal eosinophilic pustules</td>
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<tr>
<td>3</td>
<td>EP</td>
<td>Flame figures</td>
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<tr>
<td>4</td>
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<td>5</td>
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<td>Flame figures</td>
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<tr>
<td>6</td>
<td>EP</td>
<td>Ulcer</td>
</tr>
<tr>
<td>7</td>
<td>EP</td>
<td>Flame figures</td>
</tr>
<tr>
<td>8</td>
<td>EP</td>
<td>Ulcer, eosinophilic infiltrates in the subcutis</td>
</tr>
<tr>
<td>9</td>
<td>EP</td>
<td>Flame figures</td>
</tr>
<tr>
<td>10</td>
<td>EG</td>
<td>Spongiosis, intraepidermal eosinophilic pustules</td>
</tr>
<tr>
<td>11</td>
<td>EG</td>
<td>Flame figures</td>
</tr>
<tr>
<td>12</td>
<td>EG</td>
<td>Large foci of ‘collagen degeneration’</td>
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<tr>
<td>13</td>
<td>EG</td>
<td>Mild eosinophilic infiltrate, epithelioid macrophages and multinucleated cells</td>
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<tr>
<td>14</td>
<td>EG</td>
<td>Large foci of ‘collagen degeneration’</td>
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<tr>
<td>15</td>
<td>EG</td>
<td>Epithelioid macrophages and multinucleated cells</td>
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<tr>
<td>16</td>
<td>EG</td>
<td>Spongiosis</td>
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<tr>
<td>17</td>
<td>EG</td>
<td>Flame figures</td>
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<tr>
<td>18</td>
<td>EG</td>
<td>Large foci of ‘collagen degeneration’</td>
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<td>19</td>
<td>EG</td>
<td>Transepidermal elimination of foci of ‘collagen degeneration’</td>
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<td>20</td>
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<td>Flame figures</td>
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<td>21</td>
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<td>Ulcer</td>
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<tr>
<td>22</td>
<td>IU</td>
<td>Flame figures</td>
</tr>
<tr>
<td>23</td>
<td>IU</td>
<td>Ulcer, eosinophilic infiltrates in the muscle</td>
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<tr>
<td>24</td>
<td>IU</td>
<td>Large foci of ‘collagen degeneration’</td>
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RESULTS

No abnormalities were detected in H&E-stained specimens in normal feline skin. The results of the histopathological findings in H&E-stained sections are summarized in Table 1. Histopathological findings are reported for each clinical entity.

In H&E-stained specimens, flame figures were detected in 19 cases (nine EP, eight EG and two IU) (Fig. 1) and large foci of ‘collagen degeneration’ were detected in eight cases (seven EG and one IU) (Fig. 2). Both flame figures and large areas of ‘collagen degeneration’ were recognized in three cases (all EG). The epidermis was variably ulcerated and/or spongotic, and intraepidermal eosinophilic pustules were detected in two EP. A dermal, perivascular to diffuse, eosinophilic inflammatory infiltrate was observed in the entire dermis and extended to the subcutaneous tissue in one EP and one EG, and to the underlying muscle in one IU. Eosinophils were invariably present in the infiltrate and represented the predominant inflammatory cells in all but two specimens (one EG and one IU). Other inflammatory cells recognized were mast cells, usually perivascular and confined to the superficial dermis, lymphocytes and histiocytes. Neutrophils were present in the dermis underlying ulcers. Epithelioid macrophages and multinucleated cells were observed in seven specimens (six EG and one IU), surrounding both flame figures and large areas of ‘collagen degeneration’. In two biopsies (both EG), respectively, transfollicular (Fig. 3) and transepidermal elimination of foci of ‘collagen degeneration’ was detected.

In Gallego’s trichrome-stained sections, no abnormalities were observed in the skin specimens from the normal cat. In all EGC specimens examined, blue-stained collagen fibres were identified in the middle of both flame figures and large foci of ‘collagen degeneration’ (Figs 4, 5).

Figure 1. Granular eosinophilic debris accumulates along fragments of single collagen bundles (flame figures). Eosinophils and macrophages predominate in the inflammatory infiltrate (H&E, ×40).

Figure 2. Large and irregularly shaped deposit of granular eosinophilic to partly basophilic debris (large area of ‘collagen degeneration’). Multinucleated cells surround the deposit (H&E, ×10).

Figure 3. Multiple foci of ‘collagen degeneration’ are extruded from the dermis through the infundibular epithelium (transfollicular elimination of foci of ‘collagen degeneration’) (H&E, ×10).

Figure 4. Same microscopic field as in Figure 2. In a large focus of ‘collagen degeneration’ blue collagen fibres are surrounded by granular greenish debris that contains purple nuclear remnants. Note that collagen bundles stain identically to those present in the surrounding dermis (Gallego’s stain, ×20).
Collagen fibres present in the middle of both flame figures and large areas of ‘collagen degeneration’ stained identically to collagen fibres observed elsewhere in the dermis. No size, surface contour or staining collagen abnormalities were detected in any of the specimens. In Gallego’s trichrome-stained sections, the eosinophilic debris observed in the H&E-stained sections appeared as a granular greenish material containing variable amounts of purple nuclear remnants, in both flame figures and large foci of ‘collagen degeneration’. In addition, green extracellular eosinophil granules were observed surrounding the greenish debris.

**DISCUSSION**

In this study, flame figures and/or large foci of ‘collagen degeneration’ were observed in all the EGC specimens examined. These histopathological findings are considered typical of EG and IU in veterinary dermatopathology textbooks, whereas ‘collagen degeneration’ is not reported in EP. In our study, flame figures, but not large areas of ‘collagen degeneration’, were detected in all nine EP specimens examined. Moreover, in one IU, large foci of ‘collagen degeneration’ were observed and they appeared histologically indistinguishable from those classically described in EG. This finding does not agree with the statement that ‘collagen degeneration’ detected in IU is less prominent than in EG.

Based on these observations, it appears that the histopathological features of the EGC are not always predictive of the clinical aspects of the disease complex and vice versa. The three entities of the EGC appear indistinguishable histopathologically. It may be suggested, therefore, that the terms EP, EG and IU should not be used in the dermatopathological diagnosis of feline EGC, because of the absence of a correlation between specific histopathological findings and clinical lesions. We propose that the terms EP, EG and IU, which correspond to clinically distinctive lesions, should be confined to clinical dermatology, whereas in diagnostic dermatopathology the term eosinophilic dermatoses should be used.

Feline eosinophilic dermatoses show histological similarities with human Wells’ syndrome, an uncommon chronic dermatitis, characterized clinically by well-demarcated, oedematous and erythematous plaques. Histopathological changes in Wells’ syndrome may evolve through three stages. The acute stage reveals dermal oedema and marked dermal eosinophilic infiltration, the subacute stage is characterized by the presence of flame figures and in the resolving stage histiocytes and multinucleated cells surround flame figures.

Current opinion about the pathogenesis of flame figures in Wells’ syndrome is that eosinophil recruitment and degranulation represent the primary event. Collagen bundles in the middle of flame figures do not show staining abnormalities with Van Gieson stain and have a normal ultrastructure on transmission electron microscopy examination. This means that collagen is not primarily altered nor is it the primary target structure for damage in Wells’ syndrome, but rather it appears to be an innocent bystander entrapped in the middle of eosinophil granule products. When examined by immunofluorescence for major basic protein (MBP), flame figures show bright extracellular staining, suggesting that extensive eosinophil degranulation has occurred. Poor soluble eosinophil granule proteins, rather than degenerated collagen, are considered to provoke the granulomatous reaction around flame figures.

In this study, in Gallego’s trichrome-stained sections, both flame figures and large foci of ‘collagen degeneration’, appeared to consist of blue collagen fibres surrounded by an amorphous to granular greenish debris and purple nuclear remnants. Feline flame figures and large areas of ‘collagen degeneration’ appear therefore to have the same structure, in spite of the different dimension. The debris surrounding collagen bundles showed the same trichrome tinctorial properties as eosinophil granules. In addition, the presence of purple nuclear fragments mixed with the debris likely indicates eosinophil destruction, which is a commonly reported mechanism of eosinophil granule content release.

Based on these observations, we speculate that the
Greenish debris observed in Gallego’s trichrome-stained sections most likely represents eosinophil granule products, rather than degenerated collagen. In fact, it seems unlikely that, if the greenish material represented degenerated collagen, collagen fibres without any size, outline and staining abnormalities would be detected in the middle of this debris. Collagen fibres located in the centre of the lesions would probably be the first to undergo degeneration. The pathogenesis of both feline flame figures and large foci of ‘collagen degeneration’, which appear to have similar histogenesis, might therefore be analogous to that of human flame figures, i.e. eosinophil recruitment and degranulation around structurally normal collagen bundles.11 Consequently, we propose avoiding use of the term ‘collagen degeneration’ to designate the eosinophilic debris observed in H&E-stained sections in feline eosinophilic dermatoses. The term flame figures might be used, as proposed by Fernandez et al.,8 to designate both small and large foci of ‘collagen degeneration’, due to apparent similarity with the histopathogenesis of human flame figures. Nevertheless, findings in trichrome-stained sections do not confirm the eosinophil origin of the greenish debris or rule out the presence of collagen alterations not detectable with this stain. Immunological, as well as electron microscopy, studies are needed to confirm the hypotheses formulated, which are based mainly on histopathological studies and analogy with human medicine.

The similar structure of flame figures and large foci of ‘collagen degeneration’, as well as the presence of both lesions in the same biopsy specimens (three EG) support the hypothesis, suggested by Fairley,17 of a progression of histological lesions from flame figures to large areas of ‘collagen degeneration’. Similar to that reported in Wells’ syndrome,11 lesions might progress from an eosinophil infiltrate to flame figures, to large foci of ‘collagen degeneration’, to granulomatous reactions around these structures. All these lesions, single or in combination, were recognized in our specimens in association with different clinical forms of EGC. A histopathological progression of the lesions implies that the disease stage in which the biopsy is taken is crucial to the histopathological picture and this might help to explain the difficulty in defining histopathological features specific for each clinical form of the EGC. However, progression of the lesions does not rule out that large foci of ‘collagen degeneration’ might form without being preceded by flame figures or that flame figures might provoke a granulomatous reaction without evolving to large foci of ‘collagen degeneration’, depending on the degree of eosinophil degranulation.

Transepidermal–transfollicular elimination of foci of ‘collagen degeneration’, detected in two EG, is a well-recognized phenomenon in a heterogeneous group of cutaneous human diseases, called ‘perforating dermatoses’. Degenerated collagen or elastic fibres, as well as keratin, may be extruded by means of transepidermal or transfollicular elimination.18 Transepidermal elimination of collagen has been reported previously in two cats and in one case it was interpreted as being the result of focal collagen degeneration due to collagen metabolic defects.19,20 However, electron microscopy studies did not reveal collagen structure abnormalities in the extruded collagen nor were systemic metabolic defects detected.19 Interestingly, both reported cases of feline transepidermal elimination of collagen histologically resembled an eosinophilic dermatosis. In our cases, the extruded material had the same microscopic and tinctorial characteristics, in both H&E- and Gallego’s trichrome-stained sections, as dermal foci of putative eosinophil degranulation around collagen bundles. The phenomenon was therefore interpreted, analogous to that reported in human ‘perforating dermatoses’, as a possible route of elimination of an irritating material acting as a foreign body, namely eosinophil granule products.18

In conclusion, from this study it appears that there is histopathological overlap among the three clinical forms of EGC and that eosinophil recruitment and degranulation probably represent the major pathogenetic events in these lesions.

REFERENCES


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Chapter 3

Ultrastructural study of cutaneous lesions in feline eosinophilic granuloma complex

Mar Bardagi, Alessandra Fondati, Dolors Fondevila and Lluís Ferrer

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Abstract
The purpose of this study was to investigate the ultrastructural appearance of flame figures, reported to comprise a mixture of degenerate collagen and degranulated eosinophils, in feline eosinophilic granuloma complex (EGC). Skin specimens from eight cats with EGC and from two clinically healthy cats, were examined by transmission electron microscopy. Flame figures appeared to comprise ultrastructurally normal collagen fibrils separated by oedema and surrounded by large numbers of degranulating eosinophils. Longitudinal sections of collagen fibrils displayed the characteristic cross-striation of normal dermal collagen. Feline eosinophils, analogous to human eosinophils, degranulated both by cytolysis and piecemeal degranulation. The results of this study suggest that flame figures form in feline EGC due to eosinophil recruitment and degranulation, and that collagen fibres are partially disrupted but collagen fibrils are not damaged. These findings suggest that eosinophil accumulation and the release of granule contents represent the primary events in feline EGC.
Introduction

Feline eosinophilic granuloma complex (EGC) is characterised histopathologically by an intense dermal eosinophilic infiltrate accompanied by deposition of amorphous to granular debris that appears eosinophilic to basophilic on haematoxylin and eosin (H&E) staining. These deposits have been termed “flame figures”, and these have been reported to comprise a mixture of degenerate collagen and degranulated eosinophils. However, on trichrome-stained sections, normally stained collagen fibres have been identified in the centre of the flame figures, and it has been speculated that the flame figures in feline EGC, analogous to those observed in Wells’ syndrome, most likely represent normal collagen fibres surrounded by eosinophil granule products. Wells’ syndrome, the human counterpart of feline EGC, is an uncommon cutaneous disorder characterised clinically by recurrent oedematous and erythematous papules and plaques and, histologically, by dermal oedema, eosinophilic infiltration and flame figures, surrounded, in the resolving stage, by a granulomatous reaction.

Eosinophil content release around normal collagen fibres is thought to represent the major pathogenic event in Wells’ syndrome and presumably also in feline EGC. When examined by immunofluorescence for major basic protein (MBP), an eosinophil granule protein, flame figures in Wells’ syndrome show bright extracellular labelling, suggesting that extensive eosinophil degranulation has occurred. Moreover, collagen bundles, in the centre of flame figures, appear to have normal ultrastructural appearance when examined by transmission electron microscopy (TEM). This observation suggests that collagen is not primarily altered, nor is it the primary target structure for damage in Wells’ syndrome. Collagen therefore appears to be an “innocent bystander” entrapped in the middle of eosinophil granule products. The ultrastructural appearance of degenerate collagen is extremely variable, however, it is invariably characterised by alteration of fibril morphology, including swelling, loss of stain affinity and cross-striation and, on transverse sections, irregular or altered diameter profiles.

Feline eosinophils are known to contain bicompartmental specific granules, with an electron dense lamellar core embedded in a less dense matrix. The mechanisms of granule content release from feline tissue eosinophils are currently unknown. Human eosinophil granule content release, takes place in vivo through two different mechanisms, eosinophil cytolysis (ECL) and piecemeal degranulation (PMD). These two degranulation pathways are distinguishable only by TEM examination and their biological significance is currently unclear. In cytolysis, the cell membrane ruptures, the cytoplasm is lost, the cell nucleus displays signs of chromatolysis and, in the late-stage, clusters of membrane-bound free specific granules are released. There is still no consensus as to whether ECL is an active secretory process or a passive degeneration of the eosinophil. In PMD, eosinophils progressively release specific granule matrix and/or core compartments through membrane-bound secretory vesicles and retain emptied granule chambers.
Vesicles, which bud off from the specific granule, transport proteins to the plasma membrane, open to the cell surface and release free granule content into the extracellular environment. The end morphological result of PMD is the presence of partially to completely empty granules in viable eosinophils.

The purpose of this study was to investigate the ultrastructural appearance of flame figures in feline EGC lesions. Specifically, the morphology of collagen fibres and fibrils was examined, and eosinophils and eosinophil degranulation pathways were evaluated.

**Materials and Methods**

*Specimens.*

Eight adult cats (numbered 1 to 8) with clinical and histopathological features of EGC, and two clinically healthy cats (numbered 9 and 10) were included in the study. All cats were domestic shorthairs. Ages ranged between 5 months and 12 years, with a mean age of 3 years and 5 months. Four cats were males (three intact and one castrated) and four were females (two intact and two spayed). Clinical findings included variably eroded papules and plaques, or ulcers. Lesions were localized to the head in four cats (periorally in cats 7 and 8, and on the pinnae in cats 2 and 5), the ventral abdomen in cat 1 and 3, one forearm in cat 3 and the caudal thigh in cats 4 and 6. Four to six mm biopsy punches from cat 1, 2, 5, 6, 7, 8 and 9, and one to two excisional biopsies from cat 3, 4, and 10 were obtained. All specimens were bisected, one half was processed and stained by H&E stain in a routine fashion for light microscopic examination and the other half was processed for TEM examination. Skin biopsies from cats 1, 2, 4, 5, 7 and 9 were processed for TEM examination at the time the biopsy was taken, whereas skin samples from cats 3, 6 and 10 were recovered from biopsies fixed in 10% neutral buffered formalin and those from cat 8 were recovered from paraffin wax blocks.

*Electron microscopy specimen preparation.* All skin samples obtained for TEM examination were cut into small pieces (approximately 3 x 3 x 5 mm), fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (SCB) (pH 7.4) for 2 hours at 4°C, rinsed twice in SCB for 5 minutes, resuspended in fresh SCB and stored at 4°C. They were then postfixed in 1% osmium tetroxide in SCB for 2 hours at 4°C, washed in SCB, dehydrated in 30 and 50% ethanol, stained with 2% uranyl acetate in 70% ethanol, further dehydrated in ethanol solutions (90% and absolute ethanol) and embedded in Spurr’s resin. One µm-thick sections were cut with an ultramicrotome (Ultracut E, Reichert-Jung, Wien, Austria), stained with toluidine blue in 1% borax and examined by light microscopy. Areas with flame figures from two electron microscopy blocks from cats 1, 2, 3, 4, 7 and 8 and from one block from cats 5.
and 6 were selected, ultrasectioned at 80 nm, mounted on 200-mesh copper grids, stained with lead citrate and examined with a Hitachi 7000 transmission electron microscope (Hitachi LTD, Tokyo, Japan). Areas with dermal collagen were selected from one block from cats 9 and 10, ultrasectioned, mounted, stained and examined as for cats 1 to 8.

**TEM examination of collagen fibres and fibrils.**

Longitudinal and transverse sections of collagen fibres surrounded by degranulated eosinophils were photographed at magnifications of 5000 to 40,000 times. Equivalent magnification microphotographs were taken of dermal collagen fibres from control cats. Using computer assisted image analysis (MetaMorph Version 4.6, Universal Imaging Corp.), 40 to 44 axial distances of the D-periodic cross-striation pattern of different collagen fibrils were measured in one photomicrograph per cat, in seven cats (1 to 4 and 6 to 8) and in both control cats, at a magnification of 15,000 times.

**TEM examination of eosinophil degranulation pathways.**

Eosinophils (40 to 146 per cat) from two electron microscopy blocks from cats 1, 2, 3, 4, 7 and 8 and from one block from cats 5 and 6 were recorded on electron photomicrographs at a magnification of 5000 and classified according to the criteria reported by Erjefält and Persson. Resting eosinophils were defined as eosinophils with no ultrastructural signs of activation (i.e., normal cell morphology and all specific granules showing an electron-dense core surrounded by an intact matrix). Cytolytic eosinophils were defined as eosinophils showing signs of chromatolysis, with loss of plasma membrane integrity, partly dissolved cytoplasm and release of membrane bound granules into the extracellular matrix. Granules derived from cytolytic eosinophils were defined, according to Persson et al., as clusters of free eosinophil granules (Cfegs). Piecemeal-degranulated eosinophils were defined as eosinophils with intact plasma membrane and partly empty intracellular granules. In each eosinophil showing signs of PMD, specific granules were divided into two groups according to the criteria proposed by Erjefält et al. The first group included intact granules with no signs of degranulation (intact core and matrix) and the second group comprised activated granules with structural changes of PMD. These latter included ragged loss of core material but intact matrix, intact or nearly intact core but partly to completely empty matrix with early stages of matrix changes identified as coarsening of the matrix, and nearly complete or complete loss of both core and matrix material. To express the degree of degranulation of each eosinophil undergoing PMD, a degranulation index (DI) was calculated as proposed by Erjefält et al. This system defines DI as the percentage of altered granules (granules exhibiting ultrastructural signs of protein release) in a given eosinophil and is calculated as follows: DI = 100 x (number of activated granules / total granules). DI results were classified as: low PMD (<20% altered granules), moderate PMD (20-60% altered granules) and extensive PMD (>60% altered granules).
Chapter 3

Results

On light microscopic examination, an interstitial to diffuse dermal eosinophilic infiltrate with flame figure formation was observed in all specimens. In cats 5 and 6 a granulomatous reaction with multinucleate giant cells was observed surrounding the flame figures.

At TEM examination, flame figures in all samples appeared to comprise collagen fibres surrounded by large numbers of degranulating eosinophils (Fig. 1). Collagen fibres appeared to be partially disrupted with separation of collagen fibrils due to the presence of oedema and abundant cellular debris. However, no ultrastructural abnormalities of the collagen fibrils were detected. Longitudinal sections of collagen fibrils in flame figures displayed the characteristic cross-striational staining pattern of normal dermal collagen (Fig. 2). Transverse sections of collagen fibrils in all samples showed uniform diameters and a regular cross-sectional profile. Dermal collagen fibrils of control cats had the same ultrastructural morphology of those observed in flame figures of study cats. The mean axial distances of the \( D \)-periodic cross-striation pattern of collagen fibrils in the examined micrographs ranged from 43.08 to 61.24 nm and appeared to be similar both in the study cats and the controls, however, due to the small sample size, data obtained could not be statistically analysed (Fig. 3). Measurements taken in each cat had a normal distribution, tested by the Kolmogorov and Smirnov test.

In all cats, eosinophils were either lytic (Fig. 4) or displaying signs of PMD (Fig. 5). No resting eosinophils were observed in flame figures and no eosinophils were seen in the skin of control cats. ECL was the main feature of eosinophils in cats 2, 3, 5, 6 and 7, and cytolytic eosinophils make up more than 80% of the population examined in cats 2, 3 and 6 (Fig. 6). Two cats showed similar numbers of eosinophils undergoing PMD or lysis (cats 1 and 4). In cat 8, eosinophil granule content release by PMD was greater than by cytolysis. ECL was always associated with the presence of Cfegs and the majority of Cfegs showed features of PMD. DI study showed predominant extensive PMD in cats 2, 5, 6, 7 and 8, being 100% in cats 2 and 7. Cats 3 and 4 had a main pattern of moderate PMD and cat 1 had similar percentages of moderate and extensive PMD (Fig. 7). Macrophages with intracytoplasmic eosinophil granules (Fig. 8) were noted in specimens from cats 1, 4 and 8. In addition, some basophils were present in samples from cats 4 and 7, and occasional mast cells were observed in samples from cat 8.

Discussion

In this ultrastructural study, the flame figures of feline EGC appeared to comprise morphologically normal collagen fibrils, separated by oedema and surrounded by eosinophils undergoing degranulation, both via cytolysis and PMD. Macrophages with intracytoplasmic eosinophil granules were also observed.
Fibrils in the centre of the flame figures, on transverse sections, showed uniform cross-sectional profiles and little variation of fibril diameters. The accurate estimation of the periodicity of collagen fibrils in electron micrographs is known to be difficult, due to different causes, including minimal differences in sample processing and obliquity of sectioning, on longitudinal sections. Despite of that, the mean of the \( D \) measurements did not apparently differ and had a normal distribution both in the study and the control cats. All of these findings demonstrate the lack of ultrastructural damage of collagen fibrils in flame figures of feline EGC, therefore, we suggest that the terms “collagenolysis” and “collagen degeneration” are not appropriate when referring to flame figures on histological examination of the lesions of feline EGC.

The lack of collagen damage in a disease associated with eosinophil infiltration is in accordance with what is reported in human eosinophil-mediated diseases which are associated with fibrosis rather than with collagen degeneration.\(^\text{15}\) Eosinophils and eosinophil lysates have been shown to stimulate fibroblast proliferation and collagen synthesis, mainly through transforming growth factor beta release, but not to cause collagen degradation.\(^\text{16}\) Furthermore, even though \textit{in vitro} studies have shown that human and Guinea pig eosinophils possess matrix metalloproteinases (MMP), namely MMP-1 and MMP-9,\(^\text{17,18}\) the activity of these collagenases has normally been related to connective tissue remodelling rather than to collagen lysis.\(^\text{19}\)

Oedema between collagen fibrils was observed in all the sections examined and this might contribute, together with Cfegs and eosinophil debris from ECL, to give the amorphous to granular, eosinophilic to basophilic appearance of flame figures on light microscopic examination. The association between cutaneous oedema and eosinophil degranulation has been observed in several human inflammatory cutaneous disorders, including episodic angioedema.\(^\text{20}\) It has been demonstrated that MBP induces histamine release from basophils and mast cells, produces a wheal-and-flare skin reaction and directly provokes increased vasopermeability.\(^\text{20,21}\) Moreover, the numerous inflammatory mediators released by eosinophils, platelet-activating factor and leukotriene C, among others, are also able to produce tissue oedema by increasing vascular permeability.\(^\text{15}\) Therefore, the presence of tissue oedema in feline flame figures could be explained, at least in part, as a result of eosinophil degranulation, specially considering that granule content release by ECL was observed in all the examined samples.

In a previous study, examining Gallego’s trichrome-stained sections of feline flame figures, it was speculated that the granular greenish debris surrounding blue collagen fibres represented eosinophil granule products and that the purple nuclear fragments mixed with that debris likely indicated eosinophil cytolysis.\(^\text{1}\) The present study confirms this hypothesis, in fact, all the eosinophils studied showed ultrastructural signs of degranulation. We report for the first time that eosinophil degranulation, in feline EGC, follows two different ultrastructural pathways, namely, ECL and
PMD. ECL and PMD also represent the two major modes of eosinophil degranulation in damaged human tissues.\textsuperscript{9,14}

The proportion of the two types of degranulation varied among study cats. Markedly different degranulation patterns have also been reported in certain human mucosal eosinophil-associated diseases such as asthma, allergic rhinitis, and nasal polyposis.\textsuperscript{14} In addition, ECL has been selectively evoked \textit{in vivo} upon allergen exposure in allergic rhinitis\textsuperscript{12} and, \textit{in vitro}, by exposing eosinophils to secretory IgA-coated Sephadex beads\textsuperscript{22}, whereas eosinophil PMD has been selectively induced by gamma interferon.\textsuperscript{23} Based on the overall data, it has been suggested that PMD and ECL represent two functionally different processes, probably induced by different stimuli, by which eosinophils release their granule mediators.\textsuperscript{9} PMD would provide a long lasting and selective release of granule contents whereas ECL would produce a rapid and entire release of granule products, being probably more important in defence against parasites and in eosinophilic allergic diseases.\textsuperscript{9,11} We might therefore speculate that differences in the degranulation patterns observed in our cats could be related to different inciting factors of feline EGC. Nevertheless, other factors might have influenced the type of eosinophil degranulation, such as the age of the lesions or the effects of previous treatments. Unfortunately, these data from the clinical history were not available in the majority of the studied cats.

ECL has been reported by some authors as the passive result of intense eosinophil PMD, inferring that high cytoplasmic levels of toxic granule products may lead to cytolysis.\textsuperscript{9} Our finding of numerous Cfegs showing signs of PMD, apparently, could support the theory that Cfegs represent granules from lytic eosinophils, which have previously undergone PMD. However, the presence of Cfegs with signs of PMD has also been proposed as being part of the ECL process in which eosinophil lysis would generate Cfegs that would leak their contents into the target tissue assuming the morphology of granules undergoing PMD.\textsuperscript{11} If PMD is considered to cause ECL, in our cats, high ECL counts would be expected to be associated with high DIs in eosinophils undergoing PMD and vice versa. Nevertheless, in our results, high DIs were found in cats with low ECL counts and a moderate pattern of PMD was associated with high ECL counts. Although highly speculative, these findings do not support the hypothesis of PMD preceding ECL, but would rather support the hypothesis that ECL and PMD represent distinct mechanisms of eosinophil degranulation and that cytolysis constitutes a primary and active mechanism of degranulation. Furthermore, it has been recently demonstrated that granule protein release, in PMD, occurs via cytoplasmic membrane-coated transporting vesicles, which would protect the cell from the cytotoxic effects of granule proteins,\textsuperscript{10,24} making it further unlikely that ECL is an evolutive phase of eosinophil damage induced by free cytoplasmic granule proteins released by PMD. However, the fact that ECL might be secondary to the presence of highly cytotoxic eosinophil granule proteins extracellularly released by massive eosinophil degranulation, possibly by PMD, is still a reasonable
possibility. Further studies in the field of biological and physiological control of eosinophil degranulation are awaited.

In humans, the biological regulation of eosinophil cytolysis, including the mode of protein release from Cfegs, has been suggested to represent a promising area for the study of novel treatments for eosinophil-associated diseases. Since human and feline tissue eosinophils have similar degranulation patterns, there is hope that currently ongoing research in humans can bring new treatments for both human and feline eosinophil-mediated diseases, the latter being commonly reported in the skin, the gastrointestinal and respiratory tract.

Poorly soluble eosinophil granule proteins are considered to provoke the granulomatous reaction around flame figures both in Wells’ syndrome and in feline EGC. In this study, ultrastructural evidence of macrophages with intracytoplasmic eosinophil granules has been depicted. This finding and the lack of ultrastructural alterations of collagen fibrils, further contributes to the rejection of the traditional theory of damaged collagen inciting the granulomatous reaction that can be observed in certain cases of feline EGC.

In conclusion, it appears from this study that the flame figure formation observed in feline EGC on light microscopic examination is due to massive eosinophil degranulation and that the structure of collagen fibres is partially disrupted but collagen fibrils are not damaged. In addition, eosinophils in feline EGC, analogously to human eosinophils, appear to release their granule contents both by cytolysis and PMD. Taken together, these findings suggest that eosinophil recruitment and degranulation represent the primary events in feline EGC and that collagen fibres do not play any active role in the pathogenesis of this disease complex. Further studies are needed both to identify the initiating stimuli capable of inducing selective migration of circulating eosinophils into the skin of cats suffering from EGC and to better define feline eosinophil functions.

Acknowledgements

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References


**Fig. 1.** Ultrastructural appearance of a flame figure. The flame figure comprises collagen fibres (asterisk) and cell debris in which free eosinophil granules are visible (arrowhead). The intense oedema leads to separation of collagen fibrils (arrow). Bar = 0.75 \( \mu \text{m} \)

**Fig. 2.** Longitudinal section of collagen fibrils. Note the characteristic cross-striation pattern of fibrillar collagen. Bar = 0.09 \( \mu \text{m} \)
Fig. 3. Mean axial distances and standard deviation of the $D$-periodic cross-striation pattern of collagen fibrils in study cats (1-8) and controls (9, 10).

Fig. 4. Eosinophil cytolysis. Cell nuclei (asterisks) and numerous clusters of free eosinophil granules (Cfegs) are visible (arrows). Bar = 1.50 µm
**Fig. 5.** Eosinophil piecemeal degranulation (PMD). Partially empty granules (arrows) can be observed in these two viable eosinophils. Bar = 0.75 μm

**Fig. 6.** Morphological study of eosinophil degranulation pathways. Total number of eosinophils counted and percentage of cytolytic (ECL) and piecemeal degranulated (PMD) eosinophils in each cat. ■ ECL; □ PMD.
Fig. 7. Degranulation index study. Different degree of degranulation of eosinophils undergoing piecemeal degranulation (PMD). Low PMD: <20% altered granules; moderate PMD: 20-60% altered granules; extensive PMD: >60% altered granules.

- Low PMD
- Moderate PMD
- Extensive PMD

Fig. 8. Activated macrophage with intracytoplasmic phagolysosomes containing osmiidense material resembling granule core content (arrowheads). Extracellular clusters of free eosinophil granules (Cfegs) (arrow) are also visible. Bar = 0.62 μm
Chapter 4

Piecemeal degranulation (PMD) morphology in feline circulating eosinophils

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In press
Piecemeal degranulation (PMD) morphology in feline circulating eosinophils

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Abstract

Buffy coat preparation from six cats with 600–4560 circulating eosinophils/μL was collected by either blood centrifugation or sedimentation, fixed in 2.5% glutaraldehyde, post-fixed in either 1% osmium or in 1.5% potassium ferrocyanide-reduced osmium, ultra-sectioned and examined by transmission electron microscopy. Ultrastructural changes of piecemeal degranulation (PMD), which is a mechanism of eosinophil granule contents release indicative of eosinophil activation, were observed in specific granules from all the samples examined. The spectrum of PMD included coarsening of the granule matrix, budding vesicles, fragmented granule cores and lucent granules. The number of presumably activated eosinophils with ultrastructural evidence of PMD did not correlate with the level of eosinophilia. The lack of correlation suggested that, analogously with humans, blood eosinophil count might not represent the best criterion to evaluate the contribution of eosinophils to tissue damage in certain feline eosinophil-associated diseases.

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1. Introduction

Tissue and circulating eosinophilia (blood eosinophil counts exceeding 1500 eosinophils/μL) may be observed in numerous inflammatory processes of the feline gastrointestinal tract, respiratory tract and the skin (Day and Shaw, 2000b; Day and Hall, 2000). Feline eosinophils, analogous to human eosinophils, are known to contain bicompartamental specific granules with an electron-dense core, composed of concentric rings, embedded in a less dense matrix (Ward et al., 1972; Presentey et al., 1980). The content of these granules in the cat is currently unknown, as is the mechanism of granule content release. Human eosinophils selectively house cytotoxic proteins in specific granules and they are known to release their granule contents by classical exocytosis, piecemeal degranulation (PMD) and cytolysis (Dvorak, 1994; Dvorak and Weller, 2000). In classical simple exocytosis, membrane proteins fuse, membrane pores are formed and membrane-free granule contents are extruded in toto to the exterior of cells. The result is electron-microscopic images of granule-poor viable eosinophils (Dvorak, 1994; Dvorak and Weller, 2000). In PMD, eosinophils progressively release specific granule matrix and/or core compartments through membrane-bound secretory vesicles and retain emptied granule chambers. Vesicles, which bud off from the specific granule, transport proteins to the plasma membrane, open to the cell surface and release free granule contents into the extracellular environment (Dvorak, 1994; Dvorak and Weller, 2000). The end morphological result of PMD is the presence of partially to completely empty granules in viable eosinophils (Dvorak, 1994; Dvorak and Weller, 2000). In cytolysis, the cell membrane ruptures, the cytoplasm is lost, cell nucleus displays signs of chromatolysis and, in the late-stage, clusters of membrane-bound free specific granules are released (Erjefalt et al., 1999). There is no consensus as to whether cytolysis is an active secretory process resulting in a rapid and complete granule content release or a process of passive degeneration of the eosinophil succumbing due to injurious environmental factors (Erjefalt et al., 1999). Little is known about the triggers leading to these different secretory mechanisms, however, there is evidence that PMD represents a long-
lasting and selective mechanism of granule protein release (Wardlaw et al., 1995; Erjefalt et al., 1999). PMD morphology, which is indicative of eosinophil activation, has been reported in circulating eosinophils both from humans with hypereosinophilic syndrome and active atopic disease (Caulfield et al., 1990; Karawajczyk et al., 2000) and in tissue eosinophils in a variety of inflammatory diseases (Erjefalt et al., 1999). Ultrastructural analysis of eosinophils and eosinophil granules is currently the only technique available to recognise PMD morphology and to distinguish it from the other degranulation pathways (Malm-Erjefalt et al., 2001).

The purposes of this study were to investigate and to compare the ultrastructural morphology of circulating eosinophils and eosinophil granules from cats with various blood eosinophil counts.

2. Materials and methods

2.1. Specimen obtainment and preparation

Five adult cats, with a variety of inflammatory diseases and with blood eosinophil counts ranging from 1404 to 5460 eosinophils/μL, all of them patients of the Veterinary Teaching Hospital of the Universitat Autònoma de Barcelona, were included in the study (Table 1). Blood samples were withdrawn into EDTA-containing tubes. Complete blood cell counts were performed and smears prepared from all the samples, routinely stained (Hemacolor, Merck, Germany) and examined by light microscopy. They were then centrifuged at 500g for 5min. Buffy coats were collected, fixed in 2.5% glutaraldehyde, fixed for an additional 1.5h at 4°C, washed once in SCB at 300g for 2min, resuspended in fresh SCB and stored at 4°C for 30min. The buffy coat was removed, placed in fresh 2.5% glutaraldehyde, fixed for an additional 1.5h at 4°C, washed once in SCB at 300g for 2min, resuspended in fresh SCB and stored at 4°C.

2.2. Electron microscopy

All the buffy coats were pre-embedded in 2% liquid agar. The agar pellets were post-fixed in 1% osmium tetroxide in SCB for 2h at 4°C. To evaluate cell membranes and intracellular glycogen particles, half a pellet from each of the buffy coats obtained from patients 3 and 4 was post-fixed in 1.5% potassium ferrocyanide, pH 6, in 1% osmium tetroxide for 2h at 4°C (Dvorak et al., 1972). All the pellets were then washed in SCB, stained en bloc with 2% uranyl acetate in 70% ethanol, dehydrated in graded ethanol (30, 50, 70, 90% and absolute ethanol twice) and embedded in Spurr’s resin. One μm-thick sections were cut with an ultramicrotome (Ultracut E, Reichert-Jung, Wien, Austria), stained with toluidine blue in 1% borax and examined by light microscopy. Representative areas were ultra-sectioned at 80nm, mounted on 200-mesh copper grids, stained with 0.5% lead citrate and examined with a Hitachi 7000 transmission electron microscope (Hitachi, Tokyo, Japan). Selection of eosinophils for analysis was performed in a nonbiased manner starting at one corner of a section and all the eosinophils, excluding only cells without nuclei, were photographed at magnifications of 5000–40,000times.

2.3. Morphologic analyses

Using the low-magnification electron micrographs, eosinophil diameter, number of nuclear lobes, presence of uropods, Golgi apparatus, mitochondria, vesiculotubular organelles (VTO) and glycogen particles and number of specific granules were determined. Eosinophil granule morphology was studied. Features examined included coarsening of the matrix and the presence of well-limited accumulations of electron-dense material in the granule matrix, budding vesicles, fragmented cores and lucent granules. Fragmented cores were defined as core-losses and lucent granules were defined as specific

<table>
<thead>
<tr>
<th>Patient</th>
<th>Diagnosis</th>
<th>Eosinophils (μL)</th>
<th>Number of cells analysed</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Eosinophilic granuloma complex</td>
<td>4560</td>
<td>10</td>
</tr>
<tr>
<td>2</td>
<td>Eosinophilic enteritis</td>
<td>3522</td>
<td>10</td>
</tr>
<tr>
<td>3</td>
<td>Eosinophilic granuloma complex</td>
<td>3471</td>
<td>34^b</td>
</tr>
<tr>
<td>4</td>
<td>Cutaneous mastocytosis</td>
<td>1719</td>
<td>10^b</td>
</tr>
<tr>
<td>5</td>
<td>Allergic dermatitis</td>
<td>1404</td>
<td>9</td>
</tr>
<tr>
<td>6</td>
<td>Clinically healthy</td>
<td>600</td>
<td>8</td>
</tr>
</tbody>
</table>

^a Fourteen eosinophils (10 from patient 3 and 4 from patient 4) were post-fixed with potassium ferrocyanide-reduced osmium.

^b Twelve eosinophils from patient 3 were collected by blood sedimentation.
granules containing large electron-lucent, virtually empty areas. Morphologic analysis was obtained on a total of 81 eosinophils. Ten eosinophils from patient 1, 10 from patient 2, 34 from patient 3, 10 from patient 4, 9 from patient 5 and 8 from patient 6 were examined (Table 1). Twelve eosinophils from patient 3 were collected by blood sedimentation and 14 eosinophils, 10 from sample 3 and 4 from sample 4, were post-fixed with potassium ferrocyanide-reduced osmium (Table 1).

Eosinophil diameter was calculated by computer-assisted image analysis using Scion Image Beta 4.02 Win for Windows. Differences between the two samples collected by blood sedimentation and centrifugation were analysed by Student's t test performed with Microsoft Excel 5.0. Statistical difference was assumed for P values < 0.05.

3. Results

Eosinophils were round to oval and their mean diameter ranged from 5.91 μm in sample 3–7.26 μm in sample 4. Nuclei were segmented, with one to three lobes, and nucleoli were never seen. Eosinophil surface profiles were characterised by uropods in 50% and 10% of eosinophils from specimens 2 and 4, respectively. From 12.5% of eosinophils in sample 6–66.7% of eosinophils in sample 5 had a well-developed Golgi zone. In addition, mitochondria and VTO, the latter dumbbell-, C- and reversed donut-shaped, were visible and were relatively more numerous in those eosinophils with an evident Golgi zone. In the cytoplasm of these eosinophils, abundant membrane-coated empty vesicles and elongated tubules were also observed. Electron-dense glycogen particles, scattered or diffuse, were detected in the cytoplasm of 10% and 75% of eosinophils post-fixed with potassium ferrocyanide-reduced osmium, from specimens 3 and 4, respectively. Cloudy accumulations of electron-dense material, ranging from 0.25 to 0.39 μm in diameter, were observed in the cytoplasm of eosinophils from all the specimens examined. They were apparently not entirely membrane-coated and located both in the perigranular cytoplasm and beneath the plasma membrane. In some of these accumulations, the periodicity of specific granule cores was recognised.

Eosinophils contained 6–49 specific granules and the mean number of granules per cell ranged from 18.4 in sample 2–23.8 in sample 4. Granules were extremely polymorphic, varying from round to cylindrical or irregularly rhomboid, and contained an osmiophilic core, with a lamellated structure of alternating light and dark bands, embedded in a less dense outer matrix. Cores were peripherally located in the granules and they varied in number, shape, density and size (Fig. 1).

Coarsening of the granule matrix was detected in all the samples examined, with the exception of sample 2. One to three well-limited, round to oval, electron-dense accumulations were observed in the granule matrix of eosinophils (Fig. 2) from all the specimens examined (Table 2). From 25% of eosinophils in sample 6–55% of eosinophils in sample 5 exhibited 1–3 budding vesicles per cell (Fig. 3). Fragmented cores were seen in 30–100% of the eosinophils (Table 2). Lucent granules were found in 62–90% of eosinophils in the examined samples (Fig. 4) (Table 2). In some lucent granules, remnants of electron-dense material, presumably core, were detected (Fig. 5). No significant difference in eosinophil and eosinophil granule morphology (data not shown) was found between samples 3 and 3a.
Eosinophil morphology was similar to that previously described for the cat eosinophil (Ward et al., 1972; Presentey et al., 1980). In this study, morphological changes of PMD were observed in circulating eosinophils from cats suffering from diseases, allergic dermatitis and eosinophilic granuloma complex among others, in which eosinophils are known to play a significant role. The pathogenesis of these diseases probably involves a type I hypersensitivity reaction towards parasitic, food and/or environmental allergens. In the late phase response, eosinophils are recruited from the circulation into inflammatory foci where they accumulate and serve as major effector cells inducing tissue damage by releasing their granule contents (Day and Shaw, 2000a).

Coarsened and well-limited accumulations of electron-dense material in the matrix, budding vesicles, cytoplasmic accumulations of electron-dense material, fragmented cores and lucent granules were interpreted as a continuum of PMD morphologies, similar to the

Table 2
Ultrastructural features of feline eosinophil granule morphology

<table>
<thead>
<tr>
<th>Patient</th>
<th>% Eosinophils with well-limited accumulations of dense material in the matrix</th>
<th>% Eosinophils with fragmented cores</th>
<th>% Eosinophils with lucent granules</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-(sample 1)</td>
<td>10</td>
<td>30</td>
<td>90</td>
</tr>
<tr>
<td>2-(sample 2)</td>
<td>30</td>
<td>80</td>
<td>80</td>
</tr>
<tr>
<td>3-(sample 3)</td>
<td>31</td>
<td>54</td>
<td>68</td>
</tr>
<tr>
<td>3-(sample 3a)*</td>
<td>33</td>
<td>75</td>
<td>75</td>
</tr>
<tr>
<td>4-(sample 4)</td>
<td>10</td>
<td>90</td>
<td>80</td>
</tr>
<tr>
<td>5-(sample 5)</td>
<td>44</td>
<td>88</td>
<td>66</td>
</tr>
<tr>
<td>Healthy cat-(sample 6)</td>
<td>62</td>
<td>100</td>
<td>62</td>
</tr>
</tbody>
</table>

*Presence of well-limited accumulations of electron-dense material in the matrix, fragmented cores and lucent granules.

Fig. 3. A budding vesicle is attached to the specific granule (arrowhead). The extrusion of protein-loaded vesicles from the specific granules represents a mechanism of granule content release in PMD. Bar = 0.1 μm.

Fig. 4. Activated peripheral blood eosinophil undergoing PMD. The presence of nearly empty specific granules with residual core fragments (arrow) characterises PMD morphology. Note, in the granule-poor cytoplasm, the large number of mitochondria (arrowhead), empty vesicles and elongated tubules (asterisk). Bar = 0.5 μm.

Fig. 5. Lucent granules of the eosinophil showed in Fig. 4 at higher magnification. Note that these granules contain large electron-lucent, virtually empty areas (arrowhead) and have retained core fragments (arrow). This represents the end morphological result of PMD. Bar = 0.1 μm.

4. Discussion

Eosinophil morphology was similar to that previously described for the cat eosinophil (Ward et al., 1972; Presentey et al., 1980). In this study, morphological changes of PMD were observed in circulating eosinophils from cats suffering from diseases, allergic dermatitis and eosinophilic granuloma complex among others, in which eosinophils are known to play a significant role. The pathogenesis of these diseases probably involves a type I hypersensitivity reaction towards parasitic, food and/or environmental allergens. In the late phase response, eosinophils are recruited from the circulation into inflammatory foci where they accumulate and serve as major effector cells inducing tissue damage by releasing their granule contents (Day and Shaw, 2000a).

Coarsened and well-limited accumulations of electron-dense material in the matrix, budding vesicles, cytoplasmic accumulations of electron-dense material, fragmented cores and lucent granules were interpreted as a continuum of PMD morphologies, similar to the
picture defined as PMD in human eosinophils (Dvorak, 1994). It might be hypothesised that the electron-dense material observed in the granule matrix, presumably of core origin, would be progressively extruded by budding of protein-filled vesicles, transported to the cell surface and subsequently released to the extracellular milieu, resulting in eosinophils with lucent specific granules. The cloudy accumulations of electron-dense material observed in the eosinophil cytoplasm might be interpreted as protein-loaded transporting vesicles, in fact, some of them showed the typical lamellated structure of the granule core. The lack of complete visualisation of membrane profiles around these vesicles might be due to a low membrane contrast or to clouds of granule proteins obscuring the membrane, as previously described (Karawajczyk et al., 2000; Dvorak and Weller, 2000).

PMD was considered a morphologic expression of feline eosinophil activation, analogous to what has been described in humans (Dvorak, 1994; Wardlaw et al., 1995). Other ultrastructural criteria of activation, such as increased numbers of vesicles, tubules and glycogen particles and presence of uropods (Dvorak and Weller, 2000) were also recognised. Uropods, which are cytoplasmic projections containing granules, have been previously described in activated tissue eosinophils (Cheng et al., 1997). In addition, the presence of ample Golgi structures and of numerous mitochondria might be also interpreted as a morphological criterion of eosinophil activation and it might suggest that feline circulating eosinophils retain, analogous with human eosinophils, in certain circumstances, biosynthetic capacity (Wardlaw et al., 1995; Karawajczyk et al., 2000).

Evident mitochondria and Golgi apparatus have also been reported to characterise developing feline eosinophils (Presentey et al., 1980). However, it seemed unlikely that eosinophils analysed in this study were immature, because of the absence of other criteria of immaturity, such as large cell diameter, large single nuclei with a nucleolus and distended cisterns of rough endoplasmic reticulum (Presentey et al., 1980; Dvorak, 1994). Moreover, light microscopic examination of blood smears showed eosinophils with morphologic features of mature cells.

At current writing, PMD morphology is unanimously interpreted as an ultrastructural marker of activated eosinophils (Wardlaw et al., 1995; Erjefalt et al., 1999; Karawajczyk et al., 2000; Dvorak and Weller, 2000).

However, detection of high serum levels of granule proteins would have contributed to prove that morphological changes of PMD were herein related to granule content release, which is indicative of eosinophil activation (Koller et al., 1999; Karawajczyk et al., 2000).

Analogous to what has been reported in humans (Peters et al., 1988), the percentage of presumably activated eosinophils with PMD morphology did not directly correlate with the magnitude of eosinophilia. This finding implies that enumeration of eosinophils in peripheral blood might not accurately reflect their involvement in the inflammatory process. In humans, eosinophil tissue-damaging capacities appear to be linked to the number of circulating activated eosinophils and, consequently, to the amount of released cytotoxic proteins, rather than to the magnitude of eosinophilia (Karawajczyk et al., 2000).

The presence of eosinophils with PMD morphology in sample 6, from a clinically healthy cat with circulating eosinophil number below 1500 eosinophils/μL was not surprising. Granule lucency has been described also in circulating eosinophils from healthy human subjects without eosinophilia (Peters et al., 1988). Nevertheless, morphologic studies on eosinophils from larger numbers of clinically healthy cats are needed to better assess the extent and the significance of PMD in blood samples without eosinophilia.

Contrary to humans, where procedures used for cell collection may generate eosinophil activation in vitro (Berends et al., 1994), centrifugation during buffy coat collection did not affect eosinophil and eosinophil granule morphology in this study. No significant difference was observed between specimens 3 and 3a, both of which were obtained from the same cat, respectively by blood centrifugation and sedimentation.

The possibility that artefacts caused by processing techniques might have altered eosinophil granule morphology and caused a PMD phenotype was considered. Nevertheless, the morphologic changes observed in this study were believed not to reflect artefactual changes due to the following reasons. Cells containing specific granules both with a normal appearance and with different degrees of lucency were observed adjacent in the same section and within the same cell. Moreover, damaged or necrotic eosinophils were not observed, whereas mechanical damage during sample handling has been reported to induce eosinophil cytolysis rather than PMD (Kato et al., 1998).

In conclusion, variable numbers of circulating eosinophils from cats with and without peripheral eosinophilia showed ultrastructural phenotype of PMD, which is indicative of cell activation. The number of presumably activated eosinophils with PMD changes was not correlated with the level of eosinophilia.

Acknowledgements

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References


Chapter 5

Characterization of biological activities of feline eosinophil granule proteins

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Submitted to Journal of Cellular Biochemistry
Abstract

Peritoneal eosinophilia was induced in cats using *Toxocara canis* as an antigen by challenge-exposure to intraperitoneally injected antigen in previously orally-infested cats. Biological activities of granule proteins of eosinophils collected from the peritoneal cavity were investigated. Granule proteins were acid-extracted from peritoneal eosinophils and analyzed by gel-filtration chromatography. Three protein peaks were separated in the chromatogram and peroxidase, ribonuclease and bactericidal activities of the yielded protein fractions were analyzed. The first peak possessed both peroxidase and bactericidal activities. The second peak possessed ribonuclease and bactericidal activities and the N-terminal sequence of the major protein was homologue with that of proteins of the ribonuclease A superfamily. The third protein peak was bactericidal and the N-terminal sequence of the major protein was homologue to human and murine major basic protein (MBP) sequences.
Granule protein activities

Eosinophils are regarded as important effector cells of both defense mechanisms against parasite infestation and tissue damage in allergic disease. Eosinophil functions, at least partially, are exerted through the release of specific granule proteins. The human eosinophil specific granule selectively houses four biologically active proteins, major basic protein (MBP), eosinophil cationic protein (ECP), eosinophil-derived neurotoxin (EDN) and eosinophil peroxidase (EPO). The crystalloidal core of the eosinophil granule consists of MBP, whereas ECP, EDN and EPO are localized to the granule matrix. MBP (molecular mass 13.8 kDa) is cytotoxic to mammalian cells, helminths, protozoa and bacteria (Giembycz and Lindsay, 1999). ECP (molecular mass from 16 to 21.4 kDa) has multiple functions as well. It is a potent toxin for helminths, bacteria and mammalian cells, is neurotoxic and possesses ribonuclease (RNase) activity (Giembycz and Lindsay, 1999). EDN (molecular mass 18.5 kDa), a protein with 67% amino acid sequence identity to ECP, is a poor helminthotoxin and cytotoxin but a more potent RNase than ECP and a powerful neurotoxin in experimental animals (Giembycz and Lindsay, 1999). Finally, EPO, a heme-containing protein composed of a light, 12.7 kDa, and a heavy, 53 kDa, subunits, is a potent helminthotoxin and has cytotoxic properties to bacteria and mammalian cells (Giembycz and Lindsay, 1999).

Much of the research on eosinophil proteins has been centered on humans and laboratory rodents. To date, in addition to other contributions directed to characterize these proteins, eosinophil granule proteins have been purified and/or cloned from many species. MBP has been purified and/or cloned from several species, including humans (McGrogan et al., 1988, Wasmoen et al., 1988), rats (Nittoh et al., 1998, Watanabe et al., 1995), mice (Larson et al., 1995, Macias et al., 2000) and guinea pigs (Aoki et al., 1991) and it has been partially characterized in equine (Piller and Portmann, 1993) and bovine eosinophils (Duffus et al., 1980). ECP and EDN have been purified and cloned in humans (Barker et al., 1989, Gleich et al., 1986, Rosenberg et al., 1989a, Rosenberg et al., 1989b) and numerous eosinophil-associated RNases (EAR), with homology with human ECP and EDN, have been identified in rats and mice (Larson et al., 1996, Nittoh et al., 1998, Watanabe et al., 1995, Zhang et al., 2000). In addition, ECP has been characterized in pigs (Fornhem et al., 1996). EPO has been purified and/or cloned in humans (Carlson et al., 1985, Ten et al., 1989), mice (Horton et al., 1996) and pigs (Fornhem et al., 1996). In addition, EPO has been partially characterized in rat (Archer and Hirsch, 1963), guinea pig (Desser et al., 1972), horse (Jörg et al., 1982) and bovine (Duffus et al., 1980) eosinophils.

Cat eosinophils, analogous to human eosinophils, are believed to play an important role both in the immune response to parasites and in the pathogenesis of certain hypersensitivity reactions towards parasitic, food and/or environmental allergens (Day and Hall, 2000, Day and Shaw, 2000). However, the specific functions of the cat eosinophil remains obscure in spite of a considerable amount of publications dealing with common inflammatory eosinophil-associated diseases of the gastrointestinal tract, respiratory tract and the skin of the cat (Day and Hall, 2000, Day and Shaw,
Cat eosinophil functions, analogous to human eosinophil functions, are likely to be partly related to their granule contents. To date, cat eosinophils are known to contain bicompartmental specific granules, with an electron-dense lamellar core embedded in a less dense matrix (Fondati et al., 2003), which have failed to yield a positive reaction for peroxidase activity in cytochemical studies (Jain et al., 1989, Presentey et al., 1980). Despite the rudimentary knowledge of the cat eosinophil granule content, this animal species has been used as experimental model of human eosinophil-driven allergic airway inflammation (Padrid et al., 1996). Analysis of the constituents of the cat eosinophil granule would help both to confirm that the cat is a useful species for studying certain eosinophil-mediated human diseases and to elucidate specific functions of the cat eosinophil. Moreover, clarification of the presence and functions of eosinophil granule proteins in animal species has been proven to be important for the interpretation of the evolutionary events of eosinophils, eosinophil granule proteins and their respective functions.

The aim of this study was to characterize the activity of eosinophil granule-derived proteins in the cat. In the present work, granules were prepared from eosinophils obtained by peritoneal lavage. Granule proteins were extracted, separated by gel-filtration chromatography and examined for some of their properties. Namely, peroxidase, ribonuclease and bactericidal activities of cat eosinophil granule proteins have been investigated and the N-terminal sequence of some of these proteins has been determined and compared with homologue proteins in other species.

Methods

Induction of peritoneal eosinophilia and collection of peritoneal eosinophils

Two domestic cats (2 kg body weight), originated from an experimental cat breeding unit, were infested orally with *Toxocara canis* eggs as previously described (Moriello et al., 1993). Briefly, eggs, collected from adult female worms, were washed in distilled water, resuspended in 0.5% formalin and placed in an Erlenmeyer flask to embryonate. Cats were infested with 1750 *Toxocara canis* embryonated eggs/cat, by means of a feeding tube. On day 28 post-infestation, each cat was challenge exposed via intraperitoneal injection with 200 mg of frozen male worm paste prepared just prior to use. At 48 hours post-challenge, each cat was submitted to a peritoneal lavage with 200 mL of 0.9% NaCl warm sterile solution. The lavage fluid was removed 15 min post-lavage and washed once in 0.9% NaCl warm sterile solution. The number of nucleated cells/mL in the lavage fluid was counted in a Neubauer chamber and cell viability was assessed by trypan blue dye exclusion. A differential cell count was performed on glass slides stained by Hemacolor (Merck, Darmstadt, Germany) stain. The peritoneal cells were suspended at concentrations of 125 and 500 x 10^6 cells/mL in 95% fetal bovine serum and 5% dimethyl sulfoxide and stored under liquid nitrogen until granule protein extraction.
The experiment was approved by the Universitat Autònoma de Barcelona (Barcelona, Spain) and by the local Government Ethics Committee for animal research. All the procedures were performed under general anesthesia.

Isolation of eosinophil granules and extraction of granule proteins

Cells were thawed, washed and resuspended in freshly prepared ice-cold 0.34 M sucrose, disrupted by vigorous and repeated pipetting through a Pasteur pipette and centrifuged at 1000 g and 4°C for 10 min to remove unbroken cells and large cell debris. The sediment was resuspended in fresh 0.34 M sucrose and the procedure was repeated 5 to 6 times. The supernatants were pooled and centrifuged at 15000 g and 4°C for 20 min to sediment the granules. The resulting eosinophil granules pellet was lysed by exposure for 15 min at 4°C to 1 mL ice-cold 10mM HCl, pH 2, and by brief sonication, according to Ackerman et al. (1983). After centrifugation at 20000 g and 4°C for 30 min, the supernatant fraction, containing granule proteins, was immediately separated by gel-chromatography.

Chromatographic procedures

All the chromatographic procedures were carried out at 4°C. The supernatant obtained from granule protein extraction was fractionated on Sephadex G-50 superfine (Amersham Pharmacia Biotech, Uppsala, Sweden) column (1.5 x 90 cm) equilibrated with 25 mM sodium acetate buffer, pH 4.2, 0.15 M NaCl (Ackerman et al., 1983). Proteins were eluted at a flow rate of 12 mL/h and fractions of 1 mL were collected. Column was previously calibrated with molecular mass markers including dextrane blue (2000 kDa), cytochrome c (12.4 kDa) and vitamin B12 (1.5 kDa). Chromatographic protein profile was determined from the absorbance at 280 nm and protein concentration by means of the Bio-Rad Protein Assay Kit (Bio-Rad laboratories GmbH, München, Germany). Peroxidase, RNase and bactericidal activities of the eluted fractions were determined.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE of samples from initial granule extract and from column fractions was performed on 15% polyacrylamide gel according to the procedure described by Laemmli (1970). Human MBP (generously provided by G. Gleich) was used, in addition to a set of marker proteins, as molecular mass marker. Proteins were stained with Coomassie brilliant blue and/or silver nitrate stain.

Peroxidase activity

Peroxidase activity was spectrophotometrically determined at 25°C by following the increase in absorbance at 470 nm resulting from the oxidation of guaiacol (Kimura et al., 1983). The reaction mixture, freshly prepared, contained 13 mM guaiacol (Sigma-Aldrich, St Louis, MO, USA) and 0.33 mM H$_2$O$_2$ in 10 mM Tris/HCl, pH 7.2. The reaction was started by adding 3 to 5 µL samples from eluted fractions to the reaction mixture to reach a final volume of 1 mL and the change in absorbance at 470 nm was measured continuously for 3 to 5 min. Horseradish peroxidase, type VI-A (Sigma-Aldrich, St Louis, MO, USA) was used as positive control. Both 10 mM Tris/HCl, pH
7.2, and equilibrating column buffer (25 mM sodium acetate buffer, pH 4.2, 0.15 M NaCl) were used as negative controls.

**Ribonuclease activity**

The RNase activity was determined by negative-staining zymogram, using the method described by Bravo et al. (1994). Briefly, samples from the eluted fractions were mixed with nonreducing loading buffer (0.18 M Tris-HCl, 30% glycerol, 0.05% bromophenol blue, 9% SDS, pH 6.8) and analyzed for RNase activity by electrophoresis on SDS-15% polyacrylamide gels containing 0.6 mg/gel of either poly(C) (Sigma-Aldrich, St Louis, MO, USA) or poly(U) (Sigma-Aldrich, St Louis, MO, USA) as RNase substrate. Recombinant ECP (rECP) (Boix et al., 1999) and bovine RNase A (Sigma-Aldrich, St Louis, MO, USA) were used as references. After removal of SDS by incubation with a solution of 10 mM Tris-HCl, pH 7.5, and 20% isopropanol, gels were incubated at 25°C for 90 min in 100 mM Tris-HCl, pH 7.5, and stained with 0.2% toluidine blue in 10 mM Tris-HCl, pH 8.0. The relative intensity of the areas showing substrate degradation was analyzed by computer-assisted image analysis using Imaging Densitometer, GS-700, and Multi Analyst-software, Bio-Rad Laboratories.

**Bactericidal activity**

Bactericidal activity against *Escherichia coli* (BL21DE3 strain) (Novagen, Madison, WI, USA) was tested according to the procedure described by Lehrer et al. (1989) and Rosenberg (1995). Briefly, bacteria were grown overnight at 37°C in Luria-Bertani (LB)-broth Miller, washed twice and resuspended at 1:100 in 10 mM sodium phosphate buffer, pH 7.5. 20 to 40 µL samples of bacterial suspension were added to the experimental samples, previously subjected to buffer exchange to 10 mM sodium phosphate, pH 7.5, by means of Centricon YM-3 filters (Millipore Corporation, Bedford, MA, USA), and incubated for 2 h at 37°C. rECP (Boix et al., 1999) was used as positive control and 10 mM sodium phosphate buffer as negative control. After incubation, aliquots from the treated bacterial suspensions were serially 10-fold diluted in 10 mM sodium phosphate buffer, plated on LB agar and incubated overnight at 37°C. Then, the number of remaining bacterial colony-forming units (CFUs)/mL was determined in each case.

**N-terminal amino acid sequence analysis**

Samples for sequencing, prepared from eluted chromatographic fractions, were pooled, lyophilized, resuspended in MQ water and filtered with nitro-cellulose filters type VS 0.025 µm (Millipore Corporation, Bedford, MA, USA) before being separated by SDS-PAGE, subsequently electroblotted on Immobilon-P membranes (Millipore Corporation, Bedford, MA, USA) and stained with Coomassie brilliant blue. N-terminal amino acid sequencing was determined by automatic amino acid analysis (Servei de Proteòmica i Bioinformàtica, Universitat Autònoma de Barcelona, Spain).
Results

Collection of peritoneal eosinophils

0.447 x 10⁹ and 3.4 x 10⁹ nucleated cells were counted in 160 and 180 mL of lavage fluid removed from the peritoneal cavity of the two cats infested with *Toxocara canis*, respectively. Cell viability, assessed by trypan blue dye exclusion, was more than 90% in both cell suspensions. Eosinophils represented more than 85% of total cell population present in the peritoneal fluid from both cats, as judged by routine staining (Figure 1). The remaining cells were neutrophils, monocytes, lymphocytes and, in lower numbers, erythrocytes.

Chromatographic separation procedure of eosinophil granule proteins and estimation of molecular mass by SDS-PAGE

In a typical experiment, the content of solubilised granules from 0.125 to 0.25 x 10⁹ peritoneal cells was fractionated on columns of Sephadex G-50 superfine and, from the absorbance profile at 280 nm, three main peaks were separated (Figure 2A). Figure 2B.1 shows the SDS-PAGE analysis of total granule extract. Eosinophil granule extract showed a broad range of bands although a major band of approximately 10 kDa molecular mass was observed. Figure 2B.2 shows the SDS-PAGE analysis of eosinophil granule proteins eluted in the maximum of each peak from Figure 2A. Peak I, which emerged in the void volume fractions, contained different protein bands with molecular masses higher than 30 kDa. Peak II, which eluted near to the cytochrome *c* marker, contained three bands of approximately 15, 18 and 20 kDa. Peak III, which eluted after the cytochrome *c* marker, contained a band of approximately 10 kDa largely free of other materials. The bulk of absorbance at 280 nm emerged in peak III. Eosinophil granule proteins, derived from three different extraction and chromatographic separation procedures, showed an analogous protein pattern by SDS-PAGE.

Peroxidase activity analysis

Peroxidase activity, determined by the increase in absorbance at 470 nm resulting from the oxidation of guaiacol, was detected only in the initial fractions of peak I (Figure 2A). The peroxidase activity profile was maintained in two independent processes of extraction and chromatographic separation of eosinophil granule proteins. The typical absorbance spectrum of the heme iron group of EPO shows maximum absorbance near to 415 nm (Carlson et al., 1985). However, in fractions of peak I in which peroxidase activity was detected (Figure 2A), the presence of other proteins (Figure 2B.2) might have hindered the detection of the characteristic absorbance spectrum of the heme iron group.

RNase activity analysis

RNase activity of the elution fractions from gel-filtration chromatography (Figure 2A) was tested by means of negative-staining zymogram using poly(C) and poly(U) as substrate. The assay showed RNase activity only in peak II and a single activity band, with the same electrophoretic
mobility of rECP, was detected (Figure 3A). Comparative activity staining with both poly(U) and poly(C) substrates was used for the qualitative differential analysis of RNases. While pancreatic-type RNases show a preference for the poly(C) substrate, bot human eosinophil RNases (ECP and EDN) prefer poly(U) (Sorrentino and Libonati, 1994). The RNase activity of peak II from Figure 2A showed a clear preference for poly(U) as substrate, indicating the presence of an enzymatic activity analogous to human eosinophil RNases (Figure 3B). RNase activity was assayed in four independent experiments that included the extraction and separation procedures of eosinophil granule proteins and results were comparable in all cases.

**Bactericidal activity analysis**

Fractions from the three peaks were selected according to the highest protein content, analyzed for toxicity against *Escherichia coli* (BL21DE3 strain) and the number of CFUs/mL in bacterial suspensions treated with protein samples was determined. Bactericidal activity was detected in the three peaks and it appeared to be less pronounced in Peak I (Figure 4), possibly due to the presence of different proteins in this peak (Figure 2B.2) and/or to the small bactericidal activity of EPO without adding H$_2$O$_2$ and halides (Klebanoff et al., 1989). The bactericidal activity of samples obtained from different extractions and chromatographic separations of eosinophil granule proteins was assayed in three independent assays, two of which performed in duplicate, and results achieved were comparable.

**N-terminal amino acid sequence analysis**

Transfer to an Immobilon-P membrane and N-terminal amino acid sequencing of the major electrophoretic band of peak II (molecular mass of 15 kDa) (Figure 2B) showed the highest homology of this protein with proteins of the RNase A superfamily (Figure 5A): RNase k6 of different primate species (90%), human RNase k6 (80%), gerbil (*Meriones unguiculatus*) eosinophil-associated RNase 34 (70%), human ECP (60%) and human EDN (50%). The major band of peak III (molecular mass of 10 kDa) was also sequenced by the same procedure (Figure 5B). The sequence revealed 44% identity with the N-terminal sequence of both murine and human mature MBP-1. Finally, from electroblotting of peak I, no bands were yielded in a sufficient amount of protein to be sequenced, possibly due to the lack of a major band (Figure 2A).

**Discussion**

This is the first study directed to characterize biological activities of granule proteins of cat eosinophils. Peroxidase, RNase and bactericidal activities, reported in eosinophils of different species, including humans, were determined in cat eosinophil granule proteins previously separated by gel-filtration chromatography.

Cat eosinophils (Figure 1) were obtained by the peritoneal eosinophilia model, previously described in this species by Moriello et al. (1993). This method lead to the obtainment of
Granule protein activities

eosinophil-rich cellular populations. In addition, the methods herein applied to disrupt feline
eosinophils and to extract the granule proteins appeared to be effective in recovering adequate
amounts of protein for initial study. The acid-extraction of granule proteins was selected based on
the method described for human eosinophil proteins (Ackerman et al., 1983) and taking into
account the presumptive lack of EPO in cat eosinophils, as previously reported in cytochemical
studies (Jain et al., 1989, Presentey et al., 1980).

The elution profile of cat eosinophil granule proteins, analogous to human profile (Ackerman et al.,
1983), yielded three major protein peaks (Figure 2A). The major electrophoretic band (Mr ≈ 10
kDa) observed in the initial granule extract (Figure 2B) was correlated with the bulk of protein
eluted in peak III.

The presence of EPO in peak I, was supported by the demonstration of peroxidase activity in this
peak by following, spectrophotometrically, the increase of absorbance resulting from the oxidation
of guaiacol (Figure 2A). It seemed unlikely that peroxidase activity in this peak was associated to
myeloperoxidase deriving from neutrophils and monocytes present in the cell population. In fact,
these cells represented less than 15% of extracted peritoneal cells and eosinophils have been
reported to have higher peroxidase activity and contents than neutrophils and monocytes (Bos et
al., 1982). Therefore, based on our findings, it seems reasonable to state that, unlike what
previously reported, also cat eosinophil granules possess a protein with peroxidase activity.

RNase activity was observed as a single electrophoretic band of enzymatic activity (Figure 3A). The
fact that the enzymatic activity was higher when using poly(U) rather than poly(C) as substrate
in the zymogram (Figure 3B) indicated the presence of an RNase with an enzymatic activity
analogous to human eosinophil RNases. Although this band appeared to have the same
electrophoretic mobility of rECP, it might be difficult to evaluate molecular mass of proteins when
unreduced samples are analyzed, as in the zymogram technique. Anyhow, proteins of the RNase A
superfamily show molecular masses in a range similar to that of ECP. In our experiments, only one
EAR was detected and partially sequenced from the cat eosinophil, however, the presence of other
RNases, as it is the case of humans, primates and rodents (Zhang et al., 2000), cannot be
completely ruled out.

The fact that cat eosinophils have retained RNase activity supports the hypothesis that this
enzymatic activity is probably necessary for some crucial eosinophil physiologic functions in the
host defense that have yet to be discovered. In fact, the enzymatic activity is not apparently
involved in the bactericidal activity of human eosinophil RNases (Rosenberg, 1995) whereas it
might play a role in the antiviral effect against certain single-stranded RNA viruses (Domachowske
et al., 1998).

The cat EAR (Mr ≈ 15 kDa), analogous to one of the rat EARs (Nittoh et al., 1997), showed high
identity in the N-terminal sequence with primate and human RNase k6 and with gerbil eosinophil-
associated RNase 34, one of the numerous eosinophil-associated RNases identified in rodents (Zhang et al., 2000). All the RNase sequences compared with that of cat EAR, a part from those of ECP and EDN, have been deduced from cDNA. The deletion of the initial amino acids of the cat EAR in comparison with the corresponding human sequences (ECP and EDN) has been observed also in other proteins of this family, as human RNase 1 and 4 (Larson et al., 1996). Both the bactericidal activity and the sequence identity of the cat EAR make this protein more similar to human ECP than to human EDN. The presence of both enzymatic and bactericidal activities in the cat EAR would imply that selective evolutionary pressures, unlike what reported in primates and in rodents (Zhang et al., 2000), did not generate functional divergence of eosinophil RNases in this species.

The bulk of absorbance at 280 nm observed in peak III was attributed both to the presumptive abundance of MBP in the eosinophil granule protein, as reported in other species (Gleich et al., 1973), and to the acid-lysis of granule proteins, most efficient in extracting MBP (Abu-Ghazaleh et al., 1992). The identity between the N-terminal sequence of a 10 kDa band from peak III and both murine and human MBP-1 confirmed the presence of MBP in the cat eosinophil granule. In the cat eosinophil granule only one MBP was identified, whereas two MBPs have been found in humans (Plager et al., 1999) and some rodents (Macias et al., 2000), suggesting a gene duplication event also in MBP gene family members (Macias et al., 2000, Plager et al., 1999).

From the elution profile of peroxidase and RNase activities, the molecular mass of proteins from each peak and amino acid-sequencing results, we attributed the bactericidal activity to EPO in peak I, to EAR in peak II and to MBP in peak III.

The present procedures, although efficient to obtain an adequate amount of proteins for initial study, limits the availability of eosinophil granule proteins for further structural and functional studies, however, the N-terminal sequencing opens a new approach directed to the use of corresponding recombinant proteins.

The similarity between the protein content of the cat and human eosinophil granule can partly explain the analogy of lesions observed at light and electron microscopy examination in certain cat and human eosinophil-associated diseases, including eosinophilic dermatoses. Namely, histological lesions resembling flame figures, which represent a characteristic microscopic finding of human Wells’ syndrome and which are constituted by ultrastructurally normal collagen fibers surrounded by cytolytic eosinophils and free eosinophil granules, have also been described in selected eosinophilic skin diseases of the cat, demonstrating that mechanisms of eosinophil-induced tissue-damage are similar in both species (Bardagí et al., 2003, Fondati et al., 2001).

Due to similar activities of cat and human eosinophil granule proteins, cats are confirmed to represent a useful experimental model of human allergic pulmonary diseases. In addition, the feline
respiratory tract is known to contain large numbers of eosinophils in healthy individuals, as demonstrated by the recovery of approximately 25% of eosinophils from the bronchoalveolar lavage of specific-pathogen-free cats (Padrid et al., 1991). This would make the cat a suitable species to study those eosinophil biological behaviors that remain to be determined and to find new therapeutic strategies for eosinophil-mediated diseases.

In addition, the analogy between contents and functions of human and cat eosinophils will be clearly advantageous in veterinary medicine, based on the possibility that currently ongoing pharmacological research in humans will bring new and effective treatments for both human and cat eosinophil-mediated diseases.

The similarity between biological activities of eosinophil granule proteins in the cat and in other species yield to speculate that there might be an evolutionary pressure to retain certain peculiar eosinophil functions. The primary eosinophil evolutionary mandate appears to be related to the protective function, although recently debated, that eosinophils exert in the innate host defense against most helminth infestations characterized by migrating larvae (Meeusen and Balic, 2000). As human helminth infestations in Western countries have decreased in parallel with increased allergy, eosinophils are currently considered to have switched towards a destructive role (Dombrowicz and Capron, 2001). Nevertheless, helminth infestations represent a common problem in domestic mammals world-wide and they can still be considered potentially harmful for animal health. Therefore, while highly speculative, eosinophils can be currently considered to play a more defensive role in animal species than in humans, at least in Western countries.

Acknowledgments
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References


Figure 1. Feline peritoneal eosinophils stained by Hemacolor stain (1000x magnification). Eosinophils were obtained from the peritoneal cavity of cats infested and subsequently challenged with *Toxocara canis*. 
Figure 2. Separation of eosinophil granule proteins. A. Gel-filtration chromatography on Sephadex G-50 superfine column (1.5 x 90 cm). Eosinophil granule proteins were extracted and chromatographed as described in Materials and Methods. Protein elution was detected by the absorbance at 280 nm (---) and protein fractions were pooled yielding three major peaks (I-III). Peroxidase activity (---) was measured by the increase of absorbance at 470 nm resulting from the oxidation of guaiacol. 5 µL samples of each fraction were assayed. B.1. Lane A: SDS-PAGE of 20 µL sample of granule extract (Coomassie brilliant blue stain). Molecular mass markers: from 6.5 to 66 kDa. B.2. SDS-PAGE analysis of 18 µL samples from peak I to III showed in Figure 2A (silver nitrate stain). Molecular mass markers: from 6.5 to 66 kDa. Human MBP (1.75 µg) (provided by G. Gleich) (molecular mass 13.8 kDa) was also used as molecular mass marker.
Chapter 5

Figure 3. RNase activity assay. A. Analysis of fractions of peak II (Figure 2A) by means of SDS-PAGE containing either poly(C) (top) or poly(U) (bottom) as substrate. 10 µL samples of fractions 35-37, in addition to rECP (100 ng), are shown as the activity pattern. B. The RNase activity profile of peak II was obtained using 10 µL samples and determined by the relative optical density of the areas showing substrate degradation. Because the detection signal showed slightly differences between experiments, results were expressed as the ratio between the relative optical density of the analysed sample and the value obtained for a reference sample containing 285 ng of rECP.
Figure 4. Analysis of the bactericidal activity of peak I-III obtained from Figure 2A. This activity is expressed as remaining CFUs/mL in bacterial suspensions of *E. coli* (BL21DE3 strain) treated with 2.5 and 4.8 µg of proteins from Peak I, 8.7 and 15.6 µg of proteins from Peak II, and 5.4 and 6.7 µg of proteins from Peak III. rECP (4 µg) and 10mM sodium phosphate buffer were respectively used as positive and negative control.
Chapter 5

A

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Figure 5. N-terminal amino acid sequences. A. Partial amino acid sequence of the cat eosinophil-associated RNase (EAR). The sequence showed the highest homology with primate RNase k6, human RNase k6, gerbil eosinophil-associated RNase 34, human ECP and human EDN sequences. Residues identical in all the compared proteins are indicated by bold letters. B. Partial amino acid sequence of the major band of peak III that corresponds to the cat eosinophil MBP. The sequence showed the highest homology with human mature MBP and murine MBP-1 sequences. X represents an unidentified residue. Identical residues are indicated by bold letters.
Discussion

As pointed out previously, our understanding of feline EGC and our knowledge of the cat eosinophil biology are poor in many aspects. Nevertheless, we hope that our findings will partly contribute to increase our understanding of both feline EGC and eosinophils and to open new perspectives for further study. The first part of the discussion will concern (1) feline EGC, including those clinical and pathological aspects that have been clarified based on the results of our studies. In addition, hypotheses on EGC pathogenesis, developed in the course of this thesis, will be formulated. With regard to the second part (2), our findings on functional morphology and other biological aspects of feline eosinophils will be presented and discussed.

Feline EGC

The use of the term EGC, although inaccurate because predominantly eosinophilic infiltrates and/or granulomatous reactions do not characterise the totality of lesions (chapter 2), is advocated to designate those feline cutaneous lesions characterised by the distinct clinical and histopathological appearance described in chapter 2, until a more convenient definition is found.

The delineation of EGC has become blurred when similar histopathological features have been recognised in other conditions and the term feline eosinophilic dermatoses has been introduced to designate those skin diseases characterised histologically by eosinophilic inflammation, including EGC, allergic miliary dermatitis and mosquito-bite-induced dermatitis (Foil, 1995, Power and Ihrke, 1995). However, in our opinion, the term EGC should not be used as synonymous of feline eosinophilic dermatoses, overemphasising in this way its histopathological findings and disregarding its clinical aspect.

This thesis has contributed to substantiate the similarity between EGC and human Wells’ syndrome, hypothesised by Rosenkrantz (1991) years ago. Therefore, we believe it is worthwhile, before discussing our findings on EGC, to summarise the principal features of this disease.

Wells’ syndrome is a polymorphic disease, most commonly presenting with recurrent painful and/or pruritic, well demarcated, erythematous and indurate plaques frequently localised on the legs and trunk (Fisher et al., 1985).
The particular histopathological changes of Wells’ syndrome evolve over time through three stages (Aberer et al., 1988). The acute stage reveals dermal oedema and marked eosinophilic infiltration and the subacute stage is characterised by the presence of flame figures. As mentioned previously, flame figures have been depicted as ultrastructurally normal collagen bands surrounded by degranulating eosinophils (Aberer et al., 1988, Davis et al., 1998, Stern et al., 1984). Eosinophil degranulation is considered the primary event in the formation of flame figures, supported, at TEM examination, by finding Cfegs coating normal collagen fibres and fibrils (Aberer et al., 1988, Davis et al., 1998, Stern et al., 1984) and, at immunofluorescence, by positive staining of eosinophil granule proteins within flame figures (Peters et al., 1983). In the resolving stage of Wells’ syndrome, eosinophils disappear and histiocytes and multinucleated cells predominate around flame figures (Aberer et al., 1988).

Wells’ syndrome is generally considered a distinctive clinical-pathological reaction pattern associated with a variety of stimuli (Brehmer-Andersson et al., 1986, Wells and Smith, 1979, Wood et al., 1986). The most commonly documented triggering events of Wells’ syndrome are arthropod bites (Delaporte, 2001, Schorr et al., 1984). A peculiar delayed hypersensitivity reaction might initiate leading to recurrent or persistent cutaneous lesions with localised and sometimes peripheral eosinophilia (Wood et al., 1986). However, other precipitating factors of Well’s syndrome have been reported and, as in many cases they remain unidentified (Aberer et al., 1988, Anderson et al., 1995), some authors consider Wells’ syndrome a distinct idiopathic disease (Aberer et al., 1988, Anderson et al., 1995, Wood et al., 1986). In addition, a genetic predisposition for the development of Wells’ syndrome has been hypothesised (Delaporte, 2001) and supported by the occurrence of the disease in related family members (Davis et al., 1998).

A criterion for clinical definition of EGC lesions has developed in the course of this thesis. In retrospect, and based on published information, the striking clinical phenotype of all the clinical forms of EGC is analogous to that of Wells’ syndrome and consists of firm, well demarcated and variably ulcerated papules, plaques and nodules. The terms EP, EG and IU, proposed by Scott many years ago (1975), have appeared inadequate for clinical classification of EGC lesions when overlapping features of two forms are observed, as is the case of linearly orientated ulcerated EG resembling EP (MacEwen and Hess, 1987, personal observations). This suggests that clinical forms of EGC are difficult to distinguish both clinically and histologically (chapter 2), supporting in this way their grouping, originally arbitrary, as a disease complex (Scott, 1975). In our opinion, a more logical
approach in classifying EGC lesions would be a morphological description using a correct clinical terminology (i.e. papules, plaques and nodules).

Histological lesions of EGC show great similarities with those of Wells’ syndrome. On H & E stained sections EGC lesions are characterised, independently from their clinical appearance, by the presence of small- to large-sized dermal foci of amorphous to granular eosinophilic debris (chapter 2). Small foci have been called flame figures, by microscopical analogy with flame figures in Wells’ syndrome, and, as large foci, have been traditionally considered to comprise a mixture of degenerated collagen, degranulating eosinophils and free eosinophil granules (Gross et al., 1992, Yager and Wilcock, 1994), as pointed out previously.

In this thesis, both small and large foci of eosinophilic debris, from different clinical forms of EGC, have shown to possess the same ultrastructure of flame figures in Wells’ syndrome. They appeared to comprise collagen fibrils with no electronmicroscopic signs of degeneration and collagen fibres, partly disrupted by the intense oedema, surrounded by Cfegs and cell debris originated from ECL (chapter 3). Eosinophils, in fact, were extensively degranulated, mainly via ECL and partly via PMD, indicating their activation state.

With regard to eosinophil interaction with extracellular matrix, while integrin-mediated adherence to glycoproteins, as fibronectin, has been demonstrated to be important for eosinophil survival and retention in tissue (Holub et al., 2003), interaction of eosinophils with collagen remains unclear.

There is evidence that eosinophils, in part through release of TGF-β, promote fibroblasts proliferation and fibrosis (Levi-Schaffer et al., 1999, Nomura et al., 2002). Nevertheless, results concerning eosinophil capacities to release matrix metalloproteinases (MMP) are controversial. Eosinophils have been reported to degrade interstitial collagen type I and III (Hibbs et al., 1982), indicating that they release MMP-1, and to contain MMP-9 (Levi-Schaffer et al., 1999), which degrades various extracellular matrix components, including basement membrane collagen type IV.

However, at current writing, the presence of MMP-1 in human eosinophils is uncertain (Shlipov and Hasty, 1998) and eosinophils are not considered primarily involved in degradation of collagen type I (Nomura et al., 2002), which is the most abundant collagen
Regarding MMP-9, although human eosinophils express the mRNA of this collagenase (Ohno et al., 1997), its intracellular immunolocalisation has given negative results (Becky Kelly et al., 2000, Han et al., 2003, Ohno et al., 1997). Considering that inhibition of MMP-9 might represent a therapeutic strategy in eosinophil-mediated diseases, as suggested in human asthma (Walsh, 2001), due to the fact that this MMP favours eosinophil migration across basement membranes (Han et al., 2003), it might be interesting to investigate its presence in dermal eosinophils in EGC.

As regards the intense oedema observed at TEM examination of flame figures (chapter 3), interestingly, it has been hypothesised that plasma-derived molecules, from oedema, are important for eosinophil accumulation, adherence to collagen and degranulation via ECL (Erjefält et al., 1997, Erjefält and Persson, 2000).

Clinically, the peculiar increased consistency of EGC lesions, analogous to that observed in Wells’ syndrome, might be partly due to persistent tissue oedema associated with increased vasopermeability directly caused by eosinophil granule proteins (Minnicozzi et al., 1994), which have long half-lives in tissue.

Taking into account that the ultrastructure of both small and large foci of eosinophilic debris observed on H & E stained sections of EGC lesions is analogous to that of flame figures in Wells’ syndrome, the use of this term is advocated, and will be adopted herein, to designate these foci independently from their size. In addition, due to the lack of ultrastructural signs of collagen fibril damage, the term “collagen degeneration” results inappropriate to describe the eosinophilic debris of EGC lesions.

Based on our findings, namely, the similar ultrastructure of flame figures (chapter 3) and the analogous eosinophil granule contents (chapter 5) in humans and cats, it seems reasonable to hypothesise that also the pathogenetic events of tissue damage in EGC are analogous to that proposed for Wells’ syndrome, i.e. the primary effector role is played by eosinophils, being collagen an “innocent bystander” rather than the primary target structure for damage. When recruited to the skin, eosinophils activate and originate flame figures by closely aggregating onto dermal collagen fibres and degranulating.
Giving that this probably represents the cutaneous lesional progression over time in EGC, the time at which the biopsy is taken in the course of the disease is likely to influence the histopathological findings, analogous to what is described in Wells’ syndrome (Aberer et al., 1988). The presence and the size of flame figures, as well as the granulomatous reaction around them (chapter 2), might be variably influenced by the elapsing time between disease onset and biopsy timing.

In fact, although flame figures are considered an occasional histopathological feature of EP (Scott et al., 2001), they were observed in all the EP specimens examined in our study (chapter 2). As flame figures form following eosinophil recruitment, the time elapsed between disease onset and histopathological examination might determine their recovery in skin sections. In addition, being the size of flame figures related to the number of degranulated eosinophils included in the “flame”, a size increase might be observed with the progression of the disease.

Nevertheless, the size of flame figures might be related also to primary recruitment of different numbers of eosinophils in the skin. In our study (chapter 2), granulomatous reactions have been observed around small-sized flame figures, indicating that they do not always progress to a larger size, in fact, granuloma formation is a histopathological finding suggestive of regression of EGC lesions, analogous to what observed in the resolving stage of Wells’ syndrome (Aberer et al., 1988).

Granulomatus reaction around flame figures is stimulated by variably damaged Cfegs rather than by altered collagen, unlike what previously hypothesised (Bucci, 1966). In fact, occasional macrophages with intracytoplasmic phagolysosomes containing eosinophil granule content-like material were observed in our study (chapter 3) and have been reported also in human eosinophil-associated inflammatory processes characterised by ECL (Dvorak et al., 1990, Erjefält et al., 1998, Mei et al., 2002).

Macrophages are thought to clear Cfegs, thereby neutralising cytotoxic potential of granule proteins (Mei et al., 2002). When great amounts of poor soluble granule proteins accumulate, as in flame figures, they might incite granuloma formation (Cotran et al., 1999), taking into account also that eosinophils, by cytokines release, contribute to elicit and maintain the granulomatous reaction (Rumbley et al., 1999). It is noteworthy that the peculiar yellowish gritty foci clinically described both in EG and IU (Foil, 1995, Scott 2001)
can probably be ascribed to this phenomenon, rather than to "collagen degeneration", despite what commonly assumed (Foster, 2003, Power and Ihrke, 1995).

In addition, transepidermal-transfollicular elimination of flame figures, occasionally observed in EGC lesions (chapter 2), is probably elicited by the attempt to eliminate insoluble eosinophil granule proteins acting as a foreign body, analogous to what is reported in human perforating dermatoses (Heileman and Friedman, 1997).

Based on the overall conjectures, although similar histopathological findings were observed in our study in all the examined sections (chapter 2), it cannot be ruled out that they might have changed with the stage of the lesions at the time biopsies were performed. However, this hypothesis remains highly speculative until a sequential histopathological study of untreated EGC lesions will be performed. Demonstrating a continuum of histological lesions in EGC, analogous to that of Wells’ syndrome, would confirm that the reported histological variability in the same clinical form and probably also in different clinical forms, would depend, at least in part, on the time of histopathological examination.

Interestingly, a progression of histological lesions similar to that reported in Wells’ syndrome has been described in IU of the upper lip (Power and Ihrke, 1995). Lesions evolved, in a few months, from a dermal eosinophilic infiltrate to flame figure formation to fibrosis and neutrophilic ulceration.

These findings might explain why IU is commonly described as neutrophilic fibrosing dermatitis; being clinicians reluctant to biopsy the cat’s lip, the majority of lesions might be present for months at the time they are histologically examined. It is likely that the three cases of IU included in our study (chapter 2) were characterised by flame figures because they were seen by a veterinary dermatologist, who tends to perform biopsies early in the course of the disease.

Nevertheless, it has been recently reported that also recent labial IU, adjacent to the upper canine tooth, might show histopathological features of ulcerative neutrophilic dermatitis (Colombini et al., 2001). To consistently demonstrate that single cases of ulcerative fibrosing dermatitis, although clinically suggestive of IU, are part of EGC rather than a different process, i.e. traumatic labial ulcer due to excessive licking,
immunolocalisation of eosinophil granule proteins in the affected tissue is needed, analogous to what described in Wells’ syndrome (Peters et al., 1983). Unfortunately, various antibodies reactive with human eosinophil granule proteins do not stain feline eosinophil granules (Roosje et al., 2002a, personal observations).

Despite the fact that pathological changes observed in EGC lesions are primarily related to the presence of eosinophils (chapter 2, 3), the stimulus for eosinophil recruitment and activation is still unclear, analogous to what reported in Wells’ syndrome. Hypersensitivity disorders represent a plausible inciting stimulus for eosinophil recruitment in EGC, in accordance with eosinophil functions, which are likely to be analogous in humans and cats, based on the similarities of their granule protein contents (chapter 5). Moreover, EGC lesions may be observed in combination with other clinical signs suggestive of allergic disorders, i.e. miliary dermatitis and self-inflicted alopecia (Colombini et al., 2001, Gross et al., 1986, Moriello et al., 1990), and dermal eosinophil infiltration has been recently reported as indicative of feline allergic dermatitis (Roosje et al., 2002a).

As mentioned previously, flea, environmental and food allergens have been all implicated in the pathogenesis of EGC (Scott et al., 2001). However, it appears that the allergic origin and the causative allergens of EGC are rarely identified in a convincing manner. This is partly related to our poor understanding of allergic mechanisms in cats and to the difficulty to have recourse to reliable diagnostic tools.

The association between EGC lesions and flea-bite hypersensitivity has not been specifically studied. Positive allergy testing to flea antigens, which has a doubtful validity in cats (Colombini et al. 2001, Moriello and McMurdy, 1989, Scott et al., 2001), has been reported in cats with EGC lesions (Colombini et al. 2001, Foster and O'Dair, 1993, Foster et al., 1997, Gross et al. 1986, Moriello et al., 1990, Schleifer and Willemsen, 2003). Nevertheless, no further information has been given on the clinical follow-up of these cases, except in one study in which the course of IU seemed to parallel flea exposure (Colombini et al. 2001). Nevertheless, flea control is recommended as the first step in the diagnostic-therapeutic work-up of EGC by many authors (Foster, 2003, Power and Ihrke, 1995, Scott et al., 2001), suggesting that a positive response has been empirically observed.

The causal relationship between atopic dermatitis and EGC has been commonly based on positive allergy testing (O’Dair et al., 1996, Prost, 1998, Saridomichelakis and Koutinas,
or good response to hyposensitisation following *in vivo* or *in vitro* allergy testing (Halliwell, 1997, McDougal, 1986, Reedy, 1982), when it is known that the validity of these tests in cats is unclear (Foster and O’Dair, 1993, Gilbert et al, 1999, Roosje et al., 2002b, Scott et al., 2001, Wassom and Grieve, 1998), no controlled studies to investigate the benefit of hyposensitisation have been reported in cats, and EGC cases may regress spontaneously (Scott et al., 2001). Finally, the diagnosis of adverse food reaction has been rarely confirmed by exposure to the presumed offending diet (Carlotti et al., 1990, Guaguère, 1996).

If environmental or food allergens were implicated in the aethiopathogenesis of feline EGC, the cat would represent a unique species, giving that, in human atopic dermatitis, eosinophils do not represent a prominent feature of the inflammatory infiltrate (Leiferman, 1999). Based on the analogy between human and feline atopic dermatitis (Roosje et al., 1997, Roosje et al., 1998, Roosje et al., 2002c, Roosje et al., 2002d) the unicity of the cat eosinophil response appears unlikely.

An immediate hypersensitivity to another allergen, the cat allergen Feld I, has been recently hypothesised to be involved in the pathogenesis of EGC (Wisselink et al., 2002). However, it seems unlikely that this allergen might play a role in EGC, in fact, as mentioned previously, no eosinophils were observed in the inflammatory infiltrate 48 hours after its application on abraded skin (Wisselink et al., 2002).

Based on the clinical-pathological features of EGC (chapter 2 and 3), the similarities between feline EGC and Wells’ syndrome (chapter 2 and 3), and the reported causes of cutaneous histological lesions similar to EGC in other animal species (Gross et al., 1992, Scott, 1988, Scott et al., 2001, Scott and Miller, 2003, Stannard, 2000, Yager and Wilcock, 1994), it is attractive to speculate that arthropod allergens, including those of flea-origin, are the allergens involved in the aetiopathogenesis of feline EGC.

Arthropod allergens might induce an immediate hypersensitivity reaction in sensitised cats with early mast cells participation and late recruitment of eosinophils (late phase response). However, EGC lesions show weak analogy with what reported as a typical histopathological phenotype of a late phase response (Olivry et al., 2001). In addition, EGC histological lesions do not even show similarities with those observed in cutaneous delayed hypersensitivity reactions to various intradermally injected antigens in cats (De Boer et al., 1991, Lewis et al., 1999, Nagata and Ishida, 1997).
Based on these latter observations we might conjecture that a delayed hypersensitivity response, mediated by T helper 2 (Th2) cells that selectively stimulate a second and massive influx of eosinophils, might play a role in promoting the intense eosinophil infiltration that characterises EGC lesions, analogous to what suggested for cutaneous lesions in sheep sensitised to parasitic mite allergens (van den Broek et al., 2003).

Nevertheless, all the above hypotheses are highly speculative and further studies are awaited to investigate the role played by arthropod antigens in the pathogenesis of EGC.

However, some clinical aspects of EGC, including the sporadic occurrence of lesions during cat’s life (Scott et al., 2001), the lack of pruritus observed in certain cases (Scott et al., 2001) and the clustering of cases in unrelated in-contact cats (Hess and MacEwen, 1977), are not suggestive of hypersensitivity disorders. An intriguing hypothesis might be that, in individual cases, the presumptive arthropod antigens might incite a hypersensitivity-independent direct eosinophil recruitment, as conjectured for certain arthropod and helminth antigens in sheep (Meeusen, 1999, van den Broek and Huntley, 2003).

A combined genetic and allergic aetiopathogenesis has been recently suggested for EGC (Colombini et al., 2001) and it seems plausible. A genetic predisposition to develop intense eosinophil responses might help to explain why only a few cats develop EGC lesions whereas the hypothesised underlying allergic stimuli are so largely distributed and more commonly associated with different clinical-pathological presentations (Scott et al., 2001). However, the association between EGC and other eosinophil-mediated diseases, as eosinophilic ocular diseases, has not been reported (Morgan et al., 1996, Paulsen et al., 1986, Pentlarge, 1991) and cats with EGC are not apparently predisposed to develop non-cutaneous eosinophil-associated diseases in the course of their life.

In summary, findings and observations on feline EGC may be resumed as follows:

1. The three clinical entities of feline EGC (EP, EG and IU) share common clinical and histopathological features. This makes useless their distinction and supports the concept of EGC as a disease complex.
2. Variably ulcerated papules, plaques and nodules constitute the clinical features common to all the forms of EGC. This morphologically descriptive terminology should substitute the terms EP, EG and IU.

3. The presence of small- to large-sized foci of eosinophilic debris characterises EGC lesions at histopathological examination of H & E stained sections. These foci comprise ultrastructurally normal collagen surrounded by degranulating eosinophils (via ECL and PMD) and Cfgs.

4. Due to the similar ultrastructure of these foci with that reported in flame figures of Wells’ syndrome, the term flame figures should be adopted to define these foci independently from their size, and, since ultrastructural abnormalities of collagen fibrils are lacking, the term “collagen degeneration” should be abandoned for describing them at light microscopic examination.

5. The clinical and pathological analogies between feline EGC and human Wells’ syndrome and the similar granule proteins content of human and feline eosinophils support a similar mechanism of tissue injury in EGC and Wells’ syndrome, in which eosinophils play a primary role.

Feline eosinophils

As pointed out previously, while electronmicroscopic studies of mature feline circulating eosinophils had been performed in the past, ultrastructural features indicative of eosinophil functional changes, namely, eosinophil activation and degranulation, had not been previously reported. In our studies, degranulation morphology of feline eosinophils was observed in blood, from cats with various eosinophil-associated diseases (chapter 3), and in tissue, from cats with EGC lesions (chapter 4).

A continuum of PMD morphologies, analogous to that described in cultured human eosinophils (Dvorak et al., 1991, Dvorak et al., 1992), was recognised in feline circulating eosinophils (chapter 4). Despite the fact that eosinophil functional parameters were not studied, from the viewpoint of morphology, piecemeal-degranulated eosinophils were considered activated, being degranulation the main consequence of activation.
Discussion

Other morphological criteria of activation observed in our study and considered suggestive of either secretory and/or synthetic capacities, included well-developed Golgi, numerous mitochondria, high numbers of VTO and abundant glycogen particles, the latter detected by potassium-ferrocyanide reduced osmium post-fixation (chapter 4). A part from these morphological changes, the ultrastructure of cat circulating eosinophils was similar to that previously reported (Presentey et al., 1980, Ward et al., 1972).

An intriguing finding was the lack of positive correlation between the number of eosinophils with PMD morphology and the degree of blood eosinophilia (chapter 4). Despite the fact that a limited number of cats were included in our study, some speculations might be tempted.

Assuming that eosinophils with PMD morphology are activated, and based on the lack of positive correlation between the total number of circulating eosinophils and the number of activated eosinophils in individual cases, it was speculated that the total blood eosinophil count might not represent a good marker of the intensity of ongoing eosinophilic inflammation, analogous to what reported in humans. In fact, in humans, the severity of eosinophil-associated diseases is thought to correlate more closely with the number of circulating hypodense (activated) eosinophils than with the total blood eosinophil count (Krouwels et al., 1995), having activated eosinophils a greater potential for tissue injury.

It might be argued that in our study (chapter 4) we could not state that feline eosinophils with PMD morphology were hypodense, because density-gradient separation of eosinophils was not performed. However, it has been recently demonstrated that granule content release by PMD is likely to represent the morphological basis for hypodensity (Karawajczyk et al., 2000), hence, cat eosinophils displaying changes of PMD were presumed to correspond to hypodense eosinophils.

Measuring the level of eosinophil granule proteins by immunological techniques in biological fluids, including serum, might be helpful in cats with eosinophil-associated diseases, analogous to what reported in humans (Koller et al., 1999, Miyasato et al., 1996). In fact, although not unanimously recognised (Lönnkvist et al., 2001, Meijer et al., 2002, Matsumoto et al., 2001), the degree of peripheral eosinophil activation, and, in turn, the severity of the disease, are directly mirrored in the amounts of granule proteins released, further suggesting that circulating activated eosinophils degranulate.
Unfortunately, as pointed out previously, to the authors' knowledge, antibodies able to detect feline eosinophil granule proteins are not currently available.

The presence of eosinophils with PMD morphology in blood suggests that increased plasma levels of eosinophil-activating agents are able to induce activation and degranulation of feline circulating eosinophils, analogous to what suggested in humans (Krouwels et al., 1995, Miyasato et al., 1996). However, it has been hypothesised that peripheral eosinophils from patients with eosinophil-associated diseases would be activated in vivo, showing therefore increased susceptibility to degranulate, but that granule contents release would take place only in vitro (Hoekstra et al., 1994, Miyasato et al., 1996), depending from different techniques in sample processing (Berends et al., 1994).

In our study, specimen handling-techniques did not appear to influence the number of eosinophils showing PMD morphology in an individual cat. Nevertheless, this does not permit to rule out that PMD of circulating activated eosinophils might have been selectively induced in vitro.

As regards the degranulation morphology of feline tissue eosinophils in EGC (chapter 3), it is noteworthy that ECL and PMD represented the two main degranulation pathways of feline activated tissue eosinophils, analogous to what reported in many human eosinophil-mediated diseases (Erjefält and Persson, 2000).

ECL was the predominant mechanism of granule content release in the examined samples. This finding was considered in accordance with the presumed biological role of this degranulation pathway, which has been associated to eosinophil effector rather than immunoregulatory functions (Erjefält and Persson, 2000).

Nevertheless, variable proportions of eosinophils undergoing PMD were observed in the study cats (chapter 3). Despite the fact that many Cfegs displayed PMD morphology, suggesting that PMD might have preceded ECL, our findings apparently supported the independence of ECL from PMD, in fact, no positive correlation was found between the number of eosinophils showing high degrees of PMD (high DIs) and the number of eosinophils undergoing ECL in individual specimens (chapter 3), analogous to what reported in humans (Erjefält et al., 1998). When specific markers of PMD, recently identified in humans (Lacy et al., 2001, Logan et al., 2002, Mahamudi-Azer et al., 2002),
will be immunolocalised in tissue, the relationship between PMD and ECL, if there is any, will be probably elucidated.

Although it is not known whether ECL represents a regulated process, as a programmed cell lysis, or a passive cell death (Erjefält and Persson, 2000, Logan et al., 2003, Walsh, 2001), the release of Cfegs, secondary to ECL, is considered a pro-inflammatory event with the idea that granules contain toxic and biologically active proteins, cytokines and chemokines. Whereas preliminary \textit{in vitro} studies indicate that corticosteroids inhibit eosinophil PMD (Mahmudi-Azer et al., 2002), there is no evidence that drugs currently used for treatment of eosinophil-mediated human respiratory diseases exert any specific actions against ECL (Erjefält and Persson, 2000). Therefore, biologic control of ECL, generation of Cfegs and mechanisms of content release from Cfegs deserve further investigation, being a promising area for drug development.

Taken together, our findings (chapter 2, 3, 4) strongly support a strict analogy between human and feline eosinophil biology. However, the demonstration of the similarity between feline and human eosinophils was obtained by showing that they both contain granule-derived proteins with analogous biological properties, namely peroxidase, RNase and bactericidal activities (chapter 5). In addition, N-terminal sequence homology was found between feline eosinophil-associated RNase (EAR) and proteins of the RNase A superfamily, including human eosinophil RNases and EARs from other animal species, and between feline MBP and human and murine MBP-1 (chapter 5).

It is worth of note that, unlike what previously assumed (Jain et al., 1989, Presentey et al., 1980), cat eosinophils, analogous to human (Carlson et al., 1985), bovine (Duffus et al., 1980), porcine (Fornhem et al., 1996), equine (Jörg et al., 1982) and rodents (Archer and Hirsch, 1963, Desser et al., 1972, Horton et al., 1996) eosinophils, appeared to possess peroxidase activity.

The analysis of cat eosinophil granule proteins was considered relevant, from an evolutionary viewpoint, to identify their presence and functions in an additional animal order (Carnivora). Moreover, our results helped to open new perspectives on the roles played by cat eosinophils in feline eosinophil-mediated diseases, to confirm that the cat represents a useful species for studying certain human eosinophil-associated diseases, and to create a basis for further studies on the cat eosinophil biology.

In our study (chapter 5), only one EAR with both RNase and bactericidal activities was detected and partially sequenced from the cat eosinophil granule. With regard to eosinophil RNases, a functional divergence between a more cationic protein with toxic properties (ECP) and a more effective RNase (EDN), attributed to positive selection after gene duplication (Zhang et al., 1998), has been reported to date only in humans (Zhang et al., 2000). In some species from the order Rodentia, numerous homologues of ECP and EDN have been identified, although functionally similar (Nakajima et al., 2001, Singhania et al., 1999).

Moreover, in the cat eosinophil granule only one MBP, homologue to human and murine MBP-1, was identified, whereas two MBPs have been found in humans (Plager et al., 1999) and some species of the order Rodentia (Macias et al., 2000), suggesting a gene duplication event also in MBP gene family members (Macias et al., 2000, Plager et al., 1999).

It is likely that evolutionary constraints leading to functional divergence advantageous for the host are not restricted to humans and Rodentia and probably exist also in the other animal orders in which EARs and MBP have been identified, including Artiodactyla (Duffus et al., 1980, Fornhem et al., 1996), Perissodactyla (Piller and Portmann, 1993) and Carnivora. The discrepancy in the knowledge of eosinophil proteins among different orders is probably due to the fact that most of the research in this field centred on humans and laboratory rodents. In our study (chapter 5), the presence of other EARs and of MBP variants in the cat eosinophil cannot be completely ruled out and further studies are needed in this field.
As regards eosinophil granule protein functions, the retention of RNase activity in the cat eosinophil suggests that this enzymatic activity is associated with important (i.e. conserved) physiologic functions. Taking into account that bactericidal activity of ECP is RNase-independent (Rosenberg, 1995), with the purpose to investigate a beneficial physiologic role for RNase activity and based on the observation that certain human RNA respiratory viruses, associated to human asthma exacerbations, up-regulate eosinophil chemoattractants (Teran et al., 1999, Papadopoulus et al., 2001), antiviral properties of ECP and EDN have been studied.

Preliminary results indicate that eosinophil RNases, especially the potent RNase EDN, might be involved in antiviral defence (Domachowske et al., 1998a, Domachowske et al., 1998b, Rosenberg and Domachowske, 2001). It has been therefore hypothesised that the evolutionary constraints acting on the EDN/ECP genes have promoted the acquisition of a specialised RNase-dependent antiviral activity in EDN (Rosenberg and Domachowske, 2001). These findings might have important therapeutic implications; in fact, EDN-related protein structures could be studied as novel antiviral agents.

Interestingly, feline herpesvirus 1 has been described in association with eosinophil infiltration of the skin (Hargis et al., 1999) and cornea of cats (Nasisse et al., 1998). A question raises about the role played by eosinophils in these diseases. In fact, to date, eosinophil RNases have been shown to possess antiviral activity only against RNA viruses (Domachowske et al., 1998a, Domachowske et al., 1998b, Rosenberg and Domachowske, 2001). In addition, another non-eosinophil member of the RNase A superfamily, RNase 7, does not appear to have any activity against herpes simplex virus-1 (Harder and Schröder, 2002). Eosinophil chemoattractant properties of feline herpesvirus 1 and the activity of feline EAR against feline herpesvirus 1 deserve further investigation in order to establish a causal relationship between the presence of feline herpesvirus 1 and eosinophils in cutaneous and ocular tissues.

In our study, cat granule proteins appeared to possess bactericidal activity (chapter 5). This activity was attributed to feline EPO, EAR and MBP, which are all known to be antimicrobial in humans (Borelli et al., 2003, Lehrer et al., 1989, Persson et al., 2001) and other animal species (Jong et al., 1989, Klebanoff et al., 1989, Piller and Portman, 1993). Antibacterial properties of cat eosinophils against *E. coli* suggest a role of eosinophils in innate cat defence against bacteria, analogous to what happens in other species. However, the role of eosinophils in antibacterial defence *in vivo* is not settled. In fact, *in*
vivo, great numbers of eosinophils are not usually recruited at sites of bacterial infection and eosinophils have been shown to be less bactericidal than neutrophils (DeChatelet et al., 1978, Yazdanbakhsh et al., 1986).

Eosinophils, together with other inflammatory cells, may be observed in the inflammatory infiltrate in a variety of feline bacterial skin diseases, but they do not normally constitute the predominant cell type (Gross et al., 1992, Yager and Wilcock, 1994). A bacterial pathogenetic component has been hypothesised also in feline EGC, based on the identification of filamentous bacteria in one case of EG in the oral cavity (Russell et al., 1988). In addition, selected cases of EGC lesions have been reported to respond to antibiotic therapy (Rosenkrantz, 1993, Scott et al., 2001, Song, 1994). However, bacteria might represent secondary invaders, especially in eroded and ulcerated EGC lesions (Moriello et al., 1990), and response to antibiotics might be partly related to their anti-inflammatory and/or immunomodulatory effect (Rosenkrantz, 1993).

Interestingly, numerous eosinophils have been recently reported in the inflammatory infiltrates of feline abscesses caused by methicillin-resistant staphylococci (Ozaki et al., 2003). As it appears that, in humans, antibacterial proteins of the RNase A superfamily kill antibiotic-resistant bacteria (Harder and Schröder, 2002), it might be intriguing to investigate antimicrobial properties of feline eosinophil granule proteins, and their effector mechanisms, against antibiotic-resistant bacteria. This will not only have an important impact on our understanding of specific bactericidal mechanisms of eosinophil granule proteins, which remain to be completely elucidated (Carreras et al., 2003), but might also inspire the development of new therapeutic strategies, alternative to antibiotics, for infectious diseases (Carreras et al., 2003, Harder and Schröder, 2002).

Despite being speculative, because cytotoxicity of feline eosinophil granule proteins against mammalian cells was not investigated, it seems likely that cat eosinophils possess also this activity, analogous to eosinophils from humans (Motojima et al., 1989, Shah et al., 1989) and other animal species (Jong and Klebanoff, 1980). In fact, mechanisms of toxicity of granule proteins, despite being still unclear, imply the disruption of the phospholipid bilayer of plasma membranes (Carreras et al., 2003).

Cytotoxic properties exerted towards host cells by eosinophil granule proteins represent the “other face” of the multiple beneficial roles played by eosinophils in innate defence. However, as recently suggested by Rosenberg and Domachowske (2001), it is likely that
the eosinophil response represents a “double-edged sword”, reflecting the challenging balance between beneficial and detrimental features of physiologic responses. According to this concept, detrimental effects of eosinophils on human respiratory airways in asthma would represent the damage to self of an innate immune response focused to damage different pathogens, including viruses (Rosenberg and Domachowske, 2001).

The fact that eosinophils might play a primary, unrecognised, beneficial role in feline eosinophil-mediated diseases is a fascinating field to investigate, in fact, the majority of these diseases in cats have been historically considered to be idiopathic and/or associated to hypersensitivity disorders, as asthma or EGC. Further studies to appraise eosinophil participation in feline diseases are needed.

As mentioned previously, the cat has been used as a model for human eosinophil-driven allergic airway inflammation (Padrid et al., 1995, Padrid et al., 1996) even unknowing feline eosinophil granules content and function. Based on that and on the similarities between human and feline eosinophil granule contents and functions (chapter 5), the cat might represent a potential alternative to the mouse model for eosinophil-driven airway diseases. In fact, cat bronchial eosinophils, analogous to human eosinophils but unlike murine eosinophils (Denzler et al., 2001, Malm-Erjefalt et al., 2001, Stelts et al., 1998), have been indirectly demonstrated to undergo degranulation following antigenic-challenge (Padrid et al., 1996).

In addition, feline eosinophils have been directly demonstrated to degranulate via PMD and ECL in the skin (chapter 3), therefore the cat might constitute also an interesting species for studying cutaneous eosinophil-mediated diseases, including Wells’ syndrome, besides being proposed as a model for atopic dermatitis (Roosje et al., 1997, Roosje et al., 2002d).

Nevertheless, although the cat might be considered an useful species for studying eosinophil biology and pharmacology due to the many similarities between feline and human eosinophils and eosinophil-associated diseases, in our opinion, further studies are needed before assuming that data obtained from the cat may be applied to humans, i.e. before considering the cat as a suitable model for human diseases. In fact, when comparing animal models to human conditions, caution is advised until the proper parameters of eosinophil activation and involvement are addressed. For instance, the
inciting stimuli for eosinophil recruitment and activation in cats are currently unknown and need to be studied.

It appears from this thesis that further studies are needed on feline eosinophils and eosinophil granule proteins. Although an adequate number of eosinophils may be yielded using the peritoneal eosinophilia model employed in our experiment (chapter 5), difficulties of obtaining large amounts of granule proteins may hamper further investigations. Therefore, alternative techniques to yield large numbers of feline eosinophils should be investigated, including long-living eosinophil cultures, also considering that there is a common trend in biomedicine towards a reduction in the use of living animals for research. In addition, the possibility of synthesising feline recombinant eosinophil granule proteins and subsequently obtaining specific antibodies might represent a future field in feline eosinophil research, analogous to what reported in humans.

Our findings may be clearly advantageous in feline medicine, in fact, based on both the analogy between human and feline eosinophil granule contents and functions and tissue degranulation patterns, it is hoped that currently ongoing human research will bring new effective therapies for feline eosinophil-mediated diseases, especially considering that glucocorticoids are still the mainstay in these diseases (Scott et al., 2001).

In summary, findings and considerations on feline eosinophils may be resumed as follows:

1. Feline blood eosinophils may show PMD morphology, suggestive of eosinophil activation and granule contents release. The number of peripheral eosinophils showing PMD changes does not seem to directly correlate with the degree of peripheral eosinophilia. This suggests that total blood eosinophil count might not adequately indicate the severity of the ongoing eosinophil-associated disease.

2. Activated tissue eosinophils, in EGC lesions, degranulate via ECL and PMD, being ECL the predominant mode of granules content release. The lack of correlation, in individual cases, between the number of eosinophils showing extensive PMD and the number of eosinophils undergoing ECL, suggests that ECL is a secretory process independent from PMD.
3. Cat eosinophil granule proteins, analogous to eosinophil granule proteins in humans and other animal species, possess peroxidase, RNase and bactericidal activities. Feline EPO is characterised by enzymatic and bactericidal activities, feline EAR possesses enzymatic and bactericidal properties and shows N-terminal sequence homology with proteins of the RNase A superfamily, including eosinophil RNases, and feline MBP is bactericidal and its N-terminal sequence is homologue to that of human and murine MBP-1.
References


Discussion


Conclusions

1. The different clinical forms of feline eosinophilic granuloma complex have a common histopathological appearance. The typical histopathological pattern of eosinophilic granuloma complex lesions consists of perivascular to diffuse eosinophilic dermatitis with small–to large-sized dermal foci of amorphous to granular eosinophilic debris (flame figures).

2. Flame figures present in eosinophilic granuloma complex lesions comprise ultrastructurally unaltered collagen fibrils and partly disrupted collagen fibres surrounded by degranulating eosinophils. Hence, the term “collagen degeneration” is not appropriate to designate them.

3. Typical ultrastructural features of feline mature resting eosinophils in blood include segmented nuclei with no visible nucleoli, and bicompartmental specific granules with lamellated polymorphic cores and variably shaped vesiculotubular organelles in the cytoplasm.

4. Feline eosinophils are capable to release their granule contents via piecemeal degranulation and cytolysis. Piecemeal degranulation, in cats, represents a degranulation pathway common to blood and tissue eosinophils. Eosinophil cytolysis is a mechanism of granule contents release of feline tissue eosinophils.

5. Cat eosinophil granules contain major basic protein and eosinophil-associated ribonuclease, and granule proteins possess peroxidase, ribonuclease and bactericidal activities.
Summary

Despite being commonly reported, feline eosinophil-associated disorders, including EGC, are poorly understood and generally associated to immune-mediated or parasitic causes, analogous to their human counterparts. Nevertheless, cat eosinophil functions and contents, although considered similar to those of human eosinophils, are currently unknown. Hence, the objectives of this thesis were to study feline EGC and to obtain specific information on the cat eosinophil biology.

In this thesis, the histopathological features of clinically different EGC lesions were studied on H & E and trichrome stained sections. With H & E stain, all the EGC lesions examined were characterised by a dermal eosinophilic infiltration of variable intensity and the presence of small- to large-sized foci of eosinophilic debris that, with trichrome stain, appeared to consist of normally stained collagen fibres surrounded by a debris showing the same tinctorial properties as eosinophil granules. These results showed that EGC lesions with different clinical appearance are histopathologically indistinguishable and that small- and large-sized dermal foci of eosinophilic debris have similar histogenesis. Hence, the term flame figures, normally used to define small foci of eosinophilic debris by analogy with flame figures in Wells’ syndrome, may be employed also to designate large-sized focal depositions of this debris.

Furthermore, the ultrastructure of small- and large-sized flame figures in EGC lesions was investigated. They comprised morphologically unaltered collagen fibrils, collagen fibres partly disrupted by oedema and cellular debris, and degranulating eosinophils via ECL and PMD. The ultrastructure of flame figures in EGC was similar to that reported in flame figures of human Wells’ syndrome. This suggested that eosinophils play a primary role in flame figures formation in cats, analogous to what reported in humans. In addition, this study demonstrated that tissue eosinophils in feline EGC release their granule contents by ECL and PMD, analogous to human tissue eosinophils at sites of eosinophil-mediated inflammation. ECL was the predominant mode of degranulation.

An ultrastructural study of feline circulating eosinophils from cats with various blood eosinophil counts and different eosinophil-associated diseases was also performed. PMD morphology, indicative of eosinophil activation and degranulation, was recognised in peripheral eosinophils. No direct correlation was found between the number of eosinophils showing PMD changes and the level of blood eosinophilia. This latter finding suggested
that total blood eosinophil count might not represent the best criterion to evaluate the contribution of eosinophils to the ongoing eosinophil-associated disease.

Finally, a study on cat eosinophil granule proteins was conducted. Granule proteins, extracted from cat eosinophils obtained by experimentally induced peritoneal eosinophilia, were analysed by gel-filtration chromatography and their biological activities were studied. Cat eosinophil granule proteins possessed peroxidase, RNase and bactericidal activities. Feline EAR showed N-terminal sequence homology with proteins of the RNase A superfamily, including eosinophil RNases, and the N-terminal sequence of feline MBP was homologue to that of human and murine MBP-1. These findings indicated that feline eosinophil granule proteins have biological roles similar to those reported in humans and other animal species and highlighted that the cat might represent a suitable species for studying human eosinophil-mediated diseases.
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