EOSINOPHILIC MUCUS CHRONIC RHINOSINUSITIS: AN IMMUNOLOGICAL PERSPECTIVE

Harshita Pant MBBS

Department of Surgery

Faculty of Health Sciences

Adelaide University

South Australia

2005

A thesis submitted in fulfilment of the requirements for the

Degree of Doctor of Philosophy

TABLE OF CONTENTS

CHAPTER ONE: LITERATURE REVIEW

CHAPTER TWO: MATERIALS AND METHODS

CHAPTER FOUR: FUNGAL-SPECIFIC HUMORAL RESPONSE IN EMCRS PATIENTS

CHAPTER FIVE: FUNGAL-SPECIFIC PERIPHERAL BLOOD T LYMPHOCYTE PROLIFERATION IN EMCRS PATIENTS

CHAPTER SIX: CHARACTERISATION OF LYMPHOCYTE POPULATIONS IN SINONASAL POLYPS

PRÉCIS

Immunoglobulin E (IgE) – mediated systemic fungal allergy and fungi in sinus eosinophilic mucus are considered pathologically important in patients with eosinophilic mucus chronic rhinosinusitis (EMCRS). They are used to subgroup these patients into the following: allergic fungal sinusitis (AFS), AFS-like, non-allergic fungal eosinophilic sinusitis (NAFES) and non-allergic, non-fungal eosinophilic sinusitis (NANFES). The relevance of this classification system was examined in this thesis according to the clinical characteristics, systemic immune responses and the sinonasal lymphocyte populations in EMCRS patients. In doing so, it was established that the EMCRS subgroups were not significantly different from one another. However, as a single group, EMCRS patients had a more severe form of sinus disease than patients with chronic rhinosinusitis without eosinophilic mucus (CRS).

The total IgE and fungal-specific IgE-mediated allergic parameters were not significantly different between allergic EMCRS patients and disease-control group, allergic rhinitis with fungal allergy (ARFA). This indicated that fungal allergy was not of central pathogenic importance in EMCRS. Regardless of fungal allergy or of the detection of fungi, EMCRS patients had an elevated humoral and cellular immune response to *Alternaria alternata* and *Aspergillus fumigatus* compared with healthy volunteers. EMCRS patients were distinguished from ARFA and CRS groups by elevated fungal-specific serum IgG3 levels. Fungal-specific peripheral blood mononuclear cell proliferation was also increased in EMCRS patients. While $CD4^+$ and $CD8^+$ T lymphocytes proliferated in a proportion of ARFA and CRS patients, $CD8^+$ T lymphocytes did not proliferate in EMCRS. This implied a dysregulated $CD8^+$ T cell response to fungi. Compared with CRS, polyps from EMCRS patients had a greater proportion of effector memory $CD8^+$ T lymphocytes lacking the cytotoxic phenotype defined by intracellular perforin. These findings indicated that

immunological mechanisms other than IgE-mediated fungal allergy were involved in EMCRS. These mechanisms were characterised by terminally differentiated and antigen-experienced $CD8^+$ T lymphocytes in the mucosa.

The work presented here showed that eosinophilic mucus represented a distinct clinico-pathological subset of chronic rhinosinusitis patients. The clinical subgrouping of EMCRS was unfounded as IgE-mediated allergy was not the principal pathogenic mechanism, and all the EMCRS subgroups had an elevated fungal-specific immune response. Although the nature of the fungal involvement and disease mechanisms remain to be determined, this thesis represents a major conceptual advance to determine the pathogenic processes in eosinophilic mucus chronic rhinosinusitis.

DECLARATION

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

I give consent to this copy of my thesis, when deposited in the University Library, being available for loan and photocopying.

Harshita Pant

ACKNOWLEDGEMENTS

Research in rhinology and chronic rhinosinusitis in South Australia began in the late 1990's, thanks largely to Professor Peter Wormald who encouraged otolaryngologists to endeavour beyond the clinical realms into basic science research. I sincerely thank Professor Wormald for providing the opportunity for me to undertake research in rhinology as a surgeon-scientist, for his supervision, encouragement and unwavering support. A milestone for the otolaryngology research team at the Queen Elizabeth Hospital was establishing a link with the Flow Cytometry Unit, directed by Dr Peter Macardle, and the Department of Immunology, Arthritis and Allergy, headed by Professor Peter Roberts-Thomson, at the Flinders Medical Centre in 2002. My decision to pursue PhD studies was influenced largely by Dr Macardle – it was immediately apparent that his invaluable supervision would make a world of difference. I am indebted to him for allowing me to undertake my studies in his dynamic laboratory where I was introduced to cellular immunology as I made the transition from a clinician to a student of science. This work would not have been possible without his continued support, endless patience, inspiration and friendship. I also thank my co-supervisors Dr Frank Kette and Dr William Smith for their academic guidance and support.

Special thanks goes to Ms Sheree Bailey for her guidance with flow cytometry, Ms Dimitra Beroukas for her expert advice and for performing immunohistochemical studies and Ms Carolyn Mardell for helping with the ELISA work. I acknowledge the contributions of the members of the Department of Immunology, Allergy and Arthritis, in particular, Dr Petra Neufing, Ms Chris Thompson, Dr Anthony Smith, Dr Michael Jackson and Dr Dana Cavill. I appreciate the helpful discussions with Dr Robert (Bob) Heddle and Professor Peter Roberts-Thomson. I thank the members of the Department of Otolaryngology at the Queen Elizabeth Hospital, especially Ms Lyn Martin, and Dr Ian Wong and Dr Melanie Collins for

access to their patient database. I thank Dr David Gillis (IMVS) and Mr Antonios Nikoloutsopoulos (SouthPath) for performing diagnostic immunological studies, Dr Adrian Esterman and Dr Malcolm Bond (Department of Clinical Epidemiology, Flinders Medical Centre) for their advice on statistics, Mr Paul Hakendorf (Department of Clinical Epidemiology) and Dr Robert Keane (School of Geography, Population and Environmental Management, Flinders University of South Australia) for their assistance with geographical studies, Dr John Pierides (Department of Pathology, Queen Elizabeth Hospital) and Dr Craig James (Adelaide Pathology Partners) for providing pathology samples and slides and Mr Keith Bignall and Mr Cameron Hussey (SouthPath Computing) for access to databases.

I extend my gratitude to the patients who enrolled in this study – without them all our endeavours would have been in vain. I acknowledge the financial support of the Garnett Passe and Rodney Williams Memorial Foundation by means of grant and the Adelaide University and the Royal Australasian College of Surgeons by means of scholarship. I thank my otolaryngology colleagues and the members of the South Australian Otolaryngology Training Committee, in particular, Dr Dean Southwood and Dr Michael Jay for their understanding and support throughout my candidature.

As always, the endurance and support of my grandmother, *nani ji*, brother, Paresh, and friends have intensified my efforts throughout this research. I especially thank Dr Charles Evill, Dr Grant Hennig, Ms Dimitra Beroukas, Ms Mayumi Kako, Ms Sheree Bailey and Dr Mark Schembri for their friendship and support. I was extremely fortunate to have the unconditional support, encouragement and love of my dear friend, Mr Madhan Dhami, who undertook every step of this journey with me.

PUBLICATIONS

Pant H, Kette FE, Smith WB, Wormald PJ and Macardle PJ (2005). Fungal-specific humoral response in eosinophilic mucus chronic rhinosinusitis. Laryngoscope 115: 601-606

Pant H, Kette FE, Smith WB, Macardle PJ and Wormald PJ (2005). Eosinophilic mucus chronic rhinosinusitis: clinical subgroups or a homogeneous pathological entity? Am J Rhinol. *Submitted.*

Pant H, Kette FE, Smith WB, Wormald PJ and Macardle PJ. Absence of perforin in the mucosal $CD8^+$ T lymphocytes characterises patients with eosinophilic mucus chronic rhinosinusitis. *Manuscript in preparation.*

Pant H, Kette FE, Smith WB, Wormald PJ and Macardle PJ. Sinonasal mucosal immune system and pathogenic mechanisms in chronic rhinosinusitis. Review Article. *Manuscript in preparation.*

Pant H, Kette FE, Smith WB, Wormald PJ and Macardle PJ. Eosinophilic mucus chronic rhinosinusitis: a defect in the $CD8^+$ T lymphocyte response to fungi? *Manuscript in preparation.*

PRESENTATIONS

Oral Presentations

INTERNATIONAL MEETINGS

Pant H. Fungal-specific humoral response in EMCRS. The Australian Society of Otolaryngology Head and Neck Surgery Annual Conference. Sydney, Australia. 2004.

Pant H. *Alternaria alternata* and *Aspergillus fumigatus*-specific antibodies in eosinophilic mucus chronic rhinosinusitis (EMCRS). 10th Asia-Oceania Otorhinolaryngology Head and Neck Congress. Kuala Lumpur, Malaysia. 2004.

Pant H. Evidence for fungal-specific immune responses in non-"allergic fungal sinusitis" chronic rhinosinusitis patients. Suva, Republic of Fiji. 2004. *Invited Speaker.*

NATIONAL MEETINGS

Pant H. Taking "allergy" out of "allergic fungal sinusitis". Royal Australasian College of Surgeons Annual Scientific Meeting. Adelaide. 2004.

Awarded the Ronald Gristwood Medal for best presentation.

Pant H. Eosinophilic mucus chronic rhinosinusitis: A $CD8^+$ T lymphocyte-driven mucosal inflammation? The Queen Elizabeth Hospital Research Foundation. Adelaide. 2004.

Awarded first place for best presentation.

Pant H. Immunological studies in chronic rhinosinusitis. Australian Society of Clinical Immunology and Allergy. Adelaide. 2004.

Pant H. Lymphocyte subpopulations in nasal polyps. Robert Guerin Memorial Annual National Otorhinolaryngology Registrar's Conference. Melbourne. 2004.

Pant H. Immune response to fungal antigens in EMCRS. Robert Guerin Memorial Annual National Otorhinolaryngology Registrar's Conference. Sydney. 2003.

Smith WB. New insights into nasal polyps. Australian Society of Clinical Immunology and Allergy, Annual Scientific Meeting. Brisbane. 2004.

Poster Presentations

INTERNATIONAL MEETING

Lymphocyte subpopulations in the sinonasal mucosa of patients with chronic rhinosinusitis. 8th International Conference on Human Leucocyte Differentiation Antigens / 34th Annual Scientific Meeting of the Australasian Society for Immunology Incorporated (HLDA8/ASI34). Adelaide, South Australia. 2004.

NATIONAL MEETING

Unique lymphocyte subsets in the mucosa of patients with eosinophilic mucus chronic rhinosinusitis. Garnett Passe and Rodney Williams Memorial Foundation. Frontiers in Otorhinolaryngology. Queensland. 2004.

ETHICS

Human ethics approval was obtained from the following South Australian tertiary referral centres' ethics committees: Queen Elizabeth Hospital (109/2002), Royal Adelaide Hospital (030517) and Flinders Medical Centre (150/023). Written informed consent was obtained from every individual enrolled in this study.

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

-	negative
+	positive
°C	degrees Celsius
A	absorbance
ABPA	allergic bronchopulmonary aspergillosis
AFS	allergic fungal sinusitis
AFS-like	allergic fungal sinusitis-like
ARFA	allergic rhinitis with fungal allergy
ATCC	American Type Culture Collection
BD	Becton Dickinson
BCR	B cell receptor
Bqs	Becquerel
BSA	bovine serum albumin
cpm	counts per minute
CD	cluster determinant
CFDA-SE	5-(6)-carboxyfluorescein diacetate succinimidyl ester
CFSE	carboxyfluorescein succinimidyl ester
CFTR	cystic fibrosis transmembrane conductor
CRS	chronic rhinosinusitis
CT	computed tomography
DNA	deoxyribonucleic acid
ELISA	enzyme-linked immunosorbent assay
EM	eosinophilic mucus
EMCRS	eosinophilic mucus chronic rhinosinusitis
FCS	foetal calf serum
FITC	fluorescein isothiocyanate
FL-1, FL-2 and FL-3	fluorescence channel 1, 2 and 3
FMC	Flinders Medical Centre
g	relative centrifugal force
g	gram
Gzm	granzyme
HLA	human leucocyte antigen

HV	healthy volunteer
Ig	immunoglobulin
IgM	immunoglobulin, class M
IgG	immunoglobulin, class G
IgA	immunoglobulin, class A
IgE	immunoglobulin, class E
IL	interleukin
IMVS	Institute of Medical and Veterinary Science
IQR	interquartile range
IU	international unit
kU	kilo units
kU_A	kilo units of allergen
L	litre
LAMP	lysosome-associated membrane protein
MAdCAM	mucosal addressin cell adhesion molecule
MALT	mucosa-associated lymphoid tissue
MHC	major histocompatibility complex
M	molar
M_r	molecular weight
mg, ml, mM	milligram, millilitre, millimolar
µg, µl, µM	microgram, microlitre, micromolar
n	sample size
nm	nanometre
NA	not applicable
NAFES	non-allergic, fungal eosinophilic sinusitis
NALT	nasal-associated lymphoid tissue
NANFES	non-allergic, non-fungal eosinophilic sinusitis
NBS	newborn bovine serum
NK	natural killer
NKT	natural killer T
NP	nasal polyp
NS	not significant
OD	optical density

P	P value
PB	peripheral blood
PBMC	peripheral blood mononuclear cells
NPMC	nasal polyp mononuclear cells
PBS	phosphate buffered saline
PerCP	peridinin chlorophyll protein
PHA	phytohaemagglutinin – M
PNAd	peripheral node addressin
RPMI 1640	Roswell Park Memorial Institute 1640
RF5 / RF10	RPMI 1640 supplemented with penicillin, streptomycin, glutamine and 5% or 10% foetal calf serum
rpm	rotations per minute
RNA	ribonucleic acid
R-PE	R-phycoerythrin
R-PECy5	R-phycoerythrin : Cyanine-5
SI	stimulation index
sp., spp.	species (singular), species (plural)
TC	cytotoxic T cell
TCR	T cell receptor
TH	helper T cell
TNF	tumour necrosis factor
Tris	tris (hydroxymethyl) aminomethane
w/v	percent weight by volume
v/v	percent by volume

In the loving memory of my mother Kishun Kaur, and my father Dinesh Pant,

who would have been so pleased,

To my teachers who have taught me all I know, and

To my patients who continue to inspire me.

CHAPTER ONE: LITERATURE REVIEW

1.1 INTRODUCTION

Chronic rhinosinusitis (CRS) is a clinical syndrome caused by mucosal inflammation of the nose and paranasal sinuses that persists beyond twelve weeks (Benninger et al. 2003). The aetiology, clinical manifestations and prognosis of chronic rhinosinusitis are heterogeneous. The incidence of chronic rhinosinusitis and its aggressive clinical forms is increasing (Van Cauwenberge and Watelet 2000). Sinonasal polyps (Greek *poly* – many and *pous* – feet, many feet) may be associated with severe and incurable disease, particularly in allergic fungal sinusitis (AFS), a clinically aggressive form of chronic rhinosinusitis (Schwietz and Gourley 1992). The term "allergic fungal sinusitis" encompasses a constellation of pathological features. These include sinus eosinophilic mucus (EM), type I hypersensitivity to fungi, also known as fungal allergy, (Meltzer et al. 2004) and fungi in the sinus mucus (Bent and Kuhn 1994). The pathogenesis of allergic fungal sinusitis is poorly understood. Currently there is no cure and treatment options are limited.

Type I hypersensitivity to fungi is presumed to be important in the pathogenesis of allergic fungal sinusitis. However, some patients with clinical features similar to allergic fungal sinusitis, such as extensive polyps and eosinophilic mucus, do not have fungal allergy or fungus in the eosinophilic mucus (Katzenstein et al. 1983a; Ferguson 2000a; Collins et al. 2003). It is still unclear how these patients fit into the disease spectrum of allergic fungal sinusitis and severe chronic rhinosinusitis. A comparative study of the clinical features and fungal-specific immune responses of these patient groups will better characterise and define the disease.

Localised sinonasal inflammation where eosinophils and lymphocytes predominate is a recognised feature in chronic rhinosinusitis, including allergic fungal sinusitis and polypoid chronic rhinosinusitis with eosinophilic mucus (Lara and Gomez 2001; Marple 2001; Ferguson 2004). This is not unexpected, because eosinophils are the common granulocyte involved in inflammation of those body areas exposed to the external environment, including the skin, mucosal surfaces of the upper and lower airway, the gastrointestinal and genitourinary tracts (Rothenberg 2004; Arora and Yamazaki 2004). Despite numerous studies in this area, the precise role of eosinophils and lymphocytes in the sinonasal inflammatory process and the cellular and molecular mechanisms controlling them are poorly defined.

This review will address the current knowledge of the pathogenic mechanisms in eosinophilic mucosal inflammation in chronic rhinosinusitis. Special focus is directed to allergic fungal sinusitis and other subgroups of eosinophilic mucus chronic rhinosinusitis (EMCRS). Concepts are introduced from the view of the adaptive and innate mucosal immune system. Understanding the reactions of the mucosal cells to stress and injury will lead towards unravelling the mechanisms perpetuating and maintaining the chronic inflammation. In the long term, this may enable the introduction of novel and effective therapies that target strategic points in the inflammatory cascade.

1.2 PARANASAL SINUSES

Paranasal sinuses, hereafter called sinuses, are air-filled cavities within the facial bones that communicate with the nasal cavity via small, narrow openings in the nasal wall (Stammberger 1991). The sinuses include the bilaterally paired maxillary, ethmoidal, frontal and sphenoidal sinuses that are named according to the bones in which they are housed (Figure 1.1) (Bannister et al. 1995). The anterior ethmoidal and maxillary sinuses communicate via the hiatus semilunaris into the middle meatus. The drainage pathway of the frontal sinuses into the middle meatus is variable (Wormald 2005). The posterior sinuses, sphenoidal and posterior ethmoidal, communicate with the nasal cavity via small ostia into the sphenoethmoidal recess and superior meatus respectively.

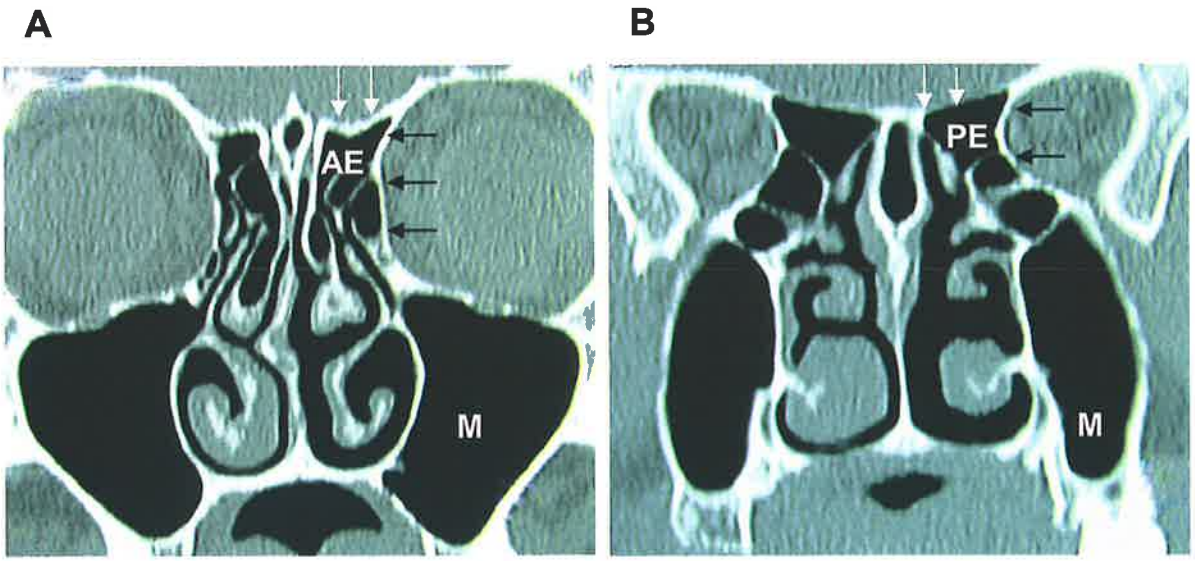


Figure 1.1 Coronal computed tomogram sections of the nose and paranasal sinuses from a healthy individual. These scans show aerated maxillary (M) and anterior ethmoid (AE) sinuses in (A) and posterior ethmoid sinuses (PE) in (B). The proximity of the sinuses to the orbit (black arrows) and the brain (white arrows) is evident.

The blood supply to the nose and sinuses derives from arterial branches of the internal and external carotid arteries and venous drainage is via the corresponding veins. The trigeminal nerve mediates somatic sensation and provides pathways for the autonomic supply. Parasympathetic secretomotor supply is from preganglionic cells in the superior salivary nucleus in the lower pons and the sympathetic supply from cells in the dorsal horn of the first to third thoracic spinal segments. Lymphatic fluid drains to the neck and the retropharyngeal lymph nodes.

The sinuses are separated from the orbit, brain, cranial nerves and major blood vessels accessing the intracranial cavity by bony walls. These walls may be paper-thin and deficient in places (Stammberger 1991). Consequently, sinus disease may readily extend into the orbit and brain, resulting in organ and life-threatening sequelae.

1.3 CHRONIC RHINOSINUSITIS

The mucosa lining the nose and paranasal sinuses is continuous and therefore inflammation of the sinuses invariably affects the nose. (Lanza and Kennedy 1997; Benninger et al. 2003). Hence, the term rhinosinusitis is more appropriate than sinusitis. The clinical spectrum of chronic rhinosinusitis spans non-troublesome symptoms to severe and recalcitrant disease associated with serious extra-sinus complications. Symptoms resulting from sinus involvement include nasal obstruction, nasal discharge, facial pain or a sensation of facial pressure, headache, hyposmia and anosmia (Benninger et al. 2003). The macroscopic mucosal pathology ranges from mucosal erythema and oedema to polyps. The clinical parameters used to subclassify chronic rhinosinusitis include: *(a)* the anatomical site of involvement, *(b)* the type of microorganism cultured from sinus contents, *(c)* presence of extra-sinus disease, *(d)* associated predisposing factors or the clinical phenotype, *(e)* the type

of mucosal pathology, including polyps and *(f)* the predominant inflammatory cell type in the mucosa (Pinheiro et al. 2001).

Chronic rhinosinusitis is estimated to affect about 14% of the population (Kaliner 1997). The exact prevalence is difficult to determine due to discrepancies in definition, missed or over diagnosis and the clinical heterogeneity of the disease (Van Cauwenberge and Watelet 2000; Benninger et al. 2003). Chronic rhinosinusitis is one of the most common chronic medical conditions that leads an individual to seek medical care; ensuing direct medical costs include office visits, diagnostic tests, antibiotics, other pharmaceuticals and surgical procedures. It also significantly impairs the quality of life by causing functional and psychological impairment. Consequently, chronic rhinosinusitis is associated with a substantial socioeconomic cost (Gliklich and Metson 1995; Benninger et al. 2003).

1.4 PATHOGENESIS OF CHRONIC RHINOSINUSITIS

Several local and systemic conditions may be involved in the pathogenesis of chronic rhinosinusitis (Table 1.1). Type I hypersensitivity has long been considered an important aetiological factor. Type I hypersensitivity refers to an immunoglobulin E (IgE) – mediated allergic reaction, where "allergy" refers to an exaggerated or inappropriate immune-mediated reaction to an antigen at a dose tolerated by normal subjects (Johansson et al. 2001). However, in the literature, "allergy" has become synonymous with type I hypersensitivity. In this thesis, the term "allergy" also refers to type I hypersensitivity. The term "atopy" describes the clinical presentations of type I hypersensitivity which include asthma, eczema, hay fever, urticaria and food allergy.

Type I hypersensitivity depends on the permanent sensitisation of mast cells in the target

Table 1.1 Aetiological factors associated with chronic rhinosinusitis

Chronic infection

- Virus, bacteria, fungi

Factors predisposing to chronic infection

- Immunodeficiency
  - Local, systemic
  - Innate, adaptive
  - Primary, secondary
- Abnormal mucociliary function
  - Primary
    - Cystic fibrosis, Young's syndrome, Kartagener's syndrome
  - Secondary
    - Infection, chronic inflammatory conditions
    - Structural abnormalities (septal deviation, Haller cells, concha bullosa)
    - Iatrogenic (nasal packs, nasal tubes, post surgical scarring)
    - Neoplasms, inflammatory polyps
  - Idiopathic

Inflammatory conditions (immune and non-immune – mediated)

- Allergy
- Aspirin sensitivity
- Autoimmune disease
- Granulomatous disease (Wegener's granulomatosis, sarcoidosis)
- Neurogenic inflammation
- Physical and chemical irritants (barotrauma, topical medications, cigarette smoke)
- Idiopathic

Modified from Pinheiro et al. 2001 and Benninger et al. 2003.

organ where high affinity receptor for IgE, FcεRI, is critical in mediating allergen-specific IgE responses (Maurer et al. 1995; Gould et al. 2003). Type I hypersensitivity results from "cross-linking" by a specific allergen of two or more IgE molecules engaged on the surface

of effector cells by FcεRI (Gould et al. 2003). Subsequent effector cell activation causes release of preformed and newly formed mediators that lead to local inflammation, and recruitment of leucocytes, including basophils and eosinophils, thereby leading to the clinical symptoms and signs (Sutton and Gould 1993). The diagnosis of type I hypersensitivity requires the detection of allergen-specific IgE in peripheral blood or a positive skin prick test to an allergen (Coombs and Gell 1968). Epidemiological studies show that the prevalence of chronic rhinosinusitis with nasal polyps is low in patients with allergic rhinitis (Settipane and Chafee 1977), signifying that type I hypersensitivity is not principally responsible for polypoid chronic rhinosinusitis (Slavin 1992). However, a worse clinical outcome is often seen in association with type I hypersensitivity than without, supporting the notion that type I hypersensitivity may aggravate underlying mucosal inflammation (reviewed by Settipane 1997).

The involvement of superantigens in chronic rhinosinusitis is also proposed (Bachert et al. 2003; Bernstein et al. 2003; Dennis 2003). Superantigens are peptides produced by many pathogens including viruses, bacteria and fungi that may colonise the sinonasal mucosa. Superantigens are so named because of their ability to activate and induce the proliferation of a substantial proportion of $CD4^+$ and $CD8^+$ T lymphocytes (Herman et al. 1991; McCormick et al. 2003; Proft and Fraser 2003; Nagashima et al. 2004). Typically, superantigens interact with the major histocompatibility complex (MHC) expressed on antigen presenting cells as intact molecules outside the peptide binding groove, followed by sequential binding to the T cell receptor (TCR) via the variable region of the TCR β chain (Seth et al. 1994). The repertoire of the variable β (Vβ) region is limited to 24 major types. Therefore, together with the mitogenic potency of the superantigen, up to 20% of T lymphocytes may be activated followed by cell anergy or deletion (Attinger et al. 2000). The

effector functions of superantigen activated T lymphocytes include cytotoxicity and cytokine secretion (Huang et al. 2002). Although the precise mechanism and role remains to be defined, superantigens may alter the repertoire and function of the mucosal lymphocyte populations and consequently, the inflammatory response.

Other immunostimulatory factors from microorganisms including bacterial endotoxins, heat shock proteins and CpG-deoxyribonucleic acid (CpG-DNA) motifs may be involved in the inflammatory process either directly or by molecular mimicry (Tlaskalova-Hogenova et al. 2004). The microorganisms may also exist as biofilms (reviewed by Post et al. 2004). Genetic factors, including those associated with the major clinical phenotypes, certain human leucocyte antigen (HLA) types, isoforms of toll-like receptors (TLR), MHC transporters and intracellular signalling mechanisms may be implicated in the chronic inflammatory state in the right microenvironment and immunological insult. These disorders may be common to the systemic immune system or limited to the mucosal system. This may, in part, explain why some patients with inflammatory bowel disease also have upper airway hyperresponsiveness (Mansi et al. 2000) and chronic rhinosinusitis (Book et al. 2003).

The pathogenesis of chronic rhinosinusitis appears to be a complex and multifactorial process that may include initiating and maintaining events. In the absence of obvious predisposing proinflammatory states, for example, selective immunodeficiency, cystic fibrosis or obstruction of the sinus drainage pathways, the initiating or maintaining events may not be apparent. Furthermore, factors that maintain the inflammation may obscure the underlying initiating event. Several processes, including type I hypersensitivity and superantigens may either initiate or maintain the inflammation and deciding which factor fits

into what category is probably impossible. Nevertheless, an explanation of why some patients have self-limiting disease whereas others have severe, polypoid sinusitis refractory to conventional treatment remains an important goal.

1.5 SINONASAL POLYPS

The term "polyp" refers to the macroscopic appearance of a pedicled tissue projection arising from a mucosal surface (Figure 1.2). The first description of sinonasal polyps was in ancient Indian and Egyptian texts dated more than 5000 years old (Brain 1997). In the general sense, polyps may contain benign or neoplastic tissue. In the context of chronic rhinosinusitis, "polyp" refers to a benign mucosal projection with an epithelial lining.

Macroscopically, sinonasal polyps are usually soft, lobular and have a smooth, shiny surface with a bluish-grey or pink translucent appearance (Hellquist 1997). A pseudostratified columnar epithelium, known as the respiratory epithelium or the Schneiderian membrane, typically lines the polyps. Squamous or cuboidal metaplasia of the epithelial cells, goblet cell hyperplasia, altered glandular architecture and thickening of the basement membrane may be present. Polyps are generally devoid of neural tissue and the stroma has varying degrees of oedema and inflammatory cells.

Polyps are generally classified based on the cell type and degree of cellularity in the tissue. Those with abundant inflammatory cells are the most common, of which eosinophilic polyps predominate followed by neutrophilic and rarely, lymphocytic polyps (Hellquist 1997). Other cells such as mast cells, basophils, macrophages and dendritic cells are also present; fibrous or glandular polyps are uncommon.

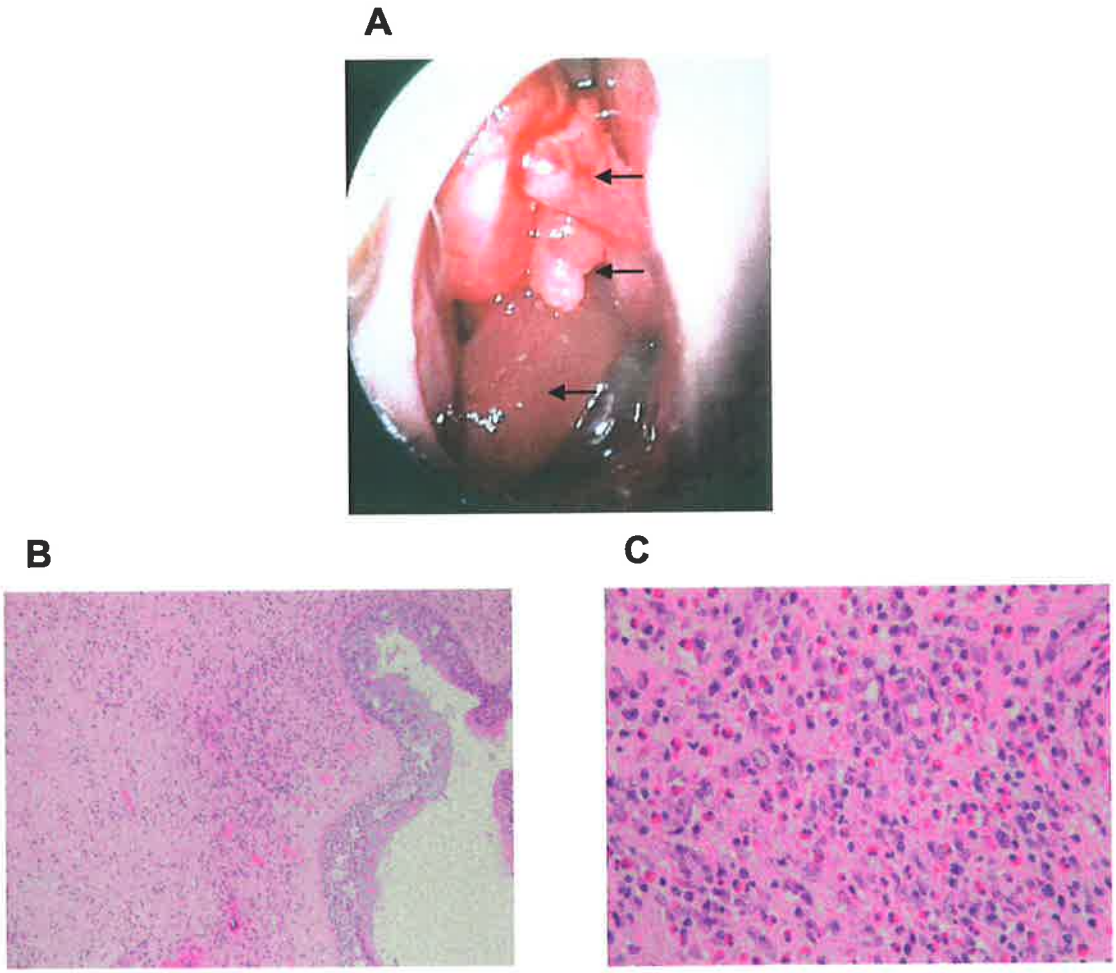


Figure 1.2 Sinonasal polyps in a patient with allergic fungal sinusitis. Extensive polyps (arrows) are seen inside the right nostril (A). Haematoxylin and eosin stained sections of a polyp showing respiratory epithelial lining and inflammatory cells (B) that are composed of principally eosinophils and lymphocytes (C). Courtesy of Professor PJ Wormald (A) and Dr J Pierides (B and C), Queen Elizabeth Hospital, South Australia.

Benign sinonasal polyps may be found in association with a number of systemic diseases (Table 1.2). The pathogenesis of polyps is unknown. The different histological types may represent a heterogeneous pathogenesis or alternatively "snap-shots" during the natural history of a polyp. Regardless, it is the inflammation and not polyps *per se* that is of primary

pathological focus. Sinonasal polyps offer a spectrum of clinical consequences ranging from asymptomatic to extensive mucosal involvement that may result in facial deformities. The latter are a recognised feature of allergic fungal sinusitis.

Table 1.2 Medical conditions associated with sinonasal polyps

Adult asthma
Intrinsic
Extrinsic
Allergic fungal sinusitis
Aspirin intolerance
Childhood asthma/rhinitis
Churg-Strauss syndrome (asthma, fever, eosinophilia)
Cystic fibrosis
Kartagener's syndrome (bronchiectasis, sinusitis, situs inversus)
Young's syndrome (sino-pulmonary disease, azoospermia)

Modified from Settipane 1997.

1.6 ALLERGIC FUNGAL SINUSITIS OR ALLERGY, FUNGI AND SINUSITIS?

Allergic fungal sinusitis is a descriptive term for an important subset of chronic rhinosinusitis patients with nasal polyps (Figure 1.3).

1.6.1 Historical background

The clinical features of nasal obstruction, hard and blood-tinged nasal casts, positive *Aspergillus fumigatus* cultures of sinus contents and nasal polyps were first described and

published as a case report in a patient suspected to be suffering from allergic bronchopulmonary aspergillosis (ABPA) (Safirstein 1976). Although chronic rhinitis (Katzenstein et al. 1975) and "nasal plugs" (McCarthy and Pepys 1971) in association with allergic bronchopulmonary aspergillosis were documented earlier, symptomatic sinonasal disease had not. It wasn't until 1981 when the first analogy of the sinus contents to the mucus plugs found in the lungs of patients with allergic bronchopulmonary aspergillosis was made (Lamb et al. 1981). These authors described the classical histopathological appearance of the sinus "inspissated debris" from five patients with allergic bronchopulmonary aspergillosis that comprised of mucus, proteins, cellular debri, eosinophils, Charcot-Leyden crystals and variable amounts fungal hyphae. *Aspergillus fumigatus* was cultured in one patient and the authors proposed that these cases represented "allergic aspergillosis" of the paranasal sinuses.

In 1983, a retrospective review of surgical specimens from the sinuses of patients with chronic rhinosinusitis demonstrated histological features indistinguishable from mucoid impaction found in the lungs of patients with allergic bronchopulmonary aspergillosis (Katzenstein et al. 1983a). This mucoid material was then termed "allergic mucin". Fungal hyphae resembling those of *Aspergillus* species was present within the mucus and there was no evidence of invasion in the mucosa. This sinus disease was defined as a distinct pathological entity from allergic bronchopulmonary aspergillosis and the term "allergic *Aspergillus* sinusitis" (AAS) was introduced in the literature. Further studies established that fungal culture of the sinus mucus specimens yielded fungi other than from the *Aspergillus* species and hence, the term "allergic fungal sinusitis" (Robson et al. 1989).

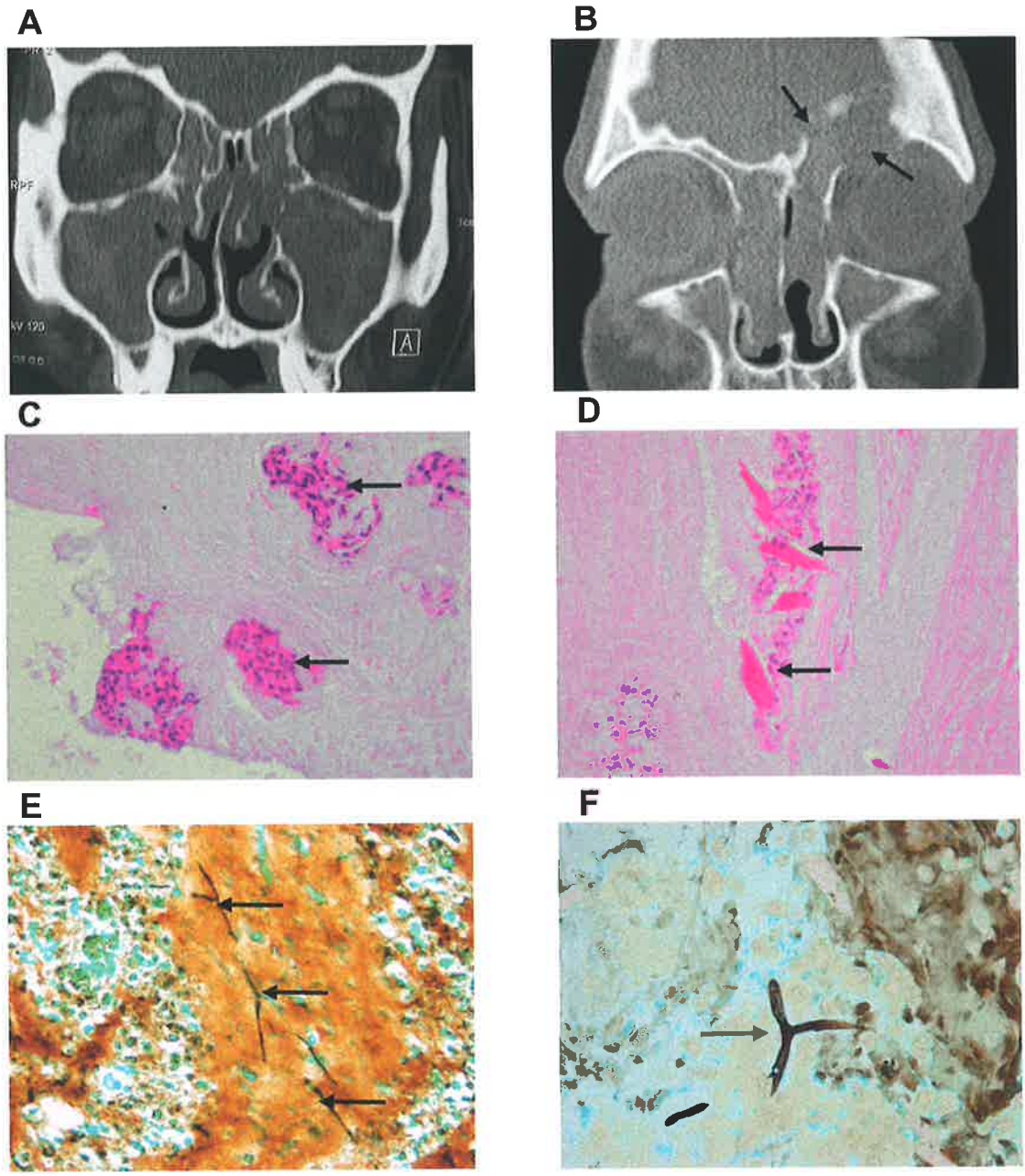


Figure 1.3 Sinus computed tomography (CT) and eosinophilic mucus histology from an allergic fungal sinusitis patient. Extensive maxillary and ethmoid sinus mucosal thickening and double densities (A) with bone erosion and pseudo-invasion of sinus contents into the orbit and brain (B) are apparent on coronal CT sections. Eosinophil clusters (C) and Charcot-Leyden crystals (D) are seen in the mucus sections stained with haematoxylin and eosin. Fungal hyphae are evident with methenamine silver stain (E and F). Courtesy of Professor PJ Wormald (A and B) and Dr J Pierides (C, D, E and F).

That allergic fungal sinusitis only existed since first recognised in the late 1970s is unlikely. "Granulomatous fungal sinusitis" caused by *Aspergillus* species (Wright 1927) and "chronic invasive fungal sinusitis in an immune-competent host" (Stringer and Ryan 2000) had been described, followed by numerous reports from North Africa, India and the Middle East. These patients were reported to have nasal polyps, thick, coloured mucus and fungi within mucus and tissue. Eosinophils often surrounded the fungi in the tissue and this was described as a granulomatous reaction (deShazo et al. 1997; Stringer and Ryan 2000). Therefore, it is a distinct possibility that allergic fungal sinusitis may have been diagnosed as chronic invasive fungal disease (Ferguson 2000b; Stringer and Ryan 2000).

1.6.2 Eosinophilic mucus

Eosinophilic mucus is a histopathological diagnosis made following examination of paraffin-embedded mucus specimens stained with haematoxylin and eosin. Eosinophilic mucus is also known as "allergic" or "fungal-like" mucin. "Mucin" is a misnomer as it refers to "any group of protein containing glyco-conjugates with high sialic acid or sulfated polysaccharide content that compose the chief constituent of mucus" (Anderson et al. 1994).

Eosinophils are the predominant and a consistent cellular component that defines eosinophilic mucus. Eosinophil breakdown products known as Charcot-Leyden crystals, sloughed respiratory epithelial cells and debris are also present (Katzenstein et al. 1983b). Macroscopically, eosinophilic mucus is typically thick, viscous to almost solid and coloured and it is often described as being of "axle grease", "peanut butter" or "cottage cheese" – like consistency (Marple 2001).

1.6.3 Diagnostic criteria for allergic fungal sinusitis

There is still controversy regarding the diagnostic criteria for allergic fungal sinusitis. The generally accepted definition is that put forward by Bent and Kuhn and encompasses: *(a)* eosinophilic mucus, *(b)* positive fungal stain of sinus contents, without fungal invasion into sinus tissue, *(c)* nasal polyposis, *(d)* characteristic radiological signs and *(e)* type I hypersensitivity to fungi (Bent and Kuhn 1994).

However, there have been several variations to this definition. In some, the macroscopic appearance of eosinophilic mucus is sufficient for diagnosis (deShazo and Swain 1995; Collins et al. 2003) while other definitions consider positive fungal cultures from either nasal (Ponikau et al. 1999) or sinus (Collins et al. 2003) mucus in the absence of fungal elements adequate. Some definitions dispute the need to demonstrate type I hypersensitivity to fungi (deShazo and Swain 1995; Ponikau et al. 1999). Recently, a multidisciplinary, international panel re-defined allergic fungal sinusitis as "histological confirmation of eosinophilic mucus and the presence of type I hypersensitivity to fungi in patients with chronic rhinosinusitis" (Meltzer et al. 2004).

The differences in definitions have arisen because the pathogenic importance of fungi and type I hypersensitivity to fungi in allergic fungal sinusitis is uncertain. The plethora of definitions of "allergic fungal sinusitis" makes it difficult to make direct comparisons between published studies and has hindered progress in understanding this disease.

1.7 CLINICAL CHARACTERISTICS OF ALLERGIC FUNGAL SINUSITIS

1.7.1 Demographics

Allergic fungal sinusitis, defined by the presence of "allergic mucin with fungal forms noted histologically or by allergic mucin with positive fungal cultures" (deShazo and Swain 1995; Ponikau et al. 1999) affects adolescents and young adults, with a mean age at diagnosis of approximately 22 years (Manning and Holman 1998) or 31 years (Ferguson 2000a). Men and women are equally affected. As definitive diagnosis can only be made by examination of surgical specimens, the true prevalence of allergic fungal sinusitis is unknown. However, based on the criteria of Bent and Kuhn, studies show that allergic fungal sinusitis affects 4–10% of patients undergoing sinus surgery (Collins et al. 2003).

1.7.2 Clinical profile

Allergic fungal sinusitis typically affects immune-competent individuals, as obvious immune disease is absent (Pratt and Burnett 1988). As reviewed by Marple 2001, the clinical presentation is of a long history of nasal obstruction, hyposmia or anosmia and of blowing out nasal casts. Nasal polyps are present in up to 100% and extra-sinus complications may be observed in a proportion of patients. The inflammatory cell component of the mucosal inflammation is characterised by eosinophils (Feger et al. 1997; Khan et al. 2000).

By definition, type I hypersensitivity to fungi is present, although not necessarily to the same fungal species identified in the eosinophilic mucus. In many cases, fungal allergy to more than one genera and species is present (Mabry et al. 1999), a common feature for fungal allergies in humans (Horst et al. 1990). Allergy to non-fungal allergens is also

common (Mabry et al. 1997; Folker et al. 1998; Collins et al. 2004). Depending on the study and definition of "allergic fungal sinusitis", total serum IgE levels, peripheral blood eosinophil count, serum eosinophil cationic protein, erythrocyte sedimentation rate and C reactive protein may be elevated (Ferguson 2000a), but none of these changes are consistent. As reviewed by Marple 2001, serum *Aspergillus* precipitins have been reported, but are more often negative. Other conditions that may coexist include asthma and aspirin sensitivity. Coexisting allergic fungal sinusitis and allergic bronchopulmonary aspergillosis is uncommon (Bhagat et al. 1993; Shah et al. 2001). However, many patients with allergic fungal sinusitis also have significant lower respiratory tract symptoms but do not fulfil the diagnostic criteria for allergic bronchopulmonary aspergillosis, including IgE levels greater than 1000 kU/L and bronchiectasis. Whether the lower respiratory tract disease in allergic fungal sinusitis patients represents a different disease process from allergic bronchopulmonary aspergillosis, is speculative.

The common fungi identified in the eosinophilic mucus are from the dematiaceous family and *Aspergillus* species (Marple 2001). Dematiaceous fungi include organisms from the genera *Alternaria, Bipolaris, Cladosporium, Curvularia, Drechslera* and *Helminthosporium.* These fungi are darkly pigmented due to dihydroxynapthalene melanin in the cell walls of the hyphae or conidia (Dixon and Polak-Wyss 1991). Typically, fungal invasion of the sinus mucosal tissue is not observed, however fungi in the sinus mucosa and bone have been documented recently (Thakar et al. 2004). Radiological examination of the sinuses with computed tomography (CT) characteristically shows areas of heterogeneous signal intensity within the affected sinuses, known as double density appearance (Mukherji et al. 1998). Double densities are due to accumulations of heavy metals including iron, manganese and calcium salt precipitation, typically associated with fungi. Expansion of the sinus cavity with

thinning of the sinus walls and bone erosion with extension of sinus disease into adjacent sites may also be observed (Nussenbaum et al. 2001). However, these computed tomography appearances are not specific for allergic fungal sinusitis.

1.7.3 Treatment and prognosis

At present, there is no long-term successful treatment for allergic fungal sinusitis. As reviewed by Marple and Mabry 1998, Schubert 2000 and Marple 2001, combinations of surgical and medical treatments are used to manage complications, provide symptomatic relief from extensive nasal polyps and to delay recurrences. Corticosteroids are the most effective agent in preventing recurrences and in selected cases used as first line treatment. Topical antifungal treatment and immunotherapy are also used although their clinical efficacy remains to be proven.

These treatments are associated with significant adverse effects. Extensive disease, sinonasal bone remodelling and bleeding increase the risk of surgical complications. Similarly, corticosteroid treatment is associated with serious adverse effects that may occur in a dose-dependent or an idiosyncratic manner (Haynes 1991).

Not withstanding the treatments, the recurrence rate of nasal polyps and eosinophilic mucus is extremely high (Schubert and Goetz 1998). Hence, there is a clear need for a better understanding of this disease and for the development of alternative therapies.

1.8 ALLERGIC FUNGAL SINUSITIS AND ALLERGIC BRONCHO-PULMONARY ASPERGILLOSIS: STRIKING SIMILARITIES AND OBVIOUS DIFFERENCES

Allergic bronchopulmonary aspergillosis was described in 1952 by Hinson et al. as a condition of the airways characterised by a prominent eosinophil and lymphocyte population in the tissue, increased levels of serum IgE and precipitating antibodies to *Aspergillus* antigens. As for allergic fungal sinusitis, the prevalence of allergic bronchopulmonary aspergillosis remains highly speculative. Depending on the study, the prevalence of allergic bronchopulmonary aspergillosis varies between 2-38% for asthmatic patients and between 35-81% for cystic fibrosis patients (Crameri 2002).

Eosinophilic mucus, eosinophils and lymphocytes in the mucosa together with fungal elements and type I hypersensitivity are present in allergic bronchopulmonary aspergillosis and allergic fungal sinusitis. Similar to allergic bronchopulmonary aspergillosis (Riley et al. 1975; Slavin et al. 1988), fungi in the tissues have been observed in allergic fungal sinusitis patients (Thakar et al. 2004). Corticosteroids are also the mainstay of medical therapy (Safirstein et al. 1973; Marple 2001). However, in allergic fungal sinusitis, fungi other than *Aspergillus* appear to be common, *Aspergillus* precipitins are not usually present and the total IgE level is often normal or only slightly elevated.

Allergic bronchopulmonary aspergillosis is thought to result from a hypersensitivity immune response to *Aspergillus fumigatus* antigens and is associated with *Aspergillus* hyphae in the lower respiratory tract (Greenberger 2002). It is typically seen in patients with cystic fibrosis and asthma. It is unclear from the literature whether other fungal species are isolated from

the lungs of these patients. Indeed, sputum samples from patients with cystic fibrosis yielded moulds other than *Aspergillus fumigatus* (Bakare et al. 2003).

Cystic fibrosis is a genetic disorder caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene that encodes the amino acid sequence for an integral membrane protein in epithelia (Knowles et al. 1983). Cystic fibrosis transmembrane conductance regulator protein functions as a cyclic adenosine monophosphate (cAMP) regulated chloride ion channel (Stutts et al. 1995). Abnormal function of this ion channel in the respiratory system results in abnormal mucus production, thickened secretions and predisposes to recurrent infections. More than 1000 mutations of the CFTR gene are identified. Together with environmental factors and polymorphisms or non-CFTR genetic modifiers, may in part explain the intra-genotype variation in the clinical phenotype of cystic fibrosis (Wang et al. 2000; Noone and Knowles 2001; Wallace et al. 2003). Allergic bronchopulmonary aspergillosis in these patients, with fungal growth in the respiratory tract could be a consequence of mucosal immune-compromise.

Total IgE levels and *Aspergillus*-specific IgE, IgG and IgA are elevated in patients with allergic bronchopulmonary aspergillosis (Brummund et al. 1987). Although type I hypersensitivity to fungi is proposed to be the main immunopathological mechanism in allergic bronchopulmonary aspergillosis and allergic fungal sinusitis, it remains to be explained why not all patients with fungal allergy develop these severe clinical phenotypes. Similar to allergic fungal sinusitis, there is no direct evidence that fungal allergy is a central pathogenic factor in allergic bronchopulmonary aspergillosis.

Examination of *Aspergillus*-specific peripheral blood mononuclear cells from allergic bronchopulmonary aspergillosis patients has shown enhanced $CD4^+$ T cell proliferation. Cytokine studies have shown either a "type 1" or "type 2" profile (section 1.15) depending on the fungal antigen extract used (Kurup et al. 1996). These studies show an enhanced systemic immune response to fungi in allergic bronchopulmonary aspergillosis. This fungal-specific immune response may be aggravating and maintaining the underlying pathological process, be it either cystic fibrosis or asthma.

1.9 EOSINOPHILIC MUCUS CHRONIC RHINOSINUSITIS

Patients with chronic polypoid rhinosinusitis and sinus eosinophilic mucus who do not have fungal elements in the sinus mucus or type I hypersensitivity to fungi have been recognised since the first description of "allergic *Aspergillus* sinusitis" (Katzenstein et al. 1983a and b). Ferguson in 2000a reviewed several patient groups and concluded that allergic fungal sinusitis patients were clinically and pathologically distinct from other patients with eosinophilic mucus, termed "eosinophilic mucin rhinosinusitis" (EMRS). However, this review was based on a retrospective analysis of studies in which the definitions of allergic fungal sinusitis were inconsistent. Currently, it is unclear whether chronic rhinosinusitis patients with eosinophilic mucus but no fungal elements or fungal allergy represent a different clinical and pathological entity from allergic fungal sinusitis.

Reviewing the allergic fungal sinusitis literature, it appears that eosinophilic mucus is the only consistent feature correlating with severe sinus disease. However, fungal allergy and fungi may be demonstrated in patients with non-polypoid sinus disease, patients with allergic rhinitis without sinusitis and in healthy individuals. The pathological and clinical significance of fungal allergy and of fungi in allergic fungal sinusitis is presently unknown.

Therefore, in this project, all patients with chronic rhinosinusitis and eosinophilic mucus have been included under one term, eosinophilic mucus chronic rhinosinusitis (EMCRS). The EMCRS patients fulfil the following clinical subclassifications documented in the literature:

1. Allergic fungal sinusitis, where fungal allergy is present and fungi are detected in eosinophilic mucus (Bent and Kuhn 1994; Ferguson 2000a).

2. Allergic fungal sinusitis-like, where fungal allergy is present but fungi are not detected in eosinophilic mucus (Collins et al. 2004).

3. Non-allergic, fungal eosinophilic sinusitis (NAFES) (Ferguson 2004) or chronic fungal sinusitis (CFS) (Collins et al. 2004), where fungal allergy is absent and fungi are detected in eosinophilic mucus.

4. Non-allergic, non-fungal, eosinophilic sinusitis (NANFES) or chronic eosinophilic sinusitis (CES) (Collins et al. 2004), where fungal allergy is absent and fungi are not detected in eosinophilic mucus.

1.10 ROLE OF FUNGI IN THE MUCOSAL INFLAMMATION IN EOSINOPHILIC MUCUS CHRONIC RHINOSINUSITIS

Pathologically, fungi may affect humans in at least three ways: (*a*) by causing hypersensitivity, (*b*) by their toxic effects or (*c*) by invasive infection (Hawranek 2002). Except invasive fungal infections, it is often difficult to attribute fungi as a cause of a disease. This is partly due to the ubiquitous nature of fungi, its variable morphology, antigenic cross-reactivity between species and the heterogeneous and changing antigenic

profile within a single fungal species (Horner et al. 1995; Kurup et al. 2002; Bisht et al. 2002; Gupta et al. 2002).

1.10.1 Fungal allergy

Type I hypersensitivity to fungi has been linked to the pathogenesis of allergic fungal sinusitis. In reviewing the literature, depending upon the definition of allergic fungal sinusitis, up to 100% of patients have fungal allergy (Manning et al. 1993; Manning and Holman 1998). However, it is well established that fungal allergy is not present in every chronic rhinosinusitis patient with sinus eosinophilic mucus and, fungal allergy may be present in healthy volunteers who have no evidence of sinonasal disease (deShazo and Swain 1995; Ponikau et al. 1999; Braun et al. 2003). In addition, when present, fungal allergy may not be to the same fungal species identified in the sinus eosinophilic mucus. Furthermore, as reviewed by Slavin 1997, epidemiological studies show a poor correlation between the prevalence of allergy and nasal polyps. Fungal allergy is estimated to affect 3-10% of adults and children world-wide (Horner et al. 1995). Accordingly, fungal allergy in allergic fungal sinusitis patients may represent the prevalence of fungal allergy in the general population.

In the absence of systemic fungal allergy, local mucosal allergy to fungi is suggested to mediate the inflammation in allergic fungal sinusitis (Collins et al. 2004). As reviewed by Smurthwaite and Durham 2002, there is good evidence of local sinonasal and lung mucosal IgE synthesis. However, mucosal studies from chronic rhinosinusitis have generated inconsistent results. Sanchez-Segura et al. 2000 showed that most nasal polyps do not contain IgE secreting plasma cells. By contrast, previous reports have demonstrated IgE in the nasal and polyp tissue; while a study showed good correlation between IgE in nasal

tissue and serum IgE levels in allergic patients (Whiteside et al. 1975), another showed that specific IgE was not present in the mucosa of every fungal-allergic patient tested (Collins et al. 2004). A comparison between allergic and non-allergic chronic rhinosinusitis patients have shown no significant differences in total IgE or antigen-specific IgE in the mucosa (Drake-Lee and Barker 1984; Liu et al. 1994). However, mucosal studies in allergic and non-allergic rhinitis patients showed that IgE-positive cells and mast cells are increased compared with asymptomatic controls (Pawankar et al. 1996; Powe et al. 2001). Interestingly, cell-bound IgE is also present in the upper airway and gut mucosa in asymptomatic individuals (Stallman et al. 1977; O'Donoghue et al. 1979). These inconsistent findings may be due, in part, to the functional diversity of mast cells and antigen-specific IgE, as they also mediate non-allergic antigen-specific and non-specific homeostatic and defense functions in the mucosa. Mast cells can be activated directly by pathogens via toll-like receptors (Marshall et al. 2003a). Mast cells and IgE are also implicated in viral and bacterial infections (Alho et al. 2003; Marshall et al. 2003b) and in autoimmune diseases including multiple sclerosis and rheumatoid arthritis (reviewed in Robbie-Ryan and Brown 2002). Monomeric IgE may bind to FcεRI without resulting in degranulation and increase mast cell survival (reviewed in Saini and MacGlashan 2002). In addition, several subsets of mast cells may be involved in allergic and non-allergic inflammatory responses (Beil et al. 2002). Thus, in the absence of systemically detectable allergen-specific IgE or a positive skin prick test, the pathogenic importance of local IgE in allergen-specific hypersensitivity responses is yet to be proven.

1.10.2 Invasive and toxic fungal disease

The demonstration of fungi in the sinuses by histology or culture is considered pathologically important in allergic fungal sinusitis (Bent and Kuhn 1994). However, given

the ubiquitous nature of fungi (Miller 1992), it is clear that using a sensitive enough technique, fungi may be detected in all sinonasal mucus samples from patients and controls, which makes detection of non-invasive fungi *per se* of limited pathological value (Ponikau et al. 1999; Catten et al. 2001; Taylor et al. 2002; Braun et al. 2003; Buzina et al. 2003). Fungal involvement in allergic fungal sinusitis is typically described as noninvasive, although fungal elements in the polyp tissue have been documented (Thakar et al. 2004). Except for their detection in the sinuses, the effect of fungi on the sinonasal mucosa and fungal-specific immune responses have not been examined thoroughly in allergic fungal sinusitis patients. Hence, the role of fungi in this disease is speculative.

Innate and adaptive immune responses are involved in providing defense against fungal organisms in the respiratory tract (Crameri and Blaser 2002). The first line of defense is the mucociliary clearance system, which can be modulated by secondary metabolites from fungi and other microorganisms (Amitani et al. 1995). Spores and mycelia are ingested and killed by mononuclear and polymorphonuclear phagocytes (Schaffner et al. 1982). Each line of defense is able to protect the host against large conidial inoculums over a long period of time. Studies with *Aspergillus* species have identified several virulence factors (Tomee and Kauffman 2000), but only if all host defense lines are overcome, can *Aspergillus* cause invasive disease (Schneemann and Schaffner 1999). Thus, "fungal invasion" might be a consequence of ongoing inflammation and mucosal immune-compromise in allergic fungal sinusitis rather than a different disease altogether.

1.11 THE APPROACH TO EXAMINING THE MUCOSAL INFLAMMATION IN CHRONIC RHINOSINUSITIS

Chronic rhinosinusitis is a complex clinical problem that is likely to be a consequence of numerous proinflammatory factors where the immunopathological process may continue even after the elimination of the triggering agent. Whereas a continued search for a particular organism or antigen that causes mucosal inflammation is an important goal, the advent of models of mucosal inflammation that collectively show that mucosal inflammation is associated with inherent immune defects in the face of an unaltered flora, indicates that this goal may prove futile (Strober et al. 2002).

Following a review of the allergic fungal sinusitis literature, several possibilities exist regarding the role of fungi and fungal allergy in this disease. These include: a primary fungal cause, an exacerbation of an underlying mucosal disease by coexisting fungal allergy or fungal toxins and fungal colonisation of already diseased sinuses. In order to understand the pathogenesis of allergic fungal sinusitis, a systematic examination of the basic inflammatory processes and of the key regulatory cells in the sinonasal mucosa is necessary.

1.12 SINONASAL MUCOSAL IMMUNE SYSTEM

The mucosal system is composed of a complex cellular and molecular network that is involved in organ-specific functions including respiration, absorption and immune functions (Macdonald 2003). This system is a unique environment that is constantly challenged by immunogenic and physical insults. The sinonasal immune system is functionally and phenotypically distinct from the peripheral immune system. The cellular components include the resident stromal cell populations and dynamic populations of cells from the myeloid and lymphoid lineage. Together, these constitute the physicochemical barrier and

are involved in cellular responses to immunogens and pathogens. The immune system needs to provide regulated active immune protection that is rapid and potent, while being compatible with commensal organisms and maintaining the integrity of the mucosal barrier (Veazey and Lackner 2003). Little is known about the sinonasal mucosal system. Although it may seem reasonable to extrapolate from one mucosal system to another, distinct differences in organogenesis of the various mucosal associated lymphoid tissues (MALT) (Harmsen et al. 2002) and differential expression of ligands for leucocyte homing receptors (Csencsits et al. 1999) from murine and human studies emphasise the "organ-specific" and "species-specific" nature of these interactions and that immune responses may be independently regulated and compartmentalised.

1.12.1 Physicochemical barrier

The respiratory epithelium lines most of the non-olfactory regions of the nose and the sinuses and is continuous with the lower respiratory tract (Bannister et al. 1995). The respiratory epithelium consists of four major cell types: ciliated columnar or cuboidal epithelial cells interspersed with goblet cells, non-ciliated columnar cells with microvilli and basal cells. The "normal" respiratory epithelium often shows scattered areas of metaplastic squamous or cuboidal epithelium. The cells contain tight junctions and rest on a basement membrane composed principally of collagen fibres (types I, III, IV, V, VI and VII) and other constituents that include heparan sulfate proteoglycan, laminin and nidogen (Agha-Mir-Salim et al. 1993). Beneath the basement membrane, the submucosa overlying the cartilage and bony sinonasal framework contains seromucinous glands, blood vessels, nerves, stromal and lymphoid tissue. Compared with the nasal cavity, the paranasal sinuses have a thinner, less specialised surface epithelium and lamina propria (Tos and Morgensen 1984). These differences in the structural and cellular components between the sinus and nasal mucosa

may reflect their different embryological origins and functional differences (Ali et al. 2002), although the physiological implications are not clearly understood (Matthias et al. 1997).

The epithelial surface is covered by mucus produced by goblet cells, submucous glands and ciliated cells. Goblet cells are stimulated by direct contact with physical and chemical stimuli whereas the submucous and seromucinous glands are typically stimulated by muscarinic (M) 1 and M3 receptor activation (Mullol 1992; Wanner 1997). Mucus is primarily composed of mucus glycoproteins, known as mucins, which are responsible for its gel-like property. The type of mucin gene expression differs between areas of the nasal cavity, the sinuses (Ali et al. 2002) and in inflammatory states (Kim et al. 2004). On the epithelial surface, mucus forms two main phases, inner sol and outer gel phase. A fine layer composed of surfactant proteins may also be present. The outer mucus layer is exposed to the external environment and traps airborne particles whereas the inner layer assists in ciliary function. Cell-bound mucins may provide attachment sites for microbes and are subsequently shed into the mucus layer. Mucus is actively propelled by the cilia towards the openings of the sinuses, enabling its drainage into the nasal cavity.

Other components of mucus that provide defense against pathogens include enzymes (lysozymes, lactoferrin), antimicrobial peptides (defensins, cathelicidins), complement proteins, C-reactive protein, immunoglobulins (IgG, IgA, IgM and IgE) and cells including leucocytes and epithelial cells (Quraishi et al. 1998). As reviewed by Fagarasan and Honjo 2004, the regulation of mucosal immunoglobulin production and function is distinct from elsewhere in the body. For example, B lymphocyte development and class switching occurs independently of T cell help and those that are T lymphocyte dependent, require different molecular interactions from systemic immunoglobulin production.

1.12.2 Lymphoid compartments

The mucosa-associated lymphoid tissue (MALT) (nasopharyngeal, palatine, tubal and lingual tonsils) surrounding the openings into the aero-digestive tracts that constitute the Waldeyer's ring, the lymph nodes in the neck and retropharyngeal areas are well described (Bannister et al. 1995). The lymphoid compartment in the sinonasal mucosa is comprised of single lymphocytes scattered among the epithelial cells and lamina propria, and the nasal-associated lymphoid tissue (NALT) (Debertin et al. 2003). The NALT are discrete un-encapsulated aggregates of lymphoid cells, akin to that in the mucosa-associated lymphoid tissue in the gut known as Peyer's patches (Bannister et al. 1995).

1.13 CHARACTERISATION OF THE MUCOSAL IMMUNE CELLS

The mucosal immune cells include lymphoid, myeloid and stromal cells. These cells may be defined by the nature of the immune response that they are typically involved in. An immune response of a specific nature is also known as an *adaptive* immune response and that of a non-specific nature is known as an *innate* immune response. The adaptive immune response is distinguished from the innate response by the following characteristics: specificity of antigen recognition, diversity of the antigen receptor repertoire, rapid clonal expansion, adaptation to the changing microenvironment and immunological memory (Roitt 1996). The lymphocyte population is composed of T cells, B cells, natural killer (NK) cells and natural killer T (NKT) cells. These cells form an integral component in the adaptive and innate immune responses. Monocytes, macrophages, dendritic cells, granulocytes, mast cells, epithelial and stromal cells typically mediate non-specific responses but may also modulate antigen-specific immune responses.

Antigen specificity is conferred by antigen-specific receptors and is the hallmark of an adaptive immune response (Sallusto et al. 2004). Typically, T and B lymphocytes are involved in adaptive immune responses by virtue of the T cell receptor and the membrane-bound immunoglobulin, the B cell receptor (BCR), respectively. The T cell receptor is a dimer of two disulphide-linked polypeptides. There are two well-defined types of T cell receptors, αβ and γδ subunits. In the peripheral blood, approximately 90% of the T cell receptors are of the αβ configuration and the remainder, γδ. In the peripheral tissues, including the gut mucosa and skin, up to 30% of the T cell receptors express the γδ subunit (Carding and Egan 2002). The T cell receptor is linked to the CD3 molecule to form the T cell receptor complex.

T lymphocytes are divided into two major subsets based on their ability to bind antigenic peptides presented by antigen presenting cells in association with the MHC molecule. The CD4 coreceptor on T lymphocytes specifically binds the MHC class II molecule and the CD8 coreceptor binds the MHC class I molecule. The CD4 molecule is a monomeric protein with four immunoglobulin-like domains and the CD8 molecule is composed of α and β subunits (CD8αβ). In the gut mucosa, a large proportion of $CD8^+$ T cells coexpress CD8αα (Gangadharan and Cheroutre 2004). The expression and upregulation of CD8αα phenotype may occur on any activated T cell. The CD8αα coreceptor is thought to modulate specific T cell receptor activation signals that facilitate T cell survival and differentiation into various specialised subsets.

Natural killer cells and natural killer T cells are lymphocyte populations of the innate immune response (Yokoyama et al. 2004). Natural killer cells are capable of lysing target cells without prior sensitisation. They are distinguished from T or B cells by an absence of T

cell receptor or B cell receptor, respectively, and by the expression of the NK molecules. Natural killer T cells express a semi-invariant T cell receptor, where the α chain configuration is highly conserved (Vα24-Jα18) and is coexpressed with the Vβ11 TCR β chain (Godfrey et al. 2004). Natural killer T cells may be $CD4^+$, $CD8^+$ or negative for CD4 and CD8 molecules (double negative). Natural killer T cells express a phenotype of effector or memory lymphocytes before encounter with any foreign antigen and may express the NK molecule. These features suggest that NKT cells may respond to conserved endogenous as well as the foreign antigens (Bendelac et al. 2001). They are restricted to CD1d-dependent antigen recognition and are primarily potent cytokine secretors including interleukin (IL) – 4 and γ-Interferon. CD1 molecules (CD1 a-e genes) are non-polymorphic evolutionary conserved antigen presenting molecules that appear capable of presenting lipid and glycolipid antigens (Brigl and Brenner 2004). Recently, CD1d-independent NKT cells called NKT-like cells have also been described in humans (Godfrey et al. 2004).

1.14 MUCOSAL IMMUNE RESPONSES

In the sinonasal mucosa, exogenous antigens are sampled by dendritic cells across the pseudostratified epithelium and by specialised membranous (M) cells overlying NALT across the simple epithelium (Karchev and Kabakchiev 1984; Neutra et al. 1996) (Figure 1.4). Pathogen recognition by the mucosal cells generally occurs by toll-like receptors that recognise conserved molecular signatures of pathogens commonly known as pathogen-associated molecular patterns (PAMP) (reviewed in Takeda et al. 2003; Akira and Takeda 2004; Mazzoni and Segal 2004). Antigen recognition by dendritic cells, macrophages, epithelial cells, B cells, T cells and eosinophils may occur in a non-specific manner via toll-

like receptors (reviewed in Armant and Fenton 2002) and in an antigen-specific manner via immunoglobulin Fc receptors.

In the NALT system, there is close association with the M cell by phagocytes, antigen presenting cells and lymphocytes. Immature dendritic cells phagocytose antigen and undergo activation-maturation, interact with mucosal lymphocytes and gain the ability to migrate to and interact with lymphocytes in the draining lymph nodes (Vermaelen et al. 2001). Mature dendritic cells typically present exogenously derived peptide antigen to $CD4^+$ T lymphocytes in association with the MHC class II molecules. Endogenously produced proteins, such as self-proteins and intracellular pathogens, are combined with the MHC class I molecule whereas lipids and lipid derivatives are combined with CD1 molecules and presented to $CD8^+$ T lymphocytes (Rosat et al. 1999).

Depending on the antigen, the microenvironment and the nature of the immune response, $CD4^+$ and $CD8^+$ T lymphocytes mediate and modulate adaptive and innate immune responses by direct cell-cell contact and by producing cytokines which provide molecular signals to other cells. Typically, $CD4^+$ T lymphocytes differentiate into predominantly cytokine secreting cells that mediate help to B cells for antibody production and modulate other immune responses whereas $CD8^+$ T lymphocytes differentiate into cytotoxic cells (Roitt 1996). Both T lymphocyte populations are capable of modulating the innate immune system by recruiting and modifying the function of granulocytes including eosinophils and neutrophils, mast cells, monocytes, basophils, antigen presenting cells, stromal cells, epithelial cells, endothelial cells, nerve cells and mucus producing cells.

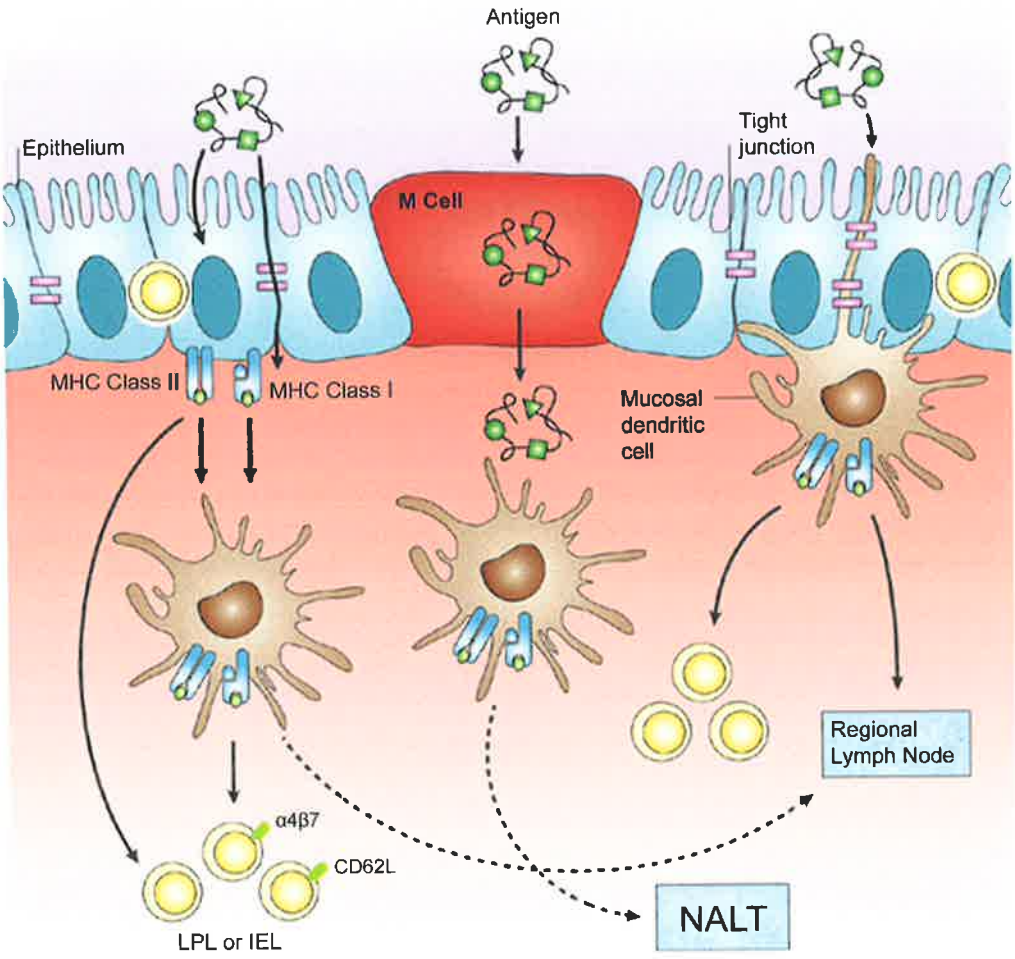


Figure 1.4 Schematic representation of antigen uptake in the sinonasal mucosa. M cells and epithelial cells mediate active uptake of antigen and present processed antigen bound to major histocompatibility complex (MHC) class I or class II molecules to mucosal dendritic cells, intraepithelial lymphocytes (IEL) and lamina propria lymphocytes (LPL). The mucosal dendritic cells can also directly sample antigen. NALT, nasal-associated lymphoid tissue. Adapted from Cheroutre and Madakamutil 2004.

Regulation of the antigen-activated immune response is critical to prevent damage to normal, uninvolved or uninfected cells in the vicinity of the immune attack (reviewed in Jiang and Chess 2004). Therefore, in addition to generating antigen-specific immune responses, immune regulation is a vital function of lymphocytes. The mechanisms involved in immune regulation include those intrinsic to antigen activation and differentiation and those mediated by suppressor, also known as regulatory, subsets of lymphocytes (Rouse and Suvas 2004).

The intrinsic immune regulatory mechanisms are mediated by changes in the T cell receptor affinity, apoptosis, antigen-induced cell death and differentiation of antigen-specific T lymphocytes into subsets expressing different arrays of cytokines (reviewed in Jiang and Chess 2004). These cells are typically described as "type 1" cells defined by the secretion of γ-Interferon, "type 2" cells defined by the secretion of IL-4, IL-5 and IL-10 and "type 3" or Tr1 cells defined by the secretion of IL-10 or transforming growth factor-β (TGF-β). These subsets of antigen-specific T lymphocytes are postulated to regulate the differentiation and function of each other.

Distinct regulatory subsets of $CD4^+$ (Walker 2004; Fehervari and Sakaguchi 2004) and $CD8^+$ T lymphocytes (Sarantopoulos et al. 2004) and NKT cells (Godfrey et al. 2004) are also recognised as important regulators of mucosal immune responses. Immune suppression by $CD4^+$ T cells is shown to be a function of a subset of $CD4^+CD25^+$ T cell population, termed $CD4^+CD25^+$ T regulatory cells (Tregs). The $CD4^+$ Tregs represent a lineage-specific suppressor population arising from the thymus and are called naturally occurring $CD4^+CD25^+$ Tregs. However, $CD4^+CD25^+$ T cells with suppressor activity are also induced in inflammatory states, and are known as peripherally-derived or inducible or adaptive

$CD4^+CD25^+$ Tregs. The Tregs are currently differentiated from conventionally activated $CD4^+$ T lymphocytes by the expression of *Foxp3*. *Foxp3* encodes a fork head or winged-helix transcription factor known as scurfin that is expressed by naturally occurring $CD4^+CD25^+$ Tregs and in some situations, by peripherally-derived $CD4^+CD25^+$ Tregs (Jiang and Chess 2004).

1.15 MUCOSAL INFLAMMATION IN CHRONIC RHINOSINUSITIS

Eosinophils and lymphocytes are abundant in the mucosal inflammation in eosinophilic chronic rhinosinusitis (reviewed in Ferguson 2004), yet there are only few mucosal studies that have examined these cells. Eosinophils are a heterogeneous population of granulocytes with diverse phenotypes, pharmacology and effector functions (reviewed in Giembycz and Lindsay 1999). Eosinophils are common to inflammation of the mucosa and the skin in allergic (IgE and non-IgE-mediated) asthma, dermatitis and rhinosinusitis and in non-allergic inflammation including asthma, dermatitis, rhinosinusitis, oesophagitis (Arora and Yamazaki 2004), inflammatory bowel disease (Carvalho et al. 2003), and infections due to viruses (Gleich 2000), bacteria (Persson et al. 2001) and parasites (Klion and Nutmen 2004). As reviewed by Alam and Busse 2004 and demonstrated in murine models (Mehlhop et al. 1997), immunological mechanisms other than IgE-mediated allergy may recruit eosinophils into sites of inflammation. The role of eosinophils extend beyond their primarily phagocytic, anti-microbial effector functions to tissue remodelling and immunomodulation via antigen presentation and cytokine release, and more recently, postulated in anti-tumour activity (Weller et al. 1993; Shi et al. 2000; reviewed by Adamko et al. 2005). Differential eosinophil phenotypes (Lacy et al. 2001; Teixeira et al. 2001) and pattern of degranulation (Erjefalt et al. 2001) depending on the type of inflammatory response is also proposed. However, defining the nature of an inflammatory response by analysis of eosinophils alone

is currently limited, in part, due to technical reasons, as cell function and cell surface antigen expression may alter depending on isolation and identification strategies (Blom et al. 1995; Sedgwick et al. 1996; Lavigne et al. 1997; Casale et al. 1999).

T lymphocytes are integral to an immune response. Hence, their functional phenotypes including clonality, antigen specificity, level of cellular differentiation and effector functions can provide insight into the type of an immune response. To date, May 2005, there are no published studies examining the lymphocyte populations in allergic fungal sinusitis. Mucosal studies in patients with rhinitis and chronic rhinosinusitis show that regardless of clinical phenotype, including allergy or aspirin sensitivity, either more $CD4^+$ cells (Larocca et al. 1989; Linder et al. 1993) or more $CD8^+$ cells (Nishimoto et al. 1988; Stoop et al. 1989; Hameleers et al. 1989; Liu et al. 1994; Driscoll et al. 1996) are present compared with controls. In contrast, a study by van Rijswijk et al. 2003 showed no significant difference in these mucosal cell populations between rhinitis and healthy controls. The dynamism of the T cell populations was illustrated by a study showing either more $CD4^+$ or $CD8^+$ cells during various clinical stages of sinonasal inflammation (Stoop et al. 1992) and with treatment (Mastruzzo et al. 2003). Together, these findings emphasise the heterogeneous nature of the inflammatory response that may be uniform across the sinonasal mucosa. This uniformity was established by studies that showed no significant differences in the lymphocyte subsets in polyp and non-polyp sinonasal mucosa (Morinaka and Nakamura 2000) and that unilateral administration of topical corticosteroids also affected inflammatory cell populations on the contralateral sinonasal mucosa (Kondo et al. 1999).

Consistent with the general proinflammatory state, upregulation of molecules associated with leucocyte recruitment, angiogenesis and remodelling have been documented in

polypoid chronic rhinosinusitis mucosa (reviewed by Bachert et al. 1998; Bachert et al. 1999; Mygind et al. 2000). As reviewed by Bachert et al. 2002, cytokines, including the "type 1" and "type 2" profile, have shown no consistent phenotype in the sinonasal mucosa. $CD4^+$ and $CD8^+$ T lymphocytes, other lymphoid and non-lymphoid cells are capable of secreting "type 1" and "type 2" profile of cytokines. When these cytokines are produced by $CD4^+$ T lymphocytes or T helper (TH) cells, they are called TH1 or TH2 type cytokines, and when produced by $CD8^+$ T lymphocytes or cytotoxic T (TC) cells, they are called TC1 or TC2 type cytokines (Mosmann et al. 1997; Woodland and Dutton 2003). However, in some systems, T cell clones may show expression of cytokine patterns that fit a random distribution, consistent with a model of a continuum of different combinations of cytokine secretion rather than discrete subsets (Kelso 1995). Consequently, the T cell pattern of cytokine production could also represent a transient stage along a differentiation pathway or a change in the microenvironment rather than a cytokine profile associated with a particular type of inflammatory response. Therefore, by only examining non-specific products including cytokines or chemokines in the inflammatory tissue, it is not possible to determine the cell type that produced it and what the end result would be. Furthermore, it is difficult to predict *in vivo* compensatory mechanisms, for example, other cells producing these cytokines, modifications in receptor expression and intracellular signalling pathways.

Although type 1 cytokines are considered pro-allergic and pro-eosinophilic, eosinophilic inflammation is common to the inflammatory bowel diseases, Crohn's disease which is typically associated with "type 1", and ulcerative colitis which is typically associated with "type 2" inflammation (reviewed by De Winter et al. 1999). The "type 1" and "type 2" paradigm may be a useful framework to predict the host response to certain infections and to investigate fundamental immunologic pathways (Romagnani 1998). However, the

discrimination is an artificial one that does not apply universally (Allen and Maizels 1997). As our understanding of immunologic phenomena becomes increasingly sophisticated, this duality may become less useful as a construct for understanding the immune system.

To dissect the nature of the immune response in chronic inflammation, an analysis of lymphocyte function is required. Several specialised functional subsets within the $CD4^+$ and $CD8^+$ T lymphocyte populations are described from studies in human immunodeficiency virus-1 (HIV-1) and other viral and non-viral disease models (reviewed in Seder and Ahmed 2003; Sallusto et al. 2004). A description of the T lymphocyte subsets in tissues may not provide an absolute indication of what the cells do but gives a starting point of what they can do. Furthermore, recent findings exemplify diseases that are often accompanied by changes in the numbers and function of "refined or discriminating" lymphocyte subsets, even when changes in the "bulk" lymphocyte populations are not evident (Giorgi et al. 1999; De Rosa et al. 2001). In certain cases, such alterations in lymphocyte subpopulations may provide powerful prognostic or diagnostic information.

1.16 ANALYSIS OF T LYMPHOCYTE FUNCTION

Simplistically, the functional status of T lymphocytes is linked to the level of cell differentiation and type of effector properties. Exposure of naïve $CD4^+$ and $CD8^+$ T lymphocytes to cognate antigen induces proliferation and functional differentiation that is linked with specific migratory and effector properties (reviewed in Seder and Ahmed 2003). The level of cell differentiation can be determined by phenotypic analysis of the following properties: antigen-experience or memory status, homing capabilities, costimulatory molecules and acquisition of effector mechanisms. Hence, evaluation of T cell function

requires an explanation of their complex phenotypes and not just the presence of effector properties.

Naïve T lymphocytes are typically restricted to re-circulation between blood and secondary lymphoid organs. However, in some conditions they can accumulate in chronically inflamed tissue (Weninger et al. 2002). Following antigen encounter, the antigen-specific cells differentiate into short-lived effector cells, lose the ability to re-circulate into lymph nodes, but gain the ability to access peripheral tissues and sites of inflammation. The long-lived memory cells migrate throughout the body to where cognate antigen may be encountered and confer immediate defense in peripheral tissues by mounting recall responses to antigens in secondary lymphoid organs (reviewed by Woodland and Randall 2004; Sallusto et al. 2004). Long-lived memory cells exist in at least two different functional states: (*a*) central memory cells that migrate to lymphoid organs and peripheral inflammation sites and (*b*) effector memory cells that localise to non-lymphoid tissues. In the B lymphocyte responses, protective memory is mediated by plasma cells whereas reactive memory is mediated by memory B cells that proliferate and differentiate into plasma cells in response to secondary antigenic stimulation. In the T lymphocyte system, protective memory is mediated by effector memory T lymphocytes that migrate to inflamed peripheral tissues and display immediate effector function. Reactive memory is mediated by central memory T lymphocytes that home to T cell areas of lymphoid organs, have little or no effector function but readily proliferate and differentiate into effector cells in response to antigenic stimulation. Hence, the memory pool of lymphocytes is not homogeneous, but contains subpopulations with distinct phenotypes and functional capabilities that reflect each cell's history of exposure to antigenic, costimulatory and inhibitory signals.

1.17 MOLECULAR MARKERS FOR T LYMPHOCYTE MEMORY, MIGRATION AND DIFFERENTIATION

1.17.1 Memory

Immunological memory results from the clonal expansion and differentiation of antigen-specific lymphocytes following a primary immune response that may persist for variable periods of time. The well known and widely used cell surface marker to determine whether a lymphocyte has undergone recent activation are changes in the isoform of the CD45 molecule (McNeill et al. 2004). CD45 phosphotyrosine phosphatase (T200 or leucocyte common antigen (LCA)) is an abundant transmembrane glycoprotein, comprising up to 10% of cell surface molecules of all nucleated haematopoietic cells (Thomas 1989; McNeill et al. 2004). Its main role is to regulate the signalling thresholds of receptors present on immune cells. CD45 is expressed as multiple isoforms resulting from alternative ribonucleic acid (RNA) splicing of exons 4-6 (Hermiston et al. 2003). The isoform nomenclature is CD45RABC, where the letter "R" denotes "restricted to" and the letters that follow refer to exon products included in that particular isoform. "CD45RO" refers to the isoform in which all three exon products have been excised. In the mature T cell repertoire CD45RO is a marker for previously activated cells. Therefore, naïve T lymphocytes may be recognised by their CD45RA expression or lack of CD45RO expression. However, the loss of A, B and C exons is reversible, thus CD45RO expression or lack of CD45RA expression may also signify relatively recent stimulation (Yang and Bell 1999).

1.17.2 Migration

T cell migration is mediated by highly regulated, multi-step cascades of molecular interactions between adhesion receptors on T lymphocytes and their ligands expressed on vascular endothelium and stromal tissues in an organ-specific manner, a process known as

lymphocyte homing (Picker 1994; Butcher and Picker 1996; Campbell et al. 2003). The nasal capillary endothelial cells express peripheral node addressin (PNAd) and mucosal addressin cell adhesion molecule-1 (MAdCAM-1) which are ligands for L-selectin (CD62L) or CCR7 and $\alpha_4\beta_7$ integrin molecules present on lymphocytes respectively (Csencsits et al. 1999). Typically, MAdCAM-1 is expressed in the Peyer's patches, mesenteric lymph nodes and gut lamina propria (Briskin et al. 1997), PNAd is typically expressed in peripheral lymph nodes, and E-selectin which binds to cutaneous leucocyte antigen (CLA) is typically expressed in skin capillary high endothelial venules (reviewed in Picker 1994). The intestinal epithelial cells express E-cadherin whose ligand is $\alpha E\beta 7$ (CD103) expressed on lymphocytes (Cepek et al. 1994). The pattern of expression of homing markers may not be significantly different in disease and non-disease states, although their level of expression may vary (Campbell et al. 2001).

1.17.3 Differentiation

The expression of the costimulatory molecules, CD27 and CD28, is used to assess the level of cell differentiation (Azuma et al. 1993; Hamann et al. 1997; Weekes et al. 1999; Tomiyama et al. 2002). These costimulatory molecules provide signals needed for the correct activation of antigen-specific T lymphocytes after T cell receptor ligation. CD27 interacts with its ligand CD70, and CD28 interacts with CD80 (B7-1) and CD86 (B7-2) expressed on antigen presenting cells. Following the interaction of CD27 and CD28 with their ligands, T lymphocytes undergo clonal expansion and develop into effector cells with the ability to respond directly to pathogens. As these T lymphocytes have "functionally matured" following antigen encounter, the costimulatory molecules are down regulated. Compared with CD28, down regulation of CD27 is irreversible and relates to end-stage differentiation status of antigen-specific T lymphocytes (Hintzen et al. 1993).

1.18 PHENOTYPIC IDENTIFICATION OF NAÏVE AND MEMORY T LYMPHOCYTE POPULATIONS

The phenotypically distinct and sequential stages of T lymphocyte differentiation is proposed based on the expression of CD45RA, CD62L, CD27 and an analysis of replicative history and clonality of the T lymphocyte populations (van Baarle et al. 2002; Weninger et al. 2002) (Figure 1.5).

1.18.1 Naïve T lymphocytes

Naïve T lymphocytes express CD45RA, as they have yet to encounter cognate antigen. In peripheral blood, naïve T cells have the ability to home to lymph nodes and peripheral lymphoid organs and thus express the ligand for PNAd, CD62L, or the ligand for MAdCAM. They also express CD27. In the peripheral tissues, the CD45RA and CD27 positive phenotype may be accompanied by loss of CD62L as the cells are sequestered in the periphery. Naïve T cells do not express effector functions (section 1.19).

1.18.2 Central memory and effector memory T lymphocytes

In humans, central memory and effector memory T lymphocytes are defined based on two distinct criteria: *(a)* the absence or presence of immediate effector function and *(b)* the expression of homing receptors that allow cells to migrate to secondary lymphoid organs versus non-lymphoid tissues (Hamann et al. 1997; reviewed by Sallusto et al. 1999; Seder and Ahmed 2003; Tough 2003; Sallusto et al. 2004). Human central memory T lymphocytes are antigen-experienced CD45RA negative cells that constitutively express CD62L. Human effector memory T lymphocytes are antigen-experienced CD45RA negative cells that have lost the constitutive expression of CD62L. The effector memory T cells may also display characteristic sets of chemokine receptors and adhesion molecules that are required for

homing to inflamed tissues. The "effector" T lymphocytes do not need another signal from CD27, as they have already functionally matured. Therefore, effector memory T lymphocytes are cells that have lost the expression of CD27. Homing molecules, including CD62L may be re-induced. Therefore reactivation may result in T lymphocytes with a central memory phenotype but with functional characteristics of full-blown effector cells corresponding to the CD27 negative phenotype.

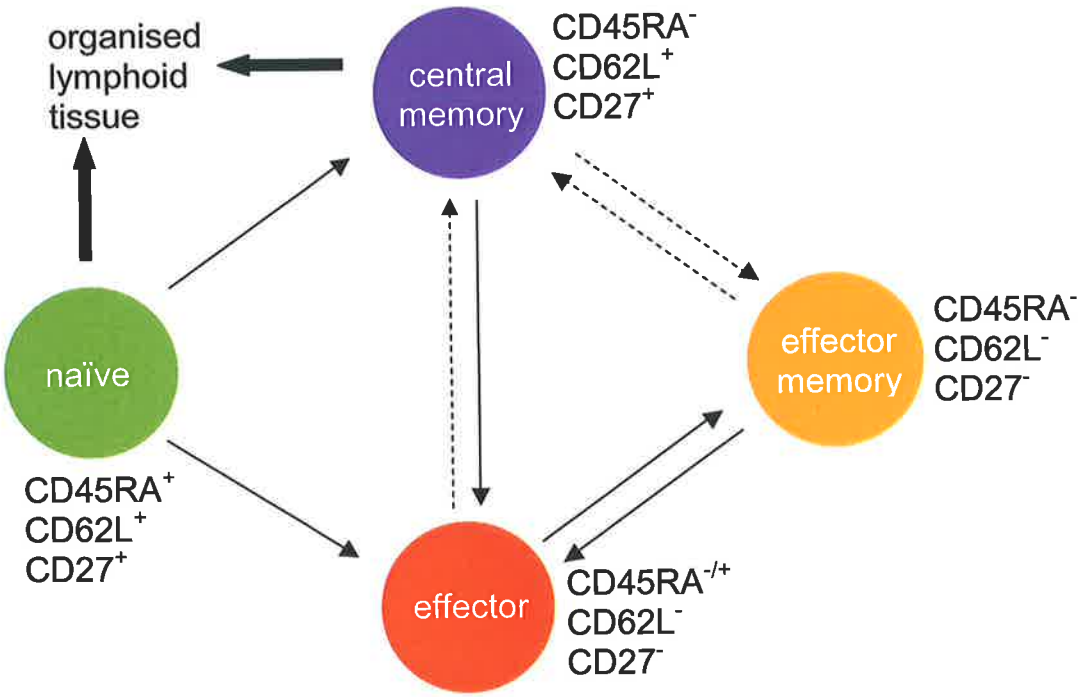


Figure 1.5 Proposed lineage differentiation model of T lymphocytes. Modified from Weninger et al. 2002.

1.19 EFFECTOR FUNCTIONS OF T LYMPHOCYTES

Activated T lymphocytes mediate effector functions through direct cell-cell cytotoxic activity and by cytokine secretion (Harty et al. 2003; Betts et al. 2004). The effector implications of cytokines are typically not clearly defined. By contrast, the basic concepts regarding cytotoxic effector cell function are essentially unchanged. However, the conception of the mechanisms mediating cytotoxic function and the role of the cytotoxic molecules in inflammatory conditions is expanding and evolving rapidly. Natural killer lymphocytes, $CD8^+$ and $CD4^+$ T lymphocytes have cytotoxic capability. The method by which lymphocytes mediate cytotoxicity include granule exocytosis, Fas/Fas ligand interaction, toll-like receptors and tumour necrosis factor (TNF)/TNF receptor pathways (Trapani and Smyth 2002; Lieberman 2003). The mechanism of cytotoxicity important for defense against intracellular pathogens and responses to tumor cells involves the granule exocytosis pathway (Grossman et al. 2004a). The cytotoxic granules are complex organelles that contain cytotoxic proteins that are restricted to expression in lymphocytes, and other ubiquitously expressed lysosomal proteins (Table 1.3) (Smyth et al. 2001). Recognition and cell interaction between lymphocytes and target cells leads to exocytosis of specific cytoplasmic granules. While the exact mechanism of granule-target cell interaction is still not clear, this interaction leads to target cell death typified by cytosolic and nuclear-apoptotic changes.

1.19.1 Perforin and granulysin

Perforin is a pore-forming protein with some homology to complement (C) component 9 (C9) (Smyth et al. 2001). At present, the mechanism of action of perforin is incompletely understood. Recent models indicate that perforin may assist in releasing the contents of the endocytosed granule contents into the target cell by endosomolytic activity rather than target

cell membrane permeability as originally described (Raja et al. 2003; Catalfamo and Henkart 2003).

Table 1.3 Contents of lymphocyte cytotoxic granules

Contents of dense core	**Specialised function**
Granzymes (A, B, H, K, M)	
Perforin	Pore formation, disruption of endosomal trafficking
Granulysin	Microbicidal agent
Calreticulin	Calcium and perforin binding
Cathepsin C	Activation of granzymes
Chondroitin sulfate proteoglycans (Serglycin)	Complexes to granzymes
Resident lysosomal proteins (in the periphery of granule)	
Mannose 6-phosphate receptor, H^+-adenosine triphosphatase, Cathepsin D, Cathepsin L, Arylsufatase, β-Hexosamidase, β-Glucuronidase, CD63, Lysosome-associated membrane protein (LAMP) 1 and 2	

Adapted from Smyth et al. 2001.

Granulysin is a member of the saposin family of lipid-binding proteins (Lieberman 2003). Although functionally related to other antimicrobial peptides, including defensins and magainins, granulysin is structurally distinct. Granulysin is active against a broad range of microbes, including bacteria, fungi and parasites. Mitochondria are the major targets and several pathways contribute to granulysin-mediated mitochondrial damage and subsequent cell death (Okada et al. 2003).

1.19.2 Granzymes

Granzymes (granule enzymes) A and B are differentially expressed in $CD4^+$ and $CD8^+$ T, NK and NKT lymphocytes in normal donors, in resting cells and upon cell stimulation (Grossman et al. 2004b). Granzymes are a family of related serine proteases belonging to the chymotrypsin superfamily that have restricted expression within cells of the lymphoid system and are absent from macrophages, neutrophils and B cells (Smyth et al. 2001). Granzyme A, B, H, K and M have been identified in humans and are inducers of apoptosis of target cells by different intracellular mechanisms. Granzymes A and B are the most abundantly expressed granzymes in humans and mice (Lieberman 2003). Murine studies suggest that granzymes A and B induce target cell death by cleaving non-overlapping sets of substrates (Bredemeyer et al. 2004). Granzyme A-mediated cell death appears independent of caspase pathways. Granzyme B can mediate apoptosis by caspase-dependent and caspase-independent pathways by disrupting mitochondrial integrity (Regner and Mullbacher 2004).

The mechanisms underpinning the synergy between granzymes and perforin remain elusive. Cytotoxic lymphocyte expression of various granzymes, perforin and other granule constituents may depend of the type of immune response, the nature of antigen or target cell and the level of functional differentiation of the lymphocyte.

1.20 RESEARCH PLAN

Based upon a review of the literature up to and including March 2002, the following problems were identified and research plan proposed.

Problems

1. The classification of EMCRS is heterogeneous.
2. Fungal allergy is considered pathogenically important in allergic EMCRS but there is no critical study to determine this.
3. It is unclear whether allergic fungal sinusitis is pathophysiologically distinct from the other "non-allergic fungal sinusitis" subgroups of EMCRS patients.
4. It is unknown whether EMCRS is different from other polypoid chronic rhinosinusitis, CRS, in terms of its pathogenesis.

Hypothesis

Eosinophilic mucus chronic rhinosinusitis is a distinct and severe form of sinus inflammatory disease that is associated with fungi. Hence, EMCRS patients have a different type of fungal-specific humoral and cellular immune response and mucosal inflammation compared with chronic rhinosinusitis patients without eosinophilic mucus (CRS).

AIMS

The aims of this thesis were to:

1. Determine the clinical and pathogenic relevance of subclassifying EMCRS patients based on the detection of fungal-specific type I hypersensitivity and fungi in sinus eosinophilic mucus.

2. Investigate fungal-specific systemic immune responses in EMCRS patients.

3. Examine the nature of the sinonasal inflammatory response in EMCRS patients.

These were achieved by:

1. Defining the clinical characteristics of the EMCRS patient subgroups (Chapter 3).

2. Examining fungal-specific IgE and non-IgE humoral responses in the EMCRS patient subgroups using a fungal antibody ELISA (Chapter 4).

3. Investigating fungal-specific cellular responses in the EMCRS patient subgroups using an *in vitro* cell proliferation assay (Chapter 5).

4. Characterising the T lymphocyte populations in the sinonasal mucosa of the EMCRS patient subgroups (Chapter 6).

CHAPTER TWO: MATERIALS AND METHODS

2.1 ANTIBODIES

2.1.1 Antibodies for ELISA experiments

Anti-human isotype-specific secondary antibodies

Antigenic determinant	Antibody clone	Isotype	Working dilution	Supplier[1] (reference[2])
IgG	HB60	IgG2b	1/2	ATCC (Kuritani and Cooper 1982)
IgA	HB63	IgG1	Neat	ATCC (Sobel et al. 1998)
IgM	HB57	IgG1	Neat	ATCC (Maruyama et al. 1985)
IgE	HB121	IgG2a	Neat	ATCC (Mansur et al. 1999)
IgG1	HP6001	IgG2b	Neat	ATCC (Reimer et al. 1984)
IgG2	HP6002	IgG1	1/3000	Sigma
IgG3	SJ-33	IgG1	1/2000	Miles Yeda
IgG4	SK-44	IgG1	1/2000	Miles Yeda

[1]A list of antibody suppliers and their addresses is provided in Table 2.1. [2]Complete references are listed in the "References" section.

Coating antibodies

Antigenic determinant	Working dilution	Source
Anti-human IgE	1/1000	Sigma
Anti-human IgGAM	1/1000	Chemicon
Anti-mouse immunoglobulin	1/1000	Chemicon

Alkaline phosphatase conjugated antibodies

Antigenic determinant	Working dilution	Source
Anti-human IgE	1/500	Sigma
Anti-mouse immunoglobulin	1/1000	Sigma

2.1.2 Antibodies for flow cytometry experiments

Unlabelled antibodies

Antigenic determinant	Antibody clone	Isotype	Supplier (reference)
CD3	OKT3	IgG2a	ATCC (Hoffman et al. 1980)
CD4	OKT4	IgG2b	ATCC (Hoffman et al. 1980)
CD8	OKT8	IgG2a	ATCC (Hoffman et al. 1980)
CD14	FMC33	IgG1	FMC (Brooks et al. 1983)
CD19	FMC63	IgG2a	FMC (Zola et al. 1991)
CD45RA	FMC71	IgG1	FMC (Liu et al. 2001)
CD62L	FMC46	IgG2b	FMC (Pilarski et al. 1991)
MHC II	FMC52	IgG1	FMC (Hart et al. 1996)

Labelled antibodies

Antigenic determinant	Label	Antibody clone	Isotype	Supplier (reference or catalogue number)
CD25	FITC	7G7/B6	IgG2a	ATCC (Rubin et al. 1985)
CD27	FITC	M-T271	IgG1	BD (555440)
CD107a	FITC	H4A3	IgG1	BD (555800)
CD107b	FITC	H4B4	IgG1	BD (555804)
Granzyme A	FITC	CB9	IgG1	BD (558905)
Granzyme B	FITC	GB11	IgG1	BD (558132)
Perforin	FITC	δG9	IgG2b	BD (556577)
γδ TCR	FITC	B1	IgG1	BD (347903)
CD3	R-PE	UCHT1	IgG1	Dako (R0810)
CD4	R-PE	MT310	IgG1	Dako (R0805)

Labelled antibodies

Antigenic determinant	Label	Antibody clone	Isotype	Supplier (reference or catalogue number)
CD8	R-PE	DK25	IgG1	Dako (R0806)
CD19	R-PE	HD37	IgG1	Dako (R0808)
CD3	R-PECy5	UCHTI	IgG1	Dako (C7067)
CD4	Biotin	Leu-3a	IgG1	BD (7321)
CD8	Biotin	Leu-2a	IgG1	BD (7311)

Tri-colour (TriTEST™) antibodies

Antigenic determinant and label	Clone	Supplier
CD3 FITC / CD4 R-PE / CD45 PerCP	SK7/SK3/2D1	BD
CD3 FITC / CD8 R-PE / CD45 PerCP	SK7/SK1/2D1	BD
CD3 FITC / CD19 R-PE / CD45 PerCP	SK7/SJ25C1/2D1	BD
CD3 FITC / CD16 + CD56 PE / CD45 PerCP	SK7/B73.1+NCAM16.2/2D1	BD

2.1.3 Antibodies for immunohistochemistry experiments

Antigenic determinant	Working dilution	Antibody clone	Isotype	Supplier
Eosinophil cationic protein	1/20	EG2	IgG1	Pharmacia
CD3	1/300	PS1	IgG2a	NeoMarkers
CD4	1/20	4B12	IgG1	Novocastra
CD8	1/20	1A5	IgG2b	Novocastra

Isotype control for unlabelled monoclonal antibodies included X63 (IgG1, mouse myeloma antibody), and Sal4 (IgG2b) and Sal5 (IgG2a) (anti-*Salmonella* antibodies) (Clark et al. 1999). Antibody clones MOPC-21 and 27-35 conjugated to FITC (BD) were used as negative controls for FITC conjugated IgG1 and IgG2b monoclonal antibodies respectively.

Table 2.1 List of suppliers

Antibody Supplier	**Abbreviation**	**Country**
American Type Culture Collection	ATCC	Manassas, VA, USA
Becton Dickinson Biosciences	BD Biosciences	San Jose, CA, USA
Chemicon Australia Pty. Ltd.	Chemicon	Victoria, Australia
DakoCytomation	Dako	Glostrup, Denmark
Flinders Medical Centre	FMC	South Australia, Australia
Miles Yeda	Miles Yeda	Rehovot, Israel
NeoMarkers	NeoMarkers	Fremont, CA, USA
Novocastra Laboratories	Novocastra	Newcastle upon Tyne, UK
Pharmacia Diagnostics AB	Pharmacia	Uppsala, Sweden
Sigma	Sigma	St Louis, MO, USA
Zymed Laboratories Inc.	Zymed	San Francisco, CA, USA

2.2 GENERAL

All reagents used were of analytical purity and all solutions were prepared with double-glass distilled water.

2.3 FUNGAL ANTIGENS

The precise epitopes of fungal antigens that may be involved in EMCRS are unknown. Therefore, a crude fungal antigen preparation in common use for desensitisation therapy and diagnosis was selected. Antigen preparations of the fungi, *Alternaria tenuis (alternata)* (catalogue number – 5009JF10) and *Aspergillus fumigatus* (catalogue number – 5021JF10), were obtained from Hollister-Stier Laboratories LLC (Spokane, WA, USA). Vials of the antigen preparation were pooled so that a single mix was available throughout the project without antigenic variability between experiments (Vailes et al. 2001). For *Alternaria alternata*, three vials of lot number F52I9664 and one vial of lot number 041G1564 and for *Aspergillus fumigatus*, two vials of lot number C52G8451, one vial of lot number C52E7735 and one vial of lot number L4207110 antigen preparations were pooled and stored in a sterile vessel at 4°C until required.

2.4 PATIENTS AND CONTROLS

2.4.1 Recruitment of patients and healthy volunteers

Patients with chronic rhinosinusitis and allergic rhinitis with fungal allergy were recruited from rhinology, immunology-allergy clinics and at the time of surgery. Healthy volunteers were recruited from laboratory and medical staff. Patients and healthy volunteers completed a questionnaire consisting of questions for sinonasal symptoms and past and current medical conditions (Appendix I). Results of sinus computed tomography, skin prick tests and

serology performed as part of routine patient management were available for stratification into study groups.

2.4.2 Selection of study groups

2.4.2.1 Eosinophilic mucus chronic rhinosinusitis (EMCRS) patients

The EMCRS patients were defined as chronic rhinosinusitis patients (Benninger et al. 2003) who had thick, tenacious and coloured mucus at surgery, confirmed to be eosinophilic mucus by histology as described in section 2.6.1.2. The EMCRS patients were divided into well-defined subgroups depending on the detection of *(a)* fungi in eosinophilic mucus by histology or culture, described in sections 2.6.1.2 and 2.6.1.3, and *(b)* type I hypersensitivity to any fungi, determined by elevated levels of mould mix-specific serum IgE or by positive skin prick tests, described in sections 2.6.3 and 2.6.4.

The term allergic fungal sinusitis (AFS) was defined as EMCRS patients who had fungi in eosinophilic mucus and had fungal allergy (Bent and Kuhn 1994). The term AFS-like was defined as EMCRS patients who did not have fungi in eosinophilic mucus and had fungal allergy (Collins et al. 2004). Non-allergic, fungal eosinophilic sinusitis (NAFES) was defined as EMCRS patients who had fungi in eosinophilic mucus and did not have fungal allergy (Ferguson 2004). Non-allergic, non-fungal eosinophilic sinusitis (NANFES) was defined as EMCRS patients who did not have fungi in eosinophilic mucus and did not have fungal allergy (Collins et al. 2004).

2.4.2.2 Chronic rhinosinusitis (CRS) patients

They were defined as chronic rhinosinusitis patients who had undergone endoscopic sinus surgery and were found not to have the characteristic macroscopic appearance of

eosinophilic mucus in their sinuses. This group included patients with polypoid mucosal inflammation.

2.4.2.3 Allergic rhinitis with fungal allergy (ARFA) patients

They were defined as patients with rhinitis and no evidence of sinus involvement determined by medical history, examination and sinus computed tomography scans. They had type I hypersensitivity to fungi determined by elevated levels of mould mix-specific serum IgE or by positive skin prick tests.

2.4.2.4 Healthy volunteers (HV)

They had no evidence of rhinitis, acute or chronic rhinosinusitis determined by a sinonasal objective test score of 1 or less and no evidence of mucopus in the nasal cavities. They had no evidence of type I hypersensitivity to mould mix determined by absent mould mix-specific serum IgE. In order for this group to represent a typical immune-control population, individuals with type I hypersensitivity to non-fungal allergens were included.

2.4.3 Study groups in the result chapters

The number of individuals in each study group varied depending on the particular study. The clinical, pathological and laboratory characteristics of the groups are presented in the result section of the relevant chapters.

2.4.4 Exclusion criteria

Except for the study described in section 3.2.2 (Chapter 3), individuals with other significant medical problems, including allergic bronchopulmonary aspergillosis, Churg-Strauss

syndrome and those treated with systemic corticosteroids (Graham and Ballas 1998) or other immunosuppressive therapy were excluded.

For the mucosal studies (Chapters 5 and 6), additional exclusion criteria were: *(a)* smoking for at least six weeks prior to surgery (Saetta 1999; Vachier et al. 2004), *(b)* history of an upper respiratory tract infection (Flynn et al. 1998; Ramadan et al. 2002; Alho et al. 2003) and (c) use of topical medications including corticosteroids (Kanai et al. 1994; Kondo et al. 1999), anti-histamines, anti-cholinergic or homeopathic preparations in the preceding four weeks. As most patients in the study groups were using topical nasal saline solutions, including Narium (Hamilton Laboratories, SA, Australia), FESS nasal spray (Paedpharm, NSW, Australia) and homemade saline solutions, they were not excluded.

2.5 COLLECTION OF BIOLOGICAL SPECIMENS

2.5.1 Peripheral venous blood samples

Peripheral blood samples were obtained by direct puncture of the antecubital vein. Blood was collected in sterile vials containing Z serum clot activator (Vacuette, Greiner-bio-one, Austria) to obtain sera or lithium-heparin (Vacuette) to obtain whole blood samples.

2.5.2 Sinonasal samples

2.5.2.1 Tissue

Nasal polyp tissue samples were obtained from EMCRS and CRS patients during routine endoscopic or open sinus surgery. Adjacent non-polyp sinus or nasal mucosal tissue was also obtained in some cases. Samples for routine pathology tests and immunohistochemistry experiments on fixed tissue were placed in a 10% formalin solution and those for tissue culture and flow cytometry experiments placed in transport medium, Roswell Park

Memorial Institute (RPMI) 1640 (Gibco BRL, Grand Island, NY, USA). Samples were processed within three hours of harvest.

2.5.2.2 Mucus

Mucus from the affected sinuses was collected for routine pathological diagnosis in a 10% formalin solution for histology (section 2.6.1.2) and a non-fixed, fresh sample for mycological studies (section 2.6.1.3).

2.6 ROUTINE DIAGNOSTIC TESTS

The following investigations were performed as part of routine patient management to determine the clinical diagnosis of the EMCRS subgroups: *(a)* examination of sinonasal tissue and mucus samples, *(b)* sinus computed tomography and *(c)* fungal-specific serum IgE levels or skin prick tests.

2.6.1 Sinonasal samples

The tissue specimens were processed by the following South Australian diagnostic laboratories, Institute of Medical and Veterinary Science (IMVS), SouthPath and Adelaide Pathology Partners, as previously described (Collins et al. 2003).

2.6.1.1 Polyp and non-polyp mucosal tissue

Serial sections of paraffin-embedded tissue samples from EMCRS and CRS patients were stained with haematoxylin and eosin (Lillie and Fullmer 1976) to examine tissue architecture and inflammatory cell populations.

2.6.1.2 Sinonasal mucus histology

Serial sections of paraffin-embedded mucus sample from every EMCRS patient were stained with (*a*) haematoxylin and eosin to examine for histological evidence of eosinophilic mucus and (*b*) silver using Grocott's methenamine silver staining method (Grocott 1955) for evidence of fungal elements. Eosinophilic mucus was confirmed when clusters of eosinophils were observed within the amorphous mucus matrix. The presence of Charcot-Leyden crystals was not essential for this diagnosis.

2.6.1.3 Sinonasal mucus mycology

A sample of sinus mucus from every EMCRS patient was processed for fungal culture in two different culture media (*a*) Sabouraud agar with chloramphenicol (240 mg/L) and gentamicin (28 mg/L) slope (Oxoid, Hampshire, UK) and (*b*) potato-dextrose agar with chloramphenicol (250 mg/L) and gentamycin (26 mg/L) (Oxoid) according to the manufacturer's instructions. The fungi were identified according to the macroscopic characteristics and the final results reported after four weeks of culture.

2.6.2 Radiology

The parameters for the unenhanced helical computed tomography scanning of the sinuses included: (*a*) prone patient position, (*b*) angulation perpendicular to the hard palate, (*c*) 14 cm field of view and (*d*) 3 mm, contiguous section thickness (Zinreich 1997). Images were examined in parasaggital and coronal reconstructions with bone and soft tissue algorithms by a radiologist and a senior otolaryngologist.

The pre-operative scans were evaluated for (*a*) extent of sinus involvement using the Lund-Mackay staging system (Lund and Mackay 1993; Oluwole et al. 1996), (*b*) double density

appearance (Mukherji et al. 1998) (*c*) expansion of the sinus cavity, (*d*) bone erosion and bone sclerosis and (*e*) extra-sinus involvement. The scans were performed by the radiology service at the Queen Elizabeth Hospital, Royal Adelaide Hospital and Perrett Medical Imaging in South Australia.

2.6.3 Peripheral blood allergen-specific IgE

Specific-IgE to fungi (mould mix) and other inhaled allergens (mixes of tree pollen, grass pollen, house dust, mite and animal epithelia) in sera was measured by the ImmunoCAP system (UniCAP® 100, Pharmacia Diagnostics AB, Uppsala, Sweden) (Appendix II A). Specific-IgE to *Aspergillus fumigatus* and *Alternaria alternata* antigens (ImmunoCAP system) was measured where indicated. Specific-IgE greater than 0.35 kU_A/L, where "A" in subscript denotes allergen-specific, was taken as a positive result (UniCAP® 100 procedure reference, Pharmacia).

2.6.4 Skin prick tests

A positive skin prick test to fungal and non-fungal allergen preparations (Hollister-Stier Laboratories LLC) (Appendix II B) was defined as a wheal equal to or greater than 3 mm in diameter.

2.6.5 Peripheral blood total immunoglobulin levels, differential leucocyte counts and *Aspergillus fumigatus* precipitin levels

Sera were tested for total serum IgE using the ImmunoCAP system (Pharmacia). The units for total IgE levels are in kilo units per litre (kU/L) where one kU/L is equivalent to one IU of IgE, that is, 2.44 ng/ml of IgE (Pharmacia). Levels of serum IgG, IgA IgM and IgG subclasses, IgG1, IgG2, IgG3 and IgG4, were measured in g/L using the "Immage"

nephelometer (Beckman Coulter, Fullerton, CA, USA). Serum IgG precipitins to *Aspergillus fumigatus* antigens (FSK1) (Microgen Bioproducts Ltd., Surrey, UK) were measured by enzyme-linked immunosorbent assay (ELISA). The peripheral blood differential leucocyte (eosinophils, lymphocytes, monocytes and basophils) counts were measured using a Coulter® MAXM haemocytometer (Beckman Coulter). These tests were performed by the IMVS and SouthPath laboratories.

2.7 DEMOGRAPHICS

Demographic data, including age, sex and geographic location in the form of postal area code was obtained at the time of patient recruitment. The number of EMCRS patients per postal area code was plotted in the Adelaide metropolitan area using the ArcGIS 9.1 software (ESRI products, Redlands, CA, USA). The geographical location of the EMCRS patients were compared between *(a)* the subgroups: AFS, AFS-like, NAFES and NANFES and *(b)* those who were fungus negative (AFS-like and NANFES) and those who were fungus positive (AFS and NAFES).

2.8 SYMPTOM SCORES

2.8.1 Chronic rhinosinusitis

Patients filled out a scale for the following pre-operative and pre-treatment symptoms: nasal obstruction, postnasal discharge, rhinorrhoea, headache, facial pain, sneeze or itch and loss of sense of smell (Appendix I). A score of zero (0) was given when asymptomatic, one (1) for mild symptoms, two (2) for moderate symptoms and three (3) for severe symptoms. (Meltzer et al. 1993). Mild symptoms were determined when patients were seldom symptomatic, moderate, when symptomatic on most days and severe, when almost-always symptomatic. The total score for the symptoms pertaining to chronic rhinosinusitis, nasal

obstruction, postnasal discharge, headache and facial pain (Meltzer et al. 2004) was determined and compared among the study groups.

2.8.2 Asthma

In this thesis, the severity of asthma was classified according to a modified method by the National Asthma Campaign 1998, in Australia (Table 2.2), where the forced expiratory volume in one second (FEV_1) and peak expiratory flow (PEF) were not evaluated. The worst individual categorisation determined the overall asthma severity. A score of one (1) was given for mild symptoms, two (2) for moderate symptoms and three (3) for severe symptoms. The asthma severity score was compared among the study groups.

Table 2.2 Classification of asthma severity

Severity	Wheeze, tightness, cough, dyspnoea	Night-time symptoms	Symptoms on waking	Admission or emergency visits	Previous life-threatening attack	Short acting beta agonist use	FEV_1	PEF
Severe	Everyday	>1/week	>1/week	Usually	May have a history	>3 to 4/day	<60%	<80%
Moderate	Most days	<1/week	<1/week	Usually not	Usually not	Most days	60-80%	60-80%
Mild	Occasional	Absent	Absent	Absent	Absent	<2/week	>80%	>80%

FEV_1, forced expiratory volume in one second; PEF, peak expiratory flow.

Adapted from the National Asthma Campaign, Asthma Management Handbook 1998.

2.9 ENZYME-LINKED IMMUNOSORBENT ASSAY

2.9.1 General ELISA method

Maxisorp, flat-bottomed, 96 well microtitre plates (Nalge Nunc International, Naperville, IL, USA) were coated with saturating concentrations of antigen or antibody in 0.03 M carbonate-sodium bicarbonate buffer (pH 9.6) for 16 hours at 4°C and subsequently blocked with 1% bovine serum albumin (BSA) (Fraction V, Sigma) for two hours. All other incubations were carried out for one hour at 37°C. Bound antibody was detected with either an anti-mouse or anti-human alkaline phosphatase conjugated antibody following the addition of substrate, p-nitrophenyl phosphate disodium (1 mg/ml, Sigma) in diethanolamine buffer (90 mM diethanolamine, 5 mM $MgCl_2$ (pH 9.6)). The amount of bound antibody was proportional to the optical density (OD) value read at 405 nm (OD_{405nm}) on a spectrophotometer (BioRAD, Hercules, CA, USA). Samples were assayed in duplicate and results expressed as the mean of duplicate OD_{405nm} value.

Between each ELISA step, the microtitre plates were washed four times with phosphate buffered saline/0.05% Tween-20 (polyoxyethylene-sorbitan monolaurate) (Sigma) (PBS/Tween). All ELISA experiments were conducted with negative and positive controls relevant to the particular assay. Other internal controls included wells that did not have the following ELISA constituents: (*a*) microtitre plate-bound antigen or antibody, (*b*) primary antibodies (*c*) secondary antibodies and (*d*) tertiary antibodies. These ELISA constituents were replaced with equal volumes of 1% BSA/PBS in their respective wells.

2.9.2 Measurement of antibody concentration in culture supernatants

Microtitre plates (Nalge Nunc International) were coated with 1.4 µg/ml of affinity purified sheep anti-mouse immunoglobulin (Chemicon). Doubling dilutions of standard mouse

immunoglobulins (Sigma) from a concentration of 10 µg/ml to 0.0005 µg/ml and of the test supernatant (neat, diluted 1/10 – 1/10000) were assayed in duplicate. Bound antibody was detected with a sheep anti-mouse alkaline phosphatase conjugated antibody (Chemicon) followed by substrate. The mean OD_{405nm} value against the standard mouse immunoglobulin concentration was plotted to construct a standard curve on Log 4 graph paper. The mean OD_{405nm} value of the test supernatant was plotted along the standard curve. The concentration (µg/ml) of the test supernatant was estimated by reading the OD_{405nm} value on the linear portion of the standard curve and multiplying by the dilution factor.

2.10 DEVELOPMENT OF THE FUNGAL ANTIBODY ELISA

The structure of the fungal antibody ELISA is shown in Figure 2.1. The optimum concentrations of reactants were determined in preliminary experiments and control steps described in section 2.9.1 were included in all experiments.

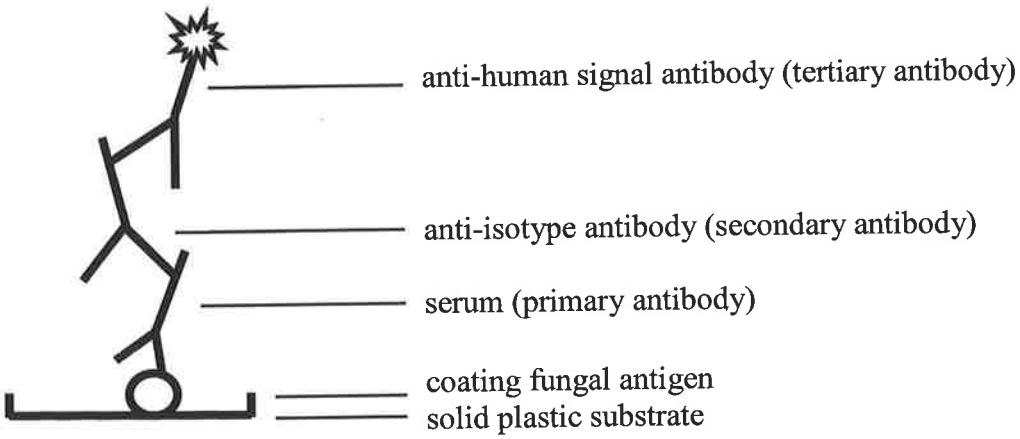


Figure 2.1 Components of the fungal antibody enzyme-linked immunosorbent assay used to detect fungal-specific IgG, IgA, IgM and IgG subclasses. Signal antibody refers to alkaline phosphatase conjugated antibody.

2.10.1 Optimisation of the coating fungal antigen concentration

Known concentrations (0.625 – 10 μg/ml) of *Alternaria alternata or Aspergillus fumigatus* antigen (Hollister-Stier LLC) were coated onto microtitre plates (Nalge Nunc International). Dilutions of serum (1/5, 1/10, 1/20, 1/50, 1/100, 1/200 and 1/500) were added to their respective wells in duplicate. The amount of bound fungal antigen-specific antibody isotype was detected with predetermined concentrations of isotype-specific secondary antibodies (section 2.1) followed by an anti-mouse alkaline phosphatase conjugated antibody (Chemicon) with substrate. The curve of the OD_{405nm} value against the concentration of fungal antigen was plotted for each serum dilution. The optimum fungal antigen concentration was determined from optical density readings in the linear portion of the curve. This was determined to be 2.5 μg/ml at a 1/80 dilution.

2.10.2 Optimisation of primary antibody dilution for each fungal-specific antibody isotype

Dilutions of sera (1/5, 1/10, 1/25 and 1/50) were tested for each fungal-specific antibody isotype and the dilution that gave the highest OD_{405nm} value for the particular isotype was selected.

The optimum serum dilution used to compare fungal-specific antibody levels between the study groups was determined as follows: 1/5 dilution for IgE, IgG3 and IgG4, 1/10 dilution for IgA, IgG1 and IgG2, and 1/25 dilution for IgM and IgG.

2.10.3 Optimisation of secondary antibody concentration

Secondary antibodies (anti-human IgG, IgA, IgM and IgG subclasses) were titrated against known concentrations of primary antibody (total serum IgG, IgA, IgM, IgG1, IgG2, IgG3

and IgG4) in g/L ("Immage" nephelometer). Microtitre plates (Nalge Nunc International) were coated with 2.5 µg/ml of goat-anti-human IgGAM (Chemicon). Two-fold dilutions of sera and secondary antibodies were added to their respective wells and bound antibody detected with an anti-mouse alkaline phosphatase conjugated antibody with substrate. The curve of OD_{405nm} value against the secondary antibody dilution was plotted for every primary antibody concentration tested. The optimum concentration of secondary antibody was determined by the OD_{405nm} value in the linear portion of the curve.

2.11 ESTABLISHMENT OF THE FUNGAL ANTIGEN-SPECIFIC IMMUNOGLOBULIN E ELISA

Microtitre plates (Nalge Nunc International) were coated with 2.5 µg/ml of goat-anti-human IgE (Sigma). Serial two-fold dilutions of serum of known total IgE concentration (49,966 - 20.75 kU/L, diluted 1/2408), determined by ImmunoCAP system, were added to the respective wells. The amount of bound antibody was detected by: (*a*) indirect ELISA, with anti-human IgE monoclonal antibody supernatant (HB121) followed by anti-mouse alkaline phosphatase conjugated antibody (Chemicon) or (*b*) direct ELISA, with sheep-anti-human IgE conjugated to alkaline phosphatase (Sigma) (Figure 2.2). The OD_{405nm} values obtained from each method were compared against the known concentration of serum IgE in kU/L.

2.11.1 Sensitivity and specificity of the IgE ELISA

The sensitivity of the direct IgE ELISA was determined by measuring the OD_{405nm} values for dilutions of sera containing known concentrations total IgE levels from 1000 kU/L to 0.19 kU/L (Pharmacia). To determine the cut-off value for a positive result by the IgE

ELISA, sera from 50 individuals who were negative for fungal-specific IgE by the ImmunoCAP system were screened.

To validate the fungal-specific IgE ELISA, sera from ten patients that were IgE positive and sera from five healthy volunteers and five patients that were IgE negative to *Alternaria alternata* and *Aspergillus fumigatus* by ImmunoCAP system were assayed by ELISA. The concentration of fungal-specific IgE measured by the ImmunoCAP system (Pharmacia) was compared against the OD_{405nm} value determined by ELISA.

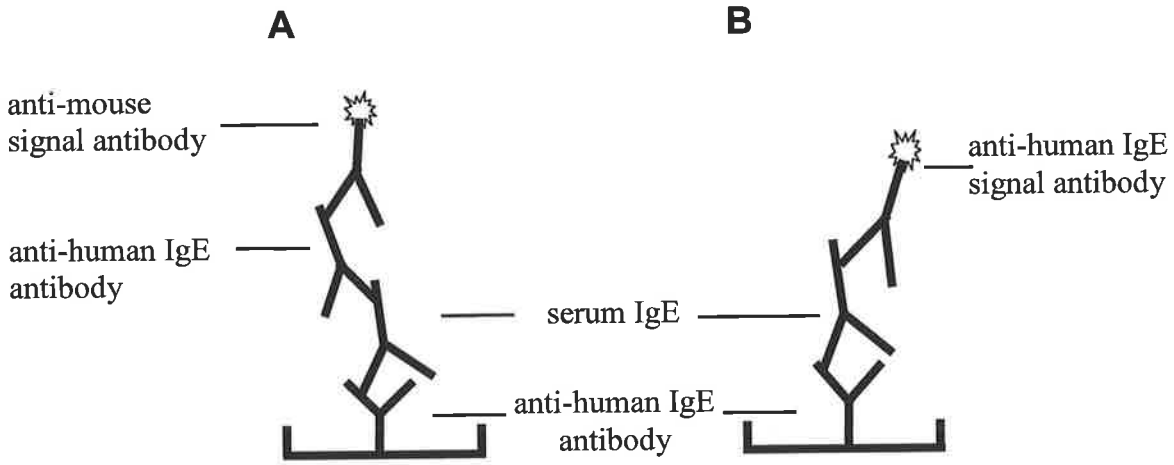


Figure 2.2 Constituents of the indirect (A) and direct (B) anti-human IgE enzyme-linked immunosorbent assay. Signal antibody refers to alkaline phosphatase conjugated antibody.

2.12 BRADFORD ASSAY

Protein concentration was estimated using the Bradford Protein Microassay (Bradford 1976) that is based on the shift in absorbance maximum of Coomassie Brilliant Blue G, from 465 to 595 nm, upon binding protein. The microassay allows accurate determination of protein

concentration between 0 to 200 µg/ml. A standard curve was constructed by the addition of 0, 5, 10, 15 and 20 µg of bovine serum albumin (Fraction V, Sigma) to Bradford reagent. Bradford reagent was prepared by dissolving 50 mg of Coomassie Brilliant Blue G-250 (BioRad Laboratories, Richmond, CA, USA) in 25 ml of 95% ethanol, 50 ml of 85% (w/v) phosphoric acid and 425 ml distilled water. Protein of unknown concentration was added to Bradford reagent and after a 5 minute incubation at room temperature the absorbance at 595 nm was measured on a spectrophotometer. A standard curve was constructed and the concentration of unknown calculated.

2.13 CELL CULTURE MEDIA AND CULTURE CONDITIONS

All tissue culture experiments were conducted in a sterile field in a laminar flow hood. Cells were grown in RPMI 1640 medium (Gibco BRL) supplemented with 5 x 10^{-5} M 2-mercaptoethanol, 2 mM L-glutamine (Gibco BRL), 100 IU/ml penicillin (Sigma), 100 µg/ml streptomycin sulphate (Sigma) and heat inactivated foetal calf serum (Gibco BRL) at 37°C in 5% CO_2 / 95% humidified air. The culture medium was known as RF5 and RF10 following the addition of 5% and 10% v/v foetal calf serum respectively.

2.14 CELL PROLIFERATION

2.14.1 Preparation of mononuclear cells from peripheral blood and nasal polyps

An equal volume of phosphate buffered saline was added to heparinized peripheral blood or nasal polyp single cell suspension (section 2.18.1) under sterile conditions. Twenty millilitres of the diluted sample was underlayed with 12 ml of Lymphoprep (density 1.077 ± 0.001 g/ml, osmolality 280 ± 15 mOsm) (Axis-Sheild ProC AS, Oslo, Norway) in a V-bottomed tube and centrifuged for 20 minutes at 800 *g* at room temperature. Mononuclear

cells that formed a distinct band at the sample/medium interphase were collected, pelleted twice prior to resuspension at a viable cell concentration, by trypan blue exclusion, at 10^7 cells/ml in phosphate buffered saline.

2.14.2 Labelling peripheral blood mononuclear cells with 5-(6)-carboxyfluorescein succinimidyl ester (CFSE)

Sufficient stock solution of precursor molecule, 5-(6)-carboxyfluorescein diacetate succinimidyl ester (CFDA-SE) (M_r 557) (Molecular Probes, Eugene, OR, USA), was added to 10^7 mononuclear cells suspended in 1 ml of phosphate buffered saline to make a final concentration of 10 μM. The suspension was mixed immediately to ensure uniform labelling of cells and allowed to incubate at 37°C for 15 minutes. The labelling process was terminated by quenching the cells in a five-fold volume of ice-cold RF5 and by incubating the cell suspension on ice for five minutes. Cells were washed three times in a five-fold volume of RF5 to ensure removal of extracellular CFDA-SE in the suspension, thereby preventing subsequent uptake into other bystander cells.

2.14.3 Assessment of cell proliferation using tritiated thymidine incorporation method

Proliferation was assessed by measuring radioactive (tritiated [6-^{3}H]) thymidine (Amersham Biosciences, Buckinghamshire, UK) incorporation into newly synthesised DNA of the dividing cells at the end of the culture period. After pulsing cells with tritiated thymidine (2.96×10^4 Bqs/ml, final concentration) for 16 hours, cells were harvested onto glass-fibre filter mats (Wallac, Turku, Finland) with a 96 well plate harvester (Tomtec MACH IIIM Harvester 96, ACC Emerging Technologies, Sydney, Australia). The filter mats were allowed to dry and the incorporated radioactivity counted on a betaplate counter (Wallac

1450 Microbeta Counter, PerkinElmer Lifesciences, Turku, Finland) following the addition of liquid scintillation (Betaplate Scint, Fisons Chemicals, Loughborough, UK). Results were expressed as either mean counts per minute (cpm) from triplicate wells or as a stimulation index (SI) (Rathore et al. 2001). The stimulation index is used to standardise and report cell proliferation results, and was calculated by dividing the mean counts per minute with stimulant by the mean counts per minute without stimulant (Froebel et al. 1999).

2.14.4 Assessment of cell division using CFSE-cytometry

Peripheral blood mononuclear cells (PBMC) were labelled with CFSE prior to setting up the fungal-specific proliferation assay. Labelling cells with CFSE allows their subsequent division history to be determined by flow cytometry (Parish 1999). The precursor, CFDA-SE, is a lipophilic molecule that is minimally fluorescent until it is transported into the intracellular compartment (Figure 2.3). Here, esterases cleave the two acetate groups from CFDA-SE to yield fluorescent CFSE. Carboxyfluorescein succinimidyl ester binds covalently to proteins and is retained in the intracellular compartment. Upon cell division, the amount of intracellular CFSE is divided equally in daughter cells. Therefore, cell division can be tracked by measuring their fluorescence intensity (Figure 2.4). Towards the end of the culture period, cells were collected and their fluorescence intensity detected on a FACScan (BD) in the fluorescence channel (FL) 1 parameter (section 2.21).

The internal controls for these experiments included (*a*) CFSE labelled cells that were incubated for the same duration without mitogen thereby allowing the position of the undivided peak to be determined and (*b*) unlabelled cells that were stimulated under the same culture conditions thereby allowing the position of the autofluorescence intensity to be determined (Figure 2.4).

At the end of the culture period, cells were colabelled with three-colour combinations of antibodies to CD3, CD4, CD8, CD14, CD19 and CD25 molecules to determine the phenotype of the dividing cells (section 2.20).

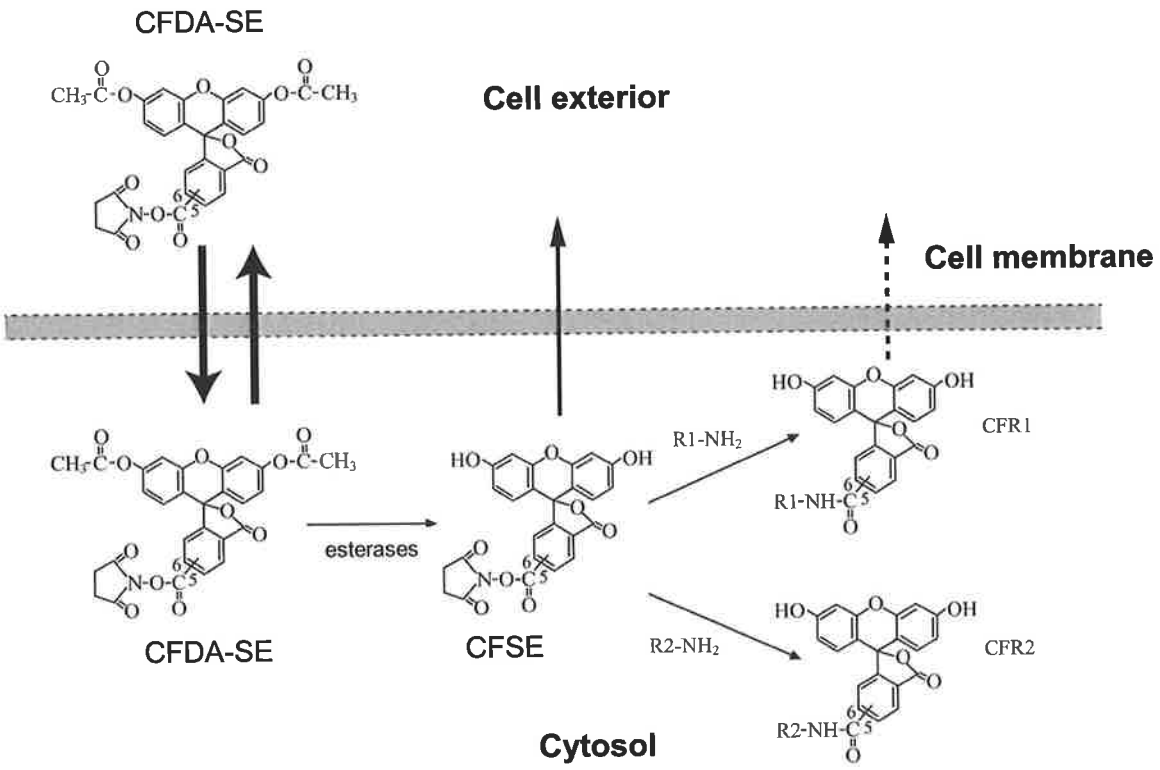


Figure 2.3 Schematic representation of the mechanism involved in the fluorescent labelling of cells using carboxyfluorescein diacetate succinimidyl ester (CFDA-SE). The CFDA-SE molecule is readily transported into the cytosol. In the intracellular compartment, fluorescent carboxyfluorescein succinimidyl ester (CFSE) is generated. The succinimidyl moiety of CFSE covalently couples carboxyfluorescein (CF) to intracellular molecules (R1-NH_2 or R2-NH_2) to form conjugates that are either short-lived (CFR1) or long-lived (CFR2). Modified from Parish 1999 and Wang et al. 2005.

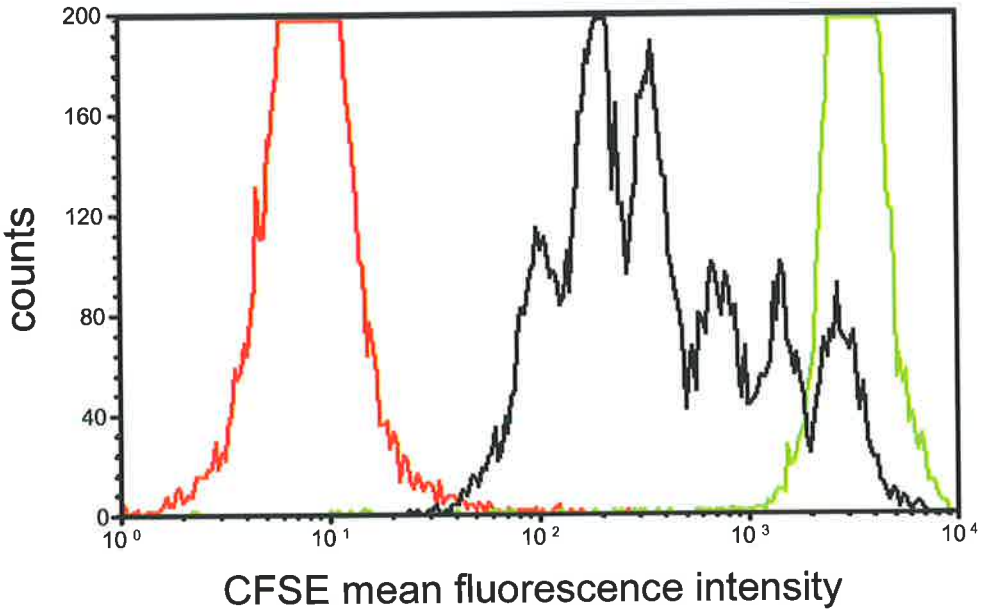


Figure 2.4 Basic features of carboxyfluorescein succinimidyl ester (CFSE)-cytometry tracking experiment for *in vitro* cell division. The histogram is gated on $CD3^+$ blast cells after four days of culture. The green line to the right illustrates the CFSE fluorescence intensity of T lymphocytes cultured unstimulated for the same period. The red line to the left portrays the autofluorescence intensity of cells that were not labelled with CFSE. The black line shows the dividing T cell population in response to phytohaemagglutinin, depicting the typical asynchronous profile. Progressive cell divisions are apparent by the two-fold dilutions of the mean fluorescence intensity. Adapted from Hasbold 1999.

2.15 ESTABLISHMENT OF THE FUNGAL-SPECIFIC MONONUCLEAR CELL PROLIFERATION ASSAY

The first method used to assess cell proliferation was that of Rathore et al. 2001 (Figure 2.5 A). Peripheral blood mononuclear cells suspended in RF5 at 10^5 cells per well were added in triplicate to sterile, flat-bottomed, 96 well, tissue culture plates (Cell Star, Greiner bio-one, Germany) in a final volume of 200 µl per well. Cells were stimulated with known concentrations of fungal antigens (diluted 1/10 – 1/160) or 12.5 µg/ml final concentration of phytohaemagglutinin (PHA) (Sigma) as a positive control and culture medium alone as a negative control. Cell proliferation was assessed by measuring cellular incorporation of tritiated thymidine after three, four, five and six days of culture.

The method above was modified to determine whether cell proliferation to fungal antigens could be enhanced (Figure 2.5 B). The PBMC were stimulated with a known concentration of fungal antigen in 96 well tissue culture plates. The remaining PBMC were kept in culture medium at $2x10^6$ cells per ml without fungal antigen in a sterile tissue culture flask (Cell Star) at 37°C until required. Following the first incubation time course (preincubation) of one half to two days, the contents of the 96 well plates were discarded. The method of discarding the well contents was compared as follows: "flicking" the contents of the tissue culture plates compared with aspirating each well, with or without a washing step. The wells were washed by adding 185 µl of culture medium to each well followed by either "flicking" the plate or re-aspirating the culture medium from each well. The non-adherent PBMC from the tissue culture flasks (kept in culture medium without fungal antigens) were collected, pelleted twice and added at 10^5 cells per well to the washed tissue culture plates. Following a second incubation time course of three, four, five, six and seven days, cell proliferation was determined by measuring cellular incorporation of tritiated thymidine.

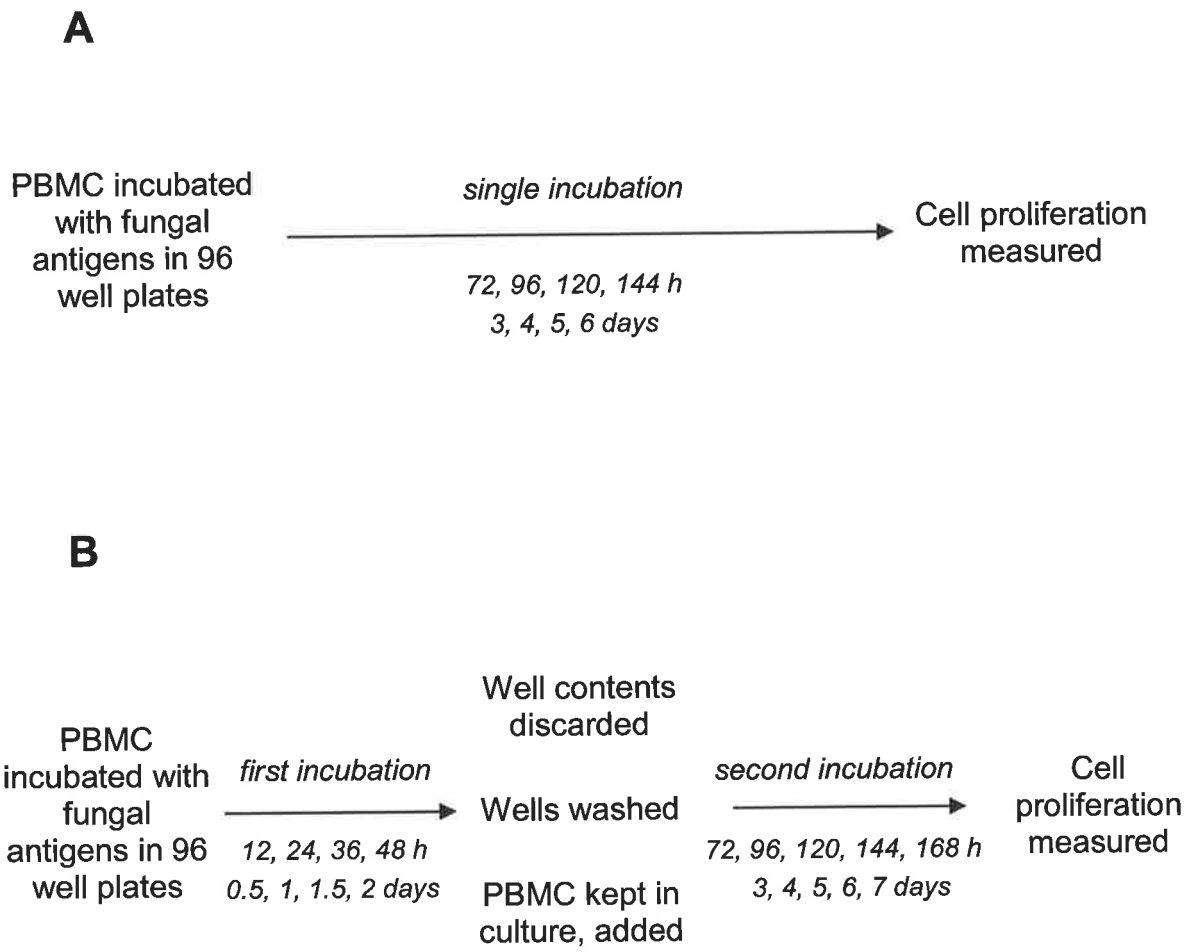


Figure 2.5 Schematic representations of the cell culture methods. Fungal-specific cell proliferation was assessed first, following a single incubation process (A) and second, by a modified method requiring two-step incubation (B). Using the two-step incubation method, the duration of the proliferation assay in which the peripheral blood mononuclear cells (PBMC) underwent a first incubation (preincubation) period of 36 hours (h) and a second incubation period of 144 hours or six days, was seven and a half days.

2.16 FUNGAL-SPECIFIC MONONUCLEAR CELL PROLIFERATION IN THE STUDY GROUPS

Cell incorporation of tritiated thymidine or CFSE-cytometry was used to measure fungal-specific proliferation using the two-step incubation method at the end of the culture period. Before setting up the proliferation assays, the proportions of $CD4^+$ and $CD8^+$ T lymphocytes, B lymphocytes and $CD14^+$ cells in each mononuclear cell sample was assessed using immunofluorescence (section 2.20). Except for the variables being tested, all fungal-specific cell proliferation assays were conducted under similar conditions. Peripheral blood mononuclear cells from two individuals that served as a negative and a positive control for fungal-specific proliferation were examined along with the test samples. The internal controls for cell proliferation included (*a*) a negative control where PBMC were incubated with tissue culture medium alone and (*b*) a positive control where PBMC were incubated with phytohaemagglutinin at a final concentration of 12.5 µg/ml.

In some experiments IL-2 was added to their respective wells at a final concentration of 25 units/ml.

2.17 MONOCLONAL ANTIBODY PRODUCTION, PURIFICATION AND CONJUGATION

2.17.1 Monoclonal antibody production from hybridomas

Hybridomas were purchased from the American Type Culture Collection (ATCC). Hybridomas were grown in RF10 media with the addition of 0.014 µg/ml hypoxanthine (Gibco BRL) and 0.004 µg/ml thymidine (Gibco BRL). Hybridoma supernatants were collected from actively growing cultures when the pH indicator of the culture medium

showed acidity; about every forty-eight hours. Cells were removed by centrifugation and supernatants were stored at –20°C in sterile aliquots until required. The monoclonal antibody concentration was determined by ELISA as described in section 2.9.2.

2.17.2 Antibody purification

Monoclonal antibodies were purified from tissue culture supernatants by affinity separation on Protein G Sepharose columns (Pharmacia). Protein G columns (5 ml) were equilibrated according to the manufacturer's instructions with 20 mM phosphate buffer (pH 7.0) containing 0.02% sodium azide. The column was attached to an LKB Uvicord II chromatography system and, to detect protein, the absorbance at 280 nm (A_{280}) of the eluate measured. Tissue culture supernatant containing the monoclonal antibody was passed through a 0.22 µm filter just prior to loading onto the column for each run at a flow rate of about 30 ml per hour. The column was washed with phosphate buffer until the baseline was re-established and bound antibody eluted with 0.1 M glycine in 0.15 M sodium chloride (pH 2.0) containing 0.01% sodium azide. On elution, purified antibody was neutralised immediately with tris buffered saline (8 g NaCl, 3 g Tris base dissolved in 1 L water, pH 7.0) and dialysed at 4°C for 48 hours against four changes of phosphate buffered saline. The protein concentration was estimated using the Bradford Assay (section 2.12) and the antibody stored in aliquots at –80°C until required.

2.17.3 Fluorescein labelling of proteins

The fluorescein conjugation method was modified from that of Goding 1986. Proteins to be conjugated with fluorescein were dialysed against 0.1 M sodium bicarbonate (pH 8.0) and the protein concentration estimated using the Bradford Assay. Fluorescein isothyiocyanate isomer 1 (FITC) (Sigma) was dissolved at 1 mg/ml in dimethylsuphoxide (Sigma). As the

protein concentration was greater than 1 mg/ml, FITC was added at a concentration of one-tenth of that of the protein concentration. The protein and FITC solution were incubated protected from light, for two hours at room temperature on a moving platform. Conjugated protein was separated from unconjugated FITC on a Sephadex G25 (PD10) column (Pharmacia) according to the manufacturer's instructions. The molar ratio of FITC to protein was calculated using the formula:

$$\text{Molar Ratio} = \frac{2.87 \times A_{495}}{A_{280} - 0.35 \times A_{495}}$$

Using this labelling method, all FITC/protein ratios were between two and four. Fluorescein isothyiocyanate-labelled proteins were stored in sterile aliquots at 4°C until required.

2.18 PREPARATION OF SINONASAL TISSUE FOR THE EXPERIMENTAL PROCEDURES

2.18.1 Preparation of single cell suspensions

Polyp and non-polyp sinonasal tissue were processed separately in a sterile field. Sinonasal tissue was placed in petri dishes containing cold RPMI 1640. The overlying blood vessels were dissected off the tissue and adherent mucus removed by washing the whole tissue thoroughly in RPMI 1640. The tissue was passed through 80 gauge sieves and the single cell suspension was washed twice with RPMI 1640 prior to suspending in newborn bovine serum (NBS) buffer (1% NBS (Gibco BRL), 0.01% sodium azide in phosphate buffered saline) for immunofluorescence studies only, or in sterile RF5 for tissue culture experiments.

2.18.2 Preparation of paraffin blocks

Sinonasal polyp and non-polyp tissue samples were embedded in paraffin blocks. Haematoxylin and eosin staining was used to examine the tissue architecture and indirect immunoperoxidase was used to examine the type and localisation of the inflammatory cell populations in these tissues (section 2.22).

2.19 CYTOSPIN PREPARATIONS OF NASAL POLYP TISSUE

Duplicate samples of nasal polyp single cell suspension (50 µl at $5x10^5$/ml) were loaded onto cytospin cuvettes (Shandon Inc., Pittsburgh, PA, USA) that were mounted with HistoGrip-coated slides (Zymed) and paper cards. The cytospin specimen chamber was centrifuged at 800 *g* for two minutes to allow a monolayer of cells to deposit on the slides. The slides were air-dried, counterstained with haematoxylin and eosin and the cell types identified by their nuclear morphology and cytoplasmic staining. All cell counts were performed using light microscopy and a haemocytometer.

2.20 IMMUNOFLUORESCENCE STAINING FOR FLOW CYTOMETRY

Flow cytometric examination of cell surface membrane and intracellular antigenic determinants were performed on single cell suspensions of sinonasal tissue, peripheral blood mononuclear cells and peripheral venous whole blood samples. Sinonasal cells and peripheral blood mononuclear cells for immunofluorescence studies were washed three times and resuspended at 10^6 to 10^7 cells per ml in NBS buffer.

Every batch of unlabelled and labelled antibodies (section 2.1) and reagents for these experiments were tested for reactivity and the titre required for maximal specific staining

determined in preliminary experiments against peripheral blood lymphocytes or cell lines. For each experiment, isotype-matched monoclonal antibody controls (section 2.1) were used. All incubations were performed at 4°C, protected from light, and all washing steps were carried out with 2 ml cold NBS buffer followed by decanting the supernatant unless otherwise stated.

2.20.1 Detection of cell surface membrane molecules

For direct, single colour immunofluorescence, the labelled antibody (primary antibody) was added to 50 µl of the cell suspension and incubated for 30 minutes. Following incubation, cells were washed once and resuspended in 50 µl of fixative (10 g D-glucose, 13 ml formaldehyde (37-40%), 2.5 ml 1 M sodium azide dissolved in phosphate buffered saline (pH 7.2-7.4)).

For indirect single colour immunofluorescence, the unlabelled antibody (primary antibody) was added to 50 µl of the cell suspension and incubated for 30 minutes. Following the incubation, cells were washed once and FITC or R-PE or biotin-labelled sheep anti-mouse immunoglobulins (Chemicon) (secondary antibody) were added for a further 30 minutes. For experiments using antibodies labelled with biotin, streptavidin-Quantum-Red (Sigma) was added for a further 30 minutes. Following the final incubation step, cells were washed once and resuspended in fixative.

Dual and three colour immunofluorescence was achieved by adding two and three antibodies directly or indirectly conjugated to fluorochromes of different emission wavelengths to the same cell sample.

In experiments with peripheral whole blood samples, red blood cell lysis was performed following immunofluorescence staining. Red blood cell lysis was accomplished by adding 2 ml of either FACS lysing solution (BD) or ammonium chloride solution (8.26 g NH_4Cl, 1 g $NaHCO_3$, 0.037 g EDTA dissolved in water (pH 7.2-7.4)) for 10 minutes at room temperature. Cells were pelleted, washed once and resuspended in fixative. In all indirect immunofluorescence experiments, additional controls included the addition of FITC or R-PE or streptavidin-Quantum Red in the absence of primary antibody.

2.20.2 Detection of intracellular molecules

Following cell surface staining with directly labelled antibodies, CD3 R-PECy5 and CD4 R-PE or CD8 R-PE, cells were fixed and permealized with the Cytofix/Cytoperm™ (BD) solution for 20 minutes at room temperature. Cells were pelleted, washed twice and resuspended in Perm/Wash™ (BD) buffer. A predetermined concentration of one of the following FITC conjugated antibodies to: granzyme A, granzyme B, perforin and the isotype-matched controls was added for 30 minutes at room temperature, protected from light. Cells were washed once, resuspended in Perm/Wash™ (BD) buffer and analysed within eight hours.

2.21 FLOW CYTOMETRIC ANALYSIS

All the flow cytometry reported in this thesis was carried out on FACScan (BD). The fluorochromes, FITC and R-PE, and the tandem dyes, Quantum Red and PE-Cy5, were excited by a 488 nm argon laser. The emitted light was detected by long pass filters in the FL-1 parameter (530/30 nm) for FITC, the FL-2 parameter (585/42 nm) for R-PE and the FL-3 parameter (650 nm) for Quantum Red and PE-Cy5. Calibration of FACS analyser was

performed before use with "Calibrite" (BD) beads according to the manufacturer's instructions. Computer analysis of the data was performed using CellQuest (BD) software.

For analysis of cell membrane surface and intracellular immunofluorescence, 10000 gated events were acquired from each sample of whole peripheral blood and mononuclear cell cells and 2500-5000 gated events from nasal tissue samples. Depending upon the experiment, cell populations were selected using side scatter (SSC) properties and $CD45^{high}$ or CD3 expression.

For cytometric analysis of CFSE labelled cells, gates were placed on the blast transformation of lymphocytes represented by increased forward scatter (FSC) and orthogonal scatter (side scatter) of light (Fulcher and Wong 1999). Populations of $CFSE^{+}$, $CD4^{+}$, $CD8^{+}$ and $CD25^{+}$ cells in this gate were examined. Routinely 50000 gated events were acquired from each sample.

2.22 IMMUNOHISTOCHEMICAL LOCALISATION OF THE INFLAMMATORY CELLS IN NASAL POLYPS

Indirect immunoperoxidase staining was used to examine the presence and distribution of the inflammatory cells in the nasal polyp tissue. The first section of tissue series from every individual was also stained with haematoxylin and eosin for histological comparison, examination of tissue architecture and cell composition. Sections were washed three times in tris buffered saline between incubation steps and all incubations were performed in a humidified chamber at room temperature.

Serial tissue sections of 4 μm thickness were cut and dried on a 37°C hotplate for three hours onto 2% HistoGrip-coated slides (Zymed). The sections were de-paraffinised in xylene, rehydrated through descending grades of ethyl alcohol (95%, 75% and 50%) through to tris buffered saline. Depending on the primary antibodies used, microwave antigen retrieval method (Shi et al 1991) in a 0.01 M citrate buffer (pH 6.0) was used to unmask epitopes on the tissue sections. Sections were cooled in water, then in citrate buffer, and blocked for 20 minutes with neat protein block serum-free (PBS-F) solution (Dako) to neutralise non-specific binding sites. Sections were incubated overnight with optimised dilutions of primary antibody (EG2, CD3, CD4, CD8 and the negative isotype control antibodies). Slides were immersed for 20 minutes into a 3% hydrogen peroxidase-absolute alcohol solution to quench endogenous peroxidases. Following washing, sections were incubated with biotinylated goat anti-mouse IgG (Dako, diluted 1/200) for 30 minutes, washed and incubated for a further 30 minutes with avidin-biotin-horseradish peroxidase (Dako, diluted 1/500). Sections were washed and incubated with 10 mg 3, 3'-diaminobezidine dissolved in imidazole-hydrochloric acid buffer (pH 7.5) containing hydrogen peroxidase as per specifications for Dako Liquid DAB solution (Dako) for five minutes. After washing, sections were counter-stained with haematoxylin to define nuclear morphology. The immunostained sections were viewed using a computer-assisted colour video image analysis system using a Leica DMLB microscope and images were captured using a Leica DC200 digital camera (Leica, NSW, Australia). Digital images were processed to match contrast and brightness and assembled into panels with Adobe Photoshop v 6.0 (Adobe Systems, Mountain View, CA, USA).

All the immunostaining was conducted in one sitting and in the presence of positive control tissue. The specificity of the primary antibody was determined by replacing the primary

antibody with buffer and by a negative control antibody. Other internal controls included omission of the secondary antibody.

2.23 DATA ANALYSIS

Statistical analysis for independent groups of data was performed with the Mann-Whitney U test for comparison between two groups and the Kruskal-Wallis test with Dunn's post-hoc test for comparison between multiple groups using GraphPad Prism software, version 4.0a for Macintosh (GraphPad, San Diego, CA, USA). For paired groups of data, the Wilcoxon signed rank test was used to compare between two groups. Data is presented as a median and the 25^{th} – 75^{th} percentiles, the limits of the interquartile range (IQR). P values are presented, together with the name of the analysis performed. A P value of less than 0.05 was considered significant.

A substantial number of measures were compared between the groups, which in itself, is a form of multiple testing. This should be taken into account when interpreting the level of statistical significance.

CHAPTER THREE: DEMOGRAPHIC, CLINICAL AND IMMUNOLOGICAL FEATURES OF EMCRS PATIENTS

3.1 INTRODUCTION

Allergic fungal sinusitis is viewed as a pathologically different disease from other subgroups of eosinophilic mucus chronic rhinosinusitis (Ferguson 2000a). This view is supported by studies that have reported significant differences in the demographic, clinical and immunological parameters and in the natural history between allergic fungal sinusitis patients and the other EMCRS subgroups (Ferguson 2000a; Marple 2001). However, studies are hampered by retrospective clinical reports, inconsistent definitions of allergic fungal sinusitis and a lack of disease-control groups. More important, the pathological significance of the criteria used to subgroup EMCRS patients, including type I hypersensitivity to fungi and detection of fungi in eosinophilic mucus, is yet to be ascertained.

The purpose of this study was to determine whether allergic fungal sinusitis patients could be distinguished from the other EMCRS subgroups: allergic fungal sinusitis-like, non-allergic, fungal eosinophilic sinusitis and non-allergic, non-fungal eosinophilic sinusitis (Chapter 2, section 2.4.2.1), by features other than the defining criteria. Therefore, the demographics, clinical characteristics, immunological parameters and other laboratory findings in the EMCRS patient subgroups were examined. To evaluate the pathogenic significance of these results in EMCRS patients, comparisons were made with healthy volunteers and two disease-control groups, chronic rhinosinusitis patients without eosinophilic mucus and allergic rhinitis with fungal allergy patients who had no evidence of rhinosinusitis.

3.2 RESULTS

3.2.1 Demographics

Demographic details were available for 188 EMCRS patients. There were 55 AFS, 12 AFS-like, 67 NAFES and 54 NANFES patients. The proportion of males to females in the EMCRS subgroups was similar (Table 3.1). However, the median age between the EMCRS subgroups was significantly different ($P = 0.0046$, Kruskal-Wallis test). Patients in the AFS subgroup were approximately 10 years younger than NAFES ($P = 0.013$) and NANFES ($P = 0.0009$) (Mann-Whitney U test). By contrast, there was no statistically significant difference between AFS and AFS-like subgroups ($P = 0.46$). Similarly, there was no significant difference in the median age between NAFES and NANFES groups ($P = 0.18$).

Table 3.1 Demographic details of the EMCRS patient subgroups

	AFS ***n*=55**	**AFS-like** ***n*=12**	**NAFES** ***n*=67**	**NANFES** ***n*=54**
Age in years	39 (31.5 – 53.0)	43 (33.5 – 55.0)	50 (41.5 – 59.5)	48 (38.5 – 60.0)
Male/Female	28/27	5/7	37/30	28/26

Age is presented as the median and the 25th-75th percentiles in parentheses

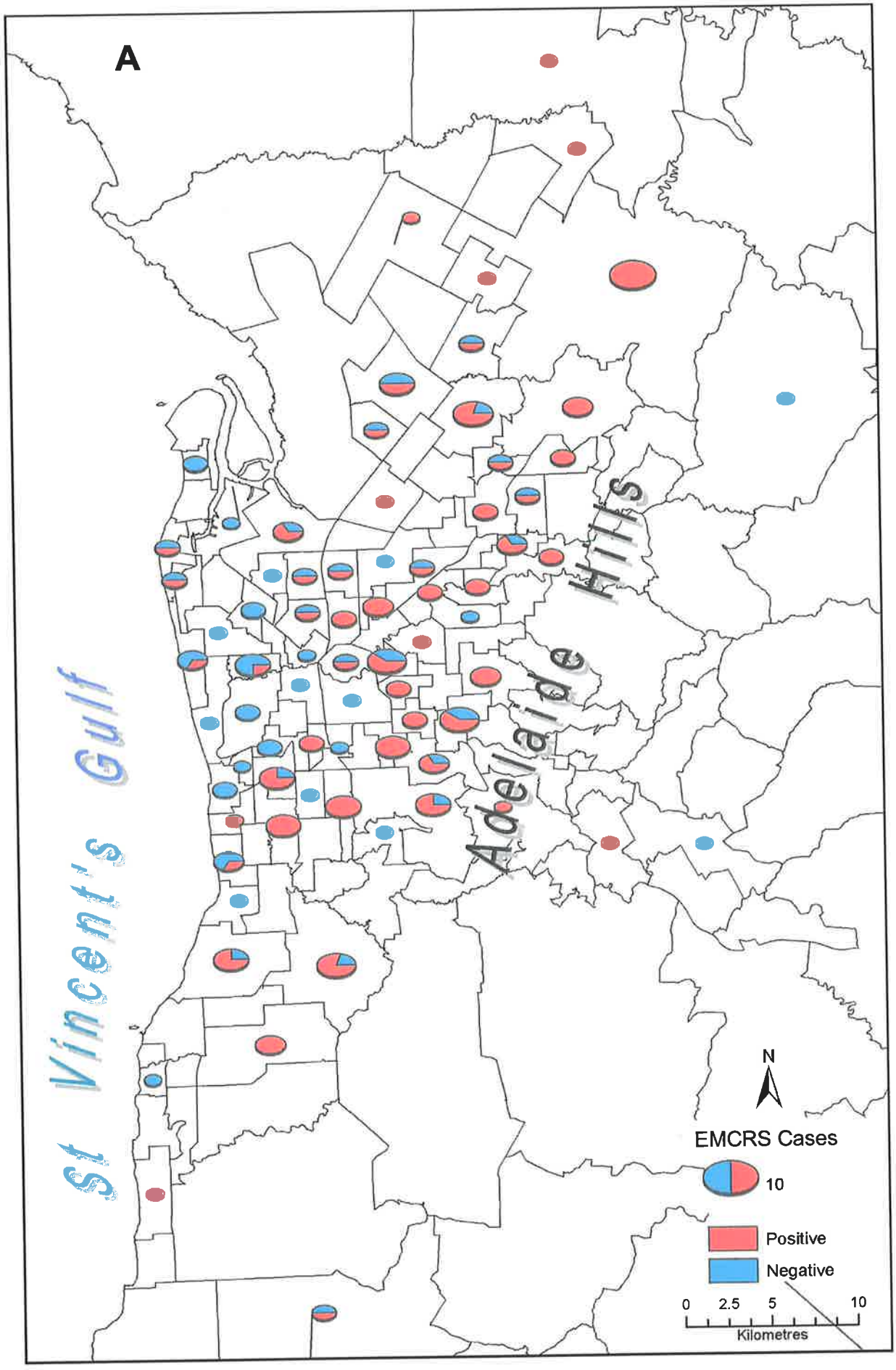
A geographic variation in the prevalence of AFS has been documented previously (Ferguson et al. 2000). To examine whether a geographic bias was present in the patient cohort in South Australia, postal code addresses of patients were used to map their location in the Adelaide metropolitan area. The count of EMCRS patients was expressed as a proportion of the population in each postal code area. There did not appear to be a geographic bias in the EMCRS patient population (Figure 3.1 A, B and C). Interestingly, a comparison between the EMCRS subgroups showed that patients who were fungus positive

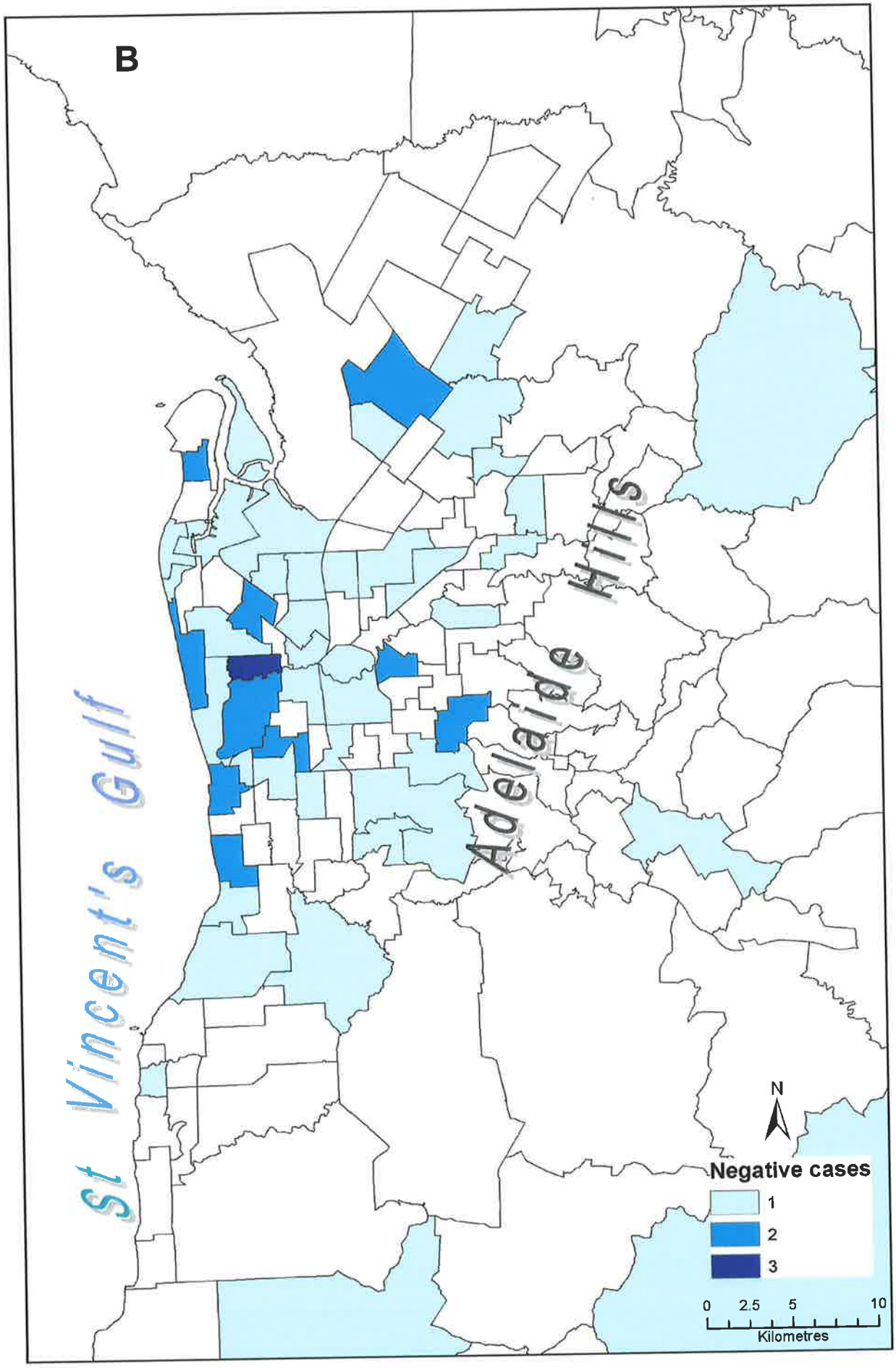
Figure 3.1 The geographic location of the EMCRS patients in metropolitan South Australia by postal code area.

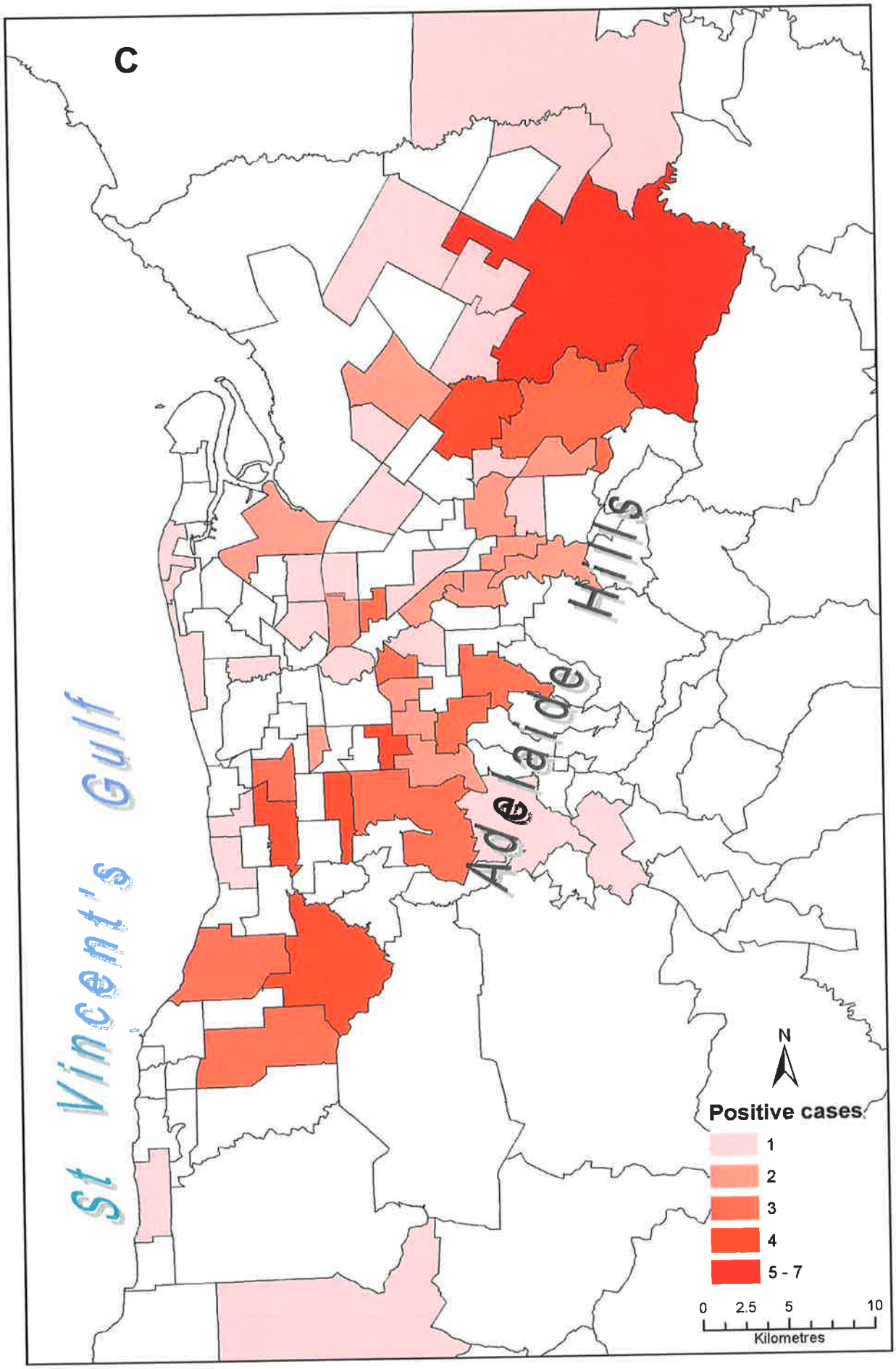
A. The pie chart of the number of EMCRS patients who were fungus negative (shown in blue) and fungus positive (shown in red) is presented as a proportion of the population in each postal code area. The number of EMCRS cases is proportional to the size of the pie as shown in the legend.

B. The geographic location of EMCRS patients who were fungus negative (AFS-like and NANFES). The colour gradient illustrates the number of fungus negative EMCRS cases per postal code address.

C. The geographic location of EMCRS patients who were fungus positive (AFS and NAFES). The colour gradient demonstrates the number of fungus positive EMCRS cases per postal code address.


A
St Vincent's Gulf
Adelaide Hills
N
EMCRS Cases
10
Positive
Negative
0 2.5 5 10
Kilometres


B
St Vincent's Gulf
Adelaide Hills
N
Negative cases
1
2
3
0 2.5 5 10
Kilometres


C
St Vincent's Gulf
Adelaide Hills
N
Positive cases
1
2
3
4
5 - 7
0 2.5 5 10
Kilometres

appeared to be located at the foothills and those who were fungus negative towards the coastline. Because AFS patients were a subgroup of fungus positive EMCRS, their geographic pattern was similar to that depicted in Figure 3.1 C.

3.2.2 Clinical history, symptoms and signs

Clinical data for 78 EMCRS patients were evaluated and compared with 23 ARFA and 26 CRS patients (Table 3.2). Because coexisting conditions were examined between the EMCRS subgroups, patients with other significant comorbidities were included (Chapter 2, section 2.4.4). The prevalence of asthma was greater in EMCRS compared with ARFA and CRS patients. A comparison of the asthma severity scores between the EMCRS subgroups showed no significant difference (P = 0.9197, Kruskal-Wallis test) (Figure 3.2). As a single group, EMCRS patients had a higher asthma severity score compared with ARFA (P = 0.0012) and CRS (P = 0.0004) (Mann-Whitney U test).

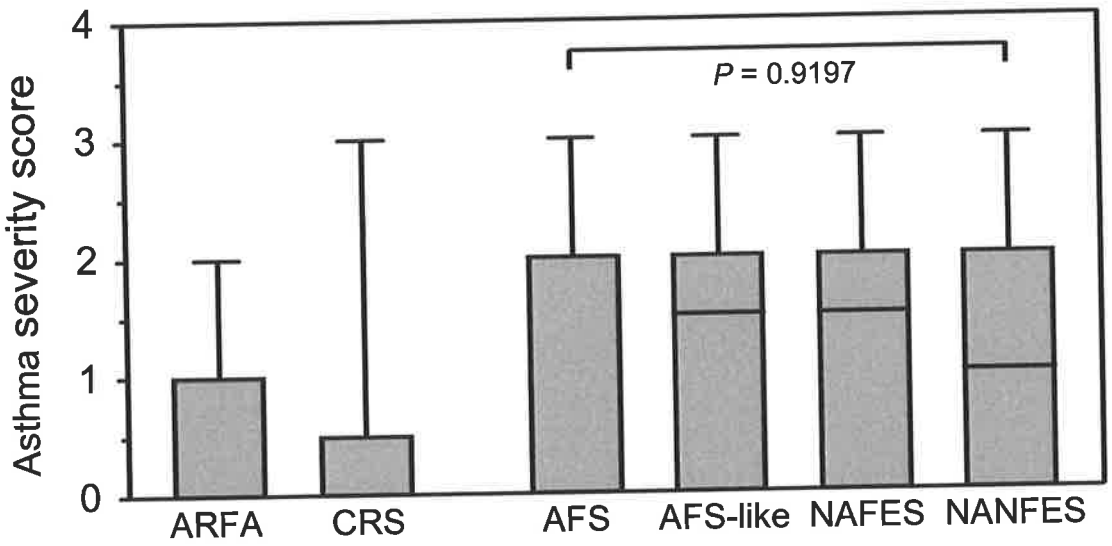


Figure 3.2 Comparison of asthma severity scores between ARFA, CRS and the EMCRS subgroups (AFS, AFS-like, NAFES and NANFES). Lower and upper limits of boxes show the 25th and 75th percentiles respectively, vertical bars show the ranges and horizontal bars the medians. The median was zero in ARFA and CRS and two in AFS.

The most common sinonasal symptoms were nasal obstruction, facial pain or headaches, blowing out "nasal casts" or nasal discharge and an altered sense of smell. Extra-sinus symptoms were present in seven patients (2 AFS, 3 NAFES and 2 NANFES). These related to ocular pain and diplopia. As expected, CRS and EMCRS patients had higher chronic rhinosinusitis symptom scores than ARFA patients (Figure 3.3). There was no significant difference in the total symptom score between the EMCRS subgroups (P = 0.24, Kruskal-Wallis test). As a group, the EMCRS patients had a higher symptom score (median = 8, IQR = 6 – 9) compared with CRS (median = 6, IQR = 5 – 7; P = 0.0008) and ARFA (median = 4, IQR = 3 – 5; $P < 0.0001$) (Mann-Whitney U test).

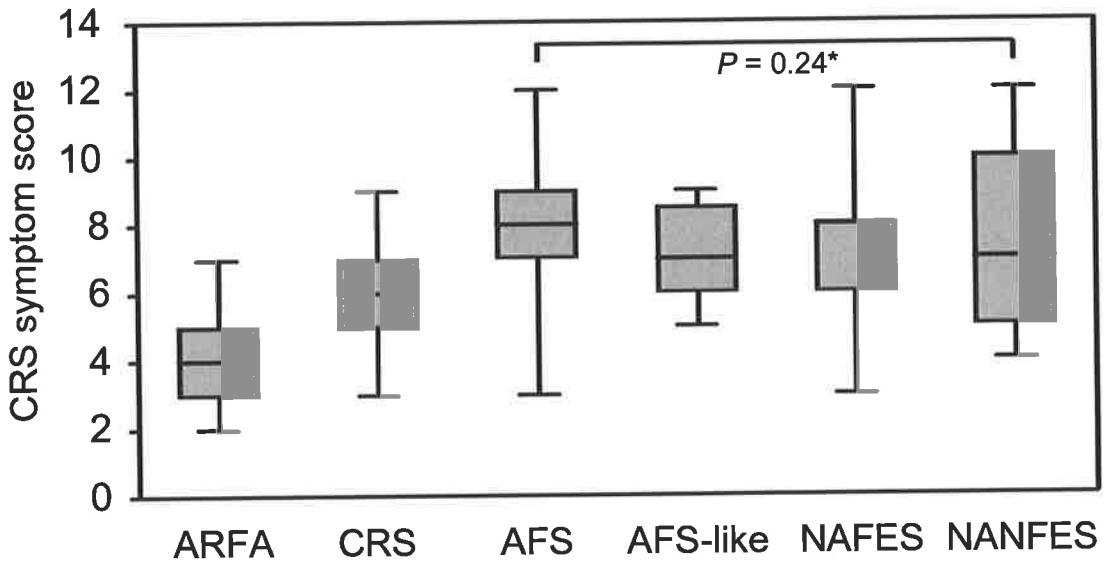


Figure 3.3 Comparison of the chronic rhinosinusitis (CRS) symptom scores between ARFA, CRS and the EMCRS subgroups (AFS, AFS-like, NAFES and NANFES). Lower and upper limits of the boxes represent the 25th and 75th percentiles respectively. The vertical bars show the ranges and horizontal bars the medians. *Kruskal-Wallis test.

Table 3.2 Comparison of the clinical features between ARFA, CRS and EMCRS patients

	ARFA *n*=23	**CRS** *n*=26	**AFS** *n*=34	**AFS-like** *n*=9	**NAFES** *n*=24	**NANFES** *n*=11
Age in years (median (IQR))	41 (34 – 52)	49 (41 – 56)	42 (32 – 56)	40 (30 – 67)	50 (46 – 59)	55 (45 – 60)
Male/Female	10/13	11/15	18/16	5/4	15/9	5/6
Nasal polyps (%)	NA	85	100	100	100	100
Previous sinus surgery[1] (%)	0	19	65	33	54	45
Asthma (%)	35	27	68	67	71	64
ABPA	0	0	2	1	NA	NA
Bronchiectasis	0	1	3	0	4	2
Churg-Strauss syndrome	1	0	1	0	1	0
Aspirin sensitive	3	3	5	1	4	2
Family history of polyps	1	4	1	0	5	2
Other coexisting medical conditions	RA (1)	RA (1)	RA (4), DM (3), Sarcoidosis (2), IBD (1), Polyps-Gastric (4), Bowel (5)	RA (1), Polyps - Bowel (1)	RA (5), DM (1), IBD (4)	RA (2), IBD (3), Polyps - Bowel (2)

[1]Patients who had at least one sinus operation before enrolling in this study. ABPA, allergic bronchopulmonary aspergillosis; DM, Type 1 Diabetes Mellitus; IBD, inflammatory bowel disease; IQR, interquartile range (25^{th}–75^{th} percentiles); NA, not applicable; RA, rheumatoid arthritis.

3.2.3 Sinus computed tomography findings

In this study, 50 EMCRS and 10 CRS patients were examined for radiological characteristics on sinus CT scans (Table 3.3). The total Lund-Mackay sinus CT scan score was not significantly different between the five study groups (P = 0.3271, Kruskal-Wallis test). Bilateral sinus disease was common to CRS and the EMCRS subgroups. Contrary to previous reports, unilateral sinus disease did not segregate to AFS patients (Ferguson 2000a). Intra-sinus double densities were seen in a minority and were not confined to cases with fungi in the sinuses. Bone erosion on CT scans was not limited to AFS patients. The most common site of bone erosion was in the ethmoid, followed by maxillary, sphenoid and frontal sinus. Bone sclerosis appeared to associate with a history of previous sinus surgery.

3.2.4 Sinus mucus and polyp histology

In this study, patients whose sinonasal mucus and polyp paraffin-blocks were available for further examination were included. Initially, sinus mucus and tissue sections stained with haematoxylin and eosin from 91 patients were examined. The sinus mucus from the 91 patients was reported by the surgeon to have resembled the typical macroscopic appearance of eosinophilic mucus. Of the 91 patients, 85 had eosinophil clusters in the mucus specimen upon histology and thus, were determined to be EMCRS patients. Of the six mucus samples that were not eosinophilic mucus, two had matted balls of fungal hyphae known as a fungal ball (Ferguson 2000c). In the remaining four samples, the only identifiable leucocyte population was the neutrophil.

Table 3.3 Comparison of the clinical characteristics and sinus computed tomography findings between CRS and EMCRS patients

	CRS ***n*=10**	**AFS** ***n*=18**	**AFS-like** ***n*=6**	**NAFES** ***n*=17**	**NANFES** ***n*=9**
Age in years (median (IQR))	44 (37 – 55)	39 (32 – 55)	35 (25 – 55)	50 (48 – 58)	53 (43 – 59)
Male/Female	5/5	10/8	3/3	11/6	5/4
Previous sinus surgery[1]	1	4	1	3	2
L-M CT score[2] (median (IQR))	12 (9 – 19)	18 (15 – 21)	15 (7 – 21)	16 (11 – 22)	12 (12 – 22)
Unilateral sinus disease	2	2	1	3	0
Double densities	0	7	2	8	3
Bone erosion[3]	0	5	0	4	1
Previous sinus surgery		1/5		1/4	0
Bone sclerosis[3]	1	6	0	5	2
Previous sinus surgery	1	3/6		3/5	2/2
Extra-sinus involvement	0	2 – orbital	0	1 – orbital	1 – orbital

[1]Number of patients who had at least one sinus operation before enrolling in this study. [2]$P = 0.3271$, Kruskal-Wallis test. [3]Number of patients with bone erosion or sclerosis is shown in the top row and the proportion of these patients that had previous surgery is provided in the bottom row. CT, computed tomography; IQR, interquartile range (25^{th}–75^{th} percentiles); L-M, Lund and Mackay.

In two out of the 85 EMCRS patients, fungal elements were demonstrated in the polyp tissue. Therefore, they were examined separately (details described below and in Table 3.4). Of the remaining 83 EMCRS patients, 23 were determined to be AFS, 8 AFS-like, 32 NAFES and 20 NANFES. Charcot-Leyden crystals were identified in 61%, 50%, 75% and 60% of eosinophilic mucus samples respectively, and therefore, called typical eosinophilic mucus. While most of the inflammatory cell population identified in the mucus samples was eosinophils, other leucocytes including lymphocytes and plasma cells were also present.

Examination of the nasal polyp tissue from the 83 EMCRS patients showed that eosinophils were common to all. However, lymphocytes, plasma cells and neutrophils were also present in most polyps. In the four patients with neutrophilic mucus samples, their polyp tissue showed a mixture of eosinophils and neutrophils. In the two patients with fungal balls, there was only a sparse infiltrate of eosinophils and lymphocytes.

Interestingly, polyps from two patients with eosinophilic mucus had fungal hyphae in the tissue. The fungal elements were confirmed to be in the plane of the tissue by phase-contrast microscopy. The leucocyte populations in the polyps from these two patients were eosinophils, lymphocytes and plasma cells. A review of these two patients' clinico-pathological characteristics showed that they were otherwise consistent with the EMCRS patient groups, AFS and NAFES (Table 3.4).

Table 3.4 Clinical characteristics of the patients who had fungal hyphae in polyp tissue

	Patient 1	**Patient 2**
Age	44	37
Sex	Male	Female
Asthma	Yes	Yes
Eosinophilic mucus	Yes	Yes
Fungal culture	*Alternaria alternata*	*Aspergillus, Drechslera* spp.
Polyp histology	Eosinophils, lymphocytes, plasma cells	Eosinophils, lymphocytes, plasma cells
Total immunoglobulin isotypes	Normal	Normal
Differential leucocyte counts	Normal	Normal
Aspergillus precipitins	Negative	Positive
Fungal allergy	*Alternaria, Cladosporium* spp.	Negative
Non-fungal allergy	Yes	Yes
Sinus CT scan		
Lund-Mackay score	22/24	20/24
Double densities	Yes	No
Bone erosion	No	No
CRS symptom score	6	5
PBMC stimulation index	212	193
Outcome	As per allergic fungal sinusitis	As per non-allergic, non-fungal eosinophilic sinusitis

CT, computed tomography; CRS, chronic rhinosinusitis, PBMC, peripheral blood mononuclear cells; spp., species.

3.2.5 Mucus mycology

Fungi were detected in sinus eosinophilic mucus in 55 out of 83 (66%) EMCRS patients by microscopy and fungal culture. Of these, fungi were demonstrated by microscopy in 10%, by culture in 58% and both microscopy and culture were positive in 32%. There was no significant difference between AFS and NAFES subgroups in the range of genera and species of fungi identified in the eosinophilic mucus samples (Table 3.5).

Fungi were also identified in three of the four patients who had neutrophilic sinus mucus. Of these three, budding yeasts were seen upon microscopy in one patient, although fungal culture was negative. In the other two patients, no fungi were demonstrated upon microscopy but fungal cultures were positive for *Penicillium* species in one patient and *Aspergillus* species in the other.

3.2.6 Peripheral blood total immunoglobulin levels, differential leucocyte counts and *Aspergillus fumigatus* precipitin levels

Pre-operative peripheral blood total immunoglobulin isotype levels and differential leucocyte counts from CRS and EMCRS patients were measured and compared with those from ARFA patients. The immunoglobulin levels from healthy volunteers were also available for comparison. There was no significant difference in the amounts of total IgG, IgA, IgM, IgE, IgG1, IgG2, IgG3 and IgG4 isotypes between the groups (Figure 3.4). Likewise, there was no significant difference in the total eosinophil, lymphocyte, monocyte and basophils counts between the groups (Figure 3.5). Precipitins to *Aspergillus fumigatus* were present in the serum of four EMCRS patients (2 AFS, 1 NAFES and 1 NANFES).

Table 3.5 Mycological findings in eosinophilic mucus of EMCRS patients

Fungi identified	**AFS** ***n*=23**	**NAFES** ***n*=32**
Number of genera[1]	2 (1 – 4)	2 (1 – 6)
Most common genus		
Alternaria	10	11
Aspergillus	8	12
Other[2]	17	21
Single fungal genus	52%	59%
Aspergillus	6	8
Alternaria	2	3
Drechslera	1	3
Fusarium	2	0
Penicillium	1	1
Scedosporium	0	1
Ulocladium	0	1
Single fungal species	13%	14%
Aspergillus fumigatus	2	2
Aspergillus niveus	1	0
Aspergillus niger	0	1
Aspergillus flavus	0	1

[1]Number of genera is presented as the median and the 25th–75th percentile, limits of the interquartile range, in parenthesis. [2]*Acremonium*, *Candida*, *Chaetomium*, *Cladosporium*, *Curvularia*, *Drechslera*, *Epicoccum Fusarium*, *Mucor*, *Penicillium*, *Phialophora*, *Scedosporium*, *Trichothecium Ulocladium*, and non-sporulating fungal species.

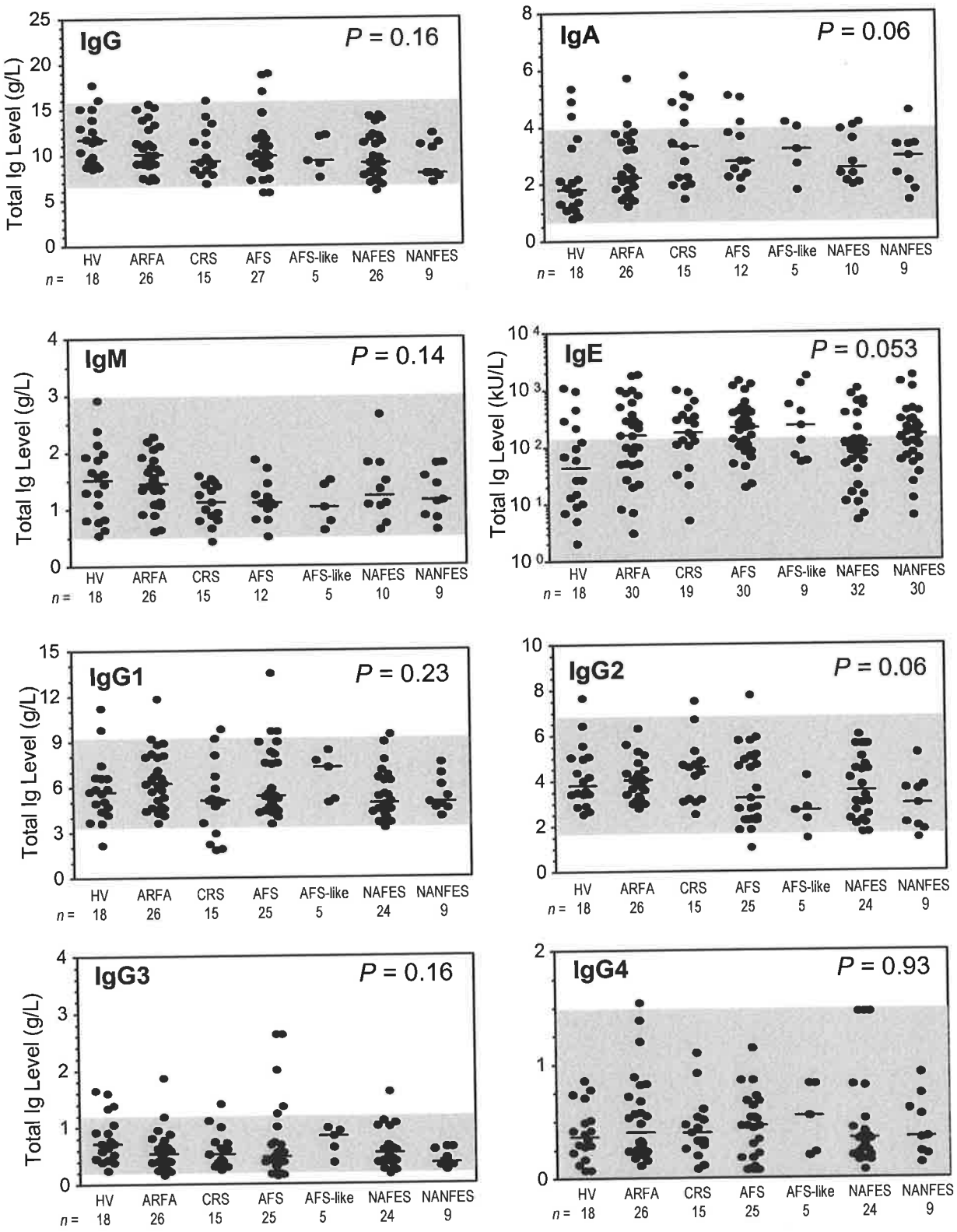


Figure 3.4 Total serum immunoglobulin levels in healthy volunteers (HV), ARFA, CRS and the EMCRS subgroups (AFS, AFS-like, NAFES and NANFES). The shaded areas indicate the reference ranges (Appendix III) for healthy adults for each immunoglobulin isotype. The bars indicate the median values and comparison of values between the groups was performed using the Kruskal-Wallis test. $P < 0.05$ was considered significant.

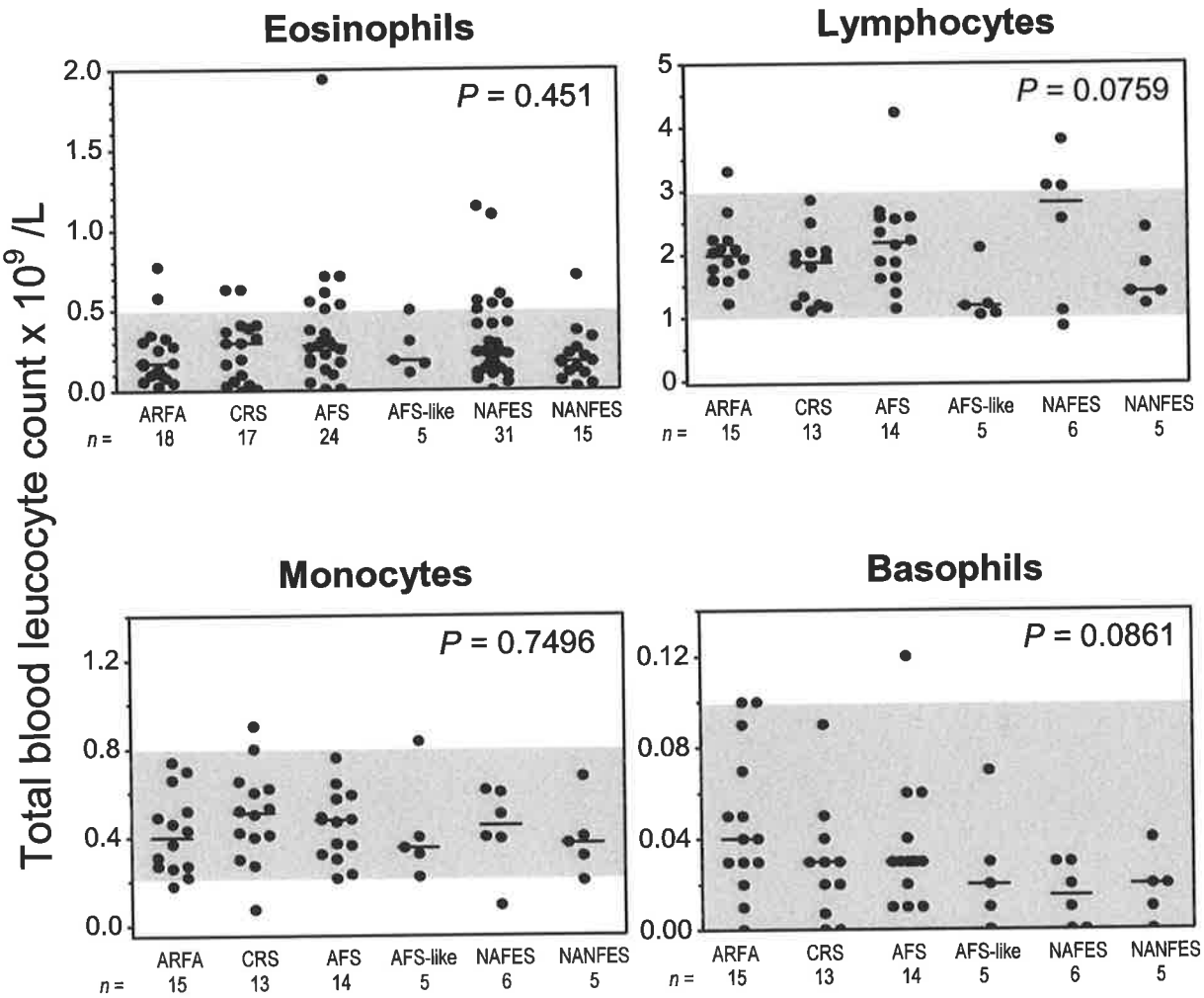


Figure 3.5 Total peripheral blood differential leucocyte counts in ARFA, CRS and the EMCRS subgroups (AFS, AFS-like, NAFES and NANFES). The shaded areas indicate the reference ranges (Appendix III) for healthy adults. The bars represent the median values and comparison of values between the groups was performed using the Kruskal-Wallis test. $P < 0.05$ was considered significant.

3.2.7 Peripheral blood mononuclear cell proliferation

The *in vitro* peripheral blood mononuclear cell proliferative response to antigen-specific or non-specific stimulants is a widely used technique to assess specific immunological memory and general immune function (Froebel et al. 1999). In this study, the magnitude of peripheral blood mononuclear cell proliferation in response to a non-specific mitogen, phytohaemagglutinin, was determined in 27 EMCRS patients (12 AFS, 3 AFS-like, 7 NAFES and 5 NANFES), 15 healthy volunteers, 15 ARFA and 15 CRS patients by measuring the cellular uptake of tritiated thymidine. Cell proliferation was expressed as a stimulation index (SI) (Chapter 2, section 2.14.3) (Figure 3.6). The proliferative response was not significantly different between the EMCRS subgroups ($P = 0.25$, Kruskal-Wallis test). As a single group, EMCRS patients had significantly greater proliferation compared with healthy volunteers ($P < 0.001$), ARFA ($P < 0.01$) and CRS ($P < 0.001$) (Mann-Whitney U test).

3.2.8 Fungal and non-fungal allergy

The prevalence of type I hypersensitivity to *Alternaria alternata* or *Aspergillus fumigatus* in AFS and AFS-like patients was similar to that observed in ARFA patients (Table 3.6). There was no obvious clustering of the allergic phenotype to AFS or other subgroups of EMCRS patients, as allergy to non-fungal aeroallergens was also common in the disease-control groups.

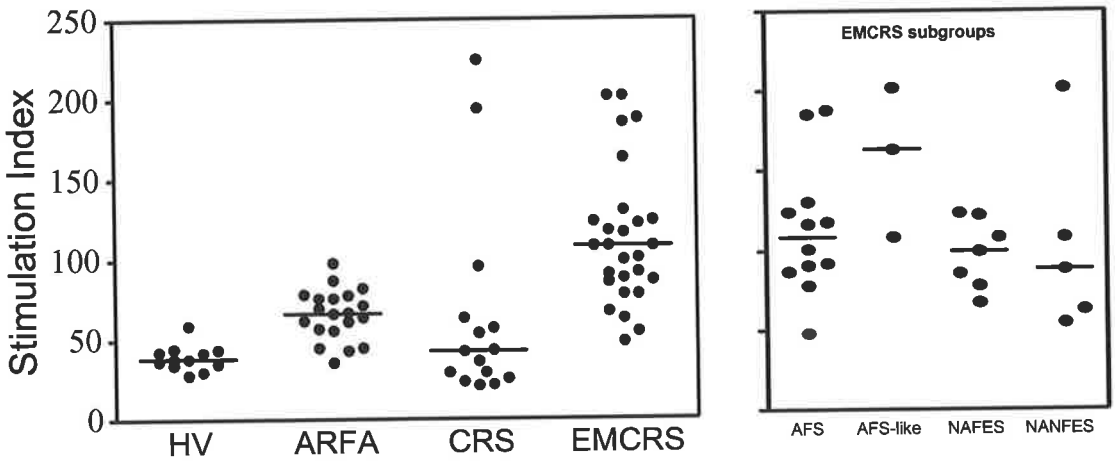


Figure 3.6 Peripheral blood mononuclear cell proliferative response to phytohaemagglutinin expressed as a stimulation index (SI). The bars indicate the median values in each group. There was no significant difference in the SI between the EMCRS subgroups: AFS (n=12), AFS-like (n=3), NAFES (n=7) and NANFES (n=5) (P = 0.25, Kruskal-Wallis test). Therefore, the SI obtained from EMCRS patients as a single group was compared with that from healthy volunteers (HV) (n=15), CRS (n=15), and ARFA (n=15) patients.

Table 3.6 Comparison of fungal and non-fungal allergy between ARFA, CRS and EMCRS patients

	ARFA ***n*=26**	**CRS** ***n*=26**	**AFS** ***n*=34**	**AFS-like** ***n*=6**	**NAFES** ***n*=24**	**NANFES** ***n*=11**
Age in years (median (IQR))	38 (25 – 42)	31 (26 – 53)	43 (32 – 56)	40 (30 – 67)	50 (46 – 59)	55 (45 – 60)
Male/Female	12/14	11/15	18/16	4/2	15/9	5/6
Fungal allergy[1]	100%	35%	100%	100%	0%	0%
Alternaria alternata	13	2	8	1		
Aspergillus fumigatus	2	1	4	1		
Both	11	2	6	2		
Other fungi[2]	23	7	21	5		
Non-fungal allergy[3]	85%	23%	88%	33%	54%	46%

[1]Positive fungal-specific serum IgE or skin prick test. [2]*Helminthosporium, Penicillium, Candida, Epicoccum* and *Cladosporium* species. [3]Mixes of tree pollen, grass pollen, house dust and mite and animal dander. IQR, interquartile range (25^{th}–75^{th} percentiles).

3.3 DISCUSSION

Work from this study determined that other than the defining criteria and the difference in the age of the patient groups, the clinical characteristics were not significantly different between the EMCRS subgroups. As a single group, a greater proportion of EMCRS patients had fungal allergy, lower respiratory tract disease and a more severe sinus disease compared with CRS patients. Together, these findings question the relevance of subgrouping EMCRS patients. Hence, eosinophilic mucus may be a marker for a pathologically distinct form of chronic rhinosinusitis, regardless of type I hypersensitivity to fungi or the detection of fungi in eosinophilic mucus.

Type I hypersensitivity to fungi was present in a larger proportion of EMCRS patients compared with CRS. However, IgE-mediated allergy to fungal and non-fungal aeroallergens was similar between EMCRS, CRS and ARFA patients. As noted previously, among the EMCRS group, the age of clinical presentation in patients with fungal allergy (AFS and AFS-like) was significantly younger compared with those without fungal allergy (NAFES and NANFES) (Ferguson 2000a). Although the duration of disease in each group could not be taken into account, the present data supported the hypothesis that coexisting allergy may exacerbate or prolong underlying inflammation, thereby resulting in an earlier clinical presentation.

A geographical bias in the prevalence of allergic fungal sinusitis has been reported in studies mainly from the United States of America, where a higher prevalence was documented in the southern states (Ferguson et al. 2000). This study did not show differences in geographic localisation of allergic fungal sinusitis patients compared with other fungus-positive EMCRS subgroups within South Australia. Significantly, there was no focus of patient groups to suggest ethnic clustering. However, there appeared to be more fungus negative

cases towards the coastal areas and fungus positive cases toward the foothills of Adelaide. This finding is of interest because most of the agricultural areas are located along the face of the Adelaide Hills. Therefore, the difference in fungal culture results may reflect a variation in the amount and type of fungal spores in the atmosphere colonising already diseased sinuses. These preliminary findings form a basis for further population-based studies, where sample size, relocation and tertiary centre referral bias are to be taken into account.

Allergic fungal sinusitis patients could not be distinguished from other subgroups of EMCRS patients based on the severity of rhinosinusitis symptoms, macroscopic and microscopic appearance of the mucosal inflammation, extra-sinus complications, CT scan findings, peripheral blood leucocyte levels and immunological studies. However, as a group, EMCRS patients had worse symptom scores and a higher prevalence of bone erosion on sinus CT scans compared with the disease-control, CRS patients. Bone erosion in EMCRS patients was not limited to allergic subjects or in patients that were fungus culture positive, and extra-sinus complications were not isolated to AFS patients. This finding, together with a history of multiple sinus operations, supported the hypothesis that EMCRS patients had a more severe disease compared with CRS patients without eosinophilic mucus.

There was no obvious association between EMCRS with autoimmune diseases, a family history of nasal polyps or with aspirin sensitivity. As noted in previous studies, coexisting diagnosis of allergic bronchopulmonary aspergillosis was not common in AFS or AFS-like patients (Shah et al. 2001). This may be explained by the inability to fulfil the stringent diagnostic criteria for allergic bronchopulmonary aspergillosis (Chapter 1, section 1.7.2). Interestingly, a higher prevalence of significant lower respiratory tract symptoms was present in EMCRS compared with CRS and ARFA patients. As in other chronic lung diseases, it is unknown whether severe lung disease with fungus in the bronchi may occur in

NAFES and NANFES patients, where an absence of allergic parameters precludes the diagnosis of allergic bronchopulmonary aspergillosis. It is also unknown whether an "allergic bronchopulmonary aspergillosis -like" condition of the lower airways occurs in the absence of allergy to fungi. Furthermore, EMCRS patients with a coexisting diagnosis of asthma had worse symptom scores than CRS and ARFA patients with asthma. Although objective measures for lung function was not performed, the present data indicates that EMCRS patients have significant upper and lower respiratory tract mucosal disease (Colice 2004). This finding is consistent with the "one-airway" hypothesis (Togias 2003) and forms a basis for future studies to determine the nature of this relationship.

Intriguingly, some EMCRS patients had previously diagnosed inflammatory bowel disease and others reported symptoms of lower bowel disease. This observation is significant as it could be marker for a predisposition to mucosal disease. This predisposition is unlikely to be due to an overt and generalised systemic immune deficiency because examination of the peripheral blood immune system in EMCRS patients showed no evidence of immune-compromise. Interestingly, measures of peripheral blood cellular response showed an enhanced activity compared with CRS, ARFA and healthy volunteers, indicating that EMCRS patients had a primed population of lymphocytes. This could reflect an ongoing underlying chronic immune stimulation, as elevated peripheral blood mononuclear proliferation to phytohaemagglutinin was not restricted to patients with fungal allergy or those with fungi in their sinuses. The presence of fungal elements in the mucosa of two EMCRS patients, also noted previously in AFS patients (Thakar et al. 2004), could be a consequence of local mucosal immune-compromise ensuing from chronic inflammation. Because the host is generally immune-competent, fulminant fungal infection is not observed.

In conclusion, work in this chapter established that most of the clinical characteristics of allergic fungal sinusitis patients were not significantly different from the other EMCRS subgroups, thereby warranting an investigation of the pathogenic significance of fungal allergy in these patients. Given the association of eosinophilic mucus with fungi, an examination of fungal-specific immunological mechanisms in EMCRS might enable a better understanding of the role of fungi in this disease. These will be the subject of further studies in Chapters 4 and 5.

CHAPTER FOUR: FUNGAL-SPECIFIC HUMORAL RESPONSE IN EMCRS PATIENTS

4.1 INTRODUCTION

Fungi in the sinus eosinophilic mucus and type I hypersensitivity to fungi are present in a proportion of EMCRS patients (Collins et al. 2003). Because of their ubiquitous nature it is not possible to attribute fungal pathogenicity simply based on their detection in eosinophilic mucus (Ponikau et al. 1999; Buzina et al. 2003). Similarly, it is not possible to attribute pathogenic importance to fungal-specific IgE-mediated hypersensitivity in EMCRS, as this is also present in patients without significant sinus disease. A critical review of the literature indicates that the role of fungi and fungal allergy in this disease is yet to be defined, as fungal-specific immune responses in EMCRS patients have not been thoroughly investigated.

The aim of this study was to determine whether EMCRS patients had a humoral response to fungi commonly isolated from their sinus eosinophilic mucus. An enzyme-linked immunosorbent assay was designed to measure fungal-specific antibody isotypes in sera. To determine the pathogenic significance of fungal-specific humoral response in EMCRS patients, comparisons with healthy volunteers and with disease-control patient groups were essential. For this purpose, sera were also examined from patients with the following diagnoses: allergic rhinitis with fungal allergy and no sinusitis, chronic rhinosinusitis without eosinophilic mucus, and from healthy volunteers.

4.2 RESULTS

4.2.1 Validation of the fungal antibody ELISA

The direct ELISA was more sensitive than the indirect ELISA (Figure 4.1). To establish the sensitivity of the IgE ELISA, the amount of IgE in a serum sample measured by ELISA was compared with the IgE levels measured by the ImmunoCAP system (Pharmacia). As shown in Figure 4.2, the direct ELISA measured IgE down to a level of 0.19 kU/L.

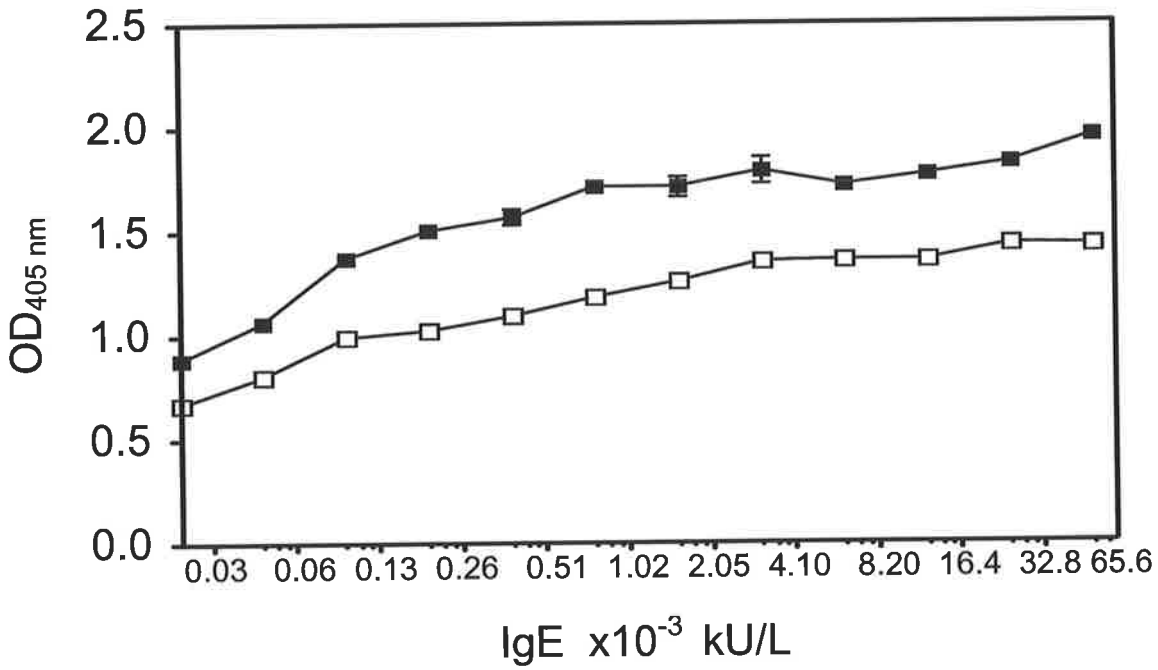


Figure 4.1 Measurement of IgE in a serum sample using a direct (■) and an indirect (□) enzyme-linked immunosorbent assay (ELISA). Serum IgE levels measured by ELISA are expressed as optical density values at 405 nm (OD_{405nm}) on the Y-axis and compared with IgE measured by the ImmunoCAP system (Pharmacia) in kU/L on the X-axis. Results are expressed as the mean OD_{405nm} values ± standard errors of the means of duplicate samples. Y intersects X-axis at X = 0.024. The X-axis is plotted on a log 2 scale.

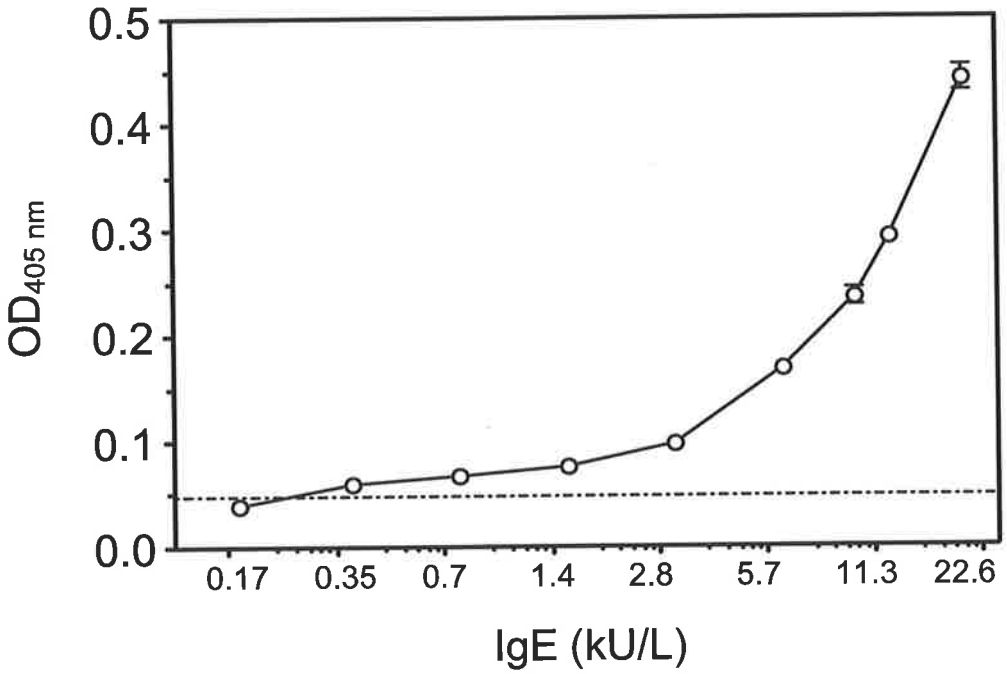


Figure 4.2 Sensitivity of the IgE enzyme-linked immunosorbent assay (ELISA). The ELISA results are expressed as the mean optical density values at 405 nm (OD_{405nm}) of three experiments conducted in duplicate ± standard errors of the means against the IgE concentration in kU/L measured by the ImmunoCAP system (Pharmacia). The OD_{405nm} values that lie above the dotted line represent positive results. As shown, the OD_{405nm} value of 0.05 corresponded to an IgE concentration of 0.19 kU/L.

Sera from 50 individuals who were negative for *Alternaria* and *Aspergillus*-specific IgE by the ImmunoCAP system were screened with the ELISA. The highest OD_{405nm} value obtained by ELISA in these subjects was 0.043. Therefore, a value of 0.05 or greater was defined as a positive result.

To determine the specificity of the fungal antibody ELISA, *Alternaria alternata* and *Aspergillus fumigatus*-specific IgE levels measured by ELISA were compared with those

measured by the ImmunoCAP system (Pharmacia). There was a positive correlation between the fungal-specific IgE results using ELISA and the ImmunoCAP system for both fungal species (Figure 4.3). However, there were some differences in the levels of IgE that were measured using the two methods. For example, two patients reported to have less than 0.35 kU_A/L of *Alternaria alternata*-specific IgE by the ImmunoCAP system gave low-positive results by ELISA, thereby indicating that the ELISA was more sensitive.

In all except for one case, sera that showed a low positive or a negative IgE result for one fungal species and high positive for the other fungal species by the ImmunoCAP method gave a similar pattern to the ELISA method. This patient who had *Alternaria* and *Aspergillus*-specific IgE of 11 kU_A/L each by the ImmunoCAP method had optical density readings of 0.258 for *Alternaria* and 0.544 for *Aspergillus* by ELISA. The difference in the measurement of fungal-specific IgE levels between the two methods, ELISA and ImmunoCAP, may be due to variations in fungal antigens in the preparations from Hollister-Stier and Pharmacia respectively (Vailes et al. 2001). As Hollister-Stier or Pharmacia would not reveal the protein profile of the fungal antigen mix, this could not be taken any further.

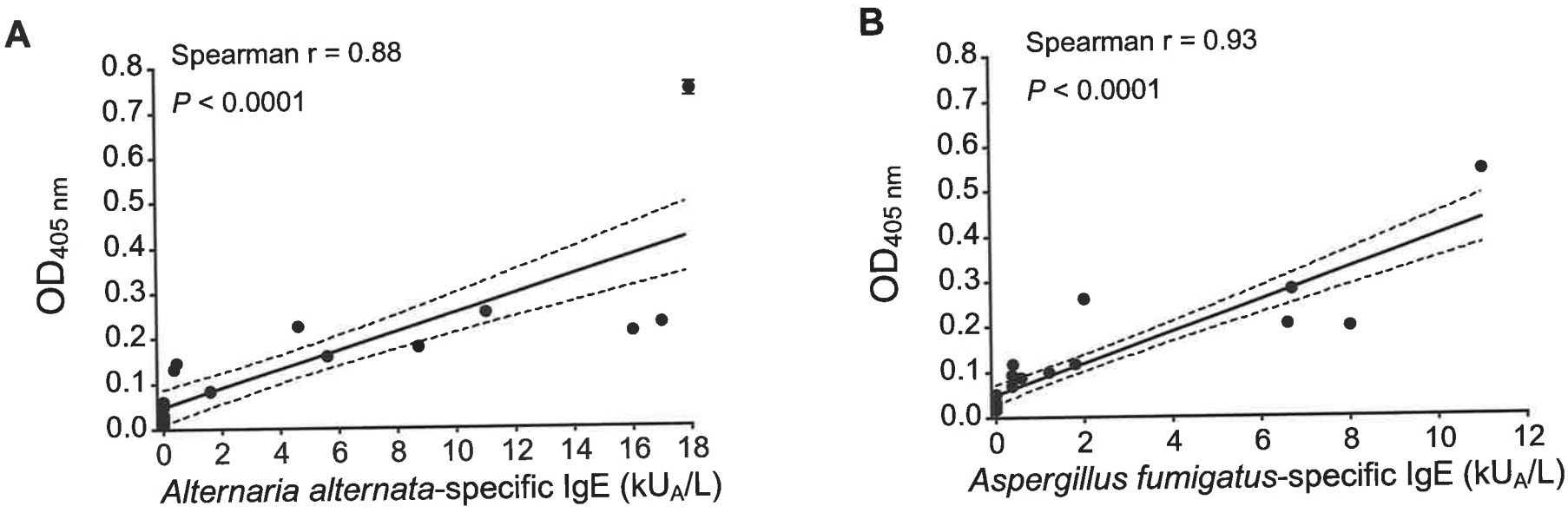


Figure 4.3 Comparison of fungal-specific serum IgE levels measured by fungal antibody enzyme-linked immunosorbent assay (ELISA) and by the ImmunoCAP system (Pharmacia). *Alternaria alternata*-specific (A) and *Aspergillus fumigatus*-specific (B) serum IgE levels were measured in 10 non-allergic individuals and 10 patients with fungal allergy. The ELISA results are expressed as the mean optical density reading at 405 nm (OD_{405nm}) of duplicate serum samples from every individual and the ImmunoCAP results expressed in kU_A/L. The continuous lines indicate the mean values for the groups and the dotted lines show the 95% confidence intervals. There was a positive correlation between the results obtained from the ELISA with those from the ImmunoCAP system (Spearman r).

4.2.2 Profile of *Alternaria alternata* and *Aspergillus fumigatus* – specific antibodies in sera of EMCRS patients

In this study, 30 EMCRS patients, 15 healthy volunteers, 26 ARFA patients and 15 CRS patients were examined. Their clinical characteristics are shown in Table 4.1. The profile of *Alternaria alternata* and *Aspergillus fumigatus* humoral response were similar. Therefore, the results presented in this section are for *Alternaria alternata* unless otherwise specified.

4.2.2.1 Total and fungal-specific IgE

The pathogenic significance of IgE-mediated systemic fungal allergy in EMCRS patients was examined by comparing total IgE levels in kU/L, and fungal-specific IgE levels in kU_A/L, between fungal-allergic EMCRS (AFS and AFS-like) and ARFA patients. There were no statistically significant differences in the total IgE levels ($P = 0.8$) and mould mix-specific IgE levels ($P = 0.6$) between fungal-allergic EMCRS and ARFA patients (Mann-Whitney U test) (Figure 4.4 A). Similarly, there was no significant difference in the ratio of mould mix-specific IgE to total IgE levels between the two fungal-allergic groups ($P = 0.9$, Mann-Whitney U test) (Figure 4.4 B).

To determine the pathogenic importance of allergy to specific fungi in EMCRS, the levels of IgE to *Alternaria alternata* and *Aspergillus fumigatus* were measured by the fungal antibody ELISA and compared with control groups. ARFA patients had higher fungal-specific IgE levels compared with healthy volunteers ($P < 0.0001$, Mann-Whitney U test) (Figure 4.4 C). Similarly, AFS ($P = 0.0036$) and AFS-like ($P = 0.0016$) patients had higher fungal-specific IgE levels compared with healthy volunteers. By contrast, there were no significant differences in fungal-specific IgE levels between fungal-allergic EMCRS and ARFA ($P = 0.8839$, Kruskal-Wallis test). Interestingly, only 42% of AFS patients had type I hypersensitivity to the same fungal species identified in their eosinophilic mucus.

Table 4.1 Clinical characteristics of the fungal-specific humoral study groups

	HV ***n*=15**	**ARFA** ***n*=26**	**CRS** ***n*=15**	**AFS** ***n*=12**	**NAFES** ***n*=8**	**AFS-like** ***n*=5**	**NANFES** ***n*=5**
Age in years (median (IQR))	29 (27 – 33)	38 (25 – 42)	43 (29 – 57)	31 (21 – 39)	36 (34 – 56)	40 (23 – 41)	33 (26 – 50)
Male/Female	9/6	11/15	8/7	7/5	4/4	3/2	4/1
Total IgE (kU/L) (median (IQR))	59 (7 – 334)	635 (322 – 997)	87 (34 – 170)	209 (40 – 1036)	46 (10 – 170)	197 (119–762)	72 (48 – 177)
Fungus in eosinophilic mucus[1]	NA	NA	NA	100%	100%	0%	0%
Alternaria alternata				6	4		
Aspergillus fumigatus				5	2		
Both				1	2		
Other fungi[2]				5	2		
Fungal allergy[3]	0%	100%	27%	100%	0%	100%	0%
Alternaria alternata		6	1	1		2	
Aspergillus fumigatus		5	0	2		1	
Both		15	3	5		2	
Other fungi[4]		17	2	8		3	
Non-fungal allergy[5] (%)	40	73	33	66	38	60	40
Aspirin sensitive (%)	0	12	27	17	0	0	20
Asthma (%)	27	31	20	75	50	80	80

[1]By histology or fungal culture. [2]*Bipolaris, Drechslera, Trichothecium, Candida, Penicillium, Scedosporium, Acremonium, Devriessi, Phialaphora* and *Cladosporium* species. [3]By positive fungal-specific serum IgE or skin prick test. [4]*Helminthosporium, Penicillium, Candida, Epicoccum* and *Cladosporium* species. [5]Mixes of tree pollen, grass pollen, house dust mite and animal dander. IQR, interquartile range (25^{th}–75^{th} percentiles); NA, not applicable.

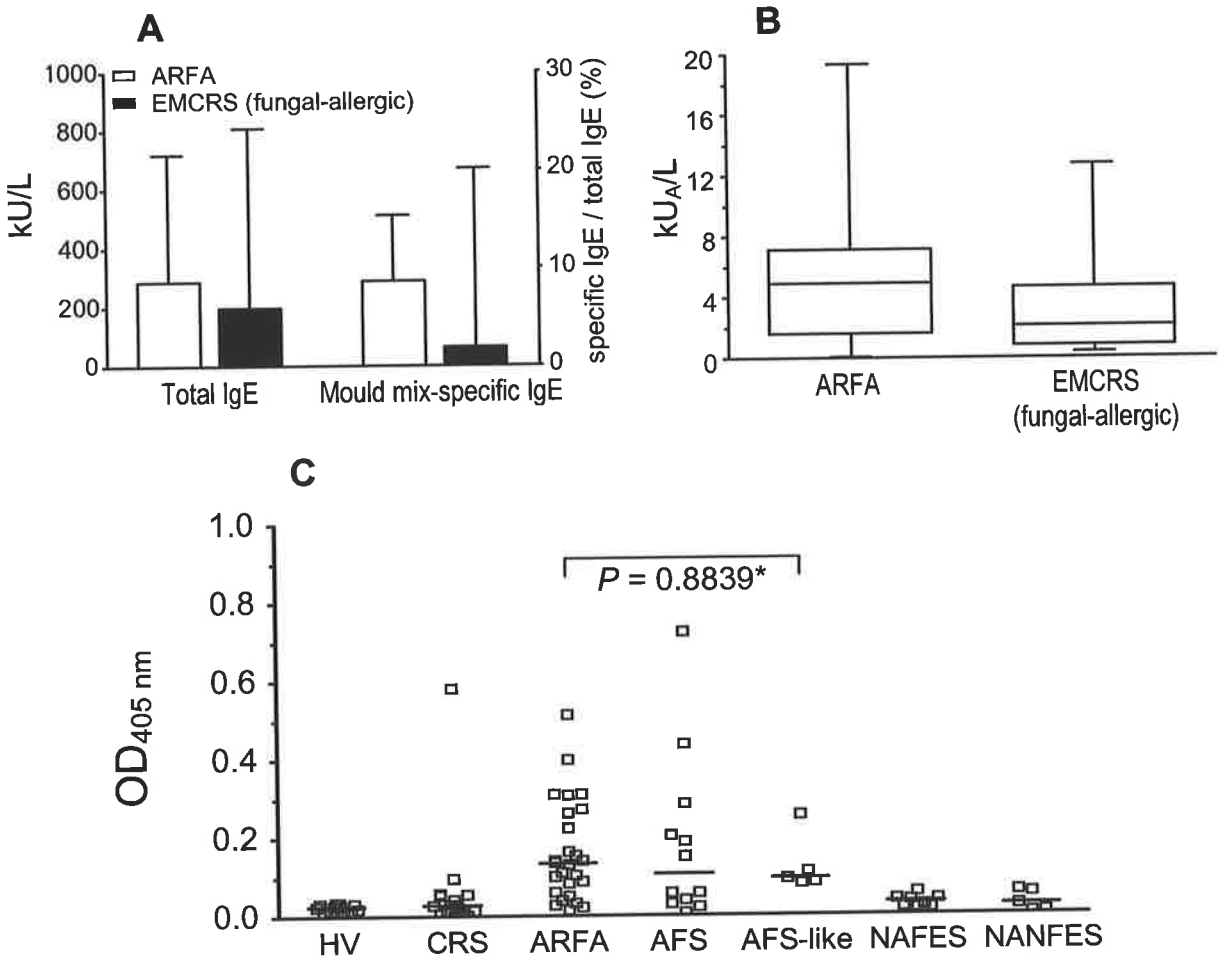


Figure 4.4 Total and fungal-specific serum IgE levels in EMCRS patients and control groups. (A) shows the comparison of total IgE (kU/L) and mould mix-specific IgE (kU$_A$/L) levels in ARFA (n=26) and fungal-allergic EMCRS patients (AFS and AFS-like) (n=17). The columns depict the median values and bars, the interquartile ranges. In (B), the ratio of mould mix-specific IgE:total IgE are expressed as percentage values in ARFA and fungal-allergic EMCRS patients. Lower and upper limits of boxes represent the 25th and 75th percentiles respectively, the vertical bars show the ranges and horizontal bars the medians. In (C), *Alternaria alternata*-specific IgE levels in control groups (HV, CRS and ARFA) and the EMCRS subgroups (AFS, AFS-like, NAFES and NANFES) assayed by ELISA is depicted. Results are expressed as the mean optical density (OD$_{405nm}$) readings of duplicate serum samples. The bars indicate the median OD$_{405nm}$ values. *Kruskal-Wallis test. The results for *Aspergillus fumigatus*-specific IgE were similar.

4.2.2.2 Fungal-specific IgG, IgA and IgM

Sera were tested for fungal-specific total IgG, IgA and IgM levels using the fungal antibody ELISA (Figure 4.5). The results for the EMCRS patients were expressed as a single group because the levels of fungal-specific IgG, IgA and IgM were not significantly different between the EMCRS subgroups (P = 0.5457, P = 0.2441 and P = 0.8358 respectively, Kruskal-Wallis test).

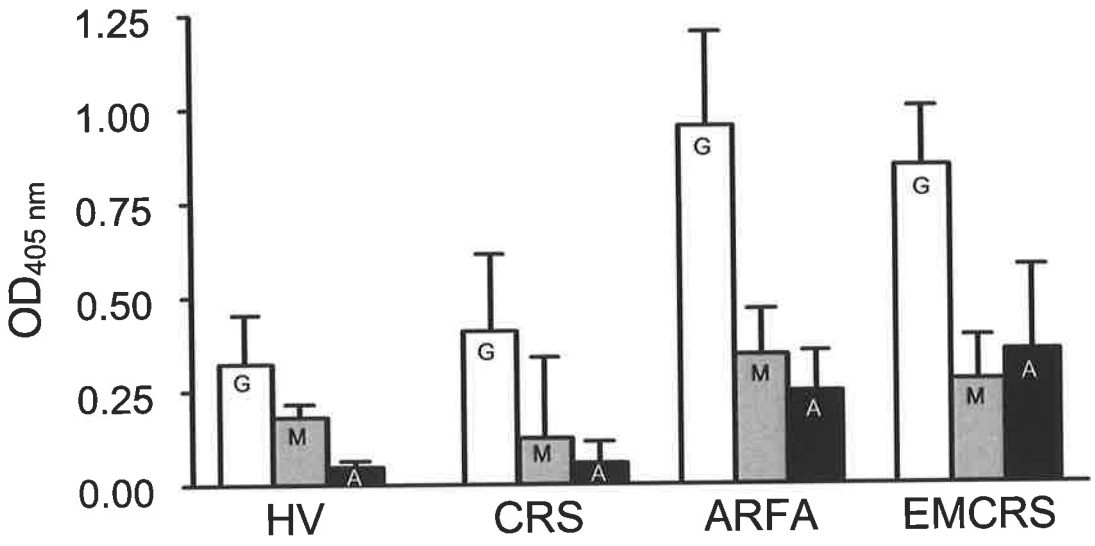


Figure 4.5 *Alternaria alternata*-specific IgG, IgM and IgA levels measured by fungal antibody ELISA in HV, CRS, ARFA and EMCRS patients. The columns represent the median optical density values at 405 nm (OD_{405nm}) in each group, and bars show the interquartile ranges. The results for *Aspergillus fumigatus*-specific IgG, IgM and IgA were similar.

Fungal-specific IgM levels were not significantly different between healthy volunteers, CRS, ARFA and EMCRS patients (P = 0.07, Kruskal-Wallis test). However, fungal-specific IgG (P < 0.0001) and IgA (P = 0.0007) levels were significantly different between the study groups. Fungal-specific IgG levels were higher in EMCRS patients compared with healthy

volunteers ($P = 0.0002$) and with CRS patients ($P = 0.0052$), but not with ARFA patients ($P = 0.2438$) (Mann-Whitney U test). Similar to the IgG data, fungal-specific IgA levels were significantly higher in EMCRS patients compared with healthy volunteers ($P = 0.0016$, Mann-Whitney U test). However, there was no significant difference between EMCRS and CRS ($P = 0.0916$) and between EMCRS and ARFA ($P = 0.8855$) (Mann-Whitney U test). Similar to the IgE results, no significant difference in the levels of fungal-specific IgG and IgA were observed between the EMCRS and ARFA patients.

4.2.2.3 Fungal-specific IgG subclasses

Levels of fungal-specific IgG1, IgG2, IgG3 and IgG4 isotypes were not significantly different among the four EMCRS subgroups ($P = 0.6969$, $P = 0.6256$, $P = 0.6019$ and $P = 0.3135$ respectively, Kruskal-Wallis test). Therefore, similar to the IgG, IgA and IgM data, the results for fungal-specific IgG subclasses were expressed as a single group for EMCRS patients.

Comparison of fungal-specific IgG subclasses between healthy volunteers, CRS, ARFA and EMCRS showed that IgG2 levels were higher across the study groups compared with IgG1, IgG3 and IgG4 isotypes (Figure 4.6). Specific IgG subclasses were significantly lower in healthy volunteers compared with CRS, ARFA and EMCRS patients ($P < 0.05$, Kruskal-Wallis test). Levels of IgG2 and IgG4 were not significantly different between EMCRS and CRS patients ($P = 0.0550$ and $P = 0.4325$ respectively) or between EMCRS and ARFA patients ($P = 0.9280$ and $P = 0.2059$ respectively) (Mann-Whitney U test). Higher levels of fungal-specific IgG1 were present in EMCRS compared with CRS ($P = 0.0089$), but not with ARFA ($P = 0.0527$) patients. However, IgG3 distinguished EMCRS patients from all the control groups, including CRS ($P = 0.0022$) and ARFA ($P = 0.0013$) (Mann-Whitney U

test). Specific IgG3 levels in EMCRS patients were independent of the detection of fungi in eosinophilic mucus or fungal allergy. Interestingly, high IgG1 levels in EMCRS patients approached statistical significance compared with ARFA patients.

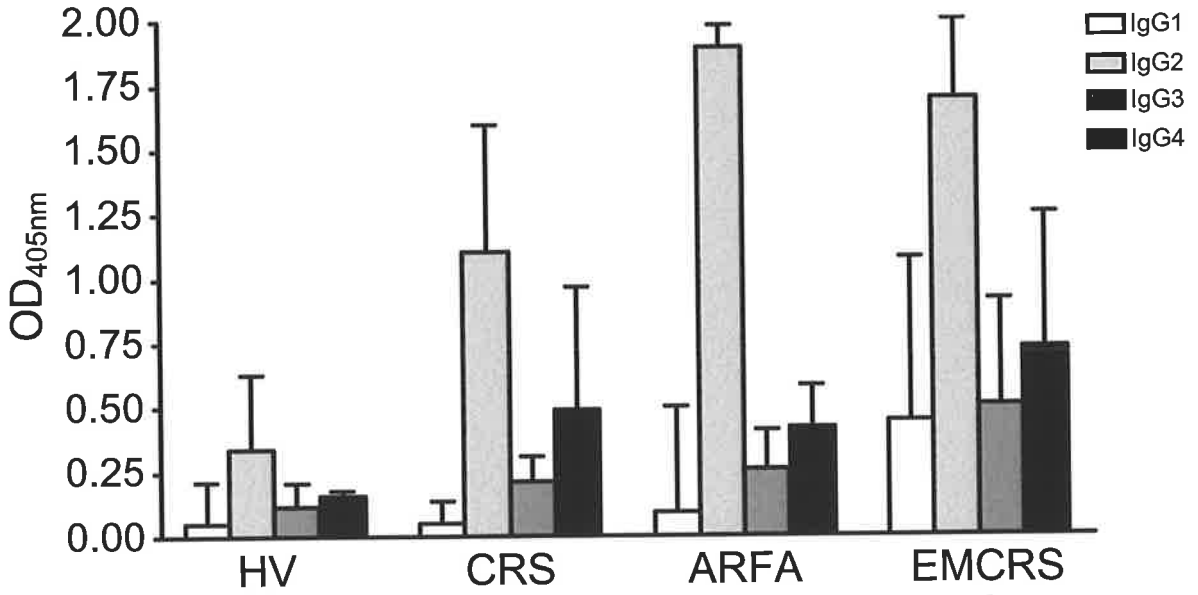


Figure 4.6 Levels of *Alternaria alternata*-specific IgG subclasses: IgG1, IgG2, IgG3 and IgG4 measured by fungal antibody ELISA in HV, CRS, ARFA and EMCRS patients. The columns indicate the median optical density values at 405 nm (OD_{405nm}) in each group, and bars show the interquartile ranges. The data for *Aspergillus fumigatus*-specific IgG subclasses were similar.

4.2.2.4 Relationship between fungal-specific immunoglobulin levels and the detection of fungus in eosinophilic mucus

To ascertain whether the detection of fungus in eosinophilic mucus affected fungal-specific serum antibody levels, results from EMCRS patients who had fungus positive eosinophilic mucus were compared with those who had fungus negative eosinophilic mucus. In parallel

with the fungal-specific IgE data, levels of specific IgG, IgA, IgM and IgG subclasses for either fungal antigen, did not differ significantly between the two groups (Table 4.2).

Table 4.2 Comparison of *Alternaria alternata*-specific serum antibody levels in EMCRS patients who had fungus positive and fungus negative sinus eosinophilic mucus

Antibody Isotype	Fungus Positive (AFS and NAFES, *n*=20)	Fungus Negative (AFS-like and NANFES, *n*=10)
IgE	0.04 (0.02 – 0.17)	0.07 (0.02 – 0.33)
IgG	0.88 (0.62 – 1.45)	0.72 (0.21 – 1.65)
IgA	0.14 (0.04 – 0.39)	0.09 (0.07 – 0.97)
IgM	0.33 (0.22 – 0.67)	0.47 (0.20 – 0.70)

Results are expressed as the median optical density values and the 25^{th}–75^{th} percentiles, limits of the interquartile range, in parentheses. These were not significantly different between the two EMCRS subgroups (Mann-Whitney U test). *Aspergillus fumigatus*-specific results were similar.

4.3 DISCUSSION

Type I hypersensitivity to fungi is argued to be the driving pathological process in allergic fungal sinusitis (Manning and Holman 1998; Meltzer et al. 2004). However, this study determined that most parameters of fungal-specific humoral immunity in fungal-allergic EMCRS did not differ significantly from fungal-allergic controls. Interestingly, fungal-specific humoral response was elevated in all the EMCRS subgroups regardless of type I hypersensitivity to fungi or the detection of fungi in eosinophilic mucus. Furthermore, high levels of fungal-specific serum IgG3 distinguished EMCRS patients from any other study group. These findings indicated that immunologic mechanisms other than allergy accounted for the elevated fungal-specific immune response in EMCRS patients. Accordingly, this raises several possibilities regarding the role of fungi in EMCRS.

Type I hypersensitivity to fungi was not pathogenically important in all the EMCRS subgroups as it was not detected in NAFES and NANFES. What then is the role of specific IgE in fungal-allergic EMCRS patients? Significantly higher levels of IgE to specific fungi was detected in allergic fungal sinusitis and AFS-like subgroups compared with healthy volunteers (Manning and Holman 1998). However, the levels of total IgE, ratio of mould mix-specific IgE to total IgE, and absolute levels of IgE to specific fungi did not differ significantly between fungal-allergic EMCRS and ARFA patients. Moreover, there was a poor correlation between the fungal species detected in eosinophilic mucus and fungal allergy. Consequently, a central pathogenic importance of fungal allergy was not supported in allergic fungal sinusitis and AFS-like patients. The data from this study was consistent with the notion that elevated fungal-specific IgE in EMCRS patients represented concurrent rhinosinusitis and fungal allergy. Coexisting allergy, fungal (Corey et al. 1997) and non-fungal, may exacerbate an underlying mucosal disease in EMCRS. This, in part, may

explain a reduction in the total IgE levels and alleviation of clinical symptoms following immunotherapy in allergic fungal sinusitis patients (Schubert and Goetz 1998). This could also explain why EMCRS subgroups with fungal allergy were younger than EMCRS subgroups without fungal allergy (Chapter 3).

In contrast with IgE, fungal-specific IgG, IgA and IgM were demonstrated in all EMCRS patients and controls, including healthy volunteers. This is consistent with mucosal exposure to common environmental fungi. Results from this study agree with previous reports of higher fungal-specific IgG levels in allergic fungal sinusitis compared with healthy volunteers (Manning and Holman 1998; Stewart and Hunsaker 2002). More important, this study demonstrated high levels of fungal-specific IgG, IgA and IgM in all the EMCRS subgroups, signifying that eosinophilic mucus marked the presence of enhanced fungal immunity. Therefore, in the absence of fungal-specific IgE in NAFES and NANFES, elevated levels of fungal-specific isotypes represented a non-allergic immune response.

High levels of fungal-specific IgG1 and IgG3 characterised the IgG subclass response in EMCRS patients compared with healthy volunteers. However, only IgG3 distinguished EMCRS from ARFA and CRS patients, thereby indicating a distinct form of fungal immunity. However, other than as a biological marker for EMCRS, the pathogenic significance of fungal-specific IgG3 was not determined in this study. To further evaluate its significance in EMCRS, fungal-specific IgG3 levels need to be compared with those from other fungal diseases of the sinuses, for example, fungal ball and invasive fungal disease.

IgG subclass response to infecting agents has been associated with severity of clinical symptoms and inflammatory responses (Hussain et al. 1995; Ashbee et al. 1997;

Dzierzanowska-Fangrat et al. 2003). This effect may be related to the distinct functional properties of the IgG subclasses. For example, IgG3 is the most effective subclass in activating complement and is capable of binding to all known IgG Fc receptors: FcγI, FcγII and FcγIII (Roitt, 1996). These receptors are differentially expressed among leucocytes, including eosinophils and the antigen presenting cells (Ravetch and Bolland 2001). In EMCRS, fungal-specific IgG3 may provide a mechanism for antibody-dependent cell (eosinophil)-mediated cytotoxicity (Kaneko et al. 1995) to fungi, noted in the form of "eosinophil granulomas" in sinonasal and cutaneous fungal diseases (Romano and Miracco 2003). IgG3 and IgG1 are the predominant antibody isotypes formed in response to protein antigens. As the half-life of IgG3 is one-third that of other IgG subclasses, it may denote ongoing stimulation by specific protein fungal antigens (Roitt 1996). The absence of fungal elements in eosinophilic mucus in AFS-like and NANFES patients could be due to insensitive fungal detection methods or due to an intense anti-fungal immune response. The latter was suggested in this study by significantly higher levels of IgG and IgA in EMCRS compared with healthy volunteers and CRS patients.

This study is the first to present evidence for non-allergic fungal immunity in EMCRS patients regardless of fungal allergy or of fungi in the eosinophilic mucus. The present data did not support the paradigm that fungal-specific IgE was a critical pathogenic marker for allergic fungal sinusitis, thus casting doubt that fungal allergy plays a unique pathogenic role in this disease. Fungal-specific IgG3 was the only marker that differentiated the EMCRS patients from those with chronic rhinosinusitis and from allergic rhinitis with fungal allergy. Collectively, these results indicate that immune mechanisms other than fungal allergy may be involved in the mucosal inflammation in EMCRS patients, including allergic fungal sinusitis. This will be examined in a further study in Chapter 5.

CHAPTER FIVE: FUNGAL-SPECIFIC PERIPHERAL BLOOD T LYMPHOCYTE PROLIFERATION IN EMCRS PATIENTS

5.1 INTRODUCTION

Direct identification and analysis of antigen-specific T lymphocytes requires knowledge of the antigen epitopes in the context of MHC-multimeres (Altman et al. 1996). Other methods independent of defined peptides and HLA-alleles include enzyme-linked immunospot (ELISPOT) assays, the cytometric cytokine secretion assays and cytotoxic assays. These studies are based on an analysis of specific T lymphocyte effector molecules after provocation of the cells with antigen *in vitro* (Kabilan et al. 1990; Assenmacher et al. 1998). However, these methods of identifying antigen-specific T lymphocytes are strictly dependent on the specific effector functions. In EMCRS, the nature of the immunodominant peptides of the fungal antigen, the fungal-specific T lymphocyte phenotype and the specific effector functions are unknown. Hence, studies were designed to examine the presence of fungal antigen-specific T lymphocytes using cell proliferation-based methods.

Clonal expansion resulting from cell proliferation is characteristic of T lymphocytes upon stimulation by cognate antigen. Proliferative responses provide an overall view of a complex cellular response that depends on a number of factors, including absolute numbers of T lymphocytes, responding cell frequency and proliferative ability, antigen presenting cell function and assay culture conditions. Therefore, measurement of T lymphocyte proliferation is an estimate of the presence of antigen-specific T lymphocytes. When interpreting these results, one should be aware that these measures do not exclude non-specific T lymphocyte activation, including bystander activation (Thiel et al. 2004), particularly when the antigen source is poorly characterised.

Measurement of cell incorporation of tritiated thymidine into DNA was used initially to determine the extent of mononuclear cell proliferation. As tritiated thymidine is added to the cultures in the last 6 to 24 hours of the cell culture, cells that underwent proliferation earlier cannot be detected. Furthermore, a more detailed analysis of the responding cell population is not possible. Cytometry was used to overcome these problems using a method to directly examine cells that underwent division (Weston and Parish 1990) (Chapter 2, section 2.14.4). In this method, the intracellular compartment is uniformly labelled with a fluorescent dye, carboxyfluorescein succinimidyl ester, before stimulating with mitogen. Cells lose fifty percent of their fluorescent label upon each division cycle. Accordingly, measurement of cellular fluorescence allows accurate identification of the divided cells and provides information of the proliferative cell history (Lyons and Parish 1994; Fazekas de St Groth et al. 1999). The CFSE-cytometry method has been used to examine cellular responses to *Candida* fungal antigens (Angulo and Fulcher 1998). In this study, it was envisaged that this technique would enable phenotypic analysis of fungal-specific T lymphocytes among the EMCRS patient groups.

The concept in this study was to compare T lymphocyte fungal antigen-specific proliferative responses in polyp and peripheral blood. However, in the main, responses of the peripheral blood lymphocytes to fungal antigens were examined because there were generally too few cells in the polyp tissue obtained during routine sinus surgery. The aim of this chapter was to determine whether the cellular response to fungi, measured by examining the magnitude and phenotype of fungal-specific mononuclear cell proliferation, was different in EMCRS patients compared with healthy volunteers, chronic rhinosinusitis patients and allergic rhinitis with fungal allergy patients.

5.2 RESULTS

5.2.1 Establishment of the fungal-specific cell proliferation assay

A patient who was allergic to *Alternaria alternata* (skin prick test wheal diameter of 4 mm) and *Aspergillus fumigatus* (wheal diameter of 7 mm) was selected for this study. Peripheral blood mononuclear cells were stimulated with either *Alternaria alternata* or *Aspergillus fumigatus* antigens, with or without the first incubation step (preincubation) and with or without a washing step (Chapter 2, section 2.15). There was a significant increase in the cell incorporation of tritiated thymidine, expressed as counts per minute, in PBMC that were preincubated with fungal antigens compared with no preincubation. With preincubation, there was a greater proliferative response when the wells were washed with media compared with no washing (Figure 5.1 A and B).

Maximum counts were obtained by *(a)* using fungal antigens at 1/20 and 1/40 dilution of stock solution (Figure 5.1 A and B), *(b)* preincubating PBMC for 36 to 48 hours (Figure 5.2 A), *(c)* washing wells by decanting each well compared with flicking-out the well contents (data not shown) and *(d)* harvesting four days after the washing step, called Day 4, of the second incubation period (Figure 5.2 B). As expected, the maximum cell division determined by CFSE-cytometry was observed on Day 6, which follows DNA synthesis measured by tritiated thymidine incorporation on Day 4 (data not shown).

Subsequent proliferation assays were standardised to a 36 hour preincubation of PBMC with 1/30 dilution of fungal antigen, washing the tissue culture wells by decanting the culture plate contents followed by adding unstimulated PBMC from day zero. Cells were harvested on Day 4 for cellular tritiated thymidine incorporation measurements and on Day 6 for determining cell division using CFSE-cytometry.

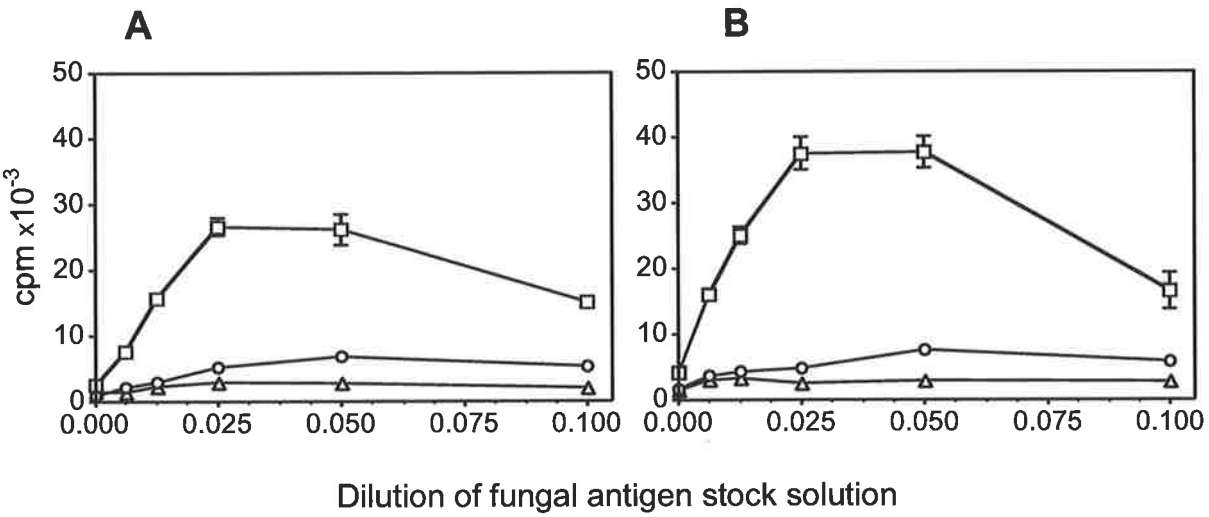


Figure 5.1 Proliferative responses of peripheral blood mononuclear cells (PBMC) from a fungal-allergic individual to fungal antigens in three different assay conditions. In the first method, cells were stimulated with *Aspergillus fumigatus* (A) and *Alternaria alternata* (B) antigens (diluted 1/10, 1/20, 1/40, 1/80 and 1/160) without preincubation (△). In the second and third methods, cells were preincubated with fungal antigens without a washing step (O) and with a washing step (□) respectively. The preincubation was performed for 36 hours and cells were harvested after four days of the second incubation period. The results are expressed as the mean counts per minute (cpm) ± standard error of the mean of three experiments.

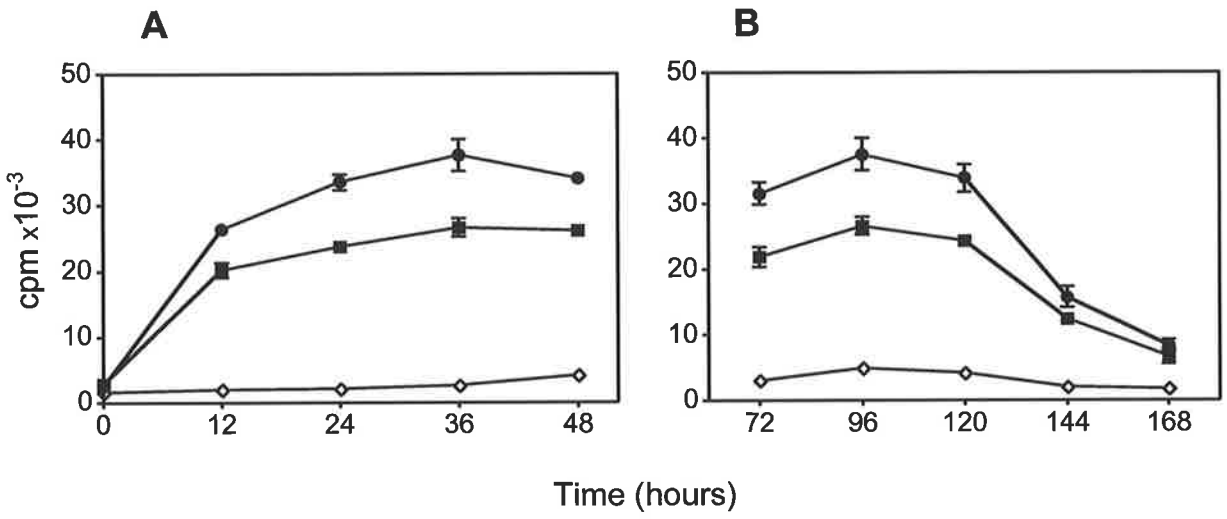


Figure 5.2 Time course experiments to determine the optimum period for preincubation (first incubation period) (A) and cell harvest (second incubation period) (B) in order to obtain maximum fungal-specific cell proliferation. Peripheral blood mononuclear cells from a fungal-allergic individual were stimulated with 1/20 dilution of *Alternaria alternata* (■) and *Aspergillus fumigatus* (●) antigens. Cells incubated without fungal antigens were used as negative control (◇). The results are expressed as the mean counts per minute (cpm) ± standard error of the mean of three experiments.

5.2.2 Magnitude of fungal-specific mononuclear cell proliferation

5.2.2.1 Peripheral blood

Tritiated thymidine incorporation into peripheral blood mononuclear cells in response to fungal antigens was measured in 27 EMCRS patients, 12 healthy volunteers, 15 ARFA patients and 15 CRS patients. The clinical characteristics of the study groups are summarised in Table 5.1. The reported value for the fungal-specific proliferative response in an individual corresponds to the highest value obtained for either *Alternaria alternata* or *Aspergillus fumigatus*-specific proliferation.

The magnitude of T lymphocyte proliferation may be proportional to the number of antigen-specific T lymphocytes and antigen presenting cells in a sample. Therefore, the percentage of T cells and antigen presenting cells in each sample was determined before setting up the proliferation assays. For each assay, peripheral blood mononuclear cells at 10^5 cells per sample were stained by indirect immunofluorescence for CD3 and either CD4 or CD8, CD14 and CD19. CD14 is expressed on monocytes, macrophages and mature dendritic cells and CD19 on B lymphocytes. The median percentage of peripheral blood $CD3^+CD4^+$, $CD3^+CD8^+$, $CD14^+$ and $CD19^+$ cells was not significantly different among the study groups ($P = 0.24$, $P = 0.413$, $P = 0.885$ and $P = 0.928$ respectively, Kruskal-Wallis test).

The extent of fungal-specific PBMC proliferation was not significantly different between the EMCRS subgroups ($P = 0.458$, Kruskal-Wallis test) (Figure 5.3 A), regardless of fungal allergy or of the detection of fungi in eosinophilic mucus. As a single group, EMCRS patients had a greater proliferative response compared with healthy volunteers ($P < 0.0001$) but not when compared with ARFA ($P = 0.3315$) or CRS ($P = 0.2937$) patients (Mann-Whitney U test).

Table 5.1 Clinical characteristics of the tritiated thymidine cell proliferation study groups

	HV ***n*=12**	**ARFA** ***n*=15**	**CRS** ***n*=15**	**AFS** ***n*=12**	**AFS-like** ***n*=3**	**NAFES** ***n*=7**	**NANFES** ***n*=5**
Age in years (median (IQR))	37 (24 – 33)	41 (35 – 52)	50 (32 – 56)	42 (25 – 46)	52 (35 – 49)	44 (23 – 51)	55 (32 – 53)
Male/Female	7/5	8/7	8/7	7/5	2/1	4/3	2/3
Fungus in EM[1]	NA	NA	NA	100%	0%	100%	0%
Alternaria alternata				4		3	
Aspergillus fumigatus				1		0	
Both				5		4	
Other fungi[2]				6		4	
Fungal allergy[3]	0%	100%	47%	100%	100%	0%	0%
Alternaria alternata		5	1	2	1		
Aspergillus fumigatus		1	1	1	2		
Both		8	2	4			
Other fungi[4]		11	3	5			
Non-fungal allergy[5] (%)	42	80	60	83	100	71	40
Aspirin sensitive (%)	0	7	7	8	0	0	20
Asthma (%)	0	33	27	58	33	57	40

[1]By histology or fungal culture. [2]*Bipolaris, Drechslera, Trichothecium, Candida, Penicillium, Scedosporium, Acremonium, Devriessi, Phialaphora* and *Cladosporium* species. [3]By positive fungal-specific serum IgE or skin prick test. [4]*Helminthosporium, Penicillium, Candida, Epicoccum* and *Cladosporium* species. [5]Mixes of tree pollen, grass pollen, house dust mite and animal dander. EM, eosinophilic mucus; IQR, interquartile range (25^{th}–75^{th} percentiles); NA, not applicable.

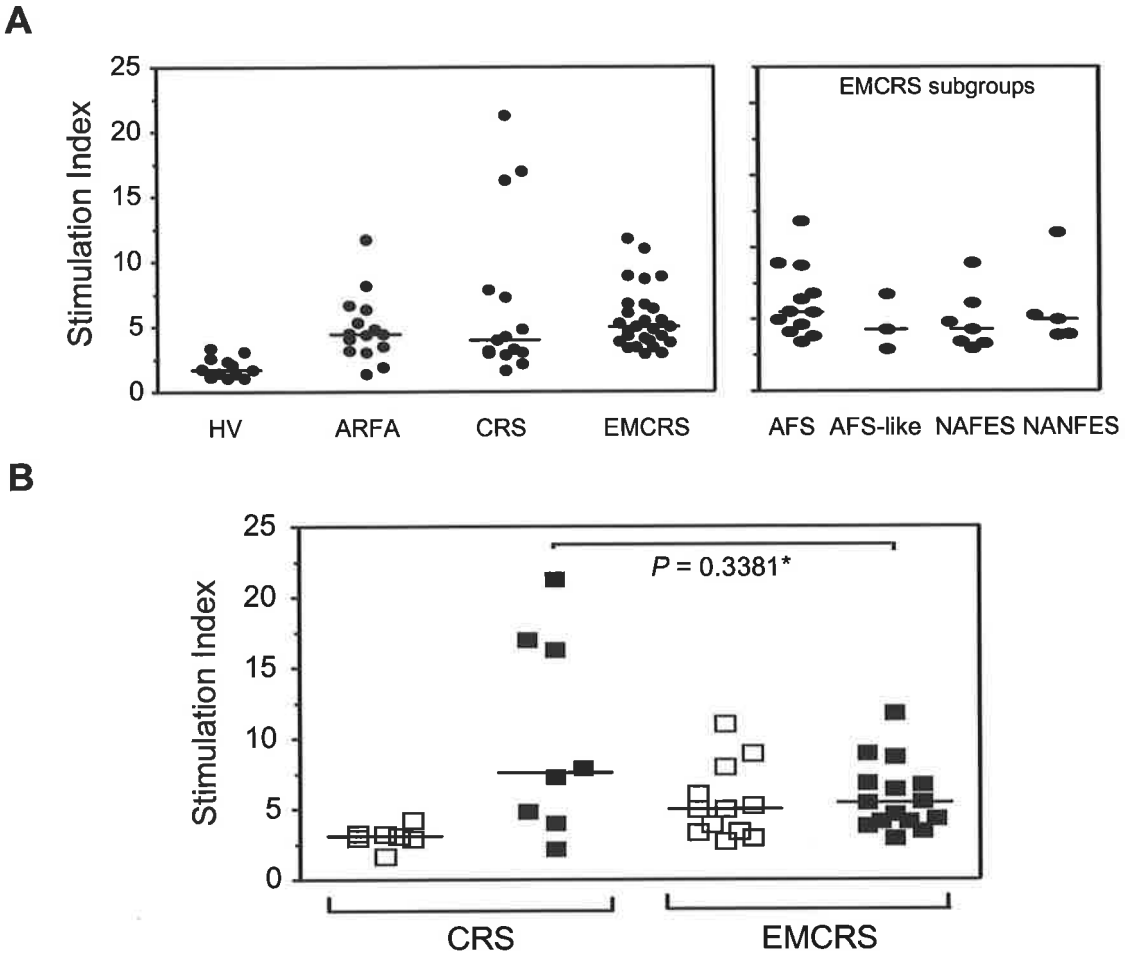


Figure 5.3 Fungal-specific peripheral blood mononuclear cell proliferation expressed as stimulation index (SI) in HV, ARFA, CRS and the EMCRS patients. The maximum proliferative response to either *Alternaria alternata* or *Aspergillus fumigatus* is plotted for every individual. The horizontal bars illustrate the median SI values. Comparison of SI between HV (n=12), ARFA (n=15), CRS (n=15) and a single group of EMCRS (n=27) patients, and between the EMCRS subgroups (AFS (n=12), AFS-like (n=3), NAFES (n=7) and NANFES (n=5)) is shown in (A). Comparison of SI between non-fungal-allergic (□) and fungal-allergic (■) CRS and EMCRS patients is depicted in (B). *Kruskal-Wallis test.

In contrast to EMCRS patients, fungal-specific PBMC proliferation was higher in fungal-allergic CRS compared with non-fungal-allergic CRS patients ($P = 0.014$, Mann-Whitney U test) (Figure 5.3 B). There was no significant difference in the stimulation index between allergic CRS patients, non-allergic EMCRS and allergic EMCRS patients ($P = 0.3381$, Kruskal-Wallis test).

5.2.2.2 Nasal polyp

Nasal polyp mononuclear cells (NPMC) from one EMCRS and from one CRS patient were stimulated with *Alternaria alternata* antigens, *Aspergillus fumigatus* antigens or phytohaemagglutinin. Matched PBMC from each patient were also examined accordingly. Fungal-specific proliferation was not observed in NPMC for either individual (Figure 5.4 A). In contrast, PBMC proliferation to either fungal antigen was observed in both individuals. This indicated that the lack of NPMC proliferation was unlikely to be due to the assay culture conditions. The number of mononuclear cells determined by cell count, and antigen presenting cells determined by the percentage of $CD19^+$ and $CD14^+$ cells, per sample were similar between NPMC and PBMC. However, the percentage of $CD3^+CD4^+$ cells and $CD3^+CD8^+$ cells appeared different (Table 5.2). Interestingly, NPMC from both individuals did not proliferate in response to PHA either (Figure 5.4 B).

Table 5.2 Percentage of $CD4^+$ and $CD8^+$ T lymphocytes in the peripheral blood and nasal polyp mononuclear cell samples from an EMCRS and a CRS patient

	Peripheral Blood	**Nasal Polyp**
EMCRS ($CD3^+CD4^+$)	52	17
EMCRS ($CD3^+CD8^+$)	22	74
CRS ($CD3^+CD4^+$)	49	48
CRS ($CD3^+CD8^+$)	24	33

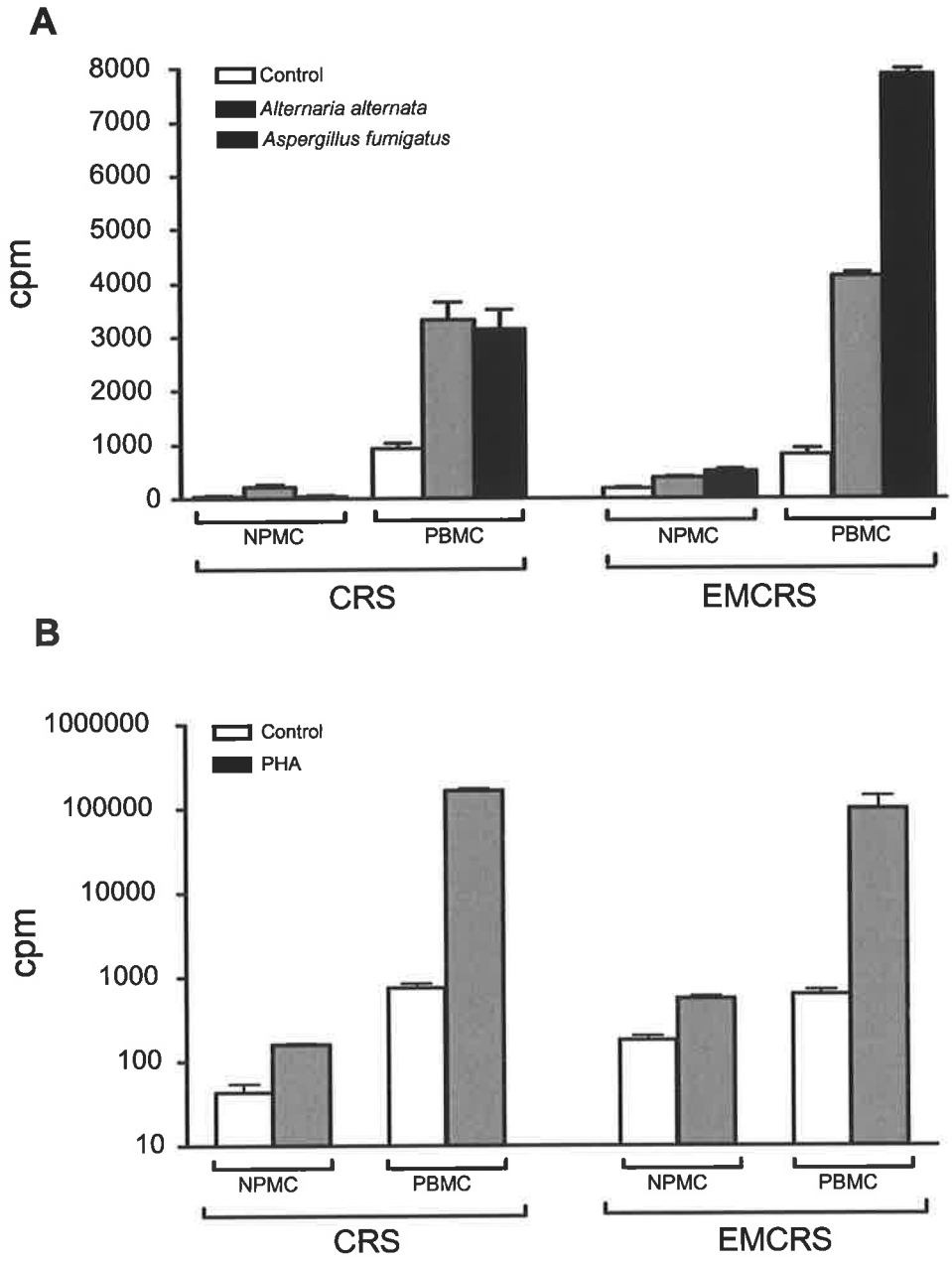


Figure 5.4 Comparison of the magnitude of nasal polyp (NP) and peripheral blood (PB) mononuclear cell (MC) proliferation measured by thymidine incorporation in a CRS and an EMCRS patient. Responses to fungal antigens, *Alternaria alternata* and *Aspergillus fumigatus*, (A) and to phytohaemagglutinin (PHA) (B) are expressed as the mean counts per minute (cpm) of triplicate values in linear and logarithmic scale respectively. The columns show the mean values and bars, the standard errors of the means.

5.2.3 Phenotype of the fungal-specific mononuclear cells

To determine the phenotype of the antigen-specific proliferating cell population, a CFSE based proliferation assay was used. In this study, 16 EMCRS patients, 5 healthy volunteers, 6 ARFA patients and 10 CRS patients were examined. Their clinical characteristics are shown in Table 5.3. Peripheral blood mononuclear cells were labelled with CFSE and stimulated with fungal antigens or with PHA as described in Chapter 2, section 2.16. Unstimulated and PHA-stimulated CFSE-labelled PBMC were used as negative and positive controls respectively. Other internal controls included unlabelled cells activated under the same culture conditions. At the end of the culture period, the PBMC were harvested and stained with directly conjugated monoclonal antibodies to CD3 and either CD4 or CD8 to examine the phenotype of the responding cell population. The PBMC were then analysed for the CFSE profiles in different culture conditions. A representative three-colour gating strategy used in this study is shown in Figure 5.5 and a summary of the proliferation results is presented in Table 5.4.

Table 5.3 Clinical characteristics of the CFSE-cytometry study groups

	HV *n*=5	**ARFA** *n*=6	**CRS** *n*=10	**AFS** *n*=6	**AFS-like** *n*=2	**NAFES** *n*=4	**NANFES** *n*=4
Age in years (median (IQR))	34 (27 – 44)	53 (24 – 54)	47 (32 – 51)	54 (21 – 53)	33 and 51	46 (39 – 58)	47 (38 – 54)
Male/Female	2/3	3/3	4/6	3/3	1/1	3/1	2/2
Fungus in EM[1]	NA	NA	NA	100%	0%	100%	0%
Alternaria alternata				2		1	
Aspergillus fumigatus				1		0	
Both				2		1	
Other fungi[2]				4		4	
Fungal allergy[3]	0%	100%	40%	100%	100%	0%	0%
Alternaria alternata		1	0	0	0		
Aspergillus fumigatus		0	1	0	0		
Both		5	2	4	1		
Other fungi[4]		4	4	5	2		
Non-fungal allergy[5] (%)	60	100	50	67	100	75	50
Aspirin sensitive (%)	0	17	10	0	0	25	0
Asthma (%)	20	33	30	50	100	50	50

[1]By histology or fungal culture. [2]*Bipolaris, Drechslera, Trichothecium, Candida, Penicillium, Scedosporium, Acremonium, Devriessi, Phialaphora* and *Cladosporium* species. [3]By positive fungal-specific serum IgE or skin prick test. [4]*Helminthosporium, Penicillium, Candida, Epicoccum* and *Cladosporium* species. [5]Mixes of tree pollen, grass pollen, house dust mite and animal dander. EM, eosinophilic mucus; IQR, interquartile range (25^{th}–75^{th} percentile); NA, not applicable.

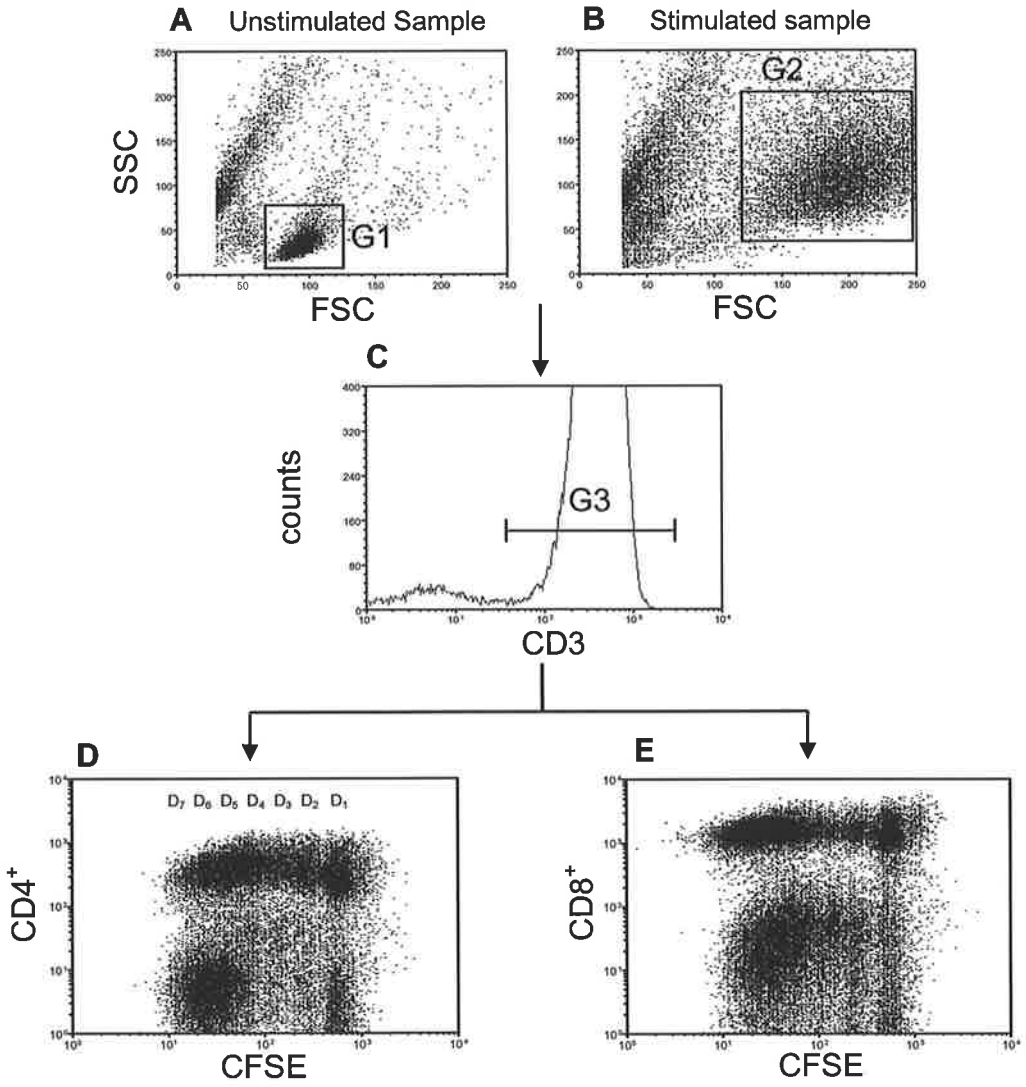


Figure 5.5 Standard three-colour gating strategy for determining carboxyfluorescein succinimidyl ester (CFSE) profiles in peripheral blood mononuclear cells (PBMC) treated with phytohaemagglutinin (PHA). The cells represented by dot plots (A) and (B) are gated for forward scatter (FSC) and side scatter (SSC) parameters. Gate (G) 1 is placed on resting PBMC in the unstimulated sample and gate 2 on blasts in the stimulated sample. The histogram is gated (G3) on CD3 positive cells in G1 and G2 (C). Depending on the experiment, cells are then analysed for cell surface expression of CD4 (D) or CD8 (E). Up to seven cell divisions, D1-D7, of $CD3^+CD4^+$ and $CD3^+CD8^+$ cells can be seen.

Table 5.4 Summary of fungal-specific peripheral blood $CD4^+$ and $CD8^+$ T cell division

Number of patients whose PBMC underwent fungal-specific cell division	**Phenotype of divided T lymphocytes**	
	$CD4^+$	**$CD8^+$**
HV (0/5)	0	0
ARFA (5/6)	5	4/5
CRS (8/10)	8	6/8
AFS (5/6)	5	0
AFS-like (2/2)	2	0
NAFES (4/4)	4	0
NANFES (4/4)	4	0

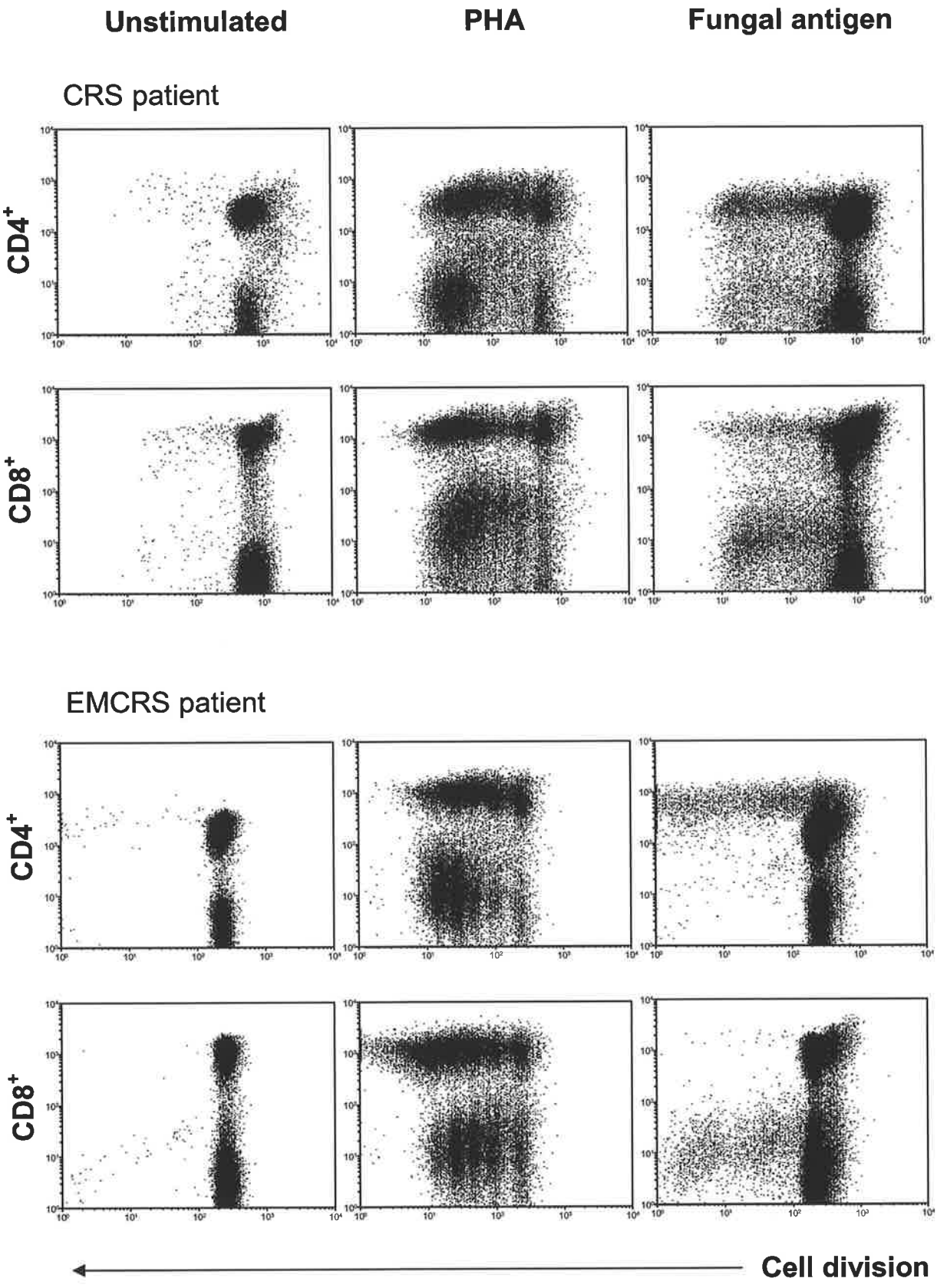
PBMC, peripheral blood mononuclear cells.

In all the patients whose PBMC underwent fungal-specific cell division, the predominant dividing T cell phenotype was $CD4^+$. Both $CD4^+$ and $CD8^+$ T lymphocytes underwent cell division in response to fungal antigens in ARFA and CRS patients. Strikingly, $CD8^+$ T cell division was not detected in any of the EMCRS patients tested. However, both $CD4^+$ and $CD8^+$ T cells divided in response to PHA in all the individuals examined. A representative experiment from a CRS and an EMCRS patient is shown in Figure 5.6. Up to seven generations of cell division was determined in the dividing $CD4^+$ or $CD8^+$ T cell populations in response to fungal antigens or to PHA.

An absence of the $CD8^+$ T cell proliferation in EMCRS could be explained by an anergic response. To examine whether this was the case, in some experiments, exogenous IL-2 was added to the cell culture, as IL-2 reverses classical T cell anergy (Essery et al. 1998). However, there was no significant increase in the percentage of $CD8^+$ T lymphocyte

proliferation with fungal antigens and IL-2 (median = 5.8, IQR = 3.3 – 6.5) compared with IL-2 alone (median = 5.0, IQR = 3.0 – 7.1) (n=8, P = 0.2184, Wilcoxon matched paired test). A representative experiment is shown in Figure 5.7.

To examine whether antigen-specific $CD8^+$ T lymphocyte activation could be detected in the absence of a blastogenic response, cell surface expression of activation markers were examined in some experiments. Expression of CD69 and CD25 (IL-2 α chain receptor) is upregulated in activated lymphocytes (Biselli et al. 1992). Because CD69 is expressed soon after cell activation, peaking at 24 hours, its expression could be missed in the assay used in this study. By contrast, CD25 expression is maximal after several days of stimulation. Therefore, in the present study, the expression of CD25 was examined on $CD8^+$ T lymphocytes. The controls included unstimulated and PHA stimulated $CD8^+$ T cells, and $CD4^+$ T cells stimulated under similar conditions. There was no significant difference between the median percentage of CD25 expression on $CD8^+$ T cells in unstimulated control samples (median = 3.6, IQR = 3.2 – 4.2) and fungal antigen stimulated samples (median = 3.4, IQR = 3.1 – 5.5) (n=6, P = 0.1875, Wilcoxon matched paired test). In contrast, most of the $CD8^+$ T cells stimulated with PHA expressed CD25 (median = 68.7, IQR = 54.0 – 89.3).


Unstimulated
PHA
Fungal antigen
CRS patient
CD4+
CD8+
EMCRS patient
CD4+
CD8+
Cell division

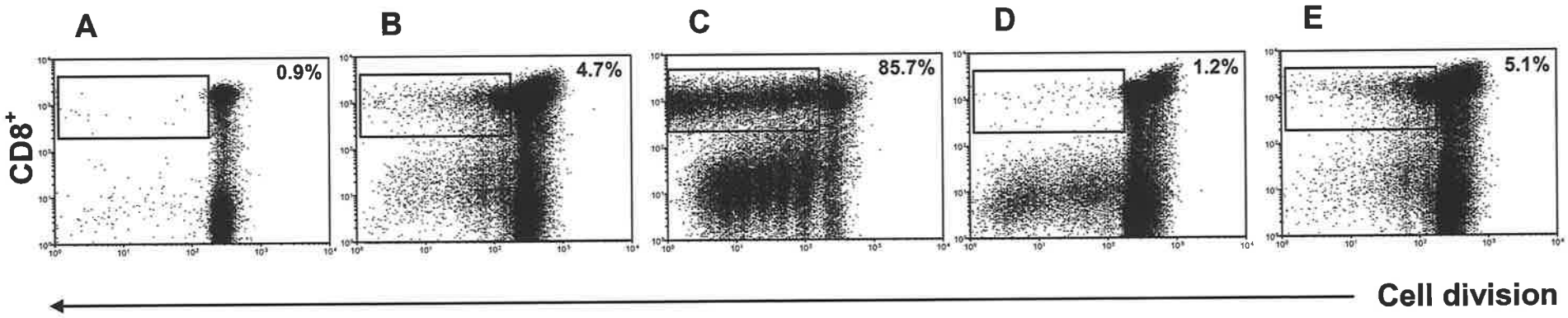


Figure 5.7 Effect of IL-2 on fungal-specific peripheral blood mononuclear cell proliferation in an EMCRS patient. A representative experiment, using carboxyfluorescein succinimidyl ester-labelled cells showing no significant increase in fungal-specific proliferation with IL-2 in the culture conditions. Cells were harvested after six days of culture in the second incubation period. Dot plots are gated on $CD3^+$ blast cells following stimulation by IL-2 alone (B), phytohaemagglutinin (PHA) (C), *Alternaria alternata* antigens (D) and IL-2 plus *Alternaria alternata* antigens (E). The unstimulated cell sample is shown in (A). The percentages refer to the proliferating $CD8^+$ T lymphocyte populations.

5.3 DISCUSSION

This study confirms and extends the observations from Chapter 4 that EMCRS patients had an elevated immune response to fungal antigens irrespective of fungal allergy or detection of fungus in eosinophilic mucus. Fungal-specific peripheral blood cell proliferation was significantly higher in EMCRS patients compared with healthy volunteers. However, the elevated cellular response observed in EMCRS patients was not significantly greater than that observed in ARFA or CRS patients. Interestingly, the nature of the cellular response was markedly different in EMCRS patients, where a lack of peripheral blood $CD8^+$ T lymphocyte proliferation to fungal antigens distinguished EMCRS from ARFA and CRS patients.

A significantly higher mononuclear cell proliferation to fungal antigens in ARFA, CRS and the EMCRS subgroups compared with healthy volunteers indicated that the number of fungal-specific T lymphocytes in the peripheral blood was greater in those patients. While the increased cell proliferation was limited to fungal-allergic subjects in the CRS group, both fungal-allergic and non-allergic EMCRS patients had an elevated fungal-specific proliferation. This finding demonstrated a separation of the cellular immune response from fungal allergy, supporting the hypothesis that mechanisms other than IgE-mediated allergy accounts for the elevated fungal-specific immune response in EMCRS patients.

In this study, $CD4^+$ T lymphocyte proliferation to fungal antigens was a common feature in ARFA, CRS and the EMCRS patients. The $CD4^+$ T lymphocyte proliferation was likely to be an antigen-specific rather than a non-specific mitogenic response to fungal antigens (Graybill and Alford 1974; Mody et al. 1999; Syme et al. 2000) because reduced cell proliferation segregated to healthy volunteers. That $CD8^+$ T lymphocytes failed to proliferate

in response to both *Alternaria alternata* and *Aspergillus fumigatus* antigens in all the EMCRS patients is a significant and novel finding. Although an absence of fungal-specific peripheral blood $CD8^+$ T lymphocytes has been reported in allergic bronchopulmonary aspergillosis, this was based on the ability to generate $CD4^+$ T cell clones only (Knutsen et al. 1994; Kurup et al. 1996; Chauhan et al. 1996; Chauhan et al. 1997; Kurup et al. 1998; Murali et al. 1998). As the methods used to generate clones may miss antigen-specific $CD8^+$ T lymphocytes, the absence of $CD8^+$ T cell clones may be due to the methodology not expanding antigen-specific $CD8^+$ T cells, thereby causing a skew of the results. In this thesis, $CD8^+$ T lymphocyte proliferation to fungal antigens in ARFA and CRS patients, and in response to phytohaemagglutinin in all the individuals tested argues against technical problems with the methodology or variations in the assay conditions.

Murine studies have shown that memory $CD8^+$ T lymphocytes are mobile and are distributed throughout the body including in peripheral blood (Klonowski et al. 2004). The magnitude of antigen-specific peripheral blood $CD8^+$ T lymphocyte proliferation correlates with active peripheral infections with *Candida albicans* and in active immune conditions including atopic contact dermatitis (Cavani et al. 1998). Therefore, if fungal-specific $CD8^+$ T lymphocytes were present in EMCRS patients, one would expect to detect them in the peripheral blood. A lack of fungal-specific $CD8^+$ T lymphocyte proliferation in EMCRS patients could be explained by T cell anergy following interaction with cognate antigen. However, T lymphocyte anergy may be abrogated by the addition of IL-2 in the culture medium (Essery et al. 1998; Chen et al. 2004). This was shown not to be the case in this study. Furthermore, the $CD8^+$ T lymphocytes from EMCRS patients proliferated to phytohaemagglutinin, making T cell anergy an unlikely explanation for a lack of fungal-specific $CD8^+$ T cell proliferation (Brenchley et al. 2003).

That fungal-specific $CD8^+$ T cells were terminally differentiated in the peripheral blood could account their lack of proliferation, as terminally differentiated lymphocytes are typically associated with limited proliferative activity (Brenchley et al. 2003; Papagno et al. 2004). If this were the case, antigen-specific activation of terminally differentiated cells may be determined by upregulation of activation markers or acquisition of effector functions such as cytotoxicity or cytokine secretion (Heintel et al. 2003). However, in this study, measurement of the expression of CD25, an activation marker, on $CD8^+$ T lymphocytes in a proportion of EMCRS patients did not show upregulation. By contrast, terminal differentiation could explain the lack of fungal-specific proliferation in nasal polyp mononuclear cells because reduced proliferative activity was also observed in response to phytohaemagglutinin.

Human and murine studies support the involvement of $CD4^+$ and $CD8^+$ T lymphocytes in immunity to fungi (Mody et al. 1994; Ashman 1998; Santoni et al. 2002; Heintel et al. 2003; Wüthrich et al. 2003). That $CD8^+$ T lymphocytes are important in fungal immunity is supported by murine studies demonstrating $CD8^+$ T cells either suppressing (Beno et al. 1995) or enhancing fungal infections (Yuan et al. 1997). These immune responses can be mediated by an MHC-restricted or non-MHC-restricted antifungal activity (Levitz et al. 1994). Furthermore, involvement of differential T cell subsets has been shown in immunity to hyphal and non-hyphal forms of the same fungal species (Heintel et al. 2003). This illustrates the complex nature of the T lymphocyte responses to fungi. Hence, a lack of $CD8^+$ T cell proliferation to fungal antigens in the present study may reflect a fundamental defect in the $CD8^+$ T cell-mediated immune response to fungal antigens. In predisposed chronic rhinosinusitis patients, a dysregulated anti-fungal cellular response may manifest as EMCRS. The defect in the $CD8^+$ T cell-mediated response to fungi may be in the antigen-

presentation process (antigen presenting cells, toll-like receptors, costimulatory process), cell differentiation process or due to an absence of fungal-specific $CD8^+$ T cells. The latter argument appears unlikely because fungal antigen-specific $CD4^+$ T cells were present in EMCRS, hence the ability to present fungal antigens and T cell receptor availability for fungal antigens. Therefore, it appears doubtful that the present data implied that fungal-specific $CD8^+$ T lymphocytes were simply absent from the peripheral blood of EMCRS patients.

The inability to demonstrate peripheral blood $CD8^+$ T lymphocyte proliferation to fungal antigens in EMCRS patients was remarkable, as preliminary examination of the T cell phenotypes from EMCRS nasal polyps (Chapter 6) and published literature on polyps showed a dominance of the $CD8^+$ T lymphocyte population (Sanchez-Segura et al. 1998). Therefore, in the following chapter, comparison of a detailed phenotype of the T lymphocyte subpopulations in the polyps and matched peripheral blood samples was performed.

CHAPTER SIX: CHARACTERISATION OF LYMPHOCYTE POPULATIONS IN SINONASAL POLYPS

6.1 INTRODUCTION

The striking observation that the $CD8^+$ T lymphocyte population in the peripheral blood of EMCRS patients did not proliferate to fungal antigens (Chapter 5) raised several questions and possible explanations. The latter included fungal-specific $CD8^+$ T cells that were anergic, terminally differentiated and non-proliferative or an as yet undefined population of $CD8^+$ T lymphocytes. Alternatively, a lack of $CD8^+$ T cell proliferation in EMCRS may signify a defective $CD8^+$ T cell immune response to fungal antigens. To clarify the nature of the T lymphocyte population and to obtain a more comprehensive understanding of the cell infiltrate in the sinonasal mucosa, matched peripheral blood and sinonasal polyp samples from each patient obtained at the time of surgery were phenotyped. Where possible, the corresponding non-polyp sinonasal tissue from the ipsilateral sinus was also examined.

The tissue architecture was examined by immunohistochemical analysis and correlated with the phenotypic analysis of the T cell populations. Lymphocytes activated by interaction with cognate antigen undergo phenotypic changes that correspond to their differentiation status. The composite model of human T lymphocyte differentiation based on changes in cell membrane expression of CD45RA, CD62L and CD27 is described in Chapter 1, section 1.18 and depicted in Figure 1.5. This model is largely compatible with the results reported in the literature and provides a background for the development of the current argument. The expression pattern of CD27 in combinations with CD45RA and CD62L reflects distinct stages of a gradual linear differentiation (van Baarle et al. 2002; Seder and Ahmed 2003). The functional development of memory T cells is paralleled by changes in CD27 expression.

For example, a typical memory T cell expressing CD27 has high proliferative capacity and low cytolytic activity compared to a highly differentiated CD27 negative T cell with strong effector functions (van Baarle et al. 2002; Zhang et al. 2003). While T lymphocyte phenotyping would not give direct evidence for the functionality of the T cell populations, it would allow one to begin a comparison of the cell populations that were present in the mucosal inflammation in EMCRS and CRS patients.

The aim of this chapter was to determine whether the nature of the sinonasal mucosal immune response in EMCRS was different to that in CRS patients by examining the phenotype of the lymphocyte populations in nasal polyps and matched peripheral blood samples.

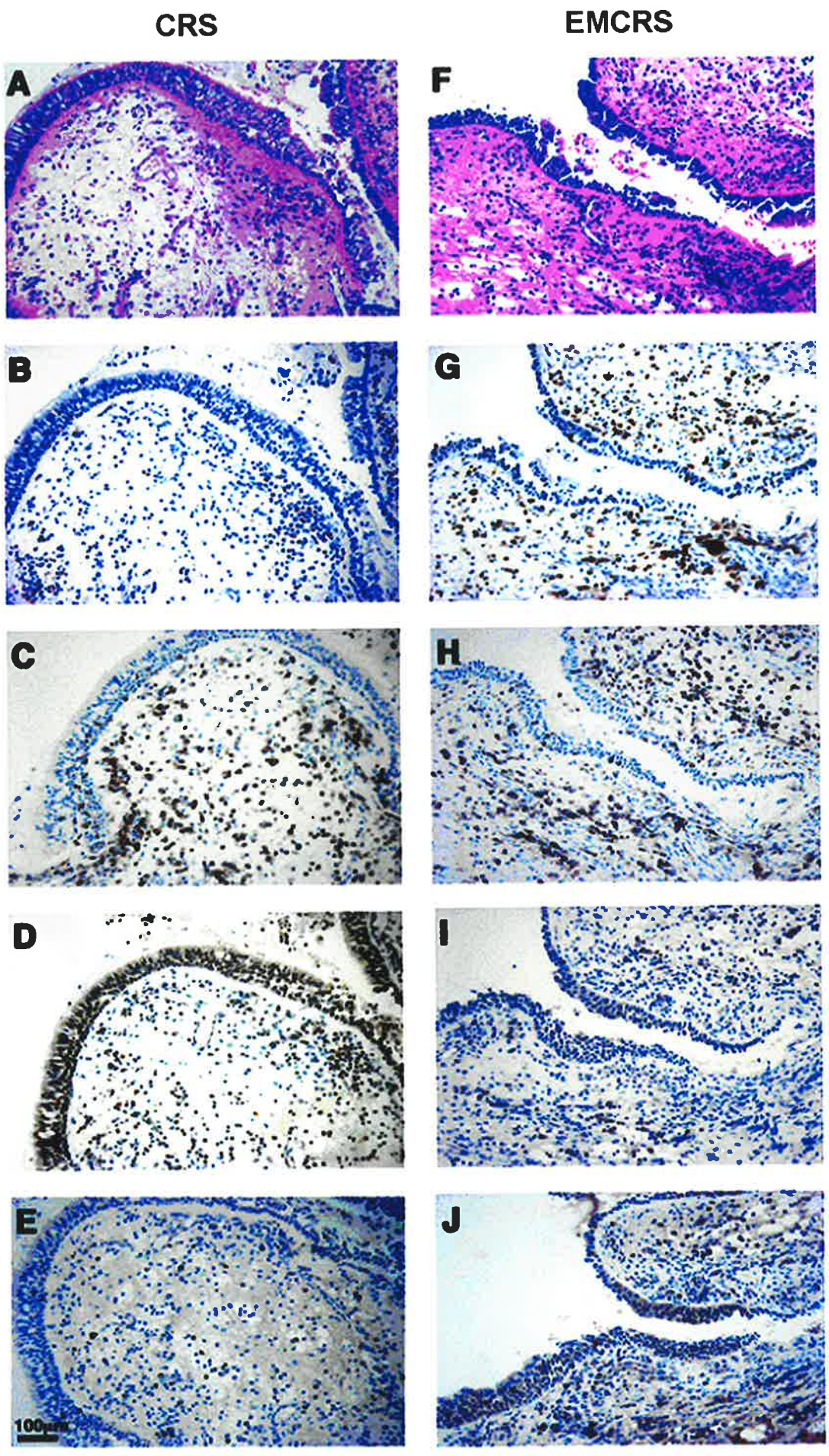
6.2 RESULTS

6.2.1 Immunohistochemical localisation of the inflammatory cells in nasal polyps

Paraffin-embedded serial sections of nasal polyps from EMCRS and CRS patients were stained with haematoxylin and eosin and examined for tissue architecture and inflammatory cell composition. The epithelial layer of the nasal polyps was better preserved in CRS than in EMCRS and glandular destruction was more prominent in polyps from EMCRS patients. A predominant eosinophil population was present in the nasal polyps from both patient groups, EMCRS and CRS. The eosinophils were found throughout the lamina propria and could be seen in the intraepithelial layer and in the mucus.

The distribution of the $CD4^+$ and $CD8^+$ cell populations were examined in EMCRS and CRS polyps by immunohistochemistry. There appeared to be more $CD8^+$ cells in EMCRS than in CRS polyps. There were no significant differences in the distribution of the lymphocyte populations between the polyps from EMCRS and CRS patients. In both patient groups, mainly $CD3^+$ cells were present in the intraepithelial region. The $CD8^+$ cells were also abundant in the intraepithelial region. In the lamina propria and periglandular areas both $CD4^+$ and $CD8^+$ cells were detected. Images from a representative experiment from a CRS and an EMCRS patient are shown in Figure 6.1.

Figure 6.1 Immunohistochemical localisation of eosinophils, $CD3^+$, $CD4^+$ and $CD8^+$ cells in nasal polyps. These images show the typical tissue architecture in sections stained with haematoxylin and eosin (A and F) and the characteristic distribution of the inflammatory cells in sections stained with immunoperoxidase in a CRS and an EMCRS patient. Cells that stained brown were positive for one of the following: eosinophil cationic protein (EG2) (B and G), CD3 (C and H), CD4 (D and I) and CD8 (E and J).


CRS
EMCRS
A
F
B
G
C
H
D
I
E
J
100μm

6.2.2 Flow cytometry – direct and indirect immunofluorescence

Matched nasal polyp and peripheral blood samples from 22 EMCRS and 11 CRS patients were examined and their clinical profiles are presented in Table 6.1. None of these patients reported topical steroid use for at least four weeks and systemic steroid use or upper respiratory tract infection for at least two months before surgery. Two EMCRS and three CRS patients were cigarette smokers. However, they had ceased smoking for at least six weeks prior to surgery.

Table 6.1 Clinical characteristics of the CRS and EMCRS patients

	CRS ***n*=11**	**AFS** ***n*=3**	**AFS-like** ***n*=1**	**NAFES** ***n*=7**	**NANFES** ***n*=11**
Age in years[1]	53 (31 – 59)	29 (28 – 42)	42	50 (30 – 64)	40 (31 – 58)
Male/Female	7/4	2/1	F	4/3	6/5
Previous operations	2	2	1	4	4
Fungi in EM[2]	NA	1	0	3	0
Neutrophils[3]	3	0	0	0	0
Fungal allergy[4]	5	3	1	0	0
Non-fungal allergy	7	3	1	3	4
Aspirin sensitive	2	0	0	0	1

[1]Age is presented as the median and the 25th–75th percentiles, limits of the interquartile range, in parenthesis. [2]By histology. [3]Where definite clusters of neutrophils were observed in tissue sections. Neutrophils, at 1-2 cells per section were present in most samples. [4]By positive fungal-specific IgE or skin prick test. EM, eosinophilic mucus; NA, not applicable.

6.2.2.1 Cellular profile of nasal polyps from EMCRS and CRS patients

The cell viability following single cell preparation from polyp tissue was greater than 97%. Comparison of the cellular material was examined from cytospin preparations (Table 6.2).

The morphological appearance of cells designated "small mononuclear cells" resembled that of lymphocytes. The lymphocytes constituted more than 20% of the inflammatory cell population in the polyps. They were characterised further with flow cytometry.

Table 6.2 Cellular profile of CRS and EMCRS polyps by light microscopy

% Inflammatory cell type	CRS *n*=8	EMCRS *n*=10	P value[1]
Eosinophils	56 (41 – 58)[2]	50 (41 – 56)	0.7
Small mononuclear cells	23 (20 – 25)	30 (23 – 32)	0.02
Large mononuclear cells	17 (16 – 19)	20 (13 – 28)	0.4
Neutrophils	3 (0 – 16)	0 (0 – 1)	0.05
Basophil/Mast cell	3 (2 – 4)	0 (0 – 2)	0.03

[1]Mann-Whitney U test. [2]Median values are presented with the 25th–75th percentiles, limits of the interquartile ranges, in parentheses.

6.2.2.2 Lymphocyte populations in polyps and peripheral blood of EMCRS and CRS patients

Cells were stained with three-colour direct immunofluorescence (TriTEST™, BD) (Chapter 2, section 2.1) to examine the lymphocyte populations. The major lymphocyte subsets were distinguished by membrane expression of CD3 (T cells), CD19 (B cells) and CD56/CD16 (Natural Killer or NK cells). The percentage of CD3, CD19, CD56/CD16 positive cells in polyps and peripheral blood was not significantly different between the EMCRS subgroups (P = 0.2113, P = 0.987 and P = 0.2817 respectively, Kruskal-Wallis test). Therefore, the results from the EMCRS subgroups were combined into a single EMCRS group (n=22) and compared with those from CRS patients (n=11) (Figure 6.2). The Mann-Whitney U test was used to examine statistically significant differences between the two groups.

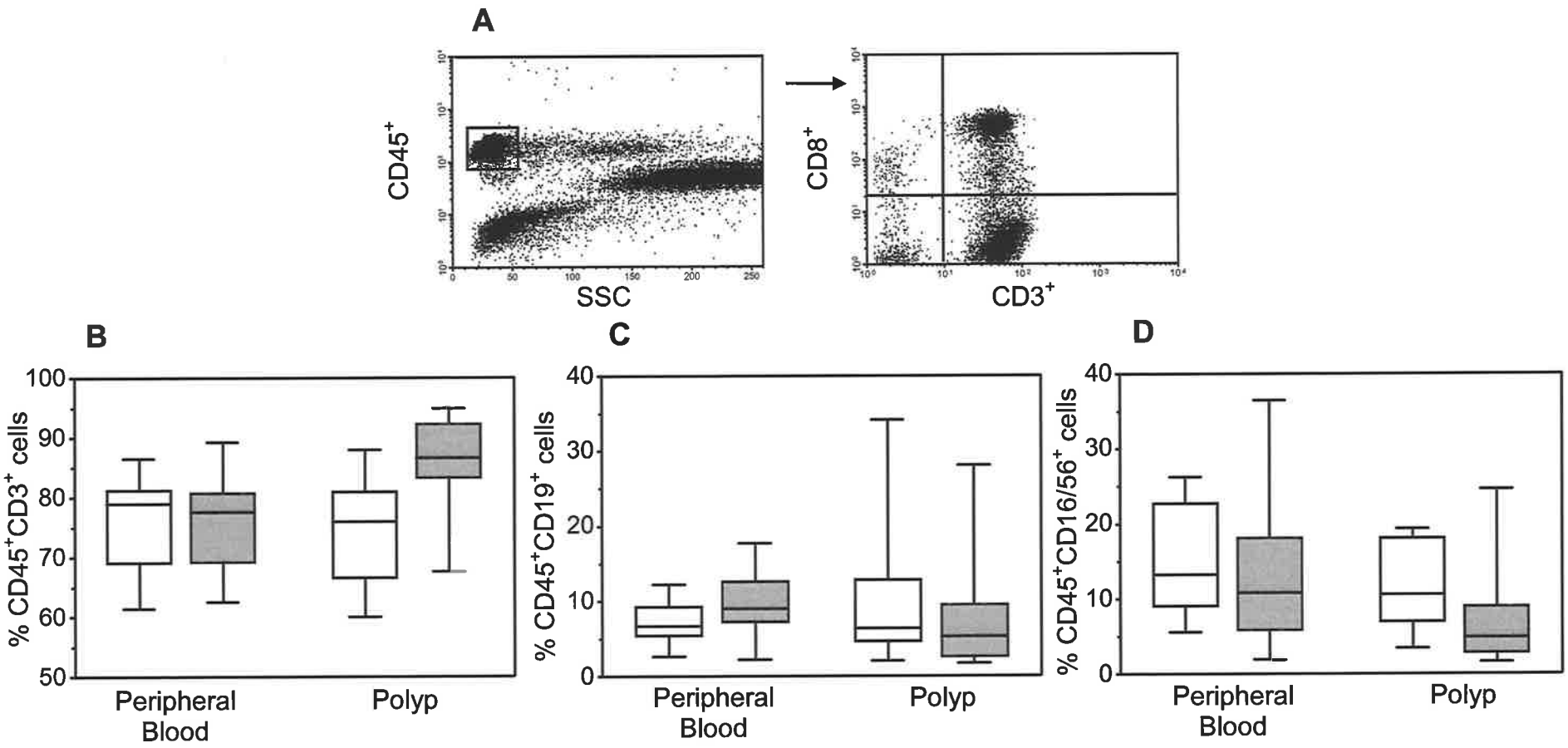


Figure 6.2 Lymphocyte subsets in the peripheral blood and nasal polyps of CRS and EMCRS patients. Dot plots are gated on $CD45^{high}$ (A) and the percentage of $CD3^+$ (B), $CD19^+$ (C) and $CD16^+/56^+$ (D) cells were determined in peripheral blood and polyps of 11 CRS (clear boxes) and 22 EMCRS (shaded boxes) patients. Lower and upper limits of the boxes indicate the 25^{th} and 75^{th} percentiles respectively. The vertical bars show the ranges and horizontal bars the medians.

The percentage of T ($CD3^+$) cells in peripheral blood was not significantly different between CRS (median = 79.0, IQR = 69.0 – 81.3) and EMCRS patients (median = 77.7, IQR = 69.2 – 80.8) ($P = 0.98$) (Figure 6.2 B). In contrast, the percentage of T cells in the nasal polyps was significantly greater in EMCRS (median = 86.6, IQR = 83.3 – 92.3) compared with CRS (median = 76.0, IQR = 66.4 – 81.0) ($P = 0.0018$).

The percentage of B cells and NK cells (Figure 6.2 C and D respectively) in the peripheral blood of CRS and EMCRS was not significantly different. However, CRS polyps had a significantly higher percentage of NK cells (median = 10.6, IQR = 6.92 – 18.1) compared with EMCRS (median = 4.9, IQR = 2.7 – 9.0) ($P = 0.0061$).

6.2.3 $CD4^+$ and $CD8^+$ T lymphocyte populations in polyps and peripheral blood

The proportion of $CD4^+$ and $CD8^+$ T cell subsets in the nasal polyps and peripheral blood was examined in CRS and EMCRS patients. There was no statistically significant difference in the percentage of peripheral blood $CD4^+$ (CRS: median = 46.0, IQR = 43.9 – 56.8; EMCRS: median = 44.6, IQR = 41.2 – 53.9) ($P = 0.4208$) and $CD8^+$ (CRS: median = 23.7, IQR = 17.2 – 28.8; EMCRS: median = 29.3, IQR = 21.6 – 32.8) ($P = 0.2394$) T cell subsets between CRS and EMCRS patients (Figure 6.3).

However, the percentage of $CD8^+$ T cells was strikingly higher in EMCRS polyps (median = 61.0, IQR = 50.2 – 67.3) compared with CRS (median = 41.1, IQR = 30.1 – 44.0) ($P = 0.0002$) and the percentage of $CD4^+$ T cells was significantly lower in EMCRS polyps (median = 25.7, IQR = 21.3 – 26.7) compared with CRS (median = 32.9, IQR = 29.0 – 44.8) ($P = 0.0041$). Interestingly, there did not appear to be a correlation with the percentage of $CD4^+$ or $CD8^+$ T cells with detection of fungus in eosinophilic mucus or with fungal allergy.

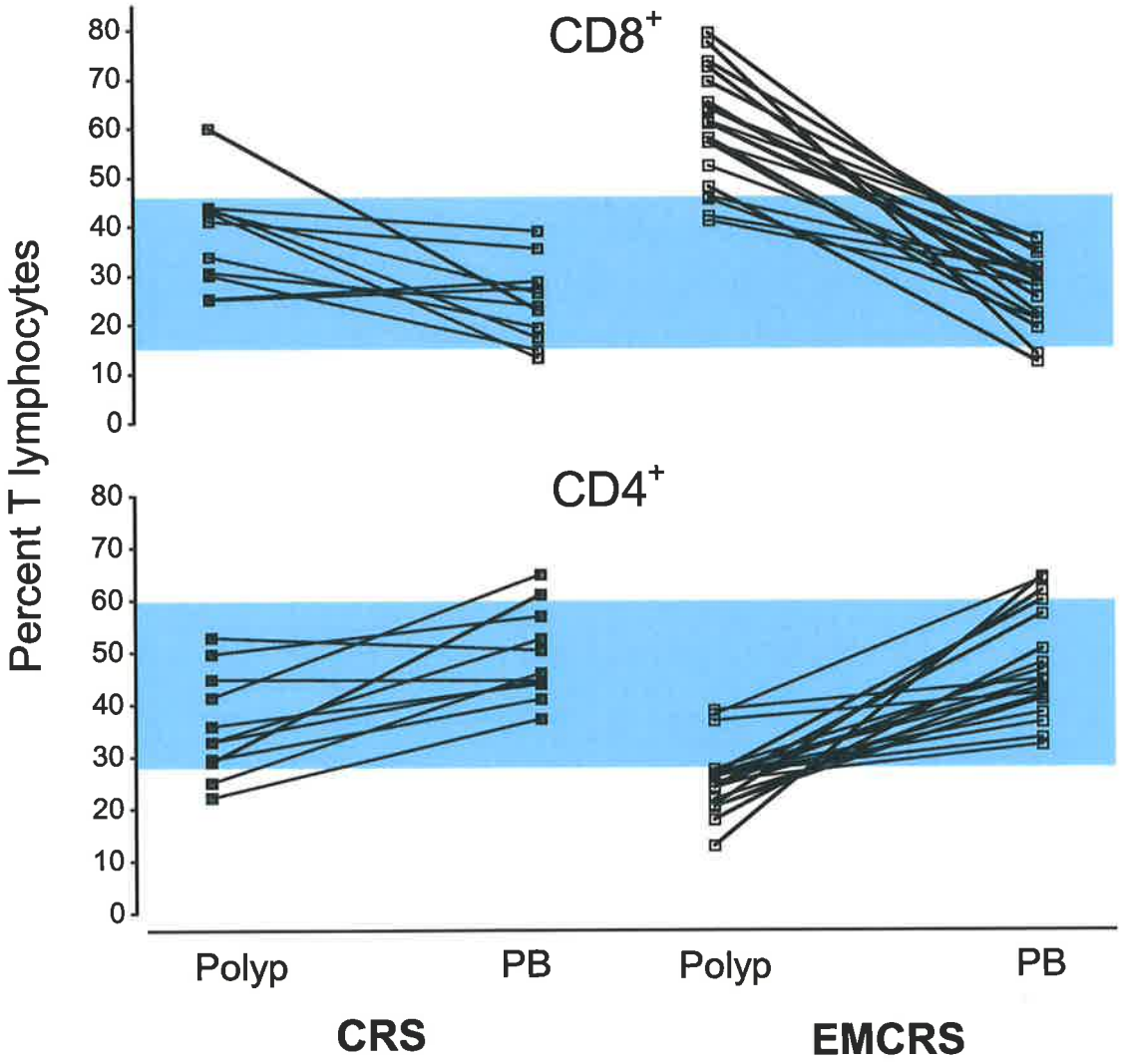


Figure 6.3 Comparison of $CD8^+$ and $CD4^+$ T lymphocytes between nasal polyp and peripheral blood in 11 CRS and 22 EMCRS patients. The lines connect the percentage values of lymphocytes between matched samples of polyp and peripheral blood (PB) for each individual. The shaded areas indicate the normal reference ranges (Appendix III) for $CD8^+$ and $CD4^+$ T lymphocytes in the peripheral blood.

T lymphocytes may express either αβ or γδ TCR (Roitt 1996). To determine the phenotype of the TCR in the nasal polyp lymphocytes, T cells were examined for their expression of γδ TCR in a group of patients. The percentage of γδ TCR in $CD4^+$ and $CD8^+$ T lymphocytes was significantly greater in the polyps than in the peripheral blood of CRS and EMCRS patients (Figure 6.4). However, a larger proportion of T cells in nasal polyps and in peripheral blood were likely to bear a αβ TCR in both CRS and EMCRS patients because most of the T lymphocytes were negative for γδ TCR.

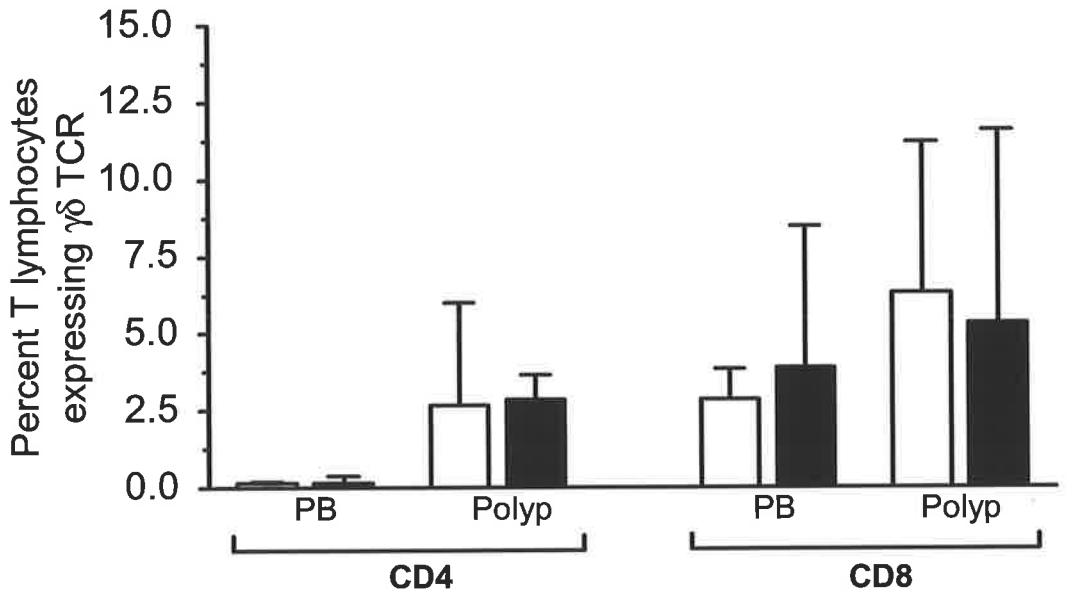


Figure 6.4 Percentage of $CD4^+$ and $CD8^+$ T lymphocytes expressing gamma delta (γδ) T cell receptor (TCR) in peripheral blood (PB) and polyps of CRS (□) and EMCRS (■) patients. The columns represent the median values and bars, the interquartile ranges.

There was no significant difference in the percentage of $CD4^+$ or $CD8^+$ T cells expressing γδ TCR between CRS and EMCRS. In CRS polyps, 2.7% (IQR = 2 – 6) of $CD4^+$ and 6.3% (IQR = 4.8 – 11.2) of $CD8^+$ T cells were γδ TCR positive and in EMCRS polyps, 2.9% (IQR = 0.9 – 3.7) of $CD4^+$ and 5.3% (IQR = 2.6 – 11.6) of $CD8^+$ T cells were γδ TCR positive.

There were more $CD8^+$ T lymphocytes expressing γδ TCR than $CD4^+$ T lymphocytes (CRS, $P = 0.0142$; EMCRS, $P = 0.022$) (Mann-Whitney U test) in the polyps in both CRS and EMCRS groups. Although this trend was also seen with the peripheral blood T cells, the difference was not statistically significant (CRS, $P = 0.053$; EMCRS, $P = 0.072$).

6.2.4 Phenotype of T lymphocytes with respect to their expression of CD45RA, CD62L and CD27 molecules

To examine the functional subpopulations of T lymphocytes in polyps and peripheral blood, the $CD4^+$ and $CD8^+$ T cells were subtyped based on their membrane expression of CD45RA, CD62L and CD27 molecules. A representative experiment is shown in Figure 6.5 and a summary of the experimental findings is presented in Figure 6.6 and Table 6.3.

There was no significant difference in the expression profile of CD45RA, CD62L and CD27 on the peripheral blood $CD4^+$ and $CD8^+$ T lymphocytes in CRS and EMCRS patients (Figure 6.6 D, E and F). However, the expression of these molecules on the nasal polyp T lymphocytes was significantly different between CRS and EMCRS (Figure 6.6 A, B and C).

The percentage of $CD4^+$ and $CD8^+$ T lymphocytes expressing CD45RA was significantly higher in polyps from CRS compared with EMCRS patients, indicating a lower proportion of an antigen-experienced T cell population in CRS than in EMCRS polyps. The percentage of $CD8^+$ T cells expressing CD62L and CD27 was significantly higher in polyps from CRS compared with EMCRS patients. By contrast, the profile of expression of these molecules on $CD4^+$ T cells was similar between CRS and EMCRS. Taken together, these results showed that a significant difference in the phenotype of nasal polyp T lymphocytes between EMCRS and CRS was in the $CD8^+$, and not in the $CD4^+$ subset.

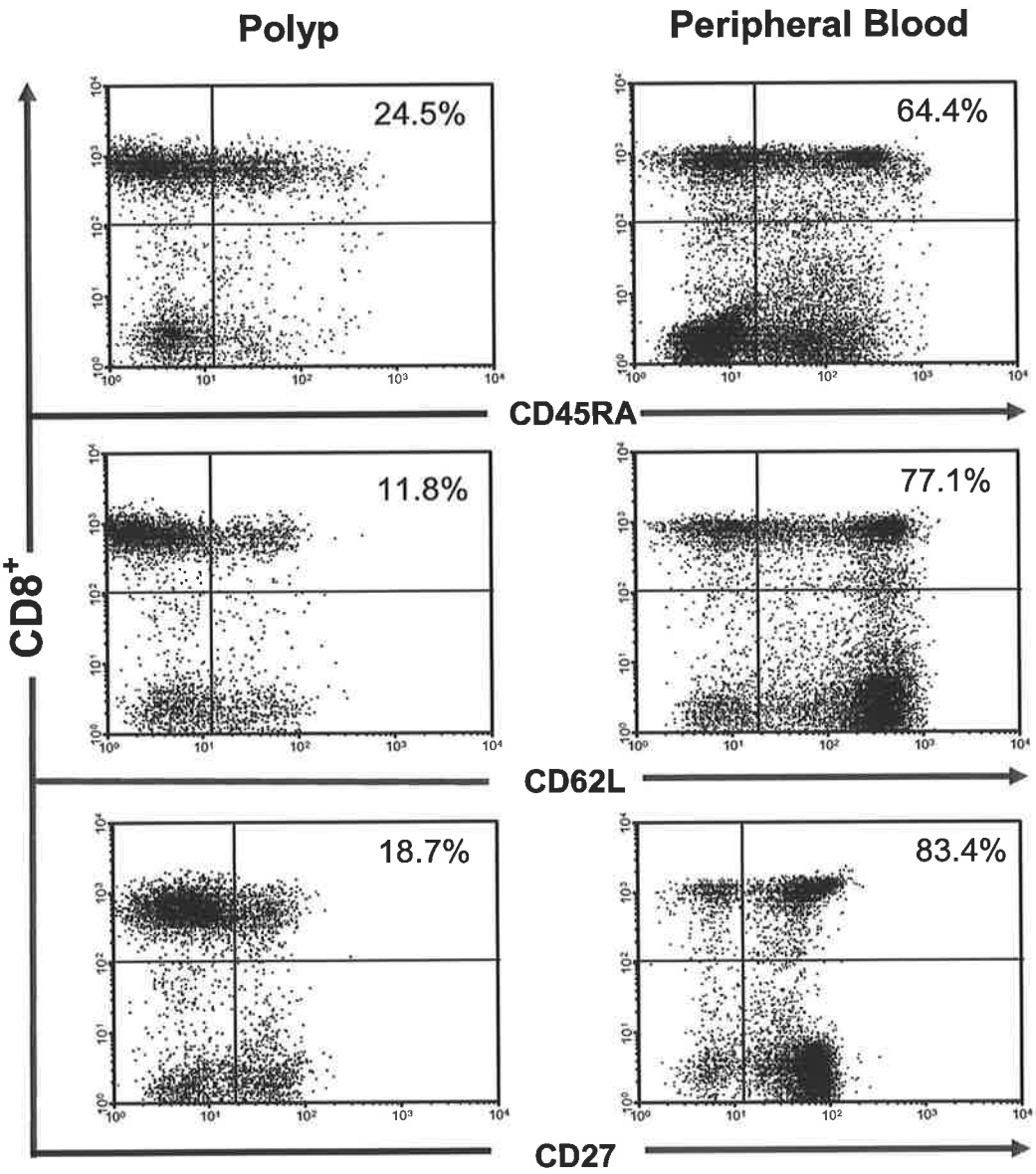


Figure 6.5 Representative dot plots showing the expression of CD45RA, CD62L and CD27 molecules on polyp and peripheral blood $CD8^+$ T lymphocytes from an EMCRS patient. Dot plots were gated on $CD3^+$ cells. The percentages in the upper right quadrants indicate double positive $CD8^+$ T lymphocytes ($CD8^+$ with one of $CD45RA^+$ or $CD62L^+$ or $CD27^+$) as a proportion of total $CD8^+$ T lymphocytes (sum of upper right and upper left quadrants).

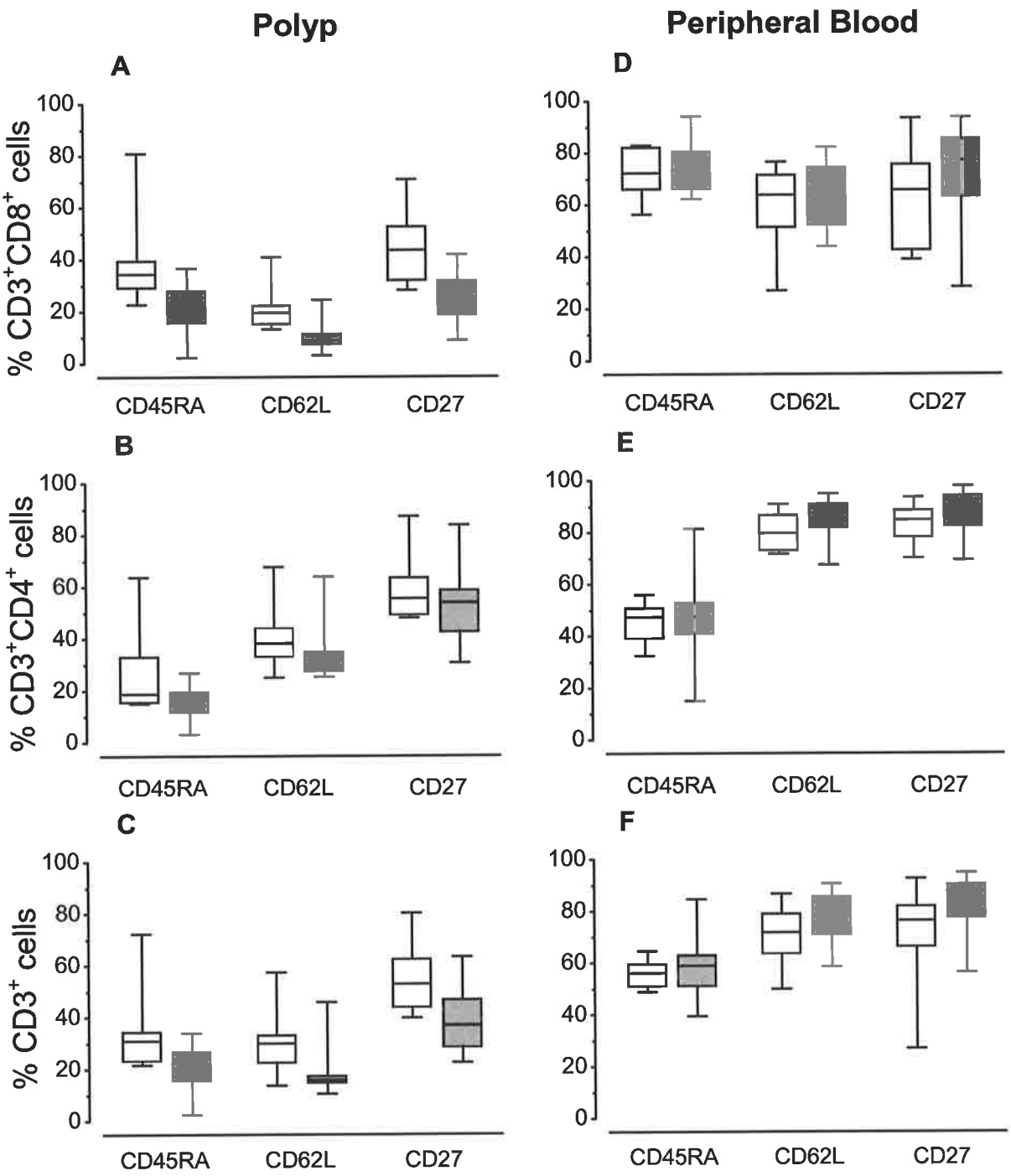


Figure 6.6 Expression of CD45RA, CD62L and CD27 molecules on $CD3^+CD8^+$, $CD3^+CD4^+$ and $CD3^+$ cells in nasal polyps and peripheral blood of CRS (clear boxes) and EMCRS (shaded boxes) patients. Lower and upper limits of the boxes indicate the 25th and 75th percentiles respectively, the vertical bars show the ranges and horizontal bars the medians. The data and statistical analysis for each graph is provided in Table 6.3.

Table 6.3 Percentage of T lymphocytes expressing CD45RA, CD62L and CD27

	CRS	EMCRS	*P* value[1]
(I) CD45RA			
Nasal Polyp			
CD3	30.9 (23.2 – 34.4)	22.4 (15.9 – 27 .1)	0.0347
CD4	18.6 (15.7 – 33.2)	14.5 (11.7 – 19.5)	0.0347
CD8	34.3 (29.0 – 39.4)	25.3 (15.6 – 27.8)	0.0044
Peripheral Blood			
CD3	56.4 (51.2 – 59.7)	59.0 (50.9 – 62.9)	0.6906
CD4	47.4 (38.9 – 50.7)	47.4 (41.0 – 52.9)	0.6461
CD8	72.2 (65.9 – 82.3)	73.2 (65.9 – 80.4)	0.7829
(II) CD62L			
Nasal Polyp			
CD3	29.8 (22.7 – 33.2)	15.7 (14.6 – 17.3)	0.0111
CD4	38.1 (33.1 – 44.0)	31.9 (27.7 – 35.1)	0.1336
CD8	19.5 (14.9 – 22.3)	8.9 (7.5 – 11.3)	0.0016
Peripheral Blood			
CD3	71.9 (63.3 – 78.9)	80.9 (70.9 – 85.6)	0.1683
CD4	79.8 (73.1 – 86.8)	87.6 (81.8 – 90.9)	0.2325
CD8	63.8 (51.5 – 71.6)	66.4 (52.1 – 74.2)	0.6461
(III) CD27			
Nasal Polyp			
CD3	52.9 (43.9 – 62.6)	36.9 (28.5 – 46.8)	0.0132
CD4	55.6 (49.2 – 63.6)	54.0 (42.7 – 58.8)	0.2325
CD8	44.5 (33.8 – 52.5)	19.7 (18.5 – 31.5)	0.0030
Peripheral Blood			
CD3	76.3 (66.0 – 81.8)	87.7 (77.7 – 90.5)	0.1502
CD4	84.8 (78.2 – 88.7)	92.5 (82.5 – 94.3)	0.1336
CD8	65.6 (42.6 – 75.6)	77.1 (63.1 – 85.5)	0.2839

The percentages are expressed as the median values and the 25th and 75th percentiles in parentheses. [1]Mann-Whitney U test.

Most T lymphocytes in EMCRS polyps were negative for CD45RA, CD62L and CD27 ($CD45RA^-$ $CD62L^-$ $CD27^-$), a phenotype that corresponds to an effector memory T cell subset. Using stoichiometric analysis, a median of 74.7% (IQR = 72.2 – 84.4) of EMCRS polyp $CD8^+$ T lymphocytes were negative for CD45RA, CD62L and CD27 compared with a median of 55.5% (IQR = 47.5 – 66.2) of $CD8^+$ T lymphocytes from CRS polyps. By contrast, this phenotype was present in a median of 40.2% (IQR = 41.2 – 57.3) and 44.4% (IQR = 36.4 – 50.8) of $CD4^+$ T lymphocytes in EMCRS and CRS polyps respectively.

In the peripheral blood, 22.9% (IQR = 14.5 – 36.9) of $CD8^+$ T lymphocytes and 7.5% (IQR = 5.7 – 17.5) of $CD4^+$ T lymphocytes from EMCRS patients were of an effector memory phenotype compared with 27.8% (IQR = 17.9 – 34.1) $CD8^+$ T lymphocytes and 15.1% (IQR = 11.3 – 21.8) of $CD4^+$ T lymphocytes from CRS patients. Hence, the proportions of peripheral blood effector memory $CD4^+$ and $CD8^+$ T lymphocytes were not significantly different between EMCRS and CRS patients.

The lymphocyte populations in the adjacent sinus mucosa were similar to that in the polyps, implying a generalised sinonasal mucosal inflammation. The greatest difference in the phenotype of nasal polyp T lymphocytes between EMCRS and CRS patients corresponded to that of an end-stage differentiation of $CD8^+$ T lymphocytes, indicated by the reduced expression of the CD27 molecule (Hintzen et al. 1993). This suggested that $CD8^+$ T lymphocytes might play an important role in the mucosal immune response in EMCRS patients. Therefore, the effector phenotype of the $CD8^+$ T lymphocytes was next examined.

6.2.5 Cytotoxic phenotype of the T lymphocytes

Intracellular expression of perforin, granzymes A and B correlate with cell-mediated cytotoxicity, a major effector function of $CD8^+$ T lymphocytes. These markers for granule-mediated cytotoxicity were examined in the T lymphocytes from the peripheral blood from five healthy volunteers and polyp and peripheral blood from seven CRS and seven EMCRS patients. Intracellular granzyme A and granzyme B was detected in both $CD8^+$ and $CD4^+$ T lymphocytes in all subjects (Table 6.4).

Table 6.4 Percentage of T lymphocytes expressing intracellular granzyme A and B

	$CD8^+$	$CD4^+$
(I) Granzyme A		
Nasal Polyp		
CRS	85.6 (80.2 – 88.8)	11.9 (10.4 - 26.5)
EMCRS	86.4 (78.9 – 88.8)	41.6 (29.6 – 52.1)
Peripheral blood		
HV	29.3 (22.3 – 40.6)	3.5 (0.5 – 4.8)
CRS	61.7 (59.8 – 72.3)	19.0 (11.1 – 25.6)
EMCRS	59.9 (47.5 – 75.2)	15.5 (10.5 – 27.8)
(II) Granzyme B		
Nasal Polyp		
CRS	17.1 (13.3 – 31.3)	9.7 (8.8 – 11.3)
EMCRS	32.3 (26.6 – 41.1)	14.5 (11.1 – 18.8)
Peripheral blood		
HV	13.1 (8.0 – 16.7)	1.02 (0.6 – 1.4)
CRS	37.2 (34.4 – 43.9)	9.7 (7.2 – 11.7)
EMCRS	38.8 (30.6 – 40.9)	16.9 (13.4 – 25.1)

The percentages are expressed as the median values and the 25th and 75th percentiles in parentheses.

As expected, granzyme A and B were expressed by a significantly higher percentage of $CD8^+$ than $CD4^+$ T lymphocytes. There was no significant difference in the percentage of T lymphocytes from CRS and EMCRS polyps expressing either granzyme A ($CD8^+$, $P = 1.0$; $CD4^+$, $P = 0.2$) or granzyme B ($CD8^+$, $P = 0.2$; $CD4^+$, $P = 0.4$) (Mann-Whitney U test). Similarly, there was no significant difference in the percentage of T lymphocytes in peripheral blood expressing granzyme A ($CD8^+$, $P = 0.6$; $CD4^+$, $P = 1.0$) and granzyme B ($CD8^+$, $P = 0.9$; $CD4^+$, $P = 0.2$).

In contrast to granzyme A and granzyme B, the profile of intracellular perforin expression in T lymphocytes was significantly different between EMCRS and CRS patients. In the peripheral blood, the percentage of perforin positive $CD8^+$ T lymphocytes was significantly higher in EMCRS (median = 47.5, IQR = 39.4 – 60.5) compared with CRS (median = 28.3, IQR = 19.4 – 43.4) ($P = 0.043$) and with healthy volunteers (median = 15.3, IQR = 10.5 – 24.8) ($P = 0.004$) (Mann-Whitney U test) (Figure 6.7). The percentage of perforin positive peripheral blood $CD4^+$ T lymphocytes in EMCRS (median = 8.6, IQR = 3.3 – 11.7) was not significantly different compared with those in CRS (median = 2.7, IQR = 0.8 – 6.2) ($P = 0.08$). Strikingly, the percentage of perforin positive $CD8^+$ ($P = 0.0022$) and $CD4^+$ ($P < 0.0001$) T lymphocytes was significantly lower in the nasal polyps of EMCRS patients compared with matched peripheral blood samples.

In the polyp mucosa, the proportion of perforin positive $CD8^+$ T lymphocytes was significantly lower in EMCRS (median = 7.3, IQR = 1.6 – 11.2) compared with CRS (median = 22.1, IQR = 10.7 – 30.9) ($P = 0.02$). The percentage of perforin positive $CD4^+$ T lymphocytes was also significantly lower in EMCRS (median = 3.0, IQR = 1.1 – 6.2) compared with CRS (median = 8.3, IQR = 6.2 – 10.7) ($P = 0.017$).

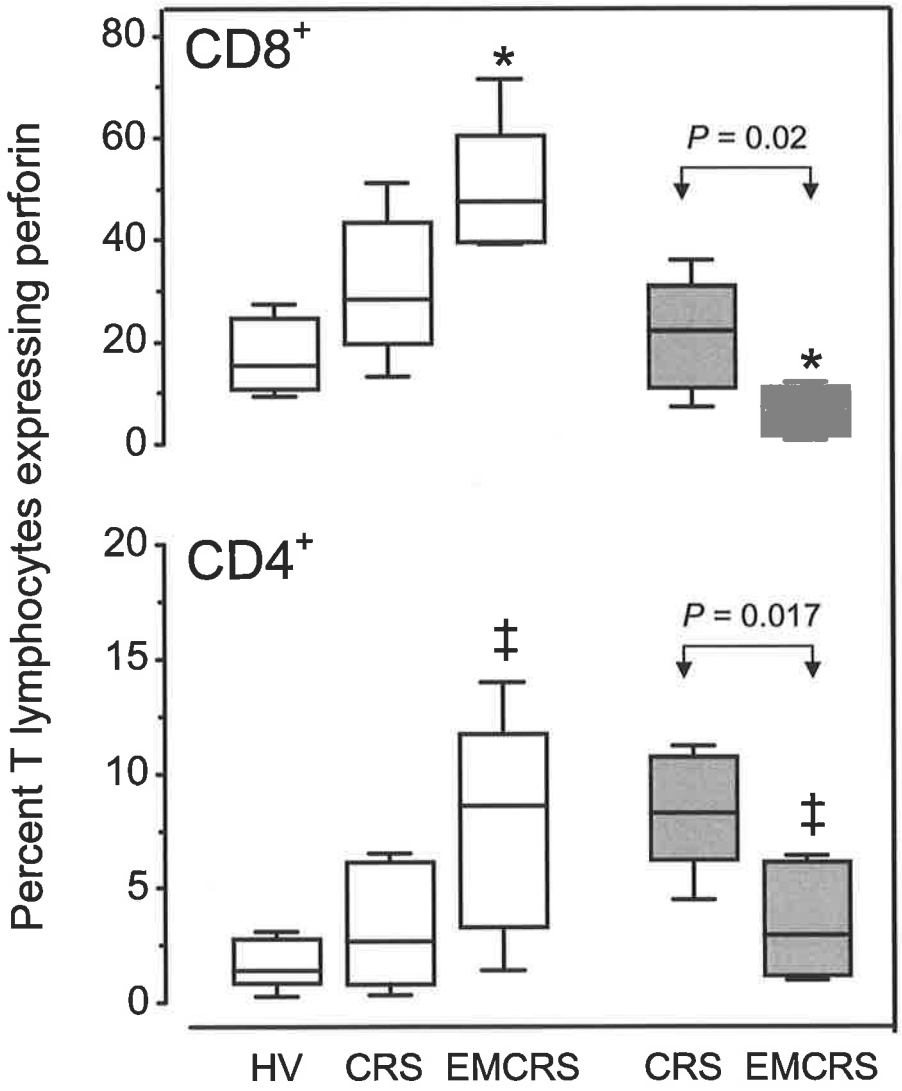


Figure 6.7 The profile of intracellular perforin expression in T lymphocytes. Peripheral blood samples (clear boxes) from five healthy volunteers (HV) and peripheral blood and nasal polyp samples (shaded boxes) from seven CRS and seven EMCRS patients were examined. Lower and upper limits of the boxes represent the 25th and 75th percentiles respectively. The vertical bars show the ranges and horizontal bars the medians. There was a significant difference in the expression of perforin among the study groups (CD8, P = 0.009; CD4, P = 0.0485, Kruskal-Wallis test). *P* = 0.02 and *P* = 0.017; *P = 0.0022 and ‡P < 0.0001 (Mann-Whitney U test).

6.2.6 MHC II and CD25 expression on T lymphocytes

To determine the proportion of activated T lymphocytes in peripheral blood and nasal polyps from CRS and EMCRS patients, cells were examined for surface expression of MHC II and CD25 molecules. If CD25 and MHC II were present on nasal polyp $CD4^+$ and $CD8^+$ T lymphocytes, then this would be evidence for *in situ* activation, compared to a lack of expression in peripheral blood lymphocytes. In the case for $CD4^+$ T lymphocytes, $CD4^+CD25^+$ phenotype may correspond to a population of regulatory T cells (Tregs) (Chapter 1, section 1.14).

The proportion of T lymphocytes expressing MHC II was not significantly different between CRS and EMCRS patients (Figure 6.8 A). The percentage of $CD4^+$ T lymphocytes expressing MHC II was significantly higher in polyps (CRS: median = 41.5, IQR = 35 – 50; EMCRS: median = 38.4, IQR = 26 – 57.1) than in peripheral blood (CRS: median = 7, IQR = 4.25 – 13.11; EMCRS: median = 4.8, IQR = 3.9 – 9.3) in both CRS and EMCRS patients (P = 0.0079, Mann-Whitney U test). Similarly, the percentage of $CD8^+$ T lymphocytes expressing MHC II was significantly higher in the polyp (CRS: median = 41.7, IQR = 37.6 – 56.9; EMCRS median = 63.3, IQR = 26.5 – 75.7) than in peripheral blood (CRS: median = 15.8, IQR = 11.9 – 18; EMCRS: median = 4.7, IQR = 4 – 14.3) in both CRS and EMCRS patients (P = 0.0079).

The fraction of T lymphocytes expressing CD25 was not significantly different between CRS and EMCRS patients (Figure 6.8 B). The percentage of $CD4^+$ T cells expressing CD25 was higher in polyp (CRS: median = 33.7, IQR = 23.5 – 47.3; EMCRS: median = 24.8, IQR = 20.9 – 37.8) than in peripheral blood (CRS: median = 4.4, IQR = 2.7 – 8.7; EMCRS: median = 4, IQR = 2.8 – 7.3) of CRS (P = 0.0022) and EMCRS (P = 0.0079) patients.

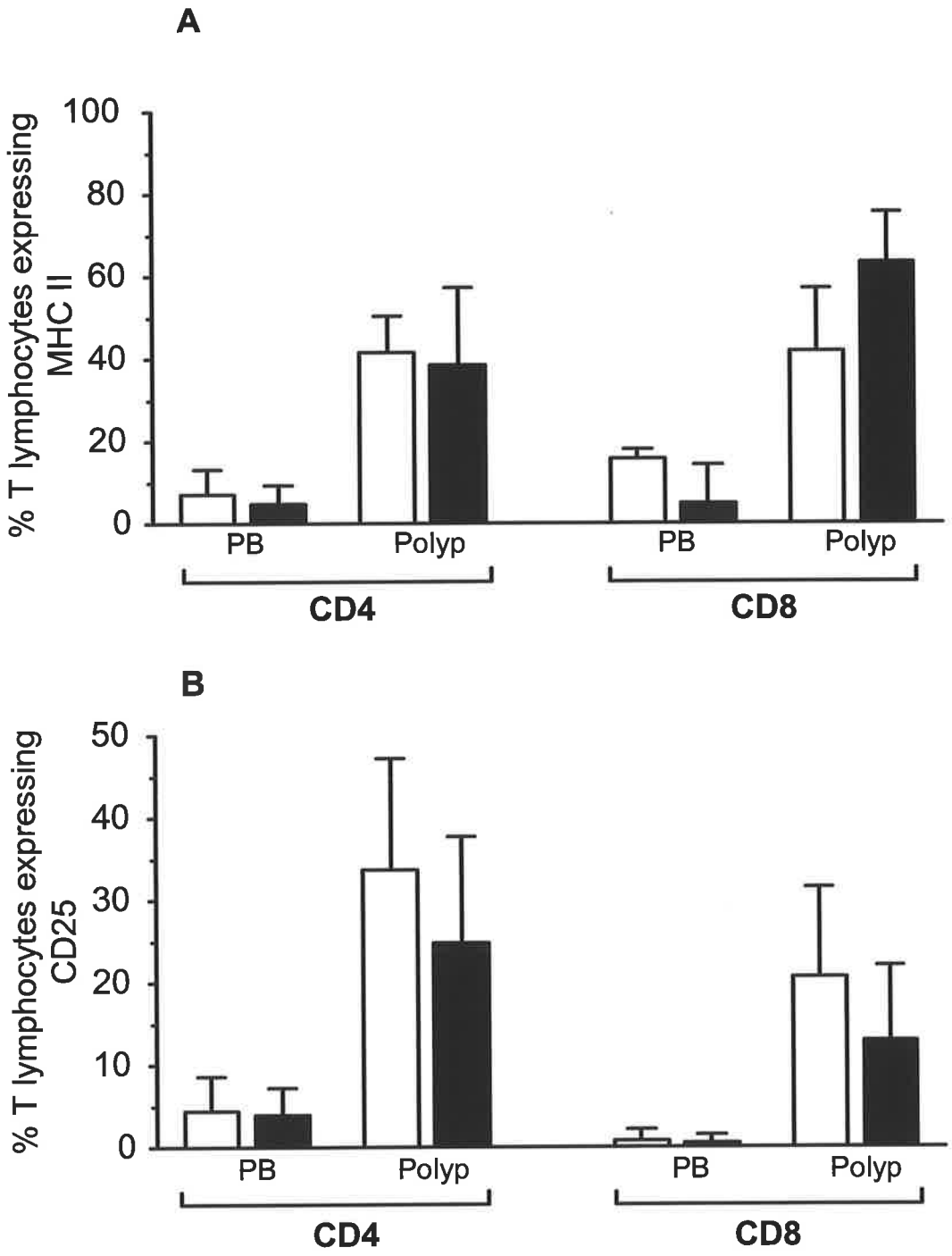


Figure 6.8 The profile of MHC II (A) and CD25 (B) expression on T lymphocytes. The $CD4^+$ and $CD8^+$ T lymphocytes from the peripheral blood and nasal polyps of five CRS (□) and five EMCRS (■) patients were examined. The columns represent the medians and bars, the interquartile ranges.

Likewise, the percentage of $CD8^+$ T lymphocytes expressing CD25 was significantly higher in polyp (CRS: median = 20.6, IQR = 11.3 – 31.6; EMCRS: median = 12.9, IQR = 6.9 – 22) than in peripheral blood (CRS: median = 0.8, IQR = 0.3 - 2.2; EMCRS: median = 0.6, IQR = 0.4 – 1.6) of CRS (P = 0.0022) and EMCRS (P = 0.0159) patients.

6.2.7 Evidence for T lymphocyte degranulation

As a means of determining whether the T lymphocyte populations had been functionally active, cell surface expression of lysosomal associated membrane proteins (LAMPs), CD107a and CD107b, was examined (Betts et al. 2003). The LAMPs are transiently incorporated into the plasma membrane following release of perforin and granzymes from the intracellular pool. Expression of cell surface CD107 can be detected as early as 30 minutes to one hour following stimulation and peaks between four and five hours (Betts et al. 2003). Consequently, CD107a and CD107b on the cell surface membrane provide a read out of recent cell degranulation. In this study, the cell surface expression of CD107a and CD107b were examined in peripheral blood and nasal polyps from five CRS and five EMCRS patients (Figure 6.9).

There was no statistically significant difference in the percentage of LAMP positive $CD4^+$ and $CD8^+$ T lymphocytes between EMCRS and CRS patients. The fraction of T lymphocytes expressing CD107a / CD107b was significantly higher in the nasal polyps than in peripheral blood ($CD4^+$: CRS and EMCRS, P = 0.0079; $CD8^+$: CRS P = 0.0022, EMCRS P = 0.0079) (Mann-Whitney U test). Thus, based on the LAMP expression profile, both $CD4^+$ and $CD8^+$ T lymphocytes from nasal polyps showed evidence of lysosome exocytosis. However, a greater proportion of $CD8^+$ T lymphocytes expressed LAMPs than $CD4^+$ T lymphocytes (CRS, P = 0.028; EMCRS, P = 0.034).

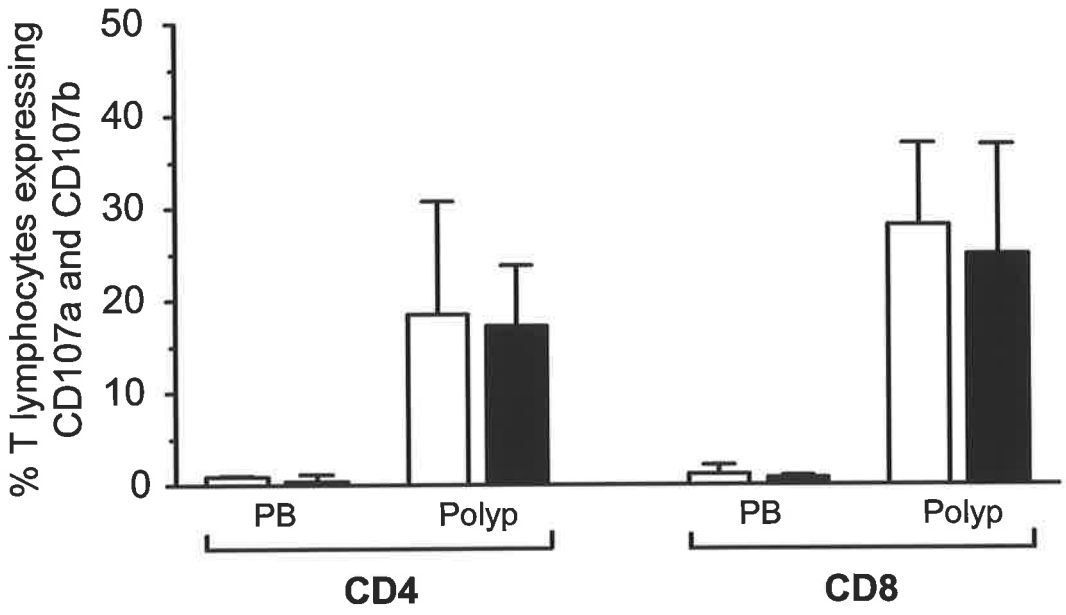


Figure 6.9 The profile of CD107a and CD107b expression on T lymphocytes. The $CD4^+$ and $CD8^+$ T lymphocytes from the peripheral blood and nasal polyps of five CRS (□) and five EMCRS (■) patients were examined. The columns depict the medians and bars, the interquartile ranges.

6.3 DISCUSSION

Despite several reports demonstrating lymphocytes in nasal polyps, their function and differentiation status have not been explored. In this study, the inflammatory cell population in polyps from EMCRS and CRS patients was characterised by eosinophils and lymphocytes. The majority of T lymphocytes in the sinonasal mucosa expressed αβ TCR (Graeme-Cook et al. 1993). Although the $CD4^+$ and $CD8^+$ T lymphocyte populations had a similar histological distribution, the proportions were markedly different between EMCRS and CRS polyps. A significantly higher percentage of $CD8^+$ T lymphocytes was present in EMCRS polyps whereas a higher percentage of $CD4^+$ T lymphocytes was demonstrated in CRS polyps. A greater proportion of the mucosal T lymphocytes in EMCRS and CRS polyps expressed surface markers consistent with the phenotype of an effector memory subset compared to that found in matched peripheral blood samples. While the percentage of $CD4^+$ effector memory T lymphocyte population (Geginat et al. 2003; Danke and Kwok 2003; Balla-Jhagjhoorsingh et al. 2004) was not significantly different between EMCRS and CRS, a greater proportion of $CD8^+$ effector memory T lymphocytes was found in EMCRS patients. Despite the phenotype corresponding to a differentiated T lymphocyte population, the $CD8^+$ T lymphocytes from EMCRS polyps lacked the cytotoxic phenotype, defined by the expression of perforin, compared with matched peripheral blood samples and with polyps from CRS patients. These studies could not take into account the phenotype of the lymphocyte populations in the sinonasal mucosa of healthy volunteers. Nevertheless, when compared with polyps from disease-control and matched peripheral blood samples, profound differences in the functional phenotype of the $CD8^+$ T cell subpopulations suggests a unique subset of $CD8^+$ T lymphocytes and a unique role for $CD8^+$ T lymphocytes in the sinonasal mucosal inflammation in EMCRS.

A greater proportion of $CD8^+$ T lymphocytes from the EMCRS mucosa expressed the CD27 negative phenotype, indicative of a highly differentiated cell population. Cell membrane expression of MHC II and CD25 and of LAMP 1 and 2 molecules indicated that the T lymphocytes were activated and had undergone recent degranulation (Betts et al. 2003). However, the cytolytic effector potential of the T cells, defined by intracellular granzymes and perforin, was significantly different in mucosal $CD8^+$ T lymphocytes from EMCRS compared with CRS and with matched peripheral blood samples. Whereas a larger fraction of the peripheral blood $CD8^+$ T lymphocytes from EMCRS patients expressed granzymes and perforin, the mucosal $CD8^+$ T lymphocytes expressed granzymes only and had an almost complete lack of perforin.

Perforin expression is reported as the defining feature of terminally differentiated antigen-specific cytolytic $CD8^+$ T lymphocytes (Hamann and al. 1997; Zhang et al. 2003). Given that a lack of CD27 expression on T lymphocytes closely correlates with end-point differentiation, the selective absence of perforin in the mucosal $CD8^+$ T lymphocytes from EMCRS and not from CRS patients was intriguing. If the EMCRS mucosal $CD8^+$ T lymphocytes utilised perforin-mediated cytotoxicity, perforin may be expressed at a later stage compared with granzymes, just prior to granule release (Kelso et al. 2002). However, the present study showed that a proportion of $CD8^+$ T lymphocytes had undergone degranulation. The absence of perforin was unlikely to be due to degranulation alone, as one would then expect both perforin and granzyme to be depleted. Therefore, it is possible that molecules other than perforin, such as granulysin, may be utilised in granule-mediated cytotoxic activity in the EMCRS mucosa (Stenger et al. 1998; Ma et al. 2002; Ma et al. 2004). That granzymes were identified in most of the $CD8^+$ T cells in the EMCRS polyps indicated that granule-mediated cytotoxic mechanisms were of importance. Hence, an

alternative explanation, that the mucosal $CD8^+$ T lymphocytes in EMCRS patients were from the non-cytolytic subpopulation was unlikely (Harty et al. 2000) (Figure 6.10).

The antigen-specificity of the EMCRS mucosal $CD8^+$ T lymphocytes is unknown. Antigen-specific, αβ TCR $CD8^+$ T lymphocytes in peripheral sites mediate protective effects from a variety of infectious agents including viruses, bacteria, mycobacteria, fungi and protozoa (Beck and Harmsen 1998; Pope et al. 2001; Leavey and Tarleton 2003; Myers et al. 2003; Wong and Pamer 2003; Badauy et al. 2005). A large population of $CD8^+$ T lymphocytes are also demonstrated in the mucosa in inflammatory bowel disease (Kappeler and Mueller 2000). Murine studies show that antigen-specific and non-antigen related $CD8^+$ T lymphocyte subpopulations mediate clearance of pathogens, cause damage to surrounding "normal" microenvironment and perform immunoregulatory or immunosuppressive functions (Scott et al. 2004; Jiang and Chess 2004; McAllister et al. 2004). Perforin is also implicated in the homeostasis of $CD8^+$ T lymphocytes (Harty et al. 2000) and a deficiency may result in an abnormal regulation of the extent of $CD8^+$ T lymphocyte clonal expansion in chronic inflammatory states (reviewed by Trapani and Smyth 2002).

If the majority of the $CD8^+$ T lymphocytes in the EMCRS mucosa were fungal-specific, a lack of perforin could indicate a defect in anti-fungal immune response. A dysregulated response to fungi was also suggested by a lack of proliferative activity in the peripheral blood $CD8^+$ T lymphocytes in Chapter 5. However, the antigen-specificity of the lymphocytes could not be determined in this study. Therefore, based on the functional phenotype of the mucosal $CD8^+$ T lymphocytes, one may speculate that the mucosal inflammation in EMCRS patients could be a consequence of failure of immune control resulting from deficient perforin (Shacklett et al. 2004; Tomiyama et al. 2004) rather than an

impairment of maturation of the $CD8^+$ T cells (van Baarle et al. 2002), as most of the EMCRS mucosal $CD8^+$ T lymphocytes were CD27 negative, showed evidence of degranulation but had selective absence of perforin.

Alternatively, reduced $CD8^+$ T lymphocyte cytotoxicity (Fuller et al. 2004) associated with a lack of perforin (Zhang et al. 2003) may have a beneficial effect on the host, as shown in the setting of chronic exposure to viral and non-viral antigens (Th. Den Boer et al. 2004). Because most $CD8^+$ T lymphocytes that have seen the antigen repeatedly are not directly cytotoxic, it protects the host from damage due to cytotoxic T cell recognition and destruction of uninfected cells that may be displaying weakly reacting self-peptides. If this were the case, the $CD8^+$ T lymphocytes in EMCRS mucosa may be incidental and not directly involved in causing the inflammation.

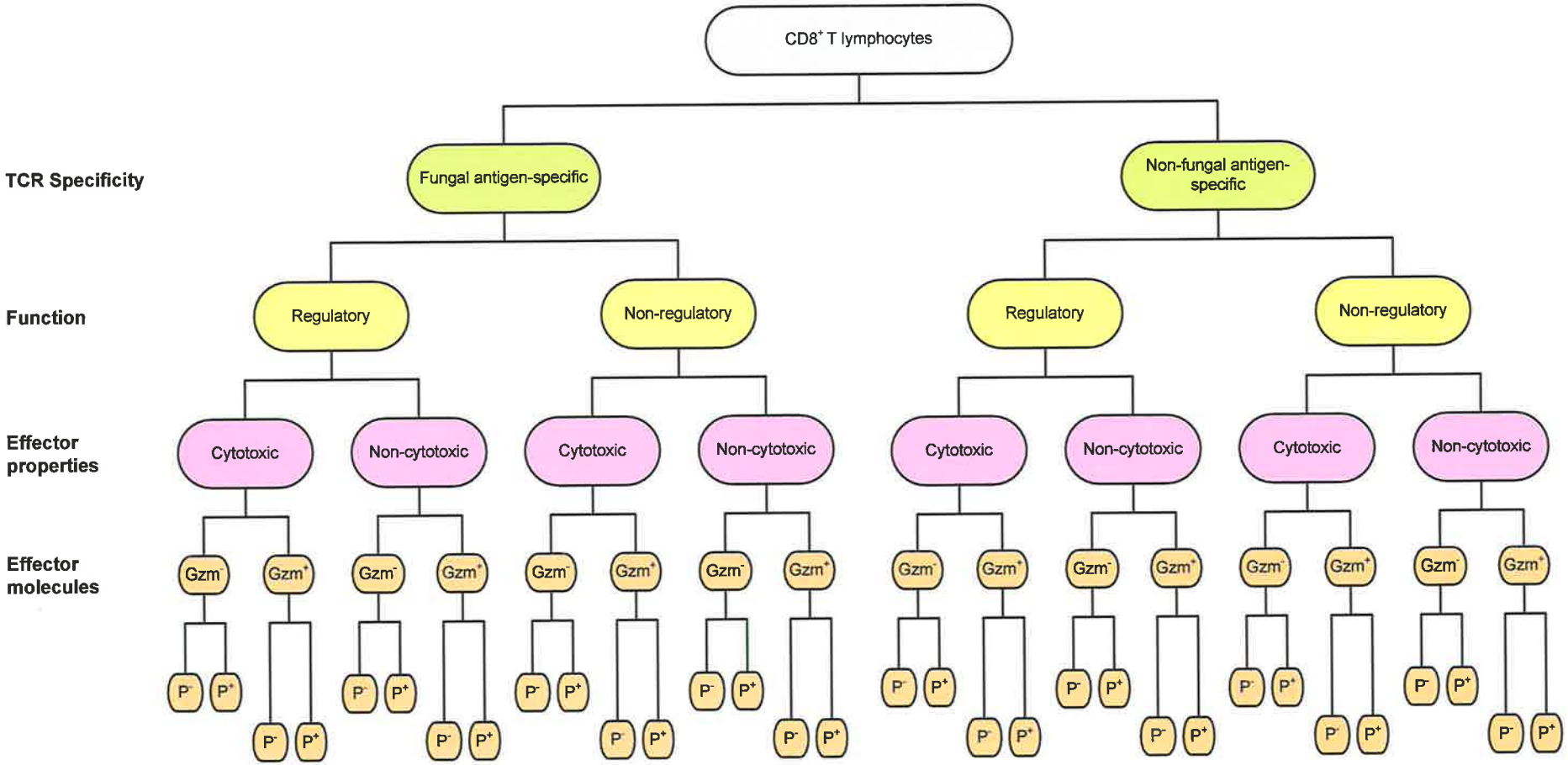
Chronic inflammation in the EMCRS mucosa could also be a consequence of a lack of mucosal $CD4^+$ T lymphocytes. The $CD4^+$ T lymphocytes may be required for the generation and maintenance of cytolytic $CD8^+$ T lymphocytes (Carbone et al. 1998; Marzo et al. 2004) and in the regulation of the overall immune response (Jiang and Chess 2004). A large proportion of mucosal $CD4^+$ T lymphocytes in EMCRS and CRS were $CD25^+$. These cells may represent a population of activated conventional $CD4^+$ T lymphocytes or $CD4^+CD25^+$ regulatory T cells (Taams et al. 2001). Activated conventional $CD4^+$ T lymphocytes may be involved in anti-pathogen activity by cytokine secretion or by mediating cytotoxicity via perforin and Fas/Fas-ligand interaction (Tazume et al. 2004). Perforin positive $CD4^+$ T lymphocytes are also described in certain autoimmune disorders (Xanthou et al. 1999; Weyland et al. 2000) and in B cell chronic lymphocytic lymphoma (Porakishvili et al. 2004).

Regulatory $CD4^+CD25^+$ T cells are increased in sites of chronic inflammation (Makita et al. 2004). Recently, these cells have been shown to express and use perforin and granzyme A (natural Tregs) or granzyme B (adaptive Tregs) for immune regulation in healthy humans (Grossman et al. 2004) and in murine models of autoimmune encephalomyelitis (Malpiero et al. 1997). It is generally accepted that Tregs influence responses to self-antigens, some tumor antigens and in an extending number of exogenous antigens. Tregs may protect the host from "collateral" tissue damage caused by protective immune response against pathogens or, may suppress anti-pathogen protective effect of the $CD8^+$ T lymphocytes (reviewed in Rouse and Suvas 2004). Further analysis of the $CD4^+CD25^+$ T cells in the EMCRS and CRS mucosa with respect to their expression of *Foxp3* may enable this cell population to be better defined.

In conclusion, the presence of T lymphocytes with a phenotype corresponding to a functional, effector memory cell population in the mucosa of EMCRS and CRS patients suggests involvement of the adaptive immune system in the inflammatory response. However, to determine the cytotoxic ability, functional studies are required (Zhang et al. 2003). To date, the bulk of evidence describing the effector memory $CD8^+$ T lymphocytes have been reported in models of infectious diseases where infections have completely resolved (Martin and Tarleton 2005). The dynamics and role of the $CD8^+$ T lymphocytes in the chronic non-infectious human diseases are being defined, where the nature of the immune response is likely to be largely regulatory, non-specific and polyclonal rather than pathogen-specific. Examination of other parameters of $CD8^+$ T lymphocyte-mediated immune responses including patterns of epitope recognition, clonotypic complexity, phenotype and function will be required to determine their role in the overall immune response. Nevertheless, it was remarkable that in this study, eosinophilic mucus marked a

population of functional $CD8^+$ T lymphocytes whose phenotype corresponded with that of a highly differentiated cell population. Future work to further phenotype these cells, determine their antigen-specificity and functional analysis could provide clues to their effector function and ultimately to the nature of the inflammatory response in the sinonasal mucosa.

Figure 6.10 Putative subpopulations of $CD8^+$ T lymphocytes in the sinonasal mucosa of EMCRS and CRS patients. The $CD8^+$ (and $CD4^+$, not shown) T lymphocyte populations may be defined by a combination of the following: their antigen specificity conferred by their T cell receptor (TCR), their role in the immune response or function, effector properties and the presence of effector molecules. The effector molecules include proteins that mediate cytotoxicity including granzyme (Gzm) and perforin (P), and chemokines (not shown). The sizes of the boxes do not bear any relationship to the sizes of the cell populations shown.


CD8+ T lymphocytes
TCR Specificity
Fungal antigen-specific
Non-fungal antigen-specific
Function
Regulatory
Non-regulatory
Regulatory
Non-regulatory
Effector properties
Cytotoxic
Non-cytotoxic
Cytotoxic
Non-cytotoxic
Cytotoxic
Non-cytotoxic
Cytotoxic
Non-cytotoxic
Effector molecules
Gzm-
Gzm+
P-
P+

CHAPTER SEVEN: GENERAL DISCUSSION AND CONCLUDING REMARKS

Chronic rhinosinusitis is a heterogeneous disease and because it is influenced by numerous environmental and genetic factors, its pathogenesis remains largely unexplained. This study began with a review of the literature that suggested allergic fungal sinusitis was a separate disease from the other subgroups of EMCRS patients (Ferguson 2000a) and was argued to be an IgE-mediated hypersensitivity reaction to fungi (Manning and Holman 1998). However, as eosinophilic mucosal inflammation and eosinophilic mucus was also present in non-allergic chronic rhinosinusitis patients, I hypothesised that allergic fungal sinusitis may not be pathogenically different from the other subgroups of EMCRS patients. Accordingly, immune mechanisms other than fungal allergy may have a major role in the eosinophilic mucosal inflammation. Indeed, there are many precedents in the development of non-IgE-mediated eosinophilic inflammation (Chapter 1, section 1.15).

Several lines of evidence presented in this thesis showed that IgE-mediated fungal allergy was not the major pathological insult in fungal-allergic EMCRS patients. In Chapter 4, an examination of total IgE and fungal-specific IgE levels showed no significant difference between the fungal-allergic EMCRS patients, allergic fungal sinusitis and allergic fungal sinusitis-like, and the fungal-allergic disease-control, ARFA patients (Pant et al. 2005). As expected, the magnitude of fungal-specific humoral responses in the fungal-allergic EMCRS patients and ARFA patients was significantly higher than from healthy volunteers. However, the nature of the fungal-specific responses in allergic EMCRS patients was different from that in ARFA patients. Both EMCRS and ARFA patients had a greater IgG and IgA antibody response to fungi compared with healthy volunteers, but the EMCRS patients were

distinguished from ARFA patients by significantly higher levels of fungal-specific IgG3 antibodies.

Similarly in Chapter 5, fungal-allergic EMCRS and ARFA patients demonstrated a significantly greater peripheral blood mononuclear cell proliferation to fungal antigens compared with healthy volunteers. Whereas both $CD4^+$ and $CD8^+$ T lymphocytes from ARFA patients proliferated in response to the fungi, $CD8^+$ T lymphocytes did not proliferate in EMCRS patients. Hence, the profile of the fungal-specific lymphocyte populations defined by their proliferative activity was markedly different between ARFA patients and fungal-allergic EMCRS patients.

IgE-mediated allergic responses are typically associated with $CD4^+$ T lymphocytes (Lambrecht 2001; Gould et al. 2003; Cohn et al. 2004) and with high levels of granzyme B positive T lymphocytes (Bratke et al. 2004). In Chapter 6, granzyme A positive $CD8^+$ T lymphocytes were abundant in the sinonasal mucosa of the fungal-allergic EMCRS patients. Finally, because a similar profile of systemic and mucosal immune responses was also present in non-allergic (fungal and non-fungal) patients, the development of allergic fungal sinusitis and the other EMCRS subgroups could not be solely attributed to a systemic IgE-mediated allergic process.

Except for the age of the patients and the defining criteria of fungal allergy and fungi in sinus eosinophilic mucus, there were no significant differences between the EMCRS subgroups with regard to the clinical parameters (Chapter 3), fungal-specific humoral (Chapter 4) and cellular (Chapter 5) immune characteristics and the phenotype of the sinonasal mucosal T lymphocytes (Chapter 6). As fungal allergy was not of central

pathogenic importance (Chapter 4), these results implied that similar immunopathological processes, other than allergy, were common to all the EMCRS patients. The finding that allergic fungal sinusitis and allergic fungal sinusitis-like patients were significantly younger than NAFES and NANFES patients could represent an accentuated underlying eosinophilic mucosal inflammation due to coexisting fungal and non-fungal allergy, thereby resulting in an earlier clinical presentation. In those patients with coexisting allergy, in addition to the IgE-mediated allergic mechanisms, the $CD8^+$ T lymphocytes in the mucosa may enhance the inflammatory responses by as yet undefined pathways (Sawicka et al. 2004; Stock et al. 2004; Rowe et al. 2004). Taken together, this thesis suggests that the fundamental immunopathological process driving the inflammatory response in EMCRS patients may not be different between the subgroups. Hence, dividing the patients into subgroups based on fungal allergy and fungi in eosinophilic mucus is not of central pathological relevance. Although the treatment of allergy, when present, may provide symptomatic improvement it would not alter the course of the underlying disease.

The results from this thesis supported the hypothesis that eosinophilic mucus marked a distinct form of chronic rhinosinusitis. A more aggressive inflammatory disease in EMCRS patients was indicated by their clinical, histopathological and immunological characteristics. EMCRS patients had severe sinus disease, were more likely to have extra-sinus complications at clinical presentation and lower respiratory tract disease than CRS patients. Histological examination of the polyps showed more inflammatory cells and loss of tissue architecture in EMCRS compared with CRS patients. A heightened inflammatory condition was also suggested by an elevated peripheral blood mononuclear cell proliferation to phytohaemagglutinin in EMCRS patients than in CRS (and ARFA) patients. The elevated cell proliferation was observed using similar proportions of peripheral blood T lymphocytes

in the samples examined, so the enhanced proliferation was not due to more T cells in EMCRS. An intense proliferative response to mitogenic stimulus indicates lymphocytes in a pre-activated state and may therefore signify a greater magnitude or duration of inflammatory stimulus in EMCRS patients.

Fungal-specific immune responses were significantly greater in EMCRS patients than in CRS patients, irrespective of fungal allergy or the detection of fungi. This supported the hypothesis that mechanisms other than fungal allergy accounted for the fungal-specific immune response in EMCRS patients. An elevated humoral and cellular response to fungi indicated ongoing exposure to fungal antigens in EMCRS patients. Elevated fungal-specific IgG and IgA from the humoral studies (Chapter 4) suggested that a mucosa-derived antigen was involved in EMCRS and CRS patients. However, the nature of the IgG subclass response was different in EMCRS compared with CRS. Similarly, an increased cellular response to the fungi was observed in EMCRS and some CRS patients (Chapter 5). In contrast to CRS (and ARFA) patients, the $CD8^+$ T cells failed to proliferate in EMCRS. In addition, the magnitude and pattern of fungal-specific immune responses in allergic CRS patients were not significantly different to ARFA patients whereas those from non-allergic CRS patients were not significantly different from healthy volunteers. This separation of fungal-specific immunity indicated that there was a unique association of fungal-specific immune response with EMCRS patients, regardless of fungal allergy or of the detection of fungi. That fungi were not detected in some EMCRS patients may reflect insensitive detection methods, hence limiting their practical or clinical value. Alternatively, molecular mimicry to fungal antigens could also rationalise the immune response in EMCRS patients, where the immunological stimulus persists in the absence of fungi (discussed below).

It is still unclear why there was a relative lack of fungal-specific $CD8^+$ T cell proliferation in EMCRS patients (Chapter 5). This was a novel and important finding that raised an intriguing possibility of a dysregulated $CD8^+$ T lymphocyte response to fungi in EMCRS patients, especially where a lack of cell proliferation was demonstrated to both fungal antigens. That fungal-specific $CD8^+$ T lymphocytes were completely absent from the peripheral blood of the EMCRS patients is an unlikely possibility. It would be interesting to determine whether the $CD8^+$ T lymphocyte population in the peripheral blood of the EMCRS patients was fungal-specific and terminally differentiated, thereby providing an explanation for their limited proliferative activity. Hence, it is apparent from this study that an in-depth analysis of the T lymphocyte response to fungal antigens is warranted.

The nature of the mucosal inflammation defined by mucosal T lymphocyte populations in EMCRS patients was strikingly different to that in CRS patients (Chapter 6). An antigen-driven and terminally differentiated $CD8^+$ T cell population that lacked perforin characterised most of the EMCRS mucosal lymphocytes. This significant finding may provide insight into the nature and regulation of the inflammatory response. Because the phenotype of these $CD8^+$ T lymphocytes corresponded to that associated with antigen-specific lymphocytes, it was likely that the adaptive immune system was involved in the mucosal inflammation. The inflammation could be mediated by immune mechanisms that directly stimulate the adaptive system or indirectly via the innate system (for example, antigen presentation or toll-like receptor polymorphisms) that subsequently affects adaptive responses. The immune stimulation may occur in the presence or absence of a pathogen. A selective loss of mucosal $CD4^+$ T lymphocytes from the sinonasal mucosa could also explain the preponderance of $CD8^+$ T lymphocytes. This situation is seen especially where mucosal immune-compromise and resulting opportunistic infections or a breakdown in immune

tolerance may occur (Veazey and Lackner 2003; Liu and Lefrançois 2004). Future studies will be directed at an examination into the function of the T lymphocyte populations described in this thesis. One approach may involve a functional evaluation of the cytotoxic ability of the mucosal $CD8^+$ T lymphocytes and an examination for other mechanisms of cytotoxicity. Another approach may involve an examination of the $CD4^+$ T lymphocyte population.

It was apparent from this study that EMCRS patients were different to CRS patients in terms of the clinical characteristics, fungal-specific immune responses and the mucosal T lymphocyte populations. However, it still remains to be elucidated whether EMCRS is a pathogenically different disease from CRS altogether, or a severe form of the chronic rhinosinusitis spectrum. It was evident that an elevated fungal-specific immune response was associated with EMCRS patients. If not fungal allergy, what other mechanisms could be responsible for the elevated fungal-specific immune response? One possibility is that in genetically susceptible individuals, fungal antigens were the major aetiological factors principally causing and maintaining the mucosal inflammation, leading to a fungal-specific mucosal immune response that was detected in the humoral and cellular components of the peripheral blood (Figure 7.1 A).

Alternatively, fungal colonisation in immune-compromised mucosa due to an underlying inflammatory process accounted for the elevated fungal-specific peripheral blood immune responses (Figure 7.1 B). If this were the case, why would the nature of the fungal-specific immune response be any different between EMCRS patients and controls, as individuals in the control groups were also exposed to fungi? EMCRS patients may be genetically susceptible or they may be exposed to a different array of immunodominant fungal antigens.

The latter may be a reflection of the environmental exposure to fungi, as suggested by the demographic data in Chapter 3. The different antigenic milieu could arise as a consequence of fungal colonisation and growth in the nose and paranasal sinuses in EMCRS patients but not in healthy volunteers or disease-controls. Future work with population-genetic typing including HLA typing between the subgroups and to define the peptides recognised by the fungal-specific immunoglobulins may determine genetic susceptibility and identify antigenic peptides associated with EMCRS (Crameri et al. 1998; Kurup et al. 2000; Knutsen et al. 2004).

A direct causal relationship between fungi and EMCRS was not established in this study. However, there are several mechanisms by which fungi may be involved in the EMCRS mucosal inflammation. Fungi have been reported in inflammation associated with $CD8^+$ T lymphocytes. A recent study by Badauy et al. 2005 demonstrated a large proportion of $CD8^+$ T lymphocytes in the mucosa of immune-competent individuals with *Candida albicans* infection. Fungi may recruit the mucosal $CD8^+$ T lymphocytes in a non-specific manner either directly due to their toxic effects (Ashman et al. 1995; Yekeler et al. 2001) or indirectly by fungal-specific $CD4^+$ T lymphocytes. In addition, it has been shown that granulysin has antifungal activity and may therefore explain the relative absence of perforin in the EMCRS mucosa. Fungi may also recruit eosinophils directly via toll-like receptors or indirectly via immunoglobulin Fc receptors and by inducing pro-eosinophilic cytokine secretion by other cells. Fungal proteins are also toxic to cilia, epithelial cells and enhance mucus production and consequently exacerbate mucosal inflammation in EMCRS patients. Although fungi are demonstrated in the sinuses in a proportion of EMCRS patients, it is unlikely that the presence of fungi is the primary cause of EMCRS because the eradication of fungi does not cure the disease. This implies an underlying inflammatory process.

The nature of the underlying inflammatory response in CRS and EMCRS patients is as yet unknown. From review of the literature and the work presented in this thesis, it is proposed that an "appropriate" immune response to common environmental antigens including viruses, bacteria or fungi, may lead to ongoing inflammation in susceptible patients (Figure 7.2). Ensuing damage from an "appropriate" immune response may result in the recognition by the mucosal immune system of an otherwise innocuous secondary antigen that persists and potentiates chronic inflammation. The secondary antigen may result from molecular mimicry or as a consequence of exposure of autoreactive $CD4^+$ T lymphocytes to excessive amounts of cryptic self-determinants, thereby resulting in an autoimmune disease in genetically susceptible individuals (Kohm et al. 2003). The latter mechanism may occur in chronic viral infections or diseases where an appropriate $CD8^+$ T lymphocyte mediated cytotoxicity results in cleavage of potentially cryptic peptides. It has been shown that many auto-antigens are cleaved by granzyme B (Casciola-Rosen et al. 1999), which was expressed in the mucosal $CD8^+$ T lymphocytes in EMCRS patients. As the mucosa is a unique system with potentially "self" reactive T cells, in the form of NKT cells, the mucosal cells could recognise the cryptic peptides and cause ongoing immune stimulation. Thus, the immunopathological process can continue after the triggering agent is eliminated. Hence, if there was molecular mimicry to fungal antigens (Crameri et al. 1996), it could explain enhanced fungal-specific immune responses in the absence of fungi in the sinuses.

The subpopulations of $CD8^+$ T lymphocytes may be implicated in mediating the damage (Shinbori et al. 2004) and in immune regulation by a perforin-independent mechanism. Alternatively, a lack of perforin may result in failure of protective effect or of immune control (discussed in Chapter 6). Ultimately, ongoing chronic inflammation is predicted to culminate in mucosal immune-compromise and colonisation by viruses, bacteria and fungi,

also known as a biofilm. Mucosal immune-compromise may account for fungi in the mucosa of EMCRS (Chapter 3; Thakar et al. 2004) and allergic bronchopulmonary aspergillosis patients (Riley et al. 1975; Slavin et al. 1988). Because the host is generally immune-competent, fulminant infection is not observed. The biofilm of pathogens may accentuate the underlying inflammation by several mechanisms. Superantigen responses (Bernstein 2003), molecular mimicry and innate responses are such examples. Hence, this may also explain the enhanced pro-inflammatory immune responses to common environmental organisms in chronic rhinosinusitis patients compared with healthy volunteers (Shin et al. 2004).

An inadequate host immune response to common environmental organisms or pathogens that constitute the biofilm would further exacerbate the inflammation. In EMCRS patients, there may be a defect in the immune response to fungal antigens as suggested by an absent fungal-specific $CD8^+$ T cell proliferation (Chapter 5). Accordingly, a dysregulated and inadequate host immune response to fungi may promote fungal growth, cause further mucosal damage, increase the inflammatory cell infiltrate and hence, eosinophilic mucus. Eosinophilic mucus may also impair the normal mucus drainage pathways in the sinuses and have proinflammatory properties while providing a nidus for more microorganisms. Because the mucosal immune system of the sinonasal and lower respiratory tract are continuous, this may explain the features of severe inflammation in the lower airways in EMCRS patients. Coexisting inflammatory conditions including allergy, aspirin sensitivity and cystic fibrosis, would also accentuate the mucosal inflammation and determine the overall clinical phenotype and severity of chronic rhinosinusitis. Therefore, treatment of one or all of the peripheral factors including anatomical problems, allergy, bacteria and fungi in the sinuses will not alleviate the underlying disease, but only alleviate the symptoms.

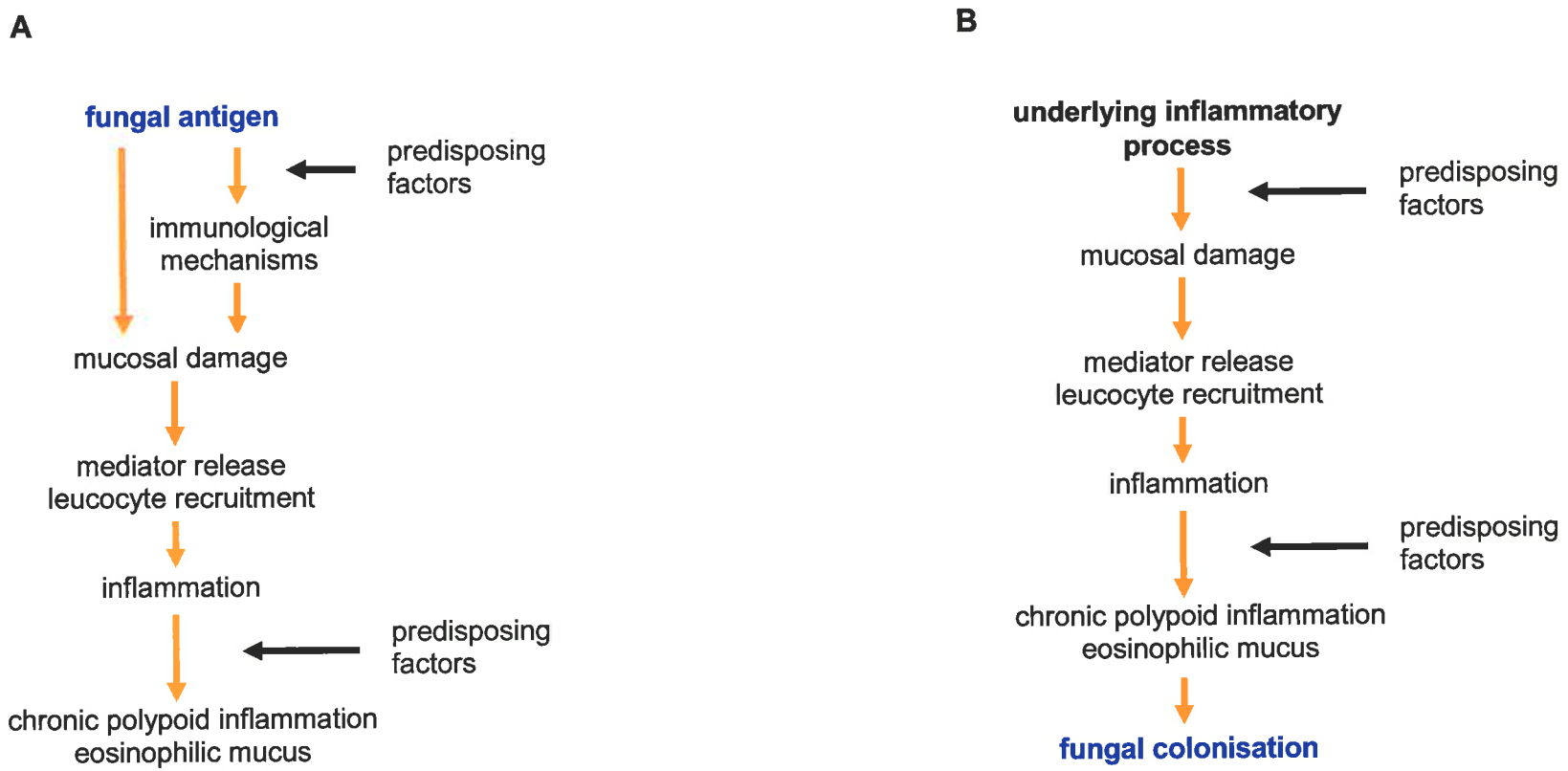


Figure 7.1 Proposed models of the putative mechanisms leading to the elevated fungal-specific immune response in EMCRS patients.

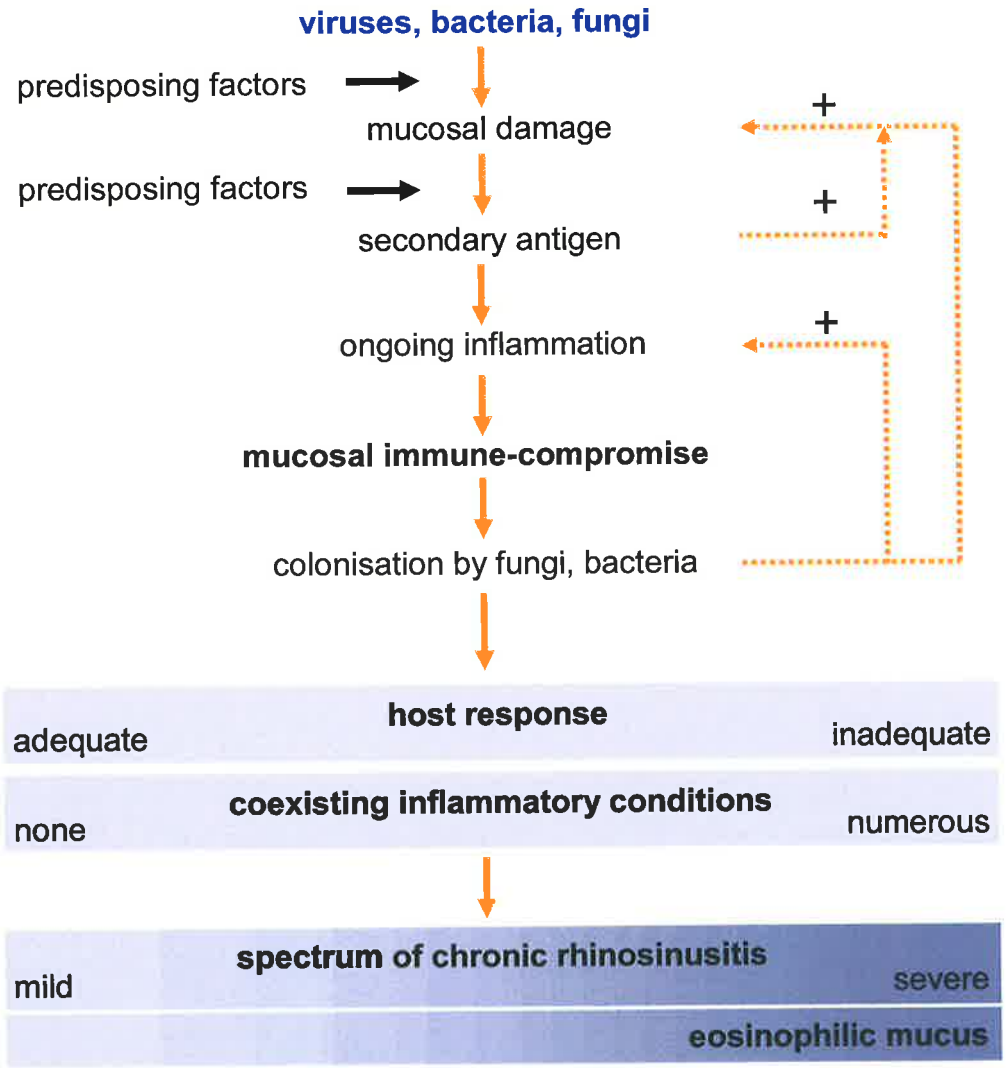


Figure 7.2 Proposed model for the development of eosinophilic mucus chronic rhinosinusitis. In susceptible individuals, inflammation is initiated by recognition of secondary antigen by the host immune cells by as yet unknown mechanism following an appropriate anti-pathogen response. Ongoing inflammation in the right microenvironment is predicted to result in mucosal immune dysfunction, culminating in mucosal immune-compromise. Subsequent colonization by microflora constitutes a biofilm, which accentuates the underlying inflammation by several mechanisms. The type of host immune responses, environment and coexisting pathology ultimately determines the clinical phenotype and severity of chronic rhinosinusitis.

REFERENCES

Adamko DJ, Odemuyiwa SO, Vethanayagam D and Moqbel R (2005). The rise of the pheonix: the expanding role of the eosinophil in health and disease. Allergy 60:13-22.

Agha-Mir-Salim P, Rauhut O and Merker HJ (1993). Electron and fluorescence microscopic investigations on composition and structure of the epithelial basement membrane of the human inferior nasal concha. Eur Arch Otorhinolaryngol 250:401-407.

Akira S and Takeda K (2004). Toll-like receptor signalling. Nat Rev Immunol 4:499-511.

Alam R and Busse WW (2004). The eosinophil-quo vadis? J Allergy Clin Immunol 113:38-42.

Alho OP, Karttunen TJ, Karttunen R, Tuokko H, Koskela M and Uhari M (2003). Lymphocyte and mast cell counts are increased in the nasal mucosa in symptomatic natural colds. Clin Exp Immunol 131:138-142.

Ali MS, Wilson JA and Pearson JP (2002). Mixed nasal mucus as a model for sinus mucin gene expression studies. Laryngoscope 112:326-331.

Allen JE and Maizels RM (1997). Th1-Th2: reliable paradigm or dangerous dogma? Immunol. Today 18:387-392.

Altman JD, Moss PA, Goulder PJ, Barouch DH, McHeyzer-Williams MG, Bell JI, McMichael AJ and Davis MM (1996). Phenotypic analysis of antigen-specific T lymphocytes. Science 274:94-96.

Amitani R, Taylor G, Elezin EN, Lewellyn-Jones C, Mitchell J, Kuse F, Cole PJ and Wilson R (1995). Purification and characterisation of factors produced by *Aspergillus fumigatus* which affect human ciliated respiratory epithelium. Infect Immun 63:3266-3271.

Anderson DM, Keith J and Novak PD, lexicographers (1994). Dorland's Illustrated Medical Dictionary, 28th ed. WB Saunders.

Angulo R and Fulcher DA (1998). Measurement of Candida-specific blastogenesis: comparison of carboxyfluorescein succinimidyl ester labelling of T cells, thymidine incorporation, and CD69 expression. Cytometry 34:143-151.

Armant MA and Fenton MJ (2002). Toll-like receptors: a family of pattern-recognition receptors in mammals. Genome Biology 3:3011.1-3011.6.

Arora AS and Yamazaki K (2004). Eosinophilic eosophagitis: asthma of the esophagus? Clin Gasteroenterol Hepatol 2:523-530.

Ashbee HR, Muir SR, Cunliffe WJ and Ingham E (1997). IgG subclasses specific to *Staphylococcus epidermidis* and *Propionibacterium acnes* in patients with acne vulgaris. Br J Derm 136:730-733.

Ashman RB, Fulurija A, Robertson TA and Papadimitriou JM (1995). Rapid destruction of skeletal muscle fibers by mycelial growth forms of *Candida albicans*. Exp Mol Pathol 62:109-117.

Ashman RB (1998). *Candida albicans*: pathogenesis, immunity and host defense. Res Immunol 149:281-8; discussion 494-496.

Assenmacher M, Lohning M, Scheffold A, Manz RA, Schmitz J and Radbruch A (1998). Sequential production of IL-2, IFN-gamma and IL-10 by individual staphylococcal enterotoxin B-activated T helper lymphocytes. Eur J Immunol 28:1534-1543.

Attinger A, Acha-Orbea H and MacDonald HR (2000). Cutting edge: Cell autonomous rather than environmental factors control bacterial superantigen-induced T cell anergy in vivo. J Immunol 165:1171-1174.

Azuma M, Phillips JH and Lanier LL (1993). $CD28^-$ T lymphocytes. Antigenic and functional properties. J Immunol 150:1147-1159.

Bachert C, Wagenmann M, Rudack C, Hopken K, Hillebrandt M, Wang D and van Cauwenberge P (1998). The role of cytokines in infectious sinusitis and nasal polyposis. Allergy 53:2-13.

Bachert C, Gevaert P and van Cauwenberge P (1999). Nasal polyposis-a new concept on the formation of polyps. ACI International 11:130-135.

Bachert C, Gevaert P, Holtappels G and van Cauwenberge P (2002). Mediators in nasal polyposis. Curr Allergy Asthma Rep 2:481-487.

Bachert C, van Zele T, Gevaert P, De Schrijver L and Van Cauwenberge P (2003). Superantigens and nasal polyps. Curr Allergy Asthma Rep 3:523-531.

Badauy CM, Barbachan JJ, Rados PV, Sant'ana Filho M and Chies JA (2005). Relationship between Candida infection and immune cellular response in inflammatory hyperplasia. Oral Microbiol Immunol 20:89-92.

Bakare N, Richkerts V, Bargon J and Just-Nubling G (2003). Prevalence of *Aspergillus fumigatus* and other fungal species in the sputum of adult patients with cystic fibrosis. Mycoses 46:19-23.

Balla-Jhagjhoorsingh S, Koopman G, Mooij P, Koornstra W, McCormack S, Weber J, Pantaleo G and Heeney J (2004). Long-term persistence of HIV-1 vaccine-induced $CD4^{+}CD45RA^{-}CD62L^{-}CCR7^{-}$ memory T-helper cells. AIDS 18:837-848.

Bannister LH, Berry MM, Collins P, Dyson M, Dussek JE and Ferguson MWJ, editors (1995). Gray's Anatomy, 38th ed. Churchill Livingstone.

Beck JM and Harmsen AG (1998). Lymphocytes in host defense against *Pneumocystis carinii*. Semin Respir Infect 13:330-338.

Beil WJ, McEuen AR, Schulz M, Wefelmeyer U, Kraml G, Walls AF, Jensen-Jarolim E, Pabst R and Pammer J (2002). Selective alterations in mast cell subsets and eosinophil

infiltration in two complementary types of intestinal inflammation: Ascariasis and Crohn's disease. Pathobiology 70:303-313.

Bendelac A, Bonneville M and Kearney JF (2001). Autoreactivity by design: innate B and T lymphocytes. Nat Rev Immunol 1:177-186.

Benninger MS, Ferguson BJ, Hadley JA, Hamilos DL, Jacobs M, Kennedy DW, Lanza DC, Marple BF, Osguthorpe JD, Stankiewicz JA, Anon J, Denneny J, Emanuel I and Levine H (2003). Adult chronic rhinosinusitis: definitions, diagnosis, epidemiology, and pathophysiology. Otolaryngol Head Neck Surg 129:(3 Suppl)1-32.

Beno D, Stover AG, Herbert L and Matthews HI (1995). Growth inhibition of *Candida albicans* hyphae by $CD8^+$ lymphocytes. J Immunol 154:5273-5281.

Bent JP 3rd and Kuhn FA (1994). Diagnosis of allergic fungal sinusitis. Otolaryngol Head Neck Surg 111:580-588.

Bernstein JM, Ballow M, Schlievert PM, Rich G, Allen C and Dryja D (2003). A superantigen hypothesis for the pathogenesis of chronic hyperplastic sinusitis with massive nasal polyposis. Am J Rhinol 17:321-326.

Betts MR, Brenchley JM, Price DA, De Rosa SC, Douek DC, Roederer M and Koup RA (2003). Sensitive and viable identification of antigen-specific $CD8^+$ T cells by a flow cytometric assay for degranulation. J Immunol Methods 281:65-78.

Betts MR, Price DA, Brenchley JM, Loré K, Guenaga JF, Smed-Sorensen A, Ambrozak DR, Migueles SA, Connors M, Roederer M, Douek DC and Koup RA (2004). The functional profile of primary human antiviral $CD8^+$ T cell effector activity is dictated by cognate peptide concentration. J Immunol 172:6407-6417.

Bhagat R, Shah A, Jaggi OP and Khan ZU (1993). Concomitant allergic bronchopulmonary aspergillosis and allergic *Aspergillus* sinusitis with an operated aspergilloma. J Allergy Clin Immunol 91:1094-1096.

Biselli R, Matricardi PM, D'Amelio R and Fattorossi A (1992). Multiparameteric flow cytometric analysis of the kinetics of surface molecule expression after polyclonal activation of human peripheral blood T lymphocytes. Scand J Immunol 35:439-447.

Bisht V, Singh BP, Arora N, Gaur SN and Sridhara S (2002). Antigenic and allergenic cross-reactivity of *Epicoccum nigrum* with other fungi. Ann Allergy Asthma Immunol 89:285-291.

Blom M, Tool AT, Mul FP, Knol EF, Roos D and Verhoeven AJ (1995). Eosinophils isolated with two different methods show different characteristics of activation. J Immunol Methods 178:183-193.

Book DT, Smith TL, McNamar JP, Saeian K, Binion DG and Toohill RJ (2003). Chronic sinonasal disease in patients with inflammatory bowel disease. Am J Rhinol 17:87-90.

Bradford MM (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248-254.

Bratke K, Bottcher B, Leeder K, Schmidt S, Kupper M, Virchow JCJ and Luttmann W (2004). Increase in granzyme B^+ lymphocytes and soluble granzyme B in bronchoalveolar lavage of allergen challenged patients with atopic asthma. Clin Exp Immunol 136:542-548.

Braun H, Buzina W, Freudenschuss K, Beham A and Stammberger H (2003). 'Eosinophilic fungal rhinosinusitis': a common disorder in Europe? Laryngoscope 113:264-269.

Brain DJ (1997). Historical background. In: Settipane GA, Lund VL, Bernstein J and Tos M, editors. Nasal polyps: epidemiology, pathogenesis and treatment, OceanSide Publications Inc., 7-15.

Bredemeyer AJ, Lewis RM, Malone JP, Davis AE, Gross J, Townsend RR and Ley TJ (2004). A proteomic approach for the discovery of protease substrates. Proc Natl Acad Sci USA 101:11785-11790.

Brenchley JM, Karandikar NJ, Betts MR, Ambrozak DR, Hill BJ, Crotty LE, Casazza JP, Kuruppu J, Migueles SA, Connors M, Roederer M, Douek DC and Koup RA (2003). Expression of CD57 defines replicative senescence and antigen-induced apoptotic death of $CD8^+$ T cells. Blood 101:2711-2720.

Brigl M and Brenner MB (2004). CD1: antigen presentation and T cell function. Annu Rev Immunol 22:817-890.

Briskin M, Winsor-Hines D, Shyjan A, Cochran N, Bloom S, Wilson J, McEvoy LM, Butcher EC, Kassam N, Mackay CR, Newman W and Ringler DJ (1997). Human mucosal addressin cell adhesion molecule-1 is preferentially expressed in intestinal tract and associated lymphoid tissues. Am J Pathol 151:97-110.

Brooks DA, Zola H, McNamara PJ, Bradley J, Bradstock KF, Hancock WW and Atkins RC (1983). Membrane antigens of human cells of the monocyte/macrophage lineage studied with monoclonal antibodies. Pathology 15:45-52.

Brummund W, Resnick A, Fink JN and Kurup VP (1987). *Aspergillus fumigatus*-specific antibodies in allergic bronchopulmonary aspergillosis and aspergilloma: evidence for a polyclonal antibody response. J Clin Microbiol 25:5-9.

Butcher EC and Picker LJ (1996). Lymphocyte homing and homeostasis. Science 272:60-66.

Buzina W, Braun H, Freudenschuss K, Lackner A, Habermann W and Stammberger H (2003). Fungal biodiversity-as found in nasal mucus. Med Mycol 41:149-161.

Campbell DJ, Debes GF, Johnston B, Wilson E and Butcher EC (2003). Targeting T cell responses by selective chemokine receptor expression. Seminars in Immunology 15:277-286.

Campbell JJ, Brightling CE, Symon FA, Qin S, Murphy KE, Hodge M, Andrew DP, Wu L, Butcher EC and Wardlaw AJ (2001). Expression of chemokine receptors by lung T cells from normal and asthmatic subjects. J Immunol 166:2842-2848.

Carbone FR, Kurts C, Bennett SR, Miller JF and Heath WR (1998). Cross-presentation: a general mechanism for CTL immunity and tolerance. Immunol Today 19:368-373.

Carding SR and Egan PJ (2002). Gammadelta T cells: functional plasticity and heterogeneity. Nat Rev Immunol 2:336-345.

Carvalho AT, Elia CC, de Souza HS, Elias PR, Pontes EL, Lukashok HP, de Freitas FC and Lapa e Silva JR (2003). Immunohistochemical study of intestinal eosinophils in inflammatory bowel disease. J Clin Gastroenterol 36:120-125.

Casale TB, Erger RA and Rozell MD (1999). Eosinophils isolated by magnetic cell sorting respond poorly to lipid chemoattractants. Ann Allergy Asthma Immunol 83:127-131.

Casciola-Rosen L, Andrade F, Ulanet D, Wong WB and Rosen A (1999). Cleavage by granzyme B is strongly predictive of autoantigen status: implications for initiation of autoimmunity. J Exp Med 190:815-826.

Catalfamo M and Henkart PA (2003). Perforin and the granule exostosis cytotoxicity pathway. Curr Opin Immunol 15:522-527.

Catten MD, Murr AH, Goldstein JA, Mhatre AN and Lalwani AK (2001). Detection of fungi in the nasal mucosa using polymerase chain reaction. Laryngoscope 111:399-403.

Cavani A, Mei D, Guerra E, Corinti S, Giani M, Pirrotta L, Puddu P and Girolomoni G (1998). Patients with allergic contact dermatitis to nickel and nonallergic individuals display different nickel-specific T cell responses. Evidence for the presence of effector $CD8^+$ and regulatory $CD4^+$ T cells. J Invest Dermatol 111:621-628.

Cepek KL, Shaw SK, Parker CM, Russell GJ, Morrow JS, Rimm DL and Brenner MB (1994). Adhesion between epithelial cells and T lymphocytes mediated by E-cadherin and the alpha E beta 7 integrin. Nature 372:190-193.

Chauhan B, Knutsen AP, Hutcheson PS, Slavin RG and Bellone CJ (1996). T cell subsets, epitope mapping, and HLA-restriction in patients with allergic bronchopulmonary aspergillosis. J Clin Invest 97:2324-2331.

Chauhan B, Santiago L, Kirschman DA, Hauptfeld V, Knutsen AP, Hutcheson PS, Woulfe SL, Slavin RG, Schwartz HJ and Bellone CJ (1997). The association of HLA-DR alleles and T cell activation with allergic bronchopulmonary aspergillosis. J Immunol 159:4072-4076.

Chen TC, Cobbold SP, Fairchild PJ and Waldmann H (2004). Generation of anergic and regulatory T cells following prolonged exposure to a harmless antigen. J Immunol 172:5900-5907.

Cheroutre H and Madakamutil L (2004). Acquired and natural memory T cells join forces at the mucosal front line. Nat Rev Immunol 4:290-300.

Clark GJ, Gunningham S, Troy A, Vuckovic S and Hart DNJ (1999). Expression of the ReIB transcription factor correlates with the activation of human dendritic cells. Immunology 98:189-196.

Cohn L, Elias JA and Chupp GL (2004). Asthma: mechanisms of disease persistence and progression. Annu Rev Immunol 22:789-815.

Colice GL (2004). Categorizing Asthma Severity: An overview of National Guidelines. Clin Med Res 2:155-163.

Collins M, Nair S, Smith W, Kette F, Gillis D and Wormald PJ (2004). Role of local immunoglobulin E production in the pathophysiology of noninvasive fungal sinusitis. Laryngoscope 114:1242-1246.

Collins MM, Nair SB and Wormald PJ (2003). Prevalence of noninvasive fungal sinusitis in South Australia. Am J Rhinol 17:127-132.

Coombs RRA and Gell PGH (1968). Clinical Aspects of Immunology, 2nd ed. Blackwell.

Corey JP, Kaiseruddin S and Gungor A (1997). Prevalence of mold-specific immunoglobulins in a Midwestern allergy practice. Otolaryngol Head Neck Surg 117:516-520.

Crameri R, Faith A, Hemmann S, Jaussi R, Ismail C, Menz G and Blaser K (1996). Humoral and cell-mediated autoimmunity in allergy to *Aspergillus fumigatus*. J Exp Med 184:265-270.

Crameri R, Hemmann S, Ismail C, Menz G and Blaser K (1998). Disease-specific recombinant allergens for the diagnosis of allergic bronchopulmonary aspergillosis. Int Immunol 10:1211-1216.

Crameri R (2002). Molecular cloning of *Aspergillus fumigatus* allergens and their role in allergic bronchopulmonary aspergillosis. Chem Immunol 81:73-93.

Crameri R and Blaser K (2002). Allergy and immunity to fungal infections and colonization. Eur Respir J 19:151-157.

Csencsits KL, Jutila MA and Pascual DW (1999). Nasal-associated lymphoid tissue: phenotypic and functional evidence for the primary role of peripheral node addressin in naive lymphocyte adhesion to high endothelial venules in a mucosal site. J Immunol 163:1382-1389.

Danke NA and Kwok WW (2003). HLA class II-restricted $CD4^+$ T cell responses directed against influenza viral antigens post influenza vaccination. J Immunol 171:3163-3169.

De Rosa SC, Herzenberg LA, Herzenberg LA and Roederer M (2001). 11-color, 13-parameter flow cytometry: identification of human naive T cells by phenotype, function, and T-cell receptor diversity. Nat Med 7:245-248.

De Winter H, Cheroutre H and Kronenberg M (1999). Mucosal immunity and inflammation. II. The yin and yang of T cells in intestinal inflammation: pathogenic and protective roles in a mouse colitis model. Am J Physiol 276:G1317-321.

Debertin AS, Tschernig T, Tonjes H, Kleemann WJ, Troger HD and Pabst R (2003). Nasal-associated lymphoid tissue (NALT): frequency and localization in young children. Clin Exp Immunol 134:503-507.

Dennis DP (2003). Chronic sinusitis: defective T-cells responding to superantigens, treated by reduction of fungi in the nose and air. Arch Environ Health 58:433-441.

deShazo RD and Swain RE (1995). Diagnostic criteria for allergic fungal sinusitis. J Allergy Clin Immunol 96:24-35.

deShazo RD, O'Brien M, Chapin K, Soto-Aguilar M, Gardner L and Swain R (1997). A new classification and diagnostic criteria for invasive fungal sinusitis. Arch Otolaryngol Head Neck Surg 123:1181-1188.

Dixon DM and Polak-Wyss A (1991). The medically important dematiaceous fungi and their identification. Mycoses 34:1-18.

Drake-Lee AB and Barker TH (1984). Free and cell bound IgE in nasal polyps. J Laryngol Otol 98:795-801.

Driscoll PV, Naclerio RM and Baroody FM (1996). $CD4^+$ lymphocytes are increased in the sinus mucosa of children with chronic sinusitis. Arch Otolaryngol Head Neck Surg 122:1071-1076.

Dzierzanowska-Fangrat K, Raeiszaheh M, Dzierzanowska D, Gladkowska-Dura M, Celinska-Cedros D and Crabtree JE (2003). IgG subclass response to *Helicobacter pylori* and CagA antigens in children. Clin Exp Immunol 134:442-446.

Erjefalt JS, Greiff L, Andersson M, Ädelroth E, Jeffery PK and Persson CGA (2001). Degranulation patterns of eosinophil granulocytes as determinants of eosinophil driven disease. Thorax 56:341-344.

Essery G, Feldmann M and Lamb JR (1998). Interleukin-2 can prevent and reverse antigen-induced unresponsiveness in cloned human T lymphocytes. Immunology 64:413-417.

Fagarasan S and Honjo T (2004). Regulation of IgA synthesis at mucosal surfaces. Curr Opin Immunol 16:277-283.

Fazekas de St Groth B, Smith AL, Koh WP, Girgis L, Cook MC and Bertolino P (1999). Carboxyfluorescein diacetate succinimidyl ester and the virgin lymphocyte: a marriage made in heaven. Immunol Cell Biol 77:530-538.

Feger TA, Rupp NT, Kuhn FA, Ford JL and Dolen WK (1997). Local and systemic eosinophil activation in allergic fungal sinusitis. Ann Allergy Asthma Immunol 79:221-225.

Fehervari Z and Sakaguchi S (2004). $CD4^+$ T regs and immune control. J Clin Invest 114:1209-1217.

Ferguson BJ (2000a). Eosinophilic mucin rhinosinusitis: a distinct clinicopathological entity. Laryngoscope 110:799-813.

Ferguson BJ (2000b). Definitions of fungal rhinosinusitis. Otolaryngol Clin North Am 33:227-235.

Ferguson BJ (2000c). Fungus balls of the paranasal sinuses. Otolaryngol Clin North Am 33:389-398.

Ferguson BJ, Barnes L, Bernstein JM, Brown D, Clark CE 3rd, Cook PR, DeWitt WS, Graham SM, Gordon B, Javer AR, Krouse JH, Kuhn FA, Levine HL, Manning SC, Marple BF, Morgan AH, Osguthorpe JD, Skedros D, Rains BM, 3rd, Ramadan HH, Terrell JE and Yonkers AJ (2000). Geographic variation in allergic fungal rhinosinusitis. Otolaryngol Clin North Am 33:441-449.

Ferguson BJ (2004). Categorization of eosinophilic chronic rhinosinusitis. Curr Opin Otolaryngol Head Neck Surg 12:237-242.

Flynn KJ, Belz GT, Altman JD, Ahmed R, Woodland DL and Doherty PC (1998). Virus-specific $CD8^+$ T cells in primary and secondary influenza pneumonia. Immunity 8:683-691.

Folker RJ, Marple BF, Mabry RL and Mabry CS (1998). Treatment of allergic fungal sinusitis: a comparison trial of postoperative immunotherapy with specific fungal antigens. Laryngoscope 108:1623-1627.

Froebel KS, Pakker NG, Aiuti F, Bofill M, Choremi-Papadopoulou H, Econonidou J, Rabian C, Roos MTL, Ryder LP, Miedema F and Raab GM (1999). Standardization and quality assurance of lymphocyte proliferation assays for use in the assessment of immune function. J Immunol Methods 227:85-97.

Fulcher DA and Wong SWJ (1999). Carboxyfluorescein succinimidyl ester-based proliferative assays for assessment of T cell function in the diagnostic laboratory. Immunol Cell Biol 77:559-564.

Fuller MJ, Khanolkar A, Tebo AE and Zajac AJ (2004). Maintenance, loss, and resurgence of T cell responses during acute, protracted, and chronic viral infections. J Immunol 172:4204-4214.

Gangadharan D and Cheroutre H (2004). The CD8 isoform CD8alphaalpha is not a functional homologue of the TCR co-receptor CD8alphabeta. Curr Opin Immunol 16:264-270.

Geginat J, Lanzavecchia A and Sallusto F (2003). Proliferation and differentiation potential of human $CD8^+$ memory T-cell subsets in response to antigen or homeostatic cytokines. Blood 101:4260-4266.

Giembycz MA and Lindsay MA (1999). Pharmacology of the eosinophil. Pharmacological reviews 51:213-339.

Giorgi JV, Hausner MA and Hultin LE (1999). Detailed immunophenotype of $CD8^+$ memory cytotoxic T-lymphocytes (CTL) against HIV-1 with respect to expression of CD45RA/RO, CD62L and CD28 antigens. Immunology Letters 66:105-110.

Gleich GJ (2000). Mechanisms of eosinophil-associated inflammation. J Allergy Clin Immunol 105:651-663.

Gliklich RE and Metson R (1995). The health impact of chronic sinusitis in patients seeking otolaryngologic care. Otolaryngol Head Neck Surg 113:104-109.

Godfrey DI, MacDonald HR, Kronenberg M, Smyth MJ and Van Kaer L (2004). NKT cells: what's in a name? Nat Rev Immunol 4:231-237.

Goding JW (1986). Monoclonal antibodies: principles and practice, 2nd ed. Academic Press.

Gould HJ, Sutton BJ, Beavil AJ, Beavil RL, McCloskey N, Coker HA, Fear D and Smurthwaite L (2003). The biology of IgE and the basis of allergic disease. Annu Rev Immunol 21:579-628.

Graeme-Cook F, Bhan AK and Harris NL (1993). Immunohistochemical characterisation of intraepithelial and subepithelial mononuclear cells of the upper airways. Am J Pathol 143:1416-1422.

Graham SM and Ballas ZK (1998). Preoperative steroids confuse the diagnosis of allergic fungal sinusitis. J Allergy Clin Immunol 101:139-140.

Graybill JR and Alford RH (1974). Cell mediated immunity in Cryptococcosis. Cell Immunol 14:12-21.

Greenberger PA (2002). Allergic bronchopulmonary aspergillosis. In: Grammer LC and Greenberger PA, editors. Patterson's Allergic Diseases, 6th ed. Lippincott Williams and Wilkins, 529-554.

Grocott RG (1955). A stain for fungi in tissue sections and smears using Gomori's methenamine-silver technic. Am J Clin Pathol 25:975-979.

Grossman WJ, Verbsky JW, Barchet W, Colonna M, Atkinson JP and Ley TJ (2004a). Human T regulatory cells can use perforin pathway to cause autologous target cell death. Immunity 21:589-601.

Grossman WJ, Verbsky JW, Tollefsen BL, Kemper C, Atkinson JP and Ley TJ (2004b). Differential expression of granzymes A and B in human cytotoxic lymphocyte subsets and T regulatory cells. Blood 104:2840-2848.

Gupta R, Singh BP, Sridhara S, Gaur SN, Kumar R, Chaudhary VK and Arora N (2002). Allergenic cross-reactivity of *Curvularia lunata* with other airborne fungal species. Allergy 57:636-640.

Hamann D, Baars PA, Rep MHG, Hooibrink B, Kerkhof-Garde SR, Klein MR and van Lier RAW (1997). Phenotypic and functional separation of memory and effector human $CD8^+$ T cells. J Exp Med 186:1407-1418.

Hameleers DM, Stoop AE, van der Ven I, Biewenga J, van der Baan S and Sminia T (1989). Intra-epithelial lymphocytes and non-lymphoid cells in the human nasal mucosa. Int Arch Allergy Appl Immunol 88:317-322.

Harmsen A, Kusser K, Hartson L, Tighe M, Sunshine MJ, Sedgwick JD, Choi Y, Littman DR and Randall TD (2002). Cutting edge: organogenesis of nasal-associated lymphoid tissue (NALT) occurs independently of lymphotoxin-α (LTα) and retinoic acid receptor-related orphan receptor-γ, but the organization of NALT is LTα dependent. J Immunol 168:986-990.

Hart PH, Bonder CS, Jones CA and Finlay-Jones JJ (1996). Control of major histocompatibility complex class II expression on human monocytes by interleukin-4: regulatory effect of lipopolysaccharide. Immunology 89:599-605.

Harty JT, Tvinnereim AR and White DW (2000). $CD8^+$ T cell effector mechanisms in resistance to infection. Annu Rev Immunol 18:275-308.

Hasbold J, Jett AV, Rush JS, Deenick E, Avery D, Jun J and Hodgkin PD (1999). Quantitative analysis of lymphocyte differentiation and proliferation in vitro using carboxyfluorescein diacetate succinimidyl ester. Immunol Cell Biol 77:516-522.

Hawranek T (2002). Cutaneous mycology. In: Breitenbach M, Crameri R and Lehrer SB, editors. Fungal Allergy and Pathogenicity, vol 81, Karger, 129-166.

Haynes RC Jr (1991). Adrenocorticotropic hormone; adrenocortical steroids and their synthetic analogs; inhibitors of the synthesis and actions of adrenocortical hormones. In: Goodman Gilman A, Rall TW, Nies AS and Taylor P, editors. The Pharmacological Basis of Therapeutics, vol 2, 8th ed. Maxwell Macmillan Pergamon Publishing Corporation, 1431-1462.

Heintel T, Breinig F, Schmitt MJ and Meyerhans A (2003). Extensive MHC class I-restricted CD8 T lymphocyte responses against various yeast genera in humans. FEMS Immunol Med Microbiol 39:279-286.

Hellquist HB (1997). Histopathology. In: Settipane GA, Lund VL, Bernstein J and Tos M, editors. Nasal polyps: epidemiology, pathogenesis and treatment, OceanSide Publications Inc., 31-39.

Herman A, Kappler JW, Marrack P and Pullen AM (1991). Superantigens: mechanism of T cell stimulation and role in immune responses. Annu Rev Immunol 9:745-772.

Hermiston ML, Xu Z and Weiss A (2003). CD45: a critical regulator of signaling thresholds in immune cells. Annu Rev Immunol 21:107-137.

Hinson KFW, Moon AJ and Plummer NS (1952). Broncho-pulmonary aspergillosis: a review and report of eight new cases. Thorax 7:317-333.

Hintzen RQ, DeJong R, Lens SM, Brouwer M, Baars P and van Lier RA (1993). Regulation of CD27 expression on subsets of mature T lymphocytes. J Immunol 151:2426-2435.

Hoffman RA, Kung PC, Hansen WP and Goldstein G (1980). Simple and rapid measurement of human T lymphocytes and their subclasses in peripheral blood. Proc Natl Acad Sci USA 77:4914-4917.

Horner WE, Helbling A, Salvaggio JE and Lehrer SB (1995). Fungal allergens. Clin Microbiol Rev 8:161-179.

Horst M, Hejjaoui A, Horst V, Michel FB and Bousquet J (1990). Double-blind, placebo-controlled rush immunotherapy with a standardized *Alternaria* extract. J Allergy Clin Immunol 85:460-472.

Huang C, Shah S, Nguyen P, Altman JD and Blackman MA (2002). Bacterial superantigen exposure after resolution of influenza virus infection perturbs the virus-specific memory $CD8^{+}$-T cell repertoire. J Virol 76:6852-6856.

Hussain R, Kifayet A and Chiang TJ (1995). Immunoglobulin G1 (IgG1) and IgG3 antibodies are markers of progressive disease in leprosy. Infect Immun 63:410-415.

Jiang H and Chess L (2004). An integrated view of suppressor T cell subsets in immunoregulation. J Clin Invest 114:1198-1208.

Johansson SGO, Hourihane JO'B, Bousquet J, Bruijnzeel-Koomen C, Dreborg S, Haahtela T, Kowalski ML, Mygind N, Ring J, van Cauwenberge P, van Hage-Hamsten M and Wüthrich B (2001). A revised nomenclature for allergy. An EAACI position statement from the EAACI nomenclature task force. Allergy 56:813-824.

Kabilan L, Anderson G, Lolli F, Ekre HP, Olsson T and Troye-Blomberg M (1990). Detection of intracellular expression and secretion of interferon-gamma at the single-cell level after activation of human T cells with tetanus toxoid in vitro. Eur J Immunol 20:1085-1089.

Kaliner MA, Osguthorpe JD, Fireman P, Anon J, Georgitis J, Davis ML, Naclerio R and Kennedy D (1997). Sinusitis: bench to bedside. Current findings, future directions. Otolaryngol Head Neck Surg 116:(6 Suppl)1-20.

Kanai N, Denburg J, Jordana M and Dolovich J (1994). Nasal polyp inflammation. Effect of topical nasal steroid. Am J Respir Crit Care Med 150:1094-1100.

Kaneko M, Swanson MC, Gleich GJ and Kita H (1995). Allergen-specific IgG1 and IgG3 through Fc gamma RII induce eosinophil degranulation. J Clin Invest 95:2813-2821.

Kappeler A and Mueller C (2000). The role of activated cytotoxic T cells in inflammatory bowel disease. Histol Histopathol 15:167-172.

Karchev T and Kabakchiev P (1984). M-cells in the epithelium of the nasopharyngeal tonsils. Rhinology 22:201-210.

Katzenstein ALA, Liebow AA and Friedman PJ (1975). Bronchocentric granulomatosis, mucoid impaction and hypersensitivity reactions to fungi. Am Rev Respir Dis 111:497-537.

Katzenstein ALA, Sale SR and Greenberger PA (1983a). Allergic *Aspergillus* sinusitis: a newly recognized form of sinusitis. J Allergy Clin Immunol 72:89-93.

Katzenstein ALA, Sale SR and Greenberger PA (1983b). Pathologic findings in allergic aspergillus sinusitis. A newly recognized form of sinusitis. Am J Surg Pathol 7:439-443.

Kelso A (1995). Th1 and Th2 subsets: paradigms lost? Immunol Today. 16:374-379.

Kelso A, Costelloe EO, Johnson BJ, Groves P, Buttigieg K and Fitzpatrick DR (2002). The genes for perforin, granzymes A-C and IFN-gamma are differentially expressed in single CD8(+) T cells during primary activation. Int Immunol 14:605-613.

Khan DA, Cody DT 2nd, George TJ, Gleich GJ and Leiferman KM (2000). Allergic fungal sinusitis: an immunohistologic analysis. J Allergy Clin Immunol 106:1096-1101.

Kim DH, Chu HS, Lee JY, Hwang SJ, Lee SH and Lee HM (2004). Up-regulation of MUC5AC and MUC5B mucin genes in chronic rhinosinusitis. Arch Otolaryngol Head Neck Surg 130:747-752.

Klion AD and Nutman TB (2004). The role of eosinophils in host defense against helminth parasites. J Allergy Clin Immunol 113:30-37.

Klonowski KD, Williams KJ, Marzo AL, Blair DA, Lingenheld EG and Lefrançois L (2004). Dynamics of blood-borne CD8 memory T cell migration in vivo. Immunity 20:551-562.

Knowles MR, Stutts MJ, Spock A, Fischer N, Gatzy JT and Boucher RC (1983). Abnormal ion permeation through cystic fibrosis respiratory epithelium. Science 221:1067-1070.

Knutsen AP, Mueller KR, Levine AD, Chouhan B, Hutcheson PS and Slavin RG (1994). Asp *f* I CD4+ T_{H2}-like T-cell lines in allergic bronchopulmonary aspergillosis. J Allergy Clin Immunol 94:215-221.

Knutsen AP, Hutcheson PS, Slavin RG and Kurup VP (2004). IgE antibody to *Aspergillus fumigatus* recombinant allergens in cystic fibrosis patients with allergic bronchopulmonary aspergillosis. Allergy 59:198-203.

Kohm AP, Fuller KJ and Miller SD (2003). Mimicking the way to autoimmunity: an evolving theory of sequence and structural homology. Trends Microbiol 11:101-105.

Kondo H, Nachtigal D, Freinkiel S, Schotman E and Hamid Q (1999). Effect of steroids on nasal inflammatory cells and cytokine profile. Laryngoscope 109:91-97.

Kuritani T and Cooper MD (1982). Human B-cell differentiation. I. Analysis of immunoglobulin heavy chain switching using monoclonal anti-immunoglobulin M, G, and A antibodies and pokeweed mitogen-induced plasma cell differentiation. J Exp Med 155:839-851.

Kurup VP, Hari V, Guo J, Murali PS, Resnick A, Krishnan M and Fink JN (1996). *Aspergillus fumigatus* peptides differentially express Th_1 and Th_2 cytokines. Peptides 17:183-190.

Kurup VP, Banerjee B, Murali PS, Greenberger PA, Krishnan M, Hari V and Fink JN (1998). Immunodominant peptide epitopes of allergen, Asp f 1 from the fungus *Aspergillus fumigatus*. Peptides 19:1469-1477.

Kurup VP, Banerjee B, Hemmann S, Greenberger PA, Blaser K and Crameri R (2000). Selected recombinant *Aspergillus fumigatus* allergens bind specifically to IgE in ABPA. Clin Exp Allergy 30:988-993.

Kurup VP, Shen HD and Vijay H (2002). Immunobiology of fungal allergens. Int Arch Allergy Immunol 129:181-188.

Lacy P, Weller PF and Moqbel R (2001). A report from the International Eosinophil Society: Eosinophils in a tug of war. J Allergy Clin Immunol 108:895-900.

Lamb D, Miller J and Johnston A (1981). Allergic Aspergillosis of the Paranasal Sinuses [Abstract]. Thorax 36:710.

Lambrecht BN (2001). The dendritic cell in allergic airway diseases: a new player to the game. Clin Exp Allergy 31:206-218.

Lanza DC and Kennedy DW (1997). Adult rhinosinusitis defined. Otolaryngol Head Neck Surg 117:(3 Suppl)1-7.

Lara JF and Gomez JD (2001). Allergic mucin with and without fungus. A comparative clinicopathologic analysis. Arch Pathol Lab Med 125:1442-1447.

Larocca LM, Maggiano N, Capelli A, Bevilacqua P, Ruscito P, Maurizi M and Bellioni P (1989). Immunopathology of nasal polyps: an immunohistochemical approach. Ann Allergy 63:508-512.

Lavigne S, Bosse M, Boulet LP and Laviolette M (1997). Identification and analysis of eosinophils by flow cytometry using the depolarized side scatter-saponin method. Cytometry 29:197-203.

Leavey JK and Tarleton RL (2003). Cutting edge: dysfunctional $CD8^+$ T cells reside in nonlymphoid tissues during chronic *Trypanosoma cruzi* infection. J Immunol 170:2264-2268.

Levitz SM, Dupont MP and Smail EH (1994). Direct activity of human T lymphocytes and natural killer cells against *Cryptococcus neoformans*. Infect Immun 62:194-202.

Lieberman J (2003). The ABCs of granule-mediated cytotoxicity: new weapons in the arsenal. Nat Rev Immunol 3:361-370.

Lillie RD and Fullmer HM (1976). Histopathologic technique and practical histochemistry, McGraw-Hill.

Linder A, Karlsson-Parra A, Hirvela C, Jonsson L, Koling A and Sjoberg O (1993). Immunocompetent cells in human nasal polyps and normal mucosa. Rhinology 31:125-129.

Liu CM, Shun CT and Hsu MM (1994). Lymphocyte subsets and antigen-specific IgE antibody in nasal polyps. Ann Allergy 72:19-24.

Liu K, Li Y, Prabhu V, Young L, Becker KG, Munson PJ and Weng N (2001). Augmentation in expression of activation-induced genes differentiates memory from naive $CD4^+$ T cells and is a molecular mechanism for enhanced cellular response of memory $CD4^+$ T cells. J Immunol 166:7335-7344.

Liu Z and Lefrançois L (2004). Intestinal epithelial antigen induces mucosal CD8 T cell tolerance, activation, and inflammatory response. J Immunol 173:4324-4330.

Lund VJ and Mackay IS (1993). Staging in rhinosinusitis. Rhinology 31:183-184.

Lyons AB and Parish CR (1994). Determination of lymphocyte division by flow cytometry. J Immunol Methods 171:131-137.

Ma LL, Spurrell JC, Wang JF, Neely GG, Epelman S, Krensky AM and Mody CH (2002). CD8 T cell-mediated killing of *Cryptococcus neoformans* requires granulysin and is dependent on CD4 T cells and IL-15. J Immunol 169:5787-5795.

Ma LL, Wang CL, Neely GG, Epelman S, Krensky AM and Mody CH (2004). NK cells use perforin rather than granulysin for anticryptococcal activity. J Immunol 173:3357-3365.

Mabry RL, Manning SC and Mabry CS (1997). Immunotherapy in the treatment of allergic fungal sinusitis. Otolaryngol Head Neck Surg 116:31-35.

Mabry RL, Marple BF and Mabry CS (1999). Mold testing by RAST and skin test methods in patients with allergic fungal sinusitis. Otolaryngol Head Neck Surg 121:252-154.

Macdonald TT (2003). The mucosal immune system. Parasite immunol 25:235-246.

Makita S, Kanai T, Oshima S, Uraushihara K, Totsuka T, Sawada T, Nakamura T, Koganei K, Fukushima T and Watanabe M (2004). $CD4^{+}CD25^{bright}$ T cells in human intestinal lamina propria as regulatory cells. J Immunol 173:3119-3130.

Malpiero U, Frei K, Spanaus KS, Agresti C, Lassmann H, Hahne M, Tschopp J, Eugster HP and Fontana A. (1997). Myelin oligodendrocyte glycoprotein-induced autoimmune encephalomyelitis is chronic/relapsing in perforin knockout mice, but monophasic in Fas- and Fas ligand-deficient 1pr and gld mice. Eur J Immunol 27:3151-3160.

Manning SC, Mabry RL, Schaefer SD and Close LG (1993). Evidence of IgE-mediated hypersensitivity in allergic fungal sinusitis. Laryngoscope 103:717-721.

Manning SC and Holman M (1998). Further evidence for allergic pathophysiology in allergic fungal sinusitis. Laryngoscope 108:1485-1496.

Mansi A, Cucchiara S, Greco L, Sarnelli P, Pisanti C, Franco MT and Santamaria F (2000). Bronchial hyperresponsiveness in children and adolescents with Crohn's Disease. Am J Respir Crit Care Med 161:1051-1054.

Mansur AH, Bishop T, Markham AF, Morton NE, Holgate ST and Morrison JFJ (1999). Suggestive evidence for genetic linkage between IgE phenotypes and chromosome 14q markers. Am J Respir Care Med 159:1796-1802.

Marple BF and Mabry RL (1998). Comprehensive management of allergic fungal sinusitis. Am J Rhinol 12:263-268.

Marple BF (2001). Allergic fungal rhinosinusitis: Current theories and management strategies. Laryngoscope 111:1006-1019.

Marshall JS, McCurdy JD and Olynych T (2003a). Toll-like receptor-mediated activation of mast cells: implications for allergic disease? Int Arch Allergy Immunol 132:87-97.

Marshall JS, King CA and McCurdy JD (2003b). Mast cell cytokine and chemokine responses to bacterial and viral infection. Curr Pharm Des 9:11-24.

Martin DL and Tarleton RL (2005). Antigen-specific T cells maintain an effector memory phenotype during persistent *Trypanosoma cruzi* infection. J Immunol 174:1594-1601.

Maruyama S, Kubagawa H and Cooper MD (1985). Activation of human B cells and inhibition of their terminal differentiation by monoclonal anti-mu antibodies. J Immunol 135:192-199.

Marzo AL, Vezys V, Klonowski KD, Lee S-J, Muralimohan G, Moore M, Tough DF and Lefrançois L (2004). Fully functional memory CD8 T cells in the absence of CD4 T cells. J Immunol 173:969-975.

Mastruzzo C, Greco LR, Nakano K, Nakano A, Palermo F, Pistorio MP, Salinaro ET, Jordana M, Dolovich J, Crimi DN and Vancheri C (2003). Impact of intranasal budesonide on immune inflammatory responses and epithelial remodeling in chronic upper airway inflammation. J Allergy Clin Immunol 112:37-44.

Matthias C, de Souza P and Merker HJ (1997). Electron microscopic and immunomorphological investigations on the mucosa of the human paranasal sinuses. Eur Arch Otorhinolaryngol 254:230-235.

Maurer D, Ebner C, Reininger B, Fiebiger E, Kraft D, Kinet JP and Stingl G (1995). The high affinity IgE receptor (Fc epsilon RI) mediates IgE-dependent allergen presentation. J Immunol 154:6285-6290.

Mazzoni A and Segal DM (2004). Controlling the Toll road to dendritic cell polarization. J Leukoc Biol 75:721-730.

McAllister F, Steele C, Zheng M, Young E, Shellito JE, Marrero L and Kolls JK (2004). T cytotoxic-1 $CD8^+$ T cells are effector cells against *Pneumocystis* in mice. J Immunol 172:1132-1138.

McCarthy DS and Pepys J (1971). Allergic bronchopulmonary aspergillosis. Clinical immunology. 2. Skin, nasal and bronchial tests. Clin Allergy 1:415-432.

McCormick JK, Tripp TJ, Llera AS, Sundberg EJ, Dinges MM, Mariuzza RA and Schlievert PM (2003). Functional analysis of the TCR binding domain of toxic shock syndrome toxin-1 predicts further diversity in MHC class II/superantigen/TCR ternary complexes. J Immunol 171:1385-1392.

McNeill L, Cassady RL, Sarkardei S, Cooper JC, Morgan G and Alexander DR (2004). CD45 isoforms in T cell signalling and development. Immunol Lett 92:125-134.

Mehlhop PD, van de Rijn M, Goldberg AB, Brewer JP, Kurup VP, Martin TR and Oettgen HC (1997). Allergen-induced bronchial hyperreactivity and eosinophilic inflammation occur in the absence of IgE in a mouse model of asthma. Proc Natl Acad Sci USA 94:1344-1349.

Meltzer EO, Orgel HA, Backhaus JW, Busse WW, Druce HM, Metzger WJ, Michell DQ, Seiner JC, Shapiro GG, Van Barel JH and Baseh C (1993). Intranasal flunisolide spray as an adjunct to oral antibiotic therapy for sinusitis. J Allergy Clin Immunol 92:812-823.

Meltzer EO, Hamilos DL, Hadley JA, Lanza DC, Marple BF, Nicklas RA, Bachert C, Baraniuk J, Baroody FM, Benninger MS, Brook I, Chowdhury BA, Druce HM, Durham S, Ferguson B, Gwaltney JM, Kaliner M, Kennedy DW, Lund V, Naclerio R, Pawankar R, Piccirillo JF, Rohane P, Simon R, Slavin RG, Togias A, Wald ER and Zinreich SJ (2004). Rhinosinusitis: Establishing definitions for clinical research and patient care. J Allergy Clin Immunol 114:(6 Suppl)155-212.

Miller JD (1992). Fungi as contaminants in indoor air. Atmos Environ 26A:2163-2172.

Mody CH, Paine R III, Jackson C, Chen GH and Toews GB (1994). CD8 cells play a critical role in delayed type hypersensitivity to intact *Cryptococcus neoformans*. J Immunol 152:3970-3979.

Mody CH, Wood CJ, Syme RM and Spurrell JC (1999). The cell wall and membrane of *Cryptococcus neoformans* possess a mitogen for human T lymphocytes. Infect Immun 67:936-941.

Morinaka S and Nakamura H (2000). Inflammatory cells in nasal mucosa and nasal polyps. Auris Nasus Larynx 27:59-64.

Mosmann TR, Li L and Sad S (1997). Functions of CD8 T-cell subsets secreting different cytokine patterns. Seminars in Immunology 9:87-92.

Mukherji SK, Figueroa RE, Ginsberg LE, Zeifer BA, Marple BF, Alley JG, Cooper LL, Nemzek WR, Yousem DM, Jones KR, Kupferberg SB and Castillo M (1998). Allergic fungal sinusitis: CT findings. Radiology 207:417-422.

Mullol J, Baraniuk JN, Logun C, Merida M, Hausfeld J, Shelhamer JH and Kaliner MA (1992). M1 and M3 muscarinic antagonists inhibit human nasal glandular secretion *in vitro*. J App Physiol 73:2069-2073.

Murali PS, Kurup VP, Bansal NK, Fink JN and Greenberger PA (1998). IgE down regulation and cytokine induction by *Aspergillus* antigens in human allergic bronchopulmonary aspergillosis. J Lab Clin Med 131:228-235.

Myers TA, Leigh JE, Arribas AR, Hager S, Clark R, Lilly E and Fidel PL Jr. (2003). Immunohistochemical evaluation of T cells in oral lesions from human immunodeficiency virus-positive persons with oropharyngeal candidiasis. Infect Immun 71:956-963.

Mygind N, Dahl R and Bachert C (2000). Nasal polyposis, eosinophil dominated inflammation, and allergy. Thorax 55 (2 Suppl):79-83.

Nagashima T, Aranami T, Iclozan C and Onoé K (2004). Analysis of T cell responses to a superantigen, staphylococcal enterotoxin-B. J Clin Exp Hematopathol 44:25-32.

National Asthma Campaign (1998). Asthma Management Handbook. National Asthma Council Australia Ltd, ACN 058 044 634, South Melbourne, Australia.

Neutra MR, Pringault E and Kraehenbuhl J-P (1996). Antigen sampling across epithelial barriers and induction of mucosal immune responses. Annu Rev Immunol 14:275-300.

Nishimoto K, Ukai K, Harada T, Jin CS and Sakakura Y (1988). Lymphocyte subsets of maxillary mucosa in chronic inflammation. Arch Otolaryngol 106:291-298.

Noone PG and Knowles MR (2001). "CFTR-opathies": disease phenotypes associated with cystic fibrosis transmembrane regulator gene mutations. Respir Res 2:328-332.

Nussenbaum B, Marple BF and Schwade ND (2001). Characteristics of bony erosion in allergic fungal rhinosinusitis. Otolaryngol Head Neck Surg 124:150-154.

O'Donoghue DP and Kumar P (1979). Rectal IgE cells in inflammatory bowel disease. Gut 20:149-153.

Okada S, Li Q, Whitin JC, Clayberger C and Krensky AM (2003). Intracellular mediators of granulysin-induced cell death. J Immunol 171:2556-2562.

Oluwole M, Russell N, Tan L, Gardiner Q and White P (1996). A comparison of computerized tomographic staging systems in chronic sinusitis. Clin Otolaryngol 21:91-95.

Pant H, Kette FE, Smith WB, Wormald PJ and Macardle PJ (2005). Fungal-specific humoral response in eosinophilic mucus chronic rhinosinusitis. Laryngoscope 115:601-606.

Papagno L, Spina CA, Marchant A, Salio M, Rufer M, Little S, Dong T, Chesney G, Waters A, Easterbrook P, Dunbar PR, Shepherd D, Cerundolo V, Emery V, Griffiths P, Conlon C, McMichael AJ, Richman DD, Rowland-Jones SL and Appay V (2004). Immune activation

and $CD8^+$ T-cell differentiation towards senescence in HIV-1 infection. PLOS Biology 2:173-185.

Paris S, Fitting C, Ramirez E, Latge JP and David B (1990). Comparison of different extraction methods of *Alternaria* allergens. J Allergy Clin Immunol 85:941-948.

Parish CR (1999). Fluorescent dyes for lymphocyte migration and proliferation studies. Immunol Cell Biol 77:499-508.

Pawankar RU, Okuda M, Suzuki K, Okumura K and Ra C (1996). Phenotypic and molecular characteristics of nasal mucosal gamma delta T cells in allergic and infectious rhinitis. Am J Respir Crit Care Med 153:1655-1665.

Persson T, Andersson P, Bodelsson M, Laurell M, Malm J and Egesten A (2001). Bactericidal activity of human eosinophilic granulocytes against *Escherichia coli.* Infect Immun 69:3591-3596.

Picker LJ (1994). Control of lymphocyte homing. Curr Opin Immunol 6:394-406.

Pilarski LM, Turley EA, Shaw AR, Gallatin WM, Laderoute MP, Gillitzer R, Beckman IG and Zola H (1991). FMC46, a cell protrusion-associated leucocyte adhesion molecule-1 epitope on human lymphocytes and thymocytes. J Immunol 147:136-143.

Pinheiro AD, Facer GW and Kern EB (2001). Rhinosinusitis: current concepts and management. In: Bailey BJ, Pillsbury III HC and Newlands SD, editors. Head and Neck Surgery - Otolaryngology, vol 1, 3rd ed. Lippincott Williams and Wilkins, 345-357.

Ponikau JU, Sherris DA, Kern EB, Homburger HA, Frigas E, Gaffey TA and Roberts GD (1999). The diagnosis and incidence of allergic fungal sinusitis. Mayo Clin Proc 74:877-884.

Pope C, Kim SK, Marzo A, Marsopust D, Williams K, Jiang J, Shen H and Lefrançois L (2001). Organ-specific regulation of the CD8 T cell response to *Listeria monocytogenes* infection. J Immunol 166:3402-3409.

Porakishvili N, Kardava L, Jewell AP, Yong K, Glennie MJ, Akbar A and Lydyard PM (2004). Cytotoxic $CD4^+$ T cells in patients with B cell chronic lymphocytic leukemia kill via a perforin-mediated pathway. Haematologica 89:435-443.

Post JC, Stoodley P, Hall-Stoodley L and Ehrlich GD (2004). The role of biofilms in otolaryngologic infections. Curr Opin Otolaryngol Head Neck Surg 12:185-190.

Powe DG, Huskisson RS, Carney AS, Jenkins D and Jones NS (2001). Evidence for an inflammatory pathophysiology in idiopathic rhinitis. Clin Exp Allergy 31:864-72.

Pratt MF and Burnett JR (1988). Fulminant *Drechslera* sinusitis in an immunocompetent host. Laryngoscope 97:1343-1347.

Proft T and Fraser JD (2003). Bacterial superantigens. Clin Exp Immunol 133:299-306.

Quraishi MS, Jones NS and Mason J (1998). The rheology of nasal mucus: a review. Clin Otolaryngol 23:403-413.

Raja SM, Metkar SS and Froelich CJ (2003). Cytotoxic granule-mediated apoptosis: unraveling the complex mechanism. Curr Opin Immunol 15:528-532.

Ramadan HH, Meek RB, Dawson GS, Spirou GA, Cuff CF and Berrebi AS (2002). Histologic and immunologic observations of viral-induced rhinosinusitis in the mouse. Am J Rhinol 16:61-67.

Rathore VB, Johnson B, Fink JN, Kelly KJ, Greenberger PA and Kurup VP (2001). T cell proliferation and cytokine secretion to T cell epitopes of Asp f 2 in ABPA patients. Clin Immunol 100:228-235.

Ravetch JV and Bolland S (2001). IgG Fc receptors. Annu Rev Immunol 19:275-290.

Regner M and Mullbacher A (2004). Granzymes in cytolytic lymphocytes - to kill a killer? Immunology and Cell Biology 82:161-169.

Reimer CB, Phillips DJ, Aloisio CH, Moore DD, Galland GG, Wells TW, Black CM and McDougal JS (1984). Evaluation of thirty-one mouse monoclonal antibodies to human IgG epitopes. Hybridoma 3:263-275.

Riley DJ, Mackenzie JW, Uhlman WE and Edelman NH (1975). Allergic bronchopulmonary aspergillosis: Evidence of limited tissue invasion. Am Rev Respir Dis 111:232-236.

Robbie-Ryan M and Brown M (2002). The role of mast cells in allergy and autoimmunity. Curr Opin Immunol 14:728-733.

Robson JM, Hogan PG, Benn RA and Gatenby PA (1989). Allergic fungal sinusitis presenting as a paranasal sinus tumour. Aust N Z J Med 19:351-353.

Roitt I, Brostoff J and Male D, editors (1996). Immunology, 4th ed. Mosby.

Romagnani S (1998). The Th1/Th2 paradigm and allergic disorders. Allergy 53:12-15.

Romano C and Miracco C (2003). Primary cutaneous aspergillosis in an immunocompetent patient. Mycoses 46:56-59.

Rosat J-P, Grant EP, Beckman EM, Dascher CC, Sieling PA, Frederique D, Modlin RL, Porcelli SA, Furlong ST and Brenner MB (1999). CD1-restricted microbial lipid antigen-specific recognition found in the $CD8^+$ $\alpha\beta$ T cell pool. J Immunol 162:366-371.

Rothenberg ME (2004). Eosinophilic gastrointestinal disorders (EGID). J Allergy Clin Immunol 113:11-28.

Rouse BT and Suvas S (2004). Regulatory cells and infectious agents: Detentes, cordiale and contraire. J Immunol 173:2211-2215.

Rowe J, Heaton T, Kusel M, Suriyaarachchi D, Serralha M, Holt BJ, de Klerk N, Sly PD and Holt PG (2004). High IFN-gamma production by $CD8^+$ T cells and early sensitization among infants at high risk of atopy. J Allergy Clin Immunol 113:710-716.

Rubin LA, Kurman CC, Biddison WE, Goldman ND and Nelson DL (1985). A monoclonal antibody 7G7/B6, binds to an epitope on the human interleukin-2 (IL-2) receptor that is distinct from that recognized by IL-2 or anti-Tac. Hybridoma 4:91-102.

Saetta M (1999). Airway inflammation in chronic obstructive pulmonary disease. Am J Respir Crit Care Med 160:(5 Suppl)17-20.

Safirstein BH, D'Souza MF, Simon G, Tai EH and Pepys J (1973). Five-year follow-up of allergic bronchopulmonary aspergillosis. Am Rev Respir Dis 108:450-459.

Safirstein BH (1976). Allergic bronchopulmonary aspergillosis with obstruction of the upper respiratory tract. Chest 70:788-790.

Saini SS and MacGlashan D (2002). How IgE upregulates the allergic response. Curr Opin Immunol 14:694-697.

Sallusto F, Lenig D, Forster R, Lipp M and Lanzavecchia A (1999). Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. Nature 401:708-712.

Sallusto F, Geginat J and Lanzavecchia A (2004). Central memory and effector memory T cell subsets: function, generation, and maintenance. Annu Rev Immunol 22:745-763.

Sanchez-Segura A, Brieva JA and Rodriguez C (1998). T lymphocytes that infiltrate nasal polyps have a specialized phenotype and produce a mixed TH1/TH2 pattern of cytokines. J Allergy Clin Immunol 102:953-960.

Sanchez-Segura A, Brieva JA and Rodriguez C (2000). Regulation of immunoglobulin secretion by plasma cells infiltrating nasal polyps. Laryngoscope 110:1183-1188.

Santoni G, Boccanera M, Adriani D, Lucciarini R, Amantini C, Morrone S, Cassone A and De Bernardis F (2002). Immune cell-mediated protection against vaginal candidiasis: evidence for a major role of vaginal $CD4^+$ T cells and possible participation of other local lymphocyte effectors. Infect Immun 70:4791-4797.

Sarantopoulos S, Lu L and Cantor H (2004). Qa-1 restriction of CD8+ suppressor T cells. J Clin Invest 114:1218-1221.

Sawicka E, Noble A, Walker C and Kemeny DM (2004). Tc2 cells respond to soluble antigen in the respiratory tract and induce lung eosinophilia and bronchial hyperresponsiveness. Eur J Immunol 34:2599-2608.

Schaffner A, Douglas H and Braude A (1982). Selective protection against conidia by mononuclear and against mycelia by polymorphonuclear phagocytes in resistance to *Aspergillus*. J Clin Invest 69:617-631.

Schneemann M and Schaffner A (1999). Host defense mechanisms in *Aspergillus fumigatus* infections. Contrib Microbiol 2: 57-68.

Schubert MS and Goetz DW (1998). Evaluation and treatment of allergic fungal sinusitis. II. Treatment and follow-up. J Allergy Clin Immunol 102:395-402.

Schubert MS (2000). Medical treatment of allergic fungal sinusitis. Ann Allergy Asthma Immunol 85:90-101.

Schwietz LA and Gourley DS (1992). Allergic fungal sinusitis. Allergy Proc 13:3-6.

Scott KGE, Yu LCH and Buret AG (2004). Role of $CD8^+$ and $CD4^+$ T lymphocytes in jejunal mucosal injury during murine giardiasis. Infect Immun 72:3536-3542.

Seder RA and Ahmed R (2003). Similarities and differences in $CD4^+$ and $CD8^+$ effector and memory T cell generation. Nat Immunol 4:835-842.

Sedgwick JB, Shikama Y, Nagata M, Brener K and Busse WW (1996). Effect of isolation protocol on eosinophil function: Percoll gradients versus immunomagnetic beads. J Immunol Methods 198:15-24.

Seth A, Stern LJ, Ottenhoff TH, Engel I, Owen MJ, Lamb JR, Clusner RD and Whiley DC (1994). Binary and ternary complexes between T-cell receptor, class II MHC and superantigen *in vitro*. Nature 369:324-327.

Settipane GA (1997). Epidemiology of nasal polyps. In: Settipane GA, Lund VL, Bernstein J and Tos M, editors. Nasal polyps: epidemiology, pathogenesis and treatment, OceanSide Publications Inc., 31-39.

Settipane GA and Chafee FH (1977). Nasal polyps in asthma and rhinitis. A review of 6,037 patients. J Allergy Clin Immunol 59:17-21.

Shacklett BL, Cox CA, Quigley MF, Kreis C, Stollman NH, Jacobson MA, Andersson J, Sandberg JK and Nixon DF (2004). Abundant expression of granzyme A, but not perforin, in granules of $CD8^+$ T cells in GALT: implications for immune control of HIV-1 infection. J Immunol 173:641-648.

Shah A, Panchal N and Agarwal AK (2001). Concomitant allergic bronchopulmonary aspergillosis and allergic *Aspergillus* sinusitis: a review of an uncommon association. Clin Exp Allergy 31:1896-1905.

Shi HZ, Humbles A, Gerard C, Jin Z and Weller PF (2000). Lymph node trafficking and antigen presentation by endobronchial eosinophils. J Clin Invest 105:945-953.

Shi SR, Key ME and Kalra KL (1991). Antigen retrieval in formalin-fixed, paraffin-embedded tissues: an enhancement method for immunohistochemical staining based on microwave oven heating of tissue sections. J Histochem Cytochem 39:741-748.

Shin SH, Ponikau JU, Sherris DA, Congdon D, Frigas E, Homburger HA, Swanson MC, Gleich GJ and Kita H (2004). Chronic rhinosinusitis: an enhanced immune response to ubiquitous airborne fungi. J Allergy Clin Immunol 114:1369-1375.

Shinbori T, Walczak H and Krammer PH (2004). Activated T killer cells induce apoptosis in lung epithelial cells and the release of pro-inflammatory cytokine TNF-alpha. Eur J Immunol 34:1762-1770.

Slavin RG, Bedrossian CW, Hutcheson PS, Pittman S, Salinas-Madrigal L, Tsai CC and Gleich GJ (1988). A pathologic study of allergic bronchopulmonary aspergillosis. J Allergy Clin Immunol 81:718-725.

Slavin RG (1992). Allergy is not a significant cause of nasal polyps. Arch Otolaryngol Head Neck Surg 118:771.

Slavin RG (1997). Nasal polyps and sinusitis. JAMA 278:1849-1854.

Smurthwaite L and Durham SR (2002). Local IgE synthesis in allergic rhinitis and asthma. Curr Allergy Asthma Rep 2:231-238.

Smyth MJ, Kelly JM, Sutton VR, Davis JE, Browne KA, Sayers TJ and Trapani JA (2001). Unlocking the secrets of cytotoxic granule proteins. J Leukoc Biol 70:18-29.

Sobel ES, Kakkanaiah VN, Schiffenbauer J, Reap EA, Cohen PL and Eisenberg RA (1998). Novel immunoregulatory B cell pathways revealed by lpr-+ mixed chimeras. J Immunol 160:1497-1503.

Stallman PJ, Wagenaar SS, Swierenga J, van der Wal RJ and Feltkemp-Vroom TM (1977). Cell-bound IgE on human mast cells and basophilic granulocytes in atopic and nonatopic subjects. Int Arch Allergy Appl Immunol 54:443-450.

Stammberger H (1991). Functional endoscopic sinus surgery: the Messerklinger Technique, Mosby-Year Book.

Stenger S, Hanson DA, Teitelbaum R, Dewan P, Niazi KR, Froelich CJ, Ganz T, Thoma-Uszynski S, Melian A, Bogdan C, Porcelli SA, Bloom BR, Krensky AM and Modlin RL (1998). An antimicrobial activity of cytolytic T cells mediated by granulysin. Science 282:121-125.

Stewart AE and Hunsaker DH (2002). Fungus-specific IgG and IgE in allergic fungal rhinosinusitis. Otolaryngol Head Neck Surg 127:324-332.

Stock P, Kallinich T, Akbari O, Quarcoo D, Gerhold K, Wahn U, Umetsu DT and Hamelmann E (2004). $CD8^+$ T cells regulate immune responses in a murine model of allergen-induced sensitization and airway inflammation. Eur J Immunol 34:1817-1827.

Stoop AE, Hameleers DMH, v Run PEM, Biewenga J and van der Baan S (1989). Lymphocytes and nonlymphoid cells in the nasal mucosa of patients with nasal polyps and of healthy subjects. J Allergy Clin Immunol 84:734-741.

Stoop AE, van der Heijden HAMD, Biewenga J and van der Baan S (1992). Clinical aspects and distribution of immunologically active cells in the nasal mucosa of patients with nasal polyps after endoscopic sinus surgery and treatment with topical corticosteroids. Eur Arch Otorhinolaryngol 249:313-317.

Stringer SP and Ryan MW (2000). Chronic invasive fungal rhinosinusitis. Otolaryngol Clin North Am 33:375-387.

Strober W, Fuss IJ and Blumberg RS (2002). The immunology of mucosal models of inflammation. Annu Rev Immunol 20:495-549.

Stutts MJ, Canessa CM, Olsen JC, Hamrick M, Cohn JA, Roissier BC and Boucher RC (1995). CFTR as a cAMP-dependent regulator of sodium channels. Science 269:847-850.

Sutton BJ and Gould HJ (1993). The human IgE network. Nature 336:421-428.

Syme RM, Wood CJ, Wong H and Mody CH (1997). Both $CD4^+$ and $CD8^+$ human lymphocytes are activated and proliferate in response to *Cryptococcus neoformans.* Immunology 92:194-200.

Syme RM, Spurrell JCL, Ma LL, Green FHY and Mody CH (2000). Phagocytosis and protein processing are required for presentation of *Cryptococcus neoformans* mitogen to T lymphocytes. Infect Immun 68:6147-6153.

Taams LS, Smith J, Rustin MH, Salmon M, Poulter LW and Akbar AN (2001). Human anergic/suppressive CD4(+)CD25(+) T cells: a highly differentiated and apoptosis-prone population. Eur J Immunol 31:1122-1133.

Takeda K, Kaisho T and Akira S (2003). Toll-like receptors. Annu Rev Immunol 21:335-376.

Tazume K, Hagihara M, Gansuvd B, Higuchi A, Ueda Y, Hirabayashi K, Hojo M, Tanabe A, Okamoto A, Kato S and Hotta T (2004). Induction of cytomegalovirus-specific CD4+ cytotoxic T lymphocytes from seropositive or negative healthy subjects or stem cell transplant recipients. Exp Hematol 32:95-103.

Taylor MJ, Ponikau JU, Sherris DA, Kern EB, Gaffey TA, Kephart G and Kita H (2002). Detection of fungal organisms in eosinophilic mucin using a fluorescein-labelled chitin-specific binding protein. Otolaryngol Head Neck Surg 127:377-383.

Teixeira MM, Robinson MK, Shock A and Hellewell PG (2001). alpha4 integrin-dependent eosinophil recruitment in allergic but not non-allergic inflammation. Br J Pharmacol 132:596-604.

Th. den Boer A, van Mierlo GJD, Fransen MF, Melief CJM, Offringa R and Toes REM (2004). The tumoricidal activity of memory CD8+ T cells is hampered by persistent systemic antigen, but full functional capacity is regained in an antigen-free environment. J Immunol 172:6074-6079.

Thakar A, Sarkar C, Dhiwakar M, Bahadur S and Dahiya S (2004). Allergic fungal sinusitis: expanding the clinicopathologic spectrum. Otolaryngol Head Neck Surg 130:209-216.

Thiel A, Scheffold A and Radbruch A (2004). Antigen-specific cytometry--new tools arrived! Clin Immunol 111:155-161.

Thomas ML (1989). The leucocyte common antigen family. Annu Rev Immunol 7:339-369.

Tlaskalova-Hogenova H, Stepankova R, Hudcovic T, Tuckova L, Cukrowska B, Lodinova-Zadnikova R, Kozakova H, Rossmann P, Bartova J, Sokol D, Funda DP, Borovska D,

Rehakova Z, Sinkora J, Hofman J, Drastich P and Kokesova A (2004). Commensal bacteria (normal flora), mucosal immunity and chronic inflammatory and autoimmune diseases. Immunology Letters 93:97-108.

Togias A (2003). Rhinitis and asthma: evidence for respiratory system integration. J Allergy Clin Immunol 111:1171-1183.

Tomee JFCH and Kauffman HF (2000). Putative virulence factors of *Aspergillus fumigatus*. Clin Exp Allergy 30:476-84.

Tomiyama H, Matsuda T and Takiguchi M (2002). Differentiation of human $CD8^+$ T cells from a memory to memory/effector phenotype. J Immunol 168:5538-5550.

Tomiyama H, Takata H, Matsuda T and Takiguchi M (2004). Phenotypic classification of human CD8+ T cells reflecting their function: inverse correlation between quantitative expression of CD27 and cytotoxic effector function. Eur J Immunol 34:999-1010.

Tos M and Morgensen C (1984). Mucus production in chronic maxillary sinusitis. A quantitative histopathological study. Acta Otolaryngol 97:151-159.

Tough DF (2003). Deciphering the relationship between central and effector memory CD8+ T cells. Trends Immunol 24:404-407.

Trapani JA and Smyth MJ (2002). Functional significance of the perforin/granzyme cell death pathway. Nat Rev Immunol 2:735-747.

Vachier I, Vignola AM, Chiappara G, Bruno A, Meziane H, Godard P, Bousquet J and Chanez P (2004). Inflammatory features of nasal mucosa in smokers with and without COPD. Thorax 59:303-307.

Vailes L, Sridhara S, Cromwell O, Weber B, Breitenbach M and Chapman M (2001). Quantitation of the major fungal allergens, Alt a 1 and Asp f 1, in commercial allergenic products. J Allergy Clin Immunol 107:641-646.

van Baarle D, Kostense S, van Oers MHJ, Hamann D and Miedema F (2002). Failing immune control as a result of impaired $CD8^+$ T-cell maturation: CD27 might provide a clue. Trends Immunol 23:586-591.

Van Cauwenberge P and Watelet JB (2000). Epidemiology of chronic rhinosinusitis. Thorax 55 (2 Suppl):20-21.

van Rijswijk JB, Blom HM, KleinJan A, Mulder PGH, Rijntjes E and Fokkens WJ (2003). Inflammatory cells seem not to be involved in idiopathic rhinitis. Rhinology 41:25-30.

Veazey R and Lackner A (2003). The mucosal immune system and HIV-1 infection. AIDS Rev 5:245-252.

Vermaelen KY, Carro-Muino I, Lambrecht BN and Pauwels RA (2001). Specific migratory dendritic cells rapidly transport antigen from the airways to the thoracic lymph nodes. J Exp Med 193:51-60.

Walker LSK (2004). $CD4^+$ $CD25^+$ Treg: divide and rule? Immunology 111:129-137.

Wallace HL, Barker PM and Southern KW (2003). Nasal airway ion transport and lung function in young people with cystic fibrosis. Am J Respir Crit Care Med 168:594-600.

Wang XZ, Moylan B, Leopold DA, Kim J, Rubenstein RC, Togias A, Proud D, Zeitlin PL and Cutting GR (2000). Mutation in the gene responsible for cystic fibrosis and predisposition to chronic rhinosinusitis in the general population. JAMA 284:1814-1819.

Wang XQ, Duan XM, Liu LH, Fang YQ and Tan Y (2005). Carboxyfluorescein diacetate succinimidyl ester fluorescent dye for cell labeling. Acta Biochim Biophys Sin 37:379-385.

Wanner A (1997). Clinical aspects of mucociliary transport. Am Rev Respir Dis 116:73-125.

Weekes MP, Carmichael AJ, Wills MR, Mynard K and Sissons JG (1999). Human $CD28^-$ $CD28^+$ T cells contain greatly expanded functional virus-specific memory CTL clones. J Immunol 162:7569-7577.

Weller PF, Rand TH, Barrett T, Elovic A, Wong DTW and Finberg RW (1993). Accessory cell function of human eosinophils. HLA-DR-dependent, MHC-restricted antigen-presentation and IL-1 alpha expression. J Immunol 150:2554-2562.

Weninger W, Manjunath N and von Andrian UH (2002). Migration and differentiation of $CD8^+$ T cells. Immunol Rev 186:221-233.

Weston SA and Parish CR (1990). New fluorescent dyes for lymphocyte migration studies. Analysis by flow cytometry and fluorescence microscopy. J Immunol Methods 133:87-97.

Weyand CM, Bryl E and Goronzy JJ (2000). The role of T cells in rheumatoid arthritis. Arch Immunol Ther Exp (Warsz) 48:429-435.

Whiteside TL, Rabin BS, Zetterberg J and Criep L (1975). The presence of IgE on the surface of lymphocytes in nasal polyps. J Allergy Clin Immunol 55:186-194.

Wong P and Pamer EG (2003). CD8 T cell responses to infectious pathogens. Annu Rev Immunol 21:29-70.

Woodland DL and Dutton RW (2003). Heterogeneity of CD4+ and CD8+ T cells. Curr Opin Immunol 15:336-342.

Woodland DL and Randall T (2004). Anatomical features of anti-viral immunity in the respiratory tract. Seminars in Immunology 16:163-170.

Wormald PJ (2005). Endoscopic Sinus Surgery. Anatomy, Three-dimensional Reconstruction, and Surgical Technique, Thieme Medical Publishers, Inc.

Wright RE (1927). Two cases of granuloma invading orbit due to aspergillosis. Br J Opthalmology 11:545-549.

Wüthrich M, Filutowicz HI, Warner T, Deepe GS Jr., and Klein BS (2003). Vaccine immunity to pathogenic fungi overcomes the requirement for CD4 help in exogenous

antigen presentation to $CD8^+$ T cells: implications for vaccine development in immune-deficient hosts. J Exp Med 197:1405-1416.

Xanthou G, Tapinos NI, Polihronis M, Nezis IP, Mararitis LH and Moutsopoulos HM (1999). CD4 cytotoxic and dendritic cells in the immunopathologic lesion of Sjogren's syndrome. Clin Exp Allergy 118:154-163.

Yang CP and Bell EB (1999). Persisting alloantigen prevents primed CD45RC- CD4 T cells from inducing allograft rejection: implications for immunological memory. Eur J Immunol 29:2177-2186.

Yekeler H, Bitmis K, Ozcelik N, Doymaz MZ and Calta M (2001). Analysis of toxic effects of *Alternaria* toxins on esophagus of mice by light and electron microscopy. Toxicol Pathol 29:492-497.

Yokoyama WM, Kim S and French AR (2004). The dynamic life of natural killer cells. Annu Rev Immunol 22:405-429.

Yuan RR, Casadevall A, Oh J and Scharff MD (1997). T cells cooperate with passive antibody to modify *Cryptococcus neoformans* infection in mice. Proc Natl Acad Sci USA 94:2483-2488.

Zhang D, Shankar P, Xu Z, Harnisch B, Chen G, Lange C, Lee SJ, Valdez H, Lederman MM and Lieberman J (2003). Most antiviral CD8 T cells during chronic viral infection do not express high levels of perforin and are not directly cytotoxic. Blood 101:226-235.

Zinreich SJ (1997). Rhinosinusitis: radiologic diagnosis. Otolaryngol Head Neck Surg 117:(3 Suppl)27-34.

Zola H, Macardle PJ, Bradford T, Weedon H, Yasui H and Kurosawa Y (1991). Preparation and characterisation of a chimeric CD19 monoclonal antibody. Immunol Cell Biol 69:411-422.

APPENDIX

APPENDIX I: PATIENT DATA SHEET

Date: _____ Treating Physician/Surgeon: _____

Name: _____ DOB: _____ URN: _____

Address: _____

SYMPTOMS:

Nil (0+), Occasional/Mild (1+), Intermittent/Moderate (2+), Constant/Severe (3+)

Rhinorrhoea _____ Post nasal discharge _____ Sneeze/Itch _____

Anosmia/Hyposmia _____ Nasal obstruction _____ Facial pain/Headaches _____

EXAMINATION:

Nasal mucosa: Normal, Oedematous, Inflamed and Oedematous

Nasal mucus: Clear, White, Stringy, Thick/glue-like, Peanut butter/solid

Nasal polyps Grade: 1a,b; 2a,b; 3a,b; 4a,b _____

SIGNIFICANT MEDICAL HISTORY:

Asthma _____ Aspirin sensitive _____ Family history _____

Other _____

INVESTIGATIONS:

1. CT Scan Sinuses: Y / N Date: _____ Double densities: Y / N

Lund and MacKay score: Normal (0), Partial opacification (1), Total opacification (2)

Disease Site	**Right**	**Left**
Maxillary sinus		
Anterior ethmoid sinus		
Posterior ethmoid sinus		
Sphenoid sinus		
Frontal sinus		
Osteo-meatal unit		
TOTAL SCORE	**____ /12**	**____ /12**

2. Sinus mucus microbiology: _____

   Repeat Cultures: _____

3. Sinus mucus histopathology: Eosinophilic Mucus Y / N Fungal Elements Y / N

   Other Cells e.g. Polymorphs _____

4. Total serum IgE _____ kU/L Fungal-specific IgE: _____ kU_A/L

   Specific IgE to other aeroallergens _____ kU_A/L

5. Positive skin pick test: Fungi _____

   Non-Fungal: _____

6. Total Ig (g/L) IgG _____ IgA _____ IgM _____ IgG subclasses: _____

7. Eosinophil count: _____

8. *Aspergillus fumigatus* precipitins: _____

9. Other: _____

DIAGNOSIS:

Allergic fungal sinusitis	❑	Non-atopic	❑
Allergic fungal sinusitis-like	❑	Atopic, no fungal allergy	❑
NAFES	❑	Allergic rhinitis, fungal allergy	❑
NANFES	❑	"Simple" Polyps	❑

Comments: _____

TREATMENT

Topical: Y / N Drug(s)&Duration _____

Systemic: Y / N Drugs(s)&Duration: _____

Sinus surgery: Y / N No. of procedures: ____ Type of surgery: _____

"Thick" mucus in sinuses: Y / N

Immunotherapy: Y /N Date Commenced: _____

Allergens: _____

_____

APPENDIX II: ALLERGEN EXTRACTS

A: Allergen-specific Immunoglobulin E (ImmunoCAP System, Pharmacia)

Mould mix-2

Alternaria alternata (m6), *Aspergillus fumigatus* (m3), *Candida albicans* (m5), *Cladosporium herbarum* (m2), *Helminthosporium halodes* (m6) and *Penicillium notatum* (m1).

Grass pollen mix

Bahia (*Paspalum notatum*, g17), Johnson (*Sorghum halepense*, g10), Kentucky blue (*Poa pratensis*, g8), rye (*Lolium perenne*, g5) and Timothy (*Phleum pretense*, g6).

Tree pollen mix – tx7

Eucalyptus (*Eucalyptus* spp., t18), melaleuca (*Melaleuca leucadentron*, t21), olive (*Olea europea*, t9), wattle (*Acacia longifolia*, t19), white pine (*Pinus strobes,* t16) and willow (*Salix caprea*, t12).

House dust and mite-mix

House dust mite (*Dermatophagoides pteronyssinus*, d1; *D. farinae*, d2; *D. microceras*, d3), house dust and *Blatella germanica.*

Animal epidermals and protein mix-ex1

Cat epithelium and dander (*Felis domesticus*, e1), cow dander *(Bos taurus*, e4), dog dander (*Canis familiaris*, e5) and horse dander (*Equus caballus*, e3).

B: Skin Prick Test (Hollister-Stier Laboratories LLC)

Moulds

Alternaria alternata, Aspergillus mix (*A. fumigatus, A. nidulans, A. niger*), *Candida albicans, Cladosporium* mix (*C. normodendrum, C. herbarum, C. cladosporoides*), *Epicoccum nigrum, Fusarium vasinsectum, Helminthosporium* spp., *Mucor racemosus, Penicillium* mix (*P. digitatum, P. expansum, P. notatum*), *Rhizopus nigricans, Pullularia pullulans* and *Trichophyton* mix (*T. mentogrophytes, T. rubrum, T. tonsuranis*).

Grass Pollen

Bahia, brome (*Bromus diandrus*), couch (*Agropyron repens*), Johnson, Kentucky blue, meadow fescue (*Festuca elatior*), redtop bent (*Agrostis alba*), rye, sweet vernal (*Anthoxantum odoratum*) and Timothy.

Tree Pollen

Ash (*Fraxinus excelsior*), birch (*Betula alba*), bottle brush (*Callistemon* spp.), swamp she-oak (*Casuarina glauca)*, Chinese elm (*Ulmus parciola*), eucalyptus, maple (*Acer pseudoplatanus*), melaleuca, olive, plane (*Platanus vulgaris*), sycamore American (*Platanus occidentalis*), wattle, white pine and willow.

Weed Pollen

Plantain (*Plantago* spp.), sorrel (*Rumex acetosa*), fat hen (*Chenopodium album*), ragweed (*Ambrosia elatior*), pellitory (*Parietaria judaica*) and sagebrush (*Artemisia douglasiana*).

House Dust Group

Dermatophagoides pteronyssinus, D. farinae, cockroach mix-6585 and house dust.

Animal

Cat pelt, dog dander, feathers (chicken (*Gallus domesticus*), duck (*Anas platyrhynca*), goose (*Anser anser*)) and horse dander.

APPENDIX III: LABORATORY REFERENCE RANGES

A

Serum Protein Panel	Normal Reference Range[1]
Total IgG	6.5 – 16 g/L
Total IgA	0.6 – 4 g/L
Total IgM	0.5 – 3 g/L
Total IgE	0 – 140 kU/L
Total IgG1	3.2 – 9.2 g/L
Total IgG2	1.6 – 6.9 g/L
Total IgG3	0.2 – 1.2 g/L
Total IgG4	Less than 1.5 g/L

B

Differential White Cell Count	Normal Reference Range[1]
Eosinophils	0.02 – 0.5 x 10^9 /L
Lymphocytes	1 – 3.5 x 10^9 /L
Monocytes	0.2 – 0.8 x10^9 /L
Basophils	0 – 0.1 x 10^9 /L

C

$CD45^{high}$ Lymphocytes	Normal Reference Range[2]
$CD3^+$	60 – 90%
$CD3^+CD4^+$	28 – 60%
$CD3^+CD8^+$	15 – 46%
$CD3^-CD19^+$	2 – 26%

[1]Institute of Medical and Veterinary Science Laboratory, Adelaide South Australia. [2]SouthPath and Cellular Immunology Laboratory, Flinders Medical Centre, Adelaide, South Australia.

Pant, H., Kette, F.E., Smith, W.B., Wormald, P.J., and Macardle, P.J., (2005) Fungal-specific humoral response in eosinophilic mucus chronic rhinosinusitis. *Laryngoscope, v. 115 (4), pp. 601-606.*