Terms and Conditions of Use of Digitised Theses from Trinity College Library Dublin

Copyright statement

All material supplied by Trinity College Library is protected by copyright (under the Copyright and Related Rights Act, 2000 as amended) and other relevant Intellectual Property Rights. By accessing and using a Digitised Thesis from Trinity College Library you acknowledge that all Intellectual Property Rights in any Works supplied are the sole and exclusive property of the copyright and/or other IPR holder. Specific copyright holders may not be explicitly identified. Use of materials from other sources within a thesis should not be construed as a claim over them.

A non-exclusive, non-transferable licence is hereby granted to those using or reproducing, in whole or in part, the material for valid purposes, providing the copyright owners are acknowledged using the normal conventions. Where specific permission to use material is required, this is identified and such permission must be sought from the copyright holder or agency cited.

Liability statement

By using a Digitised Thesis, I accept that Trinity College Dublin bears no legal responsibility for the accuracy, legality or comprehensiveness of materials contained within the thesis, and that Trinity College Dublin accepts no liability for indirect, consequential, or incidental, damages or losses arising from use of the thesis for whatever reason. Information located in a thesis may be subject to specific use constraints, details of which may not be explicitly described. It is the responsibility of potential and actual users to be aware of such constraints and to abide by them. By making use of material from a digitised thesis, you accept these copyright and disclaimer provisions. Where it is brought to the attention of Trinity College Library that there may be a breach of copyright or other restraint, it is the policy to withdraw or take down access to a thesis while the issue is being resolved.

Access Agreement

By using a Digitised Thesis from Trinity College Library you are bound by the following Terms & Conditions. Please read them carefully.

I have read and I understand the following statement: All material supplied via a Digitised Thesis from Trinity College Library is protected by copyright and other intellectual property rights, and duplication or sale of all or part of any of a thesis is not permitted, except that material may be duplicated by you for your research use or for educational purposes in electronic or print form providing the copyright owners are acknowledged using the normal conventions. You must obtain permission for any other use. Electronic or print copies may not be offered, whether for sale or otherwise to anyone. This copy has been supplied on the understanding that it is copyright material and that no quotation from the thesis may be published without proper acknowledgement.
Glycodelin:

A Novel Inflammatory Mediator in Sinus Disease

Presentation of a Thesis

By

Emer Elizabeth Lang BA MB BAO BCh (TCD, Dublin) AFRCSI

To the University of Dublin-Trinity College Dublin

For the Degree of Doctor of Medicine (M.D.)

October 2006

Work carried out in the Department of Otolaryngology, and Department of Obstetrics and
Gynaecology, Emory University School of Medicine, Atlanta, Georgia, USA

Supervisors: Giridhar Venkatraman MD, Assistant Professor Department of
Otolaryngology, Head & Neck Surgery, Emory University School of Medicine and
Sampath Parthasarath PhD, Director of Research Division, Department of Obstetrics and
Gynaecology, Emory University

T.C.D Advisor: Professor Conrad Timon
DECLARATION

I declare that this thesis has not been submitted as an exercise for a degree at this or any other University. I declare that this thesis is entirely my own work and I agree that the Library may lend or copy the thesis upon request. This permission covers only single copies made for study purposes, subject to normal conditions of acknowledgement.

Emer E. Lang
# TABLE OF CONTENTS

**ACKNOWLEDGEMENTS**  
1

**SUMMARY**  
3

**1.0  CHAPTER 1 - INTRODUCTION**  
5

**1.1 The Paranasal Sinus**  
6

**1.2 Anatomy of the Sinus**  
9

1.2.1 Prenatal Development  
9

Figure 1.1 Diagram of the Paranasal Sinuses  
10

1.2.2 Post Natal Development  
11

1.2.2 Maxillary Sinus  
11

1.2.3 Ethmoid Sinus  
13

1.2.4 Anterior / Posterior Ethmoid Arteries  
14

1.2.5 Frontal Sinus  
15

1.2.6 Sphenoid Sinus  
16

**1.3 Microscopic Anatomy**  
18

**1.4 Mucociliary Clearance**  
19

**1.5 The Sinus Function**  
20

**1.6 The Physiology of the Sinuses**  
21

**1.7 Sinus Disease**  
24

1.7.1 The Genetic Factors Associated with Sinus Disease  
26

1.7.2 The Environmental Factors Associated with Sinus Disease  
28

**1.8 Conditions Associated with Sinus Disease**  
29

1.8.1 Nasal Polyps and Rhinosinusitis  
29

1.8.2 Asthma and Rhinosinusitis  
30

1.8.3 Rhinitis and Rhinosinusitis  
31
<table>
<thead>
<tr>
<th>Section</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.9</td>
<td>Role of Infectious Agents in Acute Rhinosinusitis</td>
<td>32</td>
</tr>
<tr>
<td>1.9.1</td>
<td>Allergic Fungal Sinusitis</td>
<td>33</td>
</tr>
<tr>
<td>1.10</td>
<td>Pharmacotherapy</td>
<td>35</td>
</tr>
<tr>
<td>1.10.1</td>
<td>Antimicrobial Therapy for Rhinosinusitis in Adults</td>
<td>35</td>
</tr>
<tr>
<td>1.10.2</td>
<td>Corticosteroids</td>
<td>36</td>
</tr>
<tr>
<td>1.10.3</td>
<td>Decongestants</td>
<td>38</td>
</tr>
<tr>
<td>1.10.4</td>
<td>Anti-leukotrienes</td>
<td>38</td>
</tr>
<tr>
<td>1.10.5</td>
<td>Antihistamines</td>
<td>39</td>
</tr>
<tr>
<td>1.11</td>
<td>Radiographic Imaging</td>
<td>40</td>
</tr>
<tr>
<td>1.12</td>
<td>Endoscopy</td>
<td>40</td>
</tr>
<tr>
<td>1.13</td>
<td>Surgery</td>
<td>41</td>
</tr>
<tr>
<td>1.14</td>
<td>Glycodelin Background</td>
<td>43</td>
</tr>
<tr>
<td>1.14.1</td>
<td>Lipocalins</td>
<td>43</td>
</tr>
<tr>
<td>1.14.2</td>
<td>Glycodelin</td>
<td>45</td>
</tr>
<tr>
<td>1.14.3</td>
<td>Glycodelin and the Uterine Tract</td>
<td>45</td>
</tr>
<tr>
<td>1.14.4</td>
<td>Glycodelin Outside the Uterine Tract</td>
<td>46</td>
</tr>
<tr>
<td>1.14.5</td>
<td>Glycodelin and the Immune System</td>
<td>47</td>
</tr>
<tr>
<td>1.14.6</td>
<td>Glycodelin and T-Cell Inhibition</td>
<td>49</td>
</tr>
<tr>
<td>1.14.7</td>
<td>Glycodelin and B-Cells</td>
<td>51</td>
</tr>
<tr>
<td>1.14.8</td>
<td>Glycodelin and Pregnancy</td>
<td>52</td>
</tr>
<tr>
<td>1.14.9</td>
<td>Regulation of Glycodelin</td>
<td>52</td>
</tr>
<tr>
<td>1.14.10</td>
<td>Oxidation and Cancer</td>
<td>53</td>
</tr>
<tr>
<td>1.14.11</td>
<td>Glycodelin and Angiogenesis</td>
<td>54</td>
</tr>
<tr>
<td>1.14.12</td>
<td>Glycodelin and Tumour Growth</td>
<td>55</td>
</tr>
<tr>
<td>1.14.13</td>
<td>Glycodelin in Bone Marrow</td>
<td>56</td>
</tr>
<tr>
<td>1.14.14</td>
<td>Glycodelin in the Respiratory Tract</td>
<td>57</td>
</tr>
<tr>
<td>2.0</td>
<td>CHAPTER 2 - AIMS OF THIS THESIS</td>
<td>58</td>
</tr>
<tr>
<td>2.1</td>
<td>Aims of this Thesis</td>
<td>59</td>
</tr>
<tr>
<td>3.0</td>
<td>CHAPTER 3 - GLYCODELIN EXPRESSION IN VITRO</td>
<td>61</td>
</tr>
<tr>
<td>3.1</td>
<td>Introduction</td>
<td>62</td>
</tr>
</tbody>
</table>
3.2 Aim

3.3 Materials and Methods

3.3.1 Cell Culture
3.3.2 Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) Analysis
3.3.3 Enzyme Linked Immunosorbant Assay (ELISA)

3.4 Statistical Analysis

3.5 Glycodelin Gene Expression and Upregulation by Phorbol Myristate Acetate (PMA)

3.5.1 Results
Figure 3.1 Glycodelin Gene Expression
Figure 3.2 GAPDH Gene Expression
Figure 3.3a Densitometry Analysis
Figure 3.3b - Densitometry Analysis
Figure 3.4 ELISA Analysis

3.6 Glycodelin Gene Expression in K562 Cells Following Exposure to an Allergen Protein, House Dust Mite Antigen

3.6.1 Results
Figure 3.5 Graph of Glycodelin Production After Allergen Challenge

3.7 Discussion

4.0 CHAPTER 4 - GLYCODELIN EXPRESSION IN PERIPHERAL WHITE BLOOD CELLS (IN VIVO)

4.1 Introduction

4.2 Aim

4.3 Materials and Methods

4.3.1 Cell Culture
4.3.2 Isolation of Monocytes (Buffy Coat Layer)
Figure 4.1 Buffy Coat Isolation
4.3.3 RNA Isolation
<table>
<thead>
<tr>
<th>Section</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.3.4</td>
<td>Reverse Transcriptase-polymerase Chain Reaction (RT-PCR) Analysis</td>
<td>88</td>
</tr>
<tr>
<td>4.3.5</td>
<td>Immunocytochemistry</td>
<td>90</td>
</tr>
<tr>
<td>4.4</td>
<td><strong>Statistical Analysis</strong></td>
<td>92</td>
</tr>
<tr>
<td>4.5</td>
<td><strong>Results</strong></td>
<td>93</td>
</tr>
<tr>
<td></td>
<td>Figure 4.2               PCR Analysis Of Male and Female Normal Subjects</td>
<td>94</td>
</tr>
<tr>
<td></td>
<td>Figure 4.3               PCR Gels Of Patients with Sinus Disease</td>
<td>95</td>
</tr>
<tr>
<td></td>
<td>Figure 4.4               Densitometric Analysis of Glycodelin Gene</td>
<td>98</td>
</tr>
<tr>
<td></td>
<td>Figure 4.5               Immunocytochemistry</td>
<td>99</td>
</tr>
<tr>
<td>4.6</td>
<td><strong>Discussion</strong></td>
<td>100</td>
</tr>
<tr>
<td>5.0</td>
<td><strong>CHAPTER 5 – GLYCODELIN EXPRESSION IN NASAL MUCOSA</strong></td>
<td>103</td>
</tr>
<tr>
<td>5.1</td>
<td><strong>Introduction</strong></td>
<td>104</td>
</tr>
<tr>
<td>5.2</td>
<td><strong>Aim</strong></td>
<td>105</td>
</tr>
<tr>
<td>5.3</td>
<td><strong>Materials and Methods</strong></td>
<td>105</td>
</tr>
<tr>
<td></td>
<td>5.3.1                    Immunohistochemistry</td>
<td>106</td>
</tr>
<tr>
<td>5.4</td>
<td><strong>Results</strong></td>
<td>107</td>
</tr>
<tr>
<td></td>
<td>Figure 5.1a               Expression Patterns Of Glycodelin in the Nasal Mucosa</td>
<td>109</td>
</tr>
<tr>
<td></td>
<td>Figure 5.1b</td>
<td>110</td>
</tr>
<tr>
<td></td>
<td>Figure 5.1c</td>
<td>111</td>
</tr>
<tr>
<td></td>
<td>Figure 5.1d</td>
<td>112</td>
</tr>
<tr>
<td></td>
<td>Figure 5.1e</td>
<td>113</td>
</tr>
<tr>
<td></td>
<td>Figure 5.1f</td>
<td>114</td>
</tr>
<tr>
<td>5.5</td>
<td><strong>Discussion</strong></td>
<td>114</td>
</tr>
</tbody>
</table>
6.0 CHAPTER 6 - SERUM GLYCODELIN LEVELS IN RHINOSINUSITIS AND NASAL POLYPOSIS

6.1 Introduction

6.2 Aim

6.3 Materials and Methods

Table 6.1 Major And Minor Criteria For The Diagnosis Of Chronic Rhinosinusitis

6.3.1 Enzyme-linked Immunosorbant Assay (ELISA)

Table 6.2 Patient Clinical Details

6.4 Statistical Analysis

6.5 Results

Figure 6.1 (a) Glycodelin Concentration in Male and Female Patients and Normal Subjects

Figure 6.1 (b) Glycodelin Concentration in Patients and Normal Subjects

Figure 6.1 (c) Severe Polyposis/Purulence on Examination

Figure 6.1 (d) Patients Treated With Systemic Steroids vs. No Previous Treatment

Figure 6.1 (e) Patients Treated With Systemic Steroids vs. No Previous Treatment

6.6 Discussion

6.7 Conclusion

7.0 CHAPTER 7 - GLYCODELIN AND REACTIVE AIRWAY DISEASE

7.1 Introduction

7.2 Aim
7.3 **Materials and Methods**  

7.3.1 Patient Sample Collection 141  
7.3.2 Enzyme Linked Immunosorbant Assay (ELISA) 143  
Table 7.1 Ozone Exposure Dosage Calculation 147  
7.3.3 Ozone Exposure Calculation 148  

7.4 **Statistical Analysis** 148  

7.5 **Results** 149  

Table 7.2 Pulmonary function in study groups 151  
Figure 7.1 Nasal Lavage Glycodelin Level vs. 24 hour ozone dose 152  
Figure 7.2 Plasma Glycodelin Level vs. lung function 153  
Figure 7.3 Nasal Lavage Glycodelin Level vs. lung function 154  

7.6 **Discussion** 155  

8.0 **CHAPTER 8 - EFFECT OF SINUSITIS MEDICATIONS ON GLYCODELIN EXPRESSION IN VITRO** 159  

8.1 **Introduction** 160  

8.2 **Dexamethasone** 162  

8.3 **Loratadine** 162  

8.4 **Aim** 163  

8.5 **Materials and Methods** 164  

8.5.1 Cell Culture 164  
8.5.2 Phorbol Myristate Acetate (PMA) 165  
8.5.3 Preparation of Phorbol 12-Myristate 13-Acetate (PMA) 165  
8.5.4 Cell Culture Method 165  

8.6 **Preparation of Dexamethasone Treatment Media** 166
8.7 **Cell Treatment with Dexamethasone** 166

Figure 8.1 Plan for Cell Treatment with Dexamethasone 167

8.8 **Preparation of Loratadine** 168

8.9 **Cell Treatment with Loratadine** 168

Figure 8.2 Plan for Cell Treatment with Loratadine 169

8.10 **Polymerase Chain Reaction (PCR)** 170

8.10.1 Reverse Transcriptase - Polymerase Chain Reaction (RT_PCR) Analysis 170

8.11 **Enzyme Linked Immunosorbant Assay (ELISA)** 171

8.12 **Statistical Analysis** 174

8.13 **Results** 174

Figure 8.3.1 Glycodelin Expression in K562 Cells After Treatment with Dexamethasone 176

Figure 8.3.2 GAPDH Expression in K562 Cells After Treatment with Dexamethasone 176

Figure 8.3.3 Glycodelin Expression in K562 Cells After Treatment with Dexamethasone 177

Figure 8.3.4 GAPDH Expression in K562 Cells After Treatment with Dexamethasone 178

Figure 8.4.1 Densitometric Analysis of PCR Gels Following Treatment of K562 Cells 179

Figure 8.4.2 Dose Response Curve 180

Figure 8.5.1 Glycodelin Protein Production After Treatment with Dexamethasone 181

Figure 8.5.2 Analysis of ELISA Readings 182

Figure 8.6.1 Glycodelin Expression in K562 Cells After Treatment with Loratadine 183

Figure 8.6.2 GAPDH Expression in K562 Cells After Treatment with Loratadine 184

Figure 8.7.1 Densitometric Analysis of PCR Gels Following Treatment of K562 Cells 185

Figure 8.7.2 Dose Response Curve 186

8.14 **Discussion** 187
9.0 CHAPTER 9 - DISCUSSION

Figure 9.1 Putative Role of Glycodelin in Suppression of T-Cell Activation

APPENDICES

Appendix I

(i) Generation of Glycodelin Antibody
(ii) Purification of the Polyclonal Antibody
(iii) Specificity of Purified Antiglycodelin Antibody Tested

Appendix II

(i) RNA Isolation and RT-PCR Technique
(ii) Buffy Coat Isolation
(iii) Immunostaining of Buffy Coat Smears
(iv) Enzyme Linked Immunosorbant Assay
(v) Immunohistochemistry
(vi) Cell Culture

REFERENCES
ACKNOWLEDGEMENTS

There are many people who helped me throughout my time in the laboratory and then during the writing stage of this thesis, to all of whom I am deeply indebted. I would like to thank:

In Atlanta:

Giridhar Venkatraman, who invited me to work with him as a Rhinology Research Fellow in Emory University and who supervised this thesis. His endless enthusiasm and constant support were most gratefully appreciated.

Douglas Mattox Chief of Dept of Otolaryngology in Emory University – for supporting a Research Fellow position and his encouragement during the time I spent in the laboratory at Emory University.

Dr. Sampath Parthasarathy who welcomed me into his laboratory and whose endless love of ‘science’ made life in the laboratory most interesting. His keen interest in the testing the boundaries of knowledge was an inspiration to many.

Anne, Nyssa, Erin, Branden, Alex and Shri who made my time working in the laboratory a most pleasant experience and in particular Meera for all the practical advice. Kate Alexander, a southern belle, was a wonderful friend, coffee companion, and social advisor!!

And finally my lovely sister Máire, who along with Brian and Bláithín welcomed me into their home and into their lives. The ‘Reality TV’ and bedtime stories were a pleasant antidote to academia.
In Ireland:

Ms. H. Rowley, who encouraged me to undertake this research and who was instrumental in my obtaining the fellowship at Emory. Thank you for your friendship and support.

Mr. S. Hone has been a friend throughout my career in Otolaryngology and was always just a phone call away with support and advice.

Others that I am appreciative to for their encouragement during the writing phase of this thesis are: Mr. J. Lang, Mr. P. Gormley and Professor S. Sellers.

My brother Padraic the first doctor in our family, who is always a source of fun and good humour.

Kristofer has been my best friend, has given me constant encouragement, and has supported me throughout the process of completing this work. You are my soul mate and I look forward to spending the rest of my life with you . . .

Last but not least to my Mother and Father who have supported and encouraged me selflessly over the years. To both of you I am truly thankful.

This work is dedicated to you.
SUMMARY

Rhinosinusitis is one of the three most common disorders presenting to primary care physicians, generating high socioeconomic costs (Moss and Parsons 1986; Poole 1999). Understanding the pathomechanisms of inflammation, especially in chronic rhinosinusitis and nasal polyposis, is critical to improving disease treatment. My work focuses on a novel inflammatory mediator - glycodelin. Lipocalins such as glycodelin have been reported to be acute phase proteins and exert significant immunomodulatory effects in vitro (Logdberg and Wester 2000). Members of the lipocalin family exert a regulatory, dampening influence on the inflammatory cascade, thereby protecting against tissue damage from excessive inflammation. We hypothesise that nasal inflammation leads to nasal congestion and superinfection and that glycodelin is produced in response to this inflammation. The role of glycodelin in nasal inflammation has not previously been defined.

Using cell culture techniques we confirmed glycodelin gene expression in K562 cells and up regulation following exposure to various stimuli in vitro. We then demonstrated glycodelin gene expression in human white blood cells using PCR techniques. Glycodelin protein production by mononuclear cells in patients and controls was confirmed using immunocytochemistry techniques. This finding had not been previously described. We confirmed the presence of glycodelin in the nasal mucosa of patients with chronic rhinosinusitis and in normal subjects via immunohistochemistry on archival tissue materials. We found that glycodelin, is up regulated in endothelial cells of those with rhinosinusitis. Glycodelin had not been previously demonstrated in nasal mucosa. We examined for evidence of
glycodelin protein production in peripheral white cells of patients with chronic nasal disease and controls using ELISA techniques. We showed that glycodelin levels were highest in those patients with severe polyposis and purulence and levels were decreased in those patients who treated with steroid therapy. The ELISA assay was performed on nasal and pulmonary secretions from patients with asthma and normal controls. A positive correlation was found between 24-hr ozone exposure and glycodelin levels in all patients. Glycodelin levels along with other inflammatory markers are raised in response to ozone exposure and high oxidant stress. Plasma glycodelin levels appear to correlate with airflow limitation in unstable asthmatics. Those patients with most active disease and poorest lung function had higher levels of serum glycodelin. We then examined using cell culture, PCR, and ELISA techniques, the effect of two common treatment modalities used in sinus disease, dexamethasone and loratadine, on glycodelin gene expression and protein production in vitro. Having confirmed the presence of glycodelin within paranasal sinus mucosa and up regulation of glycodelin in patients with sinus disease, and having shown higher levels of glycodelin in patients with severe disease, we feel that glycodelin does indeed play a role in sinus inflammation. The production of glycodelin by peripheral white blood cells may prove a novel marker for nasal inflammation. Novel therapeutic methods directed against glycodelin production by peripheral blood cells may play an important role in the management of chronic nasal disease and nasal polyposis. These experiments have enhanced and extended our understanding of glycodelin’s role in the complex immune system dysfunctions that characterise sinus disease.
CHAPTER 1

INTRODUCTION
1.1 The Paranasal Sinuses

Rhinosinusitis is one of the three most common disorders presenting to primary care physicians, generating high socioeconomic costs including lost school and/or working days for both children and adults (Moss and Parsons 1986; Poole 1999). In the 1993 National Health Interview Survey, (a yearly survey of the non-institutionalised United States civilian population), rhinosinusitis was the most frequently reported chronic disease in the United States, (Benson V 1994). Nearly 15% of all respondents of the 1993 survey reported having rhinosinusitis that lasted at least three months in duration (Benson V 1994). In the United States, rhinosinusitis develops in approximately 31 million people each year, with each individual losing an average of four days from work (Spector, Bernstein et al. 1998). In Europe, rhinosinusitis affects more than 5% of the population (Kennedy 1995). However, since accurate figures from family practitioners are not available in Europe, this is most certainly an underestimate. Extrapolating the available data to the British population, rhinosinusitis is estimated to cause six million restricted working days per year (Kennedy 1995).

In the United States, McCaig et al estimated conservatively that in 1992, 13 million antibiotic prescriptions were prescribed for acute and chronic rhinosinusitis and that the direct medical costs of rhinosinusitis reached almost $2.4 billion dollars (McCaig and Hughes 1995). Direct costs include hospitalisations for acute and chronic rhinosinusitis, visits to both private physicians' offices and hospital outpatient units for chronic rhinosinusitis, and prescriptions for antimicrobial treatment for acute and chronic rhinosinusitis.
However, these costs do not include the expense of managing a coexistent illness such as allergic rhinitis or nasal polyps or the expense of surgery and its related costs.

In addition, no data has been aggregated to indicate the potentially billions of euros/dollars of indirect costs resulting from rhinosinusitis or the costs for several expensive and important treatments such as endoscopic sinus surgery and diagnostic computed tomographic (CT) imaging. When surgical procedures are included in the summation, the economic burden of rhinosinusitis rises significantly. The estimated overall health care expenditure attributable to rhinosinusitis in the United States is $5.8 billion - with nearly 90% of all of this expenditure (US $5.1 billion) associated with ambulatory or emergency care (Ray, Baraniuk et al. 1999). Thus, the total cost of rhinosinusitis is certain to be much larger, establishing rhinosinusitis as one of the most expensive disorders experienced in the general population.

Accordingly, the high prevalence of rhinosinusitis has led to an increasing interest in the pathophysiology of different forms of rhinosinusitis as the basis for better treatment modalities. Rhinosinusitis has been understood as inflammation of the paranasal mucous membranes due to bacterial infection, which might be based on impaired ventilation and drainage of the sinuses due to either viral infection in acute rhinosinusitis or obstruction of the ostiomeatal complex in chronic rhinosinusitis (Stammberger 1986; Baraniuk 1994; Gwaltney 1994). In contrast, nasal polyposis seems to be only partially related to infection, but is mainly related to eosinophilic inflammatory mechanisms as in aspirin sensitivity or asthma (Settipane and Chaffee 1987; Mygind 1990; Tos, Sasaki et al. 1992).
Despite the considerable human and socioeconomic burden associated with rhinosinusitis, many questions remain unanswered. While it is clear that infection, mucosal hyperactivity, and anatomical variation all play important parts, how the basic aetiological mechanisms responsible for the clinical picture of rhinosinusitis interact is not completely clear. Inflammatory processes in the sinus cavities constitute a serious clinical problem and research activities have mostly focused on mucociliary clearance, microbiology and morphology of the sinuses. These efforts have recently led to the development of new concepts of sinus physiology, followed by the introduction of sophisticated surgical techniques which take functional aspects into account (Stammberger 1986).

While surgical treatment for rhinosinusitis is widely practiced, with 200,000 procedures per year performed in the United States alone (Ogusthorpe 1999), failure of treatment or recurrence of disease is still seen after restitution of ventilation and drainage. The failure of treatment or the recurrence of disease is obviously linked to underlying inflammatory processes.

Today, the role of cytokines, chemokines and adhesion receptors is emerging from studies on different models of nasal inflammation such as viral or allergic rhinitis. Understanding the pathomechanisms of inflammation, especially in chronic rhinosinusitis and nasal polyposis, is critical to improving success rates in disease treatment.
1.2 Anatomy of the Sinuses

The paranasal sinuses are air-filled spaces within the bones of the head. In healthy adults, the air and bone are separated by ciliated epithelium containing goblet cells, nerves, blood vessels, lymphatic vessels, and glandular rich connective tissue. The structure of a person’s paranasal sinuses is as unique as a set of fingerprints. Even from side to side in the same person, anatomic features may vary markedly. CT studies have been performed to examine the anatomy of these normal structures in both healthy and diseased patient populations. For example, a concha bullosa may be found in 20% of normal subjects but are more frequent in patients with chronic or recurrent rhinosinusitis.

The anatomic development of the paranasal sinuses may be divided into (1) the prenatal development and (2) the postnatal aeration of the sinuses that result in the ‘mature’ anatomy.

1.2.1 Prenatal Development

We know little about the role of potential cellular growth factors in the development of the ethmoid air cells within the foetal frontal recess, suprabullar recess, and ethmoid infundibulum. Data on these growth factors would help us better understand situations such as the various permutations of drainage of the frontal sinus into the frontal recess and subsequently into the ethmoid infundibulum.
Our current understanding is that the development of the lateral nasal wall begins with a smooth, undifferentiated structure. The first outgrowth is the maxilloturbinal, which will eventually become the inferior turbinate. Subsequently, another mound of mesenchyme forms the ethmoturbinal. The ethmoturbinal is destined to become the middle, superior, and supreme turbinates by subdividing into the second and third ethmoturbinals. This growth is followed by the development of the aggar nasi cells, uncinate process, and ethmoid infundibulum.

At this stage, the sinuses then begin to develop. The resultant system of cavities, depressions, ostia, and processes is a complex system of structures. These structures must be fully understood in detail before surgical management of sinus disease can be safe and effective.
The lateral nasal wall includes portions of the ethmoid, the maxilla, the palatine, the lacrimal, the medial pterygoid plate of the sphenoid, the nasal and the inferior turbinate bones. Three to four turbinates project from the lateral nasal wall. The supreme, the superior, and the middle turbinates are all projections of the ethmoid bone. However, the inferior is considered to be an independent structure. Each of these structures overlies an airspace beneath and lateral to it known as a meatus. A small slip of bone projects from the ethmoid bone, which covers the opening(s) of the laterally placed maxillary sinus and forms a trough behind the middle turbinate. This thin bony partition is known as the uncinate process. The superior nasal sidewall consists of ethmoid sinus cells, which laterally border the olfactory epithelium and the fragile cribriform plate. Superior to the anterior most ethmoid cells lies the frontal sinus, which drains between an assortment of air cells. The posterior-superior aspect of the lateral nasal wall becomes the anterior wall of the sphenoid sinus, which nestles below the sella turcica and the cavernous sinus.

1.2.2 Post Natal Development

Maxillary Sinus

The maxillary sinus (antrum of Highmore) is the first sinus to develop. These structures are usually fluid-filled at birth. The growth of these sinuses is biphasic with growth occurring during years 0-3 and 7-12. During the later phase, pneumatisation spreads more inferiorly as the permanent teeth take their place.
Pneumatisation can be so extensive as to expose tooth roots with only a thin layer of soft tissue covering them.

The adult maxillary sinus is pyramidal in shape with a volume of approximately 15 ml (34x33x23mm). At the base of the pyramid is the nasal wall, with the peak pointing toward the zygomatic process. The anterior wall has the infraorbital foramen located at the midsuperior portion with the infraorbital nerve running over the roof of the sinus and exiting through the foramen. This nerve can be dehiscent (14%). The thinnest portion of the anterior wall is just above the canine tooth--the canine fossa. The roof is formed by the orbital floor and transected by the course of the infraorbital nerve. The posterior wall is unremarkable. Behind this wall is the pterygomaxillary fossa with the internal maxillary artery, sphenopalatine ganglion and the vidian canal, the greater palatine nerve and the foramen rotundum. The floor, as discussed above, varies in its level. From birth to age nine the floor of the sinus is above that of the nasal cavity. At age nine the floor is generally at the level of the nasal floor. The floor continues to sink as the maxillary sinus pneumatises. Because of the close relationship with the dentition, dental disease can cause maxillary infection, and tooth extraction can result in oral-antral fistulae.

Branches of the internal maxillary artery supply this sinus. These include the infraorbital (as it runs with the infraorbital nerve), lateral branches of the sphenopalatine, greater palatine, and the alveolar arteries. Venous drainage runs anteriorly into the facial vein and posteriorly into the maxillary and dural sinus systems. Branches of the trigeminal nerve, specifically, the greater palatine nerve and the branches of the infraorbital nerve innervate the maxillary sinus.
1.2.3 Ethmoid Sinuses

In a newborn child, the ethmoid sinuses are well delineated, fluid-filled structures. During foetal development, the anterior cells form first, followed by the posterior cells. These cells grow gradually and are adult size by age 12 and are not usually seen on radiographs until age one. The septa gradually thin and pneumatisation spreads as the child ages. Ethmoid cells are highly variable and can often be found above the orbit, lateral to the sphenoid, into the roof of the maxillary sinus, and anteriorly above the frontal sinus. The variations of these ethmoid cells have been named. A cell above the orbit is called a supraorbital cell and is found in 15% of patients. Invasion of an ethmoid cell into the floor of the frontal sinus is called a frontal bulla. Extension into the middle turbinate is termed concha bullosa. Cells in the roof of the maxillary sinus (infraorbital) are called, "Haller's cells," and are found in 10% of the population. These cells can obstruct the maxillary ostia and narrow the infundibulum and result in disruption of normal sinus function. Finally, a cell that extends anterolaterally to the sphenoid sinus is called an Onodi cell (10%). The common variability of these cells makes preoperative imaging essential to clarify a patient's individual anatomy.

Posterior and anterior cells combined have a volume of 15 ml (3.3x2.7x1.4cm). The ethmoids are shaped like a pyramid and are divided into multiple cells by thin septa. The roof of the ethmoids is composed of multiple important structures. The roof slopes both posteriorly (angle of 15 degrees) and medially. The anterior two-thirds of the roof is thick and strong and is composed
of the frontal bone and the foveolae ethmoidalis. The posterior third is higher laterally and slopes down medially to the cribriform plate. The junction between the lateral dense bone and the plate is one-tenth as strong as the lateral roof. The difference in height between the lateral and medial roof is variable, but can be as much as 15-17mm. The posterior aspect of the ethmoid cells border on the sphenoid sinus. The lateral wall is the lamina papyracea of the orbit.

The ethmoid sinuses are supplied by blood flow originating from both the external and internal carotid arteries. The sphenopalatine artery and the ophthalmic artery (which branches into the anterior and posterior ethmoid arteries) supply the sinus. Venous drainage follows arterial supply and thus can track infection intracranially. Both the first and second branches of the trigeminal nerve innervate this region. The first branch supplies the more superior aspect with the second branch innervating the inferior regions. Parasympathetic innervation is via the vidian nerve. Sympathetic innervation is via the cervical sympathetic ganglion and follows the arterial vasculature to the mucosa of the sinuses.

1.2.4 Anterior/Posterior Ethmoid Arteries

The anterior and posterior ethmoid arteries arise from the ophthalmic artery in the orbit. The anterior artery crosses the medial rectus and penetrates the lamina papyracea. The artery then courses across the roof the ethmoid sinus in a thin bony mesentery (usually dehiscent), eventually supplying the cribriform plate
and anterior septum. This artery is usually large and singular and may drape inferiorly into a sinus cell. Its position closely corresponds to the position of the more medial structure, ethmoidal fovea. The posterior artery crosses the medial rectus, penetrates the lamina papyracea and courses through the posterior ethmoid cells (usually corresponding with the anterior wall of the posterior-most cell) to the septum. It supplies the posterior ethmoid sinuses, part of the superior and middle turbinates and small amount of the posterior septum. This artery is usually smaller and branched. It can be dehiscent and drape down within the sinus cells. Its position is associated with the position of the optic nerve near the orbital vertex. Because the development of these structures predates the sinuses their relation to the ethmoid cells can vary. Their association with the fovea and optic nerve remain constant.

1.2.5 Frontal Sinus

The frontal sinus is likely formed by the upward movement of the anterior-most ethmoid cells. Since the frontal bone is membranous at birth there is seldom more than a recess until the bone begins to ossify around age two. Thus, radiographs seldom show this structure before that time. True growth begins at age five and continues into the late teens.

The volume of the sinus is approximately 6-7 ml (28x24x20mm). Frontal sinus anatomy is highly variable, but generally there are two sinuses that are funnel shaped and point upward. The depth of the sinus is the most surgically significant dimension as it determines the limitations of surgical approach. Both
Frontal sinuses have their ostia at the most dependant portion of the cavity (posteromedial). Many feel this is the reason that these sinuses are rarely involved with infectious disease. Both the anterior and posterior walls of this sinus are composed of diploe bone. However, the posterior wall (separates the frontal sinus from the anterior cranial fossa) is much thinner. The floor of the sinus also functions as a portion of the orbital roof.

The frontal sinus is supplied by the ophthalmic artery via supraorbital and supratrochlear arteries. Venous drainage is via the superior ophthalmic veins to the cavernous sinus and via small venulae in the posterior walls, which drain to the dural sinuses. Branches of the trigeminal nerve innervate the frontal sinus. Specifically, these nerves include the supraorbital and supratrochlear branches.

**1.2.6 Sphenoid Sinus**

The sphenoid sinuses are unique in that they do not arise from outpouchings of the nasal cavity. These sinuses arise from within the nasal capsule of the embryonic nose. They remain undeveloped until age three. By age seven the pneumatisation has reached the sella turcica. By age 18 the sinuses have reached full size. In the late teen years the sinus reaches its full size with a volume of 7.5 ml (23x20x17mm). Pneumatisation of this sinus, like that of the frontal sinus, is very variable. Generally these are bilateral structures located at the posterosuperior aspect of the nasal cavity. Pneumatisation can extend as far as the clivus, the sphenoid wings, and the foramen magnum. The walls of the sphenoid vary in thickness with the anterosuperior wall and roof being the
thinnest (0.1mm to 1.5 mm). The other walls are thicker. The thinnest part of the anterior wall is 1cm from the fovea ethmoidalis. The position of the sinus and, therefore, its anatomic relationships depend on the extent of pneumatisation. The sinus can sit far anterior to, just anterior to, or immediately under the sella turcica (conchal, presellar, sellar/postsellar). The most posterior position can place the sinus just adjacent to vital structures such as the carotid arteries, optic nerves, maxillary branch of the trigeminal nerve, the vidian nerve, the pons, sella turcica, and the cavernous sinus. These structures are often identified as indentions on the roof and walls of the sinus. A small percentage will have dehiscence of bone over such vital structures as the optic nerve and carotid arteries. Care must also be taken when removing sinus septa as these may be in continuity with the carotid and optic canal and can result in death and blindness.

The sphenoid sinus ostium drains into the sphenoethmoidal recess. The ostium is very small (0.5-4mm) and is located about 10mm above the sinus floor. A 30-degree angle drawn from the anterior nasal floor approximates the location of the ostium on the posterosuperior nasal wall. It is noted to be close to the midline at the junction of the upper 1/3 and the lower 2/3 of the anterior sinus wall. It is generally medial to the supreme/superior turbinate, and is only a few millimetres from the cribriform plate. This ostium, like that of the maxillary sinus, has a much larger bony dehiscence that is narrowed by a membranous septum.

The posterior ethmoid artery supplies the roof of the sphenoid sinus. The sphenopalatine artery supplies the rest of the sinus. Venous drainage is via the maxillary veins to the jugular and pterygoid plexus systems. Branches of the
trigeminal nerve supply the sphenoid sinus. The nasociliary nerve (from first part of trigeminal) runs into the posterior ethmoid nerve and supplies the roof. The branches of the sphenopalatine nerve (from second part of the trigeminal) supply the floor.

1.3 Microscopic Anatomy

The sinuses are lined with pseudostratified ciliated columnar epithelium, which is in continuity with the mucosa of the nasal cavities. The epithelium of the sinuses is thinner than that of the nose. There are four basic cell types. These include ciliated columnar epithelial cells, nonciliated columnar cells, basal cells, and goblet cells. The ciliated cells have 50-200 cilia per cell with the usual structure of 9+2 microtubules with dynein arms. Experimental data shows these cells to beat at 700-800 times a minute, moving mucus at a rate of 9 mm/minute. Nonciliated cells are characterised by microvilli, which cover the apical aspect of the cell and serve to increase surface area (likely to facilitate humidification and warming of inspired air). It is interesting to note that there is an increased concentration (up to 50%) at the sinus ostium. The basal cell's function is unknown. They vary in size, shape and number. Some have theorised that they serve as a stem cell, which can differentiate as needed. Goblet cells produce glycoproteins, which are responsible for the viscosity and elasticity of mucus. They are innervated by the parasympathetic and sympathetic nervous system.
Thus, parasympathetic stimulation induces thicker mucus with sympathetic stimulation leading to more watery mucus secretion.

The epithelial layer is supported by a thin basement membrane, lamina propria, and periosteum. Both serous and mucinous glands project down into the lamina propria. Anatomic studies have shown a general paucity of goblet cells and submucosal glands in the sinuses compared to the nasal mucosa. When comparing the sinuses, the maxillary sinus has the highest density of goblet cells. The ostia of the maxillary, sphenoid, and anterior ethmoid sinuses seem to have an increased number of submucosal serous and mucinous glands.

1.4 Mucociliary Clearance

The ciliated cells in each sinus beat in a specific direction resulting in a specific pattern of mucus flow. Since many of the sinuses develop in an outward and inferior fashion, the ciliated mucosa often moves material against gravity to the sinus' exit. This means that mucus produced just adjacent to a sinus ostia, if it is on the afferent side, will travel around the entire sinus cavity, often against gravity, before exiting the ostia. This is one reason that creation of accessory ostia at sites outside the physiologic ostium will not significantly improve sinus drainage. In fact, this sometimes results in mucus draining from the natural ostia re-entering the sinus via the newly created opening and cycling through the sinus again. Hilding was the first that described each sinus's mucus flow patterns, and his observations are still valid today (Hilding 1932). Later researchers described a
phenomenon of stagnation, which occurs when two ciliated surfaces come into contact (particularly applicable at the ostiomeatal complex). This disrupts mucociliary mucus clearance and can result in rhinosinusitis. Successful treatment of the disease is accomplished when this clearance mechanism has been restored.

1.5 The Sinus Function

The physiology and function of the sinuses have been the subject of much research, unfortunately, we still are unsure as to all of the functions of these air-filled spaces. In fact, multiple theories of sinus function exist. One of these theories involves the functions of warming and humidification of air. Under this theory, the functions of warming and humidification of air assist in the regulation of intranasal pressure and serum gas pressures (and subsequently minute ventilation). This in turn contributes to immune defence, increasing mucosal surface area, lightening the skull, giving resonance to the voice, absorbing shock, and facial growth.

The nose is an amazing humidifier and warmer of air. Even at seven litres/minute of airflow, the nose has not reached its maximal ability to perform this function. Nasal humidification has been shown to contribute as much as 6.9 mm Hg on serum pO2. Although the nasal mucosa is best adapted to perform this task, the sinuses contribute to mucosal surface area and warming ability. Some researchers have shown that mouth breathers have a decreased end-tidal CO2, which may increase serum CO2 and contribute to sleep apnoea.
The physiology and function of the paranasal sinuses is a subject that reflects the complexity of their anatomy. Continued research may likely reveal that all of these functions are part of a bigger, more involved picture than is now apparent.

1.6 The Physiology of the Sinuses

The nasal cycle refers to the physiological asymmetrical congestion, which alternates between sides of the nose every 2-7 hours. Various vessel types within the microvasculature of the nose regulate nasal volume, humidity and heat exchange. Respiratory airflow is regulated by venous sinusoids in nasal erectile tissue, located on the inferior turbinate and to a lesser extent on the anterior septum. Mucociliary flow occurs as a mass motion of the mucous layer in sinuses at a rate of 1 centimetre per minute. The components of the mucociliary system include respiratory epithelium, a double-layered mucous blanket and mucous producing glands. Because of the sinuses' copious mucus production, they contribute heavily to the immune defence/air filtration performed by the nose. The nasal and sinus mucosa are ciliated and function to move mucus to the choanae and the stomach beyond. The thickened superficial layer of nasal mucus serves to trap bacteria and particulate matter in a substance rich with immune cells, antibodies, and antibacterial proteins. The underlying sol layer is much thinner and serves to provide a thinner substrate in which the cilia are able to beat; their tips essentially grabbing the superficial layer and pushing it in the direction of the beat. Unless obstructed by disease or anatomical variance, the sinuses
move mucous through their cavities and out of their ostia toward the choana. Nasal mucous is composed of water, glycoproteins, salts, IgA and lysozymes.

Ostial blockage leads to a local hypoxic environment in the sinuses, resulting in the accumulation of secretions and inflammatory products. Hypo-oxygenation and hypercapnoea in the sinuses lead to a lowered pH, which when combined with the accumulation of secretions and inflammatory products, result in altered ciliary function. This creates a vicious cycle of inflammation and trapping of secretions, thereby providing an ideal medium for bacterial growth. In the majority of cases, the aetiology of mucosal inflammation is either allergic or viral.

Alterations in epithelial function may play an important role in the pathogenesis of rhinosinusitis. Not only is epithelial hyperplasia a common feature of chronic rhinosinusitis, but altered production of epithelial cell products could play a role in cellular inflammation. Epithelial cells are capable of producing a range of cytokines, such as interleukin-8 (IL-8), IL-6, IL-11, IL-4, IL-5, tumour necrosis factor (TNF) - alpha, RANTES (regulated on activation normal T cell expressed and secreted), MCP-1 (macrophage chemotactic protein) and granulocyte macrophage colony stimulating factor (GM-CSF) (Brezillon, Dupuit et al. 1995). These polypeptides attract inflammatory cells, particularly eosinophils and basophils from the microcirculation, and prolong their survival. In turn these inflammatory cells can produce cytokines such as IL-3, TNF- alpha, and GM-CSF in an autocrine up regulated fashion; these cytokines then recruit more inflammatory cells. The inflammatory mediators chiefly implicated in rhinosinusitis are cytokines and chemokines. In acute rhinosinusitis, the synthesis
of pro-inflammatory cytokines and of the neutrophil chemokine IL-8 and IL-3 appear to be up regulated (Rudack, Stoll et al. 1998). In chronic sinusitis the orchestration of cellular recruitment and activation of the inflammatory infiltrate has been largely attributed to the T helper 2 cells and their cytokines – IL-3, IL-4, IL-5, IL-9, IL-13 and GM-CSF (Borish and Rosenwasser 1996). In bilateral nasal polyposis, but not in antrochoanal polyps, the eosinophil-related cytokine IL-5 is strongly up-regulated. Altered production of such cytokines could affect the recruitment, survival and activation state of inflammatory leukocytes. Moreover, altered production of nitric oxide by the epithelium could be important in changing the antibacterial protection of the sinuses. The factors that lead to the initial insult in the lateral wall of the nose are not known but could be bacteria, viruses, allergens, or pollutants.

Infection of epithelial cells with common respiratory viruses such as rhinoviruses, respiratory syncytial virus, and influenza can induce the production of several cytokines (Elias, Zheng et al. 1994) (Noah and Becker 1993; Subauste, Jacoby et al. 1995). Studies with rhinovirus show that this response does not depend on any overt cytotoxic actions of the virus. Production of IL-8 could recruit neutrophils and some types of T-lymphocytes into the sinus mucosa, whereas RANTES is chemotactic for eosinophils. Eosinophil survival in the sinus mucosa would be supported by increased production of GM-CSF, which also primes these cells for enhanced responses to activating stimuli. Once activated, eosinophil products can alter epithelial ion transport processes and induce ciliostasis. On the basis of these observations, it is attractive to hypothesise that inflammation influenced by changes in epithelial function could in some manner
facilitate bacterial colonisation. This process could be influenced by production of nitric oxide, which is generated in large quantities by the nasal epithelium. Several bacterial products can also affect both epithelial function and cytokine production and could further extend the cycle of inflammation. Eventually this inflammatory cycle could become self-perpetuating and lead to long term epithelial thickening and goblet cell hyperplasia.

1.7 Sinus Disease

Chronic rhinosinusitis represents an ongoing inflammatory process for which inciting agents have been difficult to identify or prove. Chronic rhinosinusitis with or without nasal polyposis is characterised by inflammatory thickening and polypoid changes in the sinus mucosa, goblet cell hyperplasia, sub-epithelial fibrosis, and persistent inflammation (Wellicome, Thornhill et al. 1990; Groves, Ross et al. 1992; Bochner, Klunk et al. 1995) (Van Nostrand and Goodman 1976). The histological hallmark is marked tissue eosinophilia (Wellicome, Thornhill et al. 1990; Groves, Ross et al. 1992; Bochner, Klunk et al. 1995; Groves, Allen et al. 1995). The majority of eosinophils express the activation marker EG2, a phenotype associated with degranulation and other signs of activation, such as cytokine and mediator production. Nasal polyp biopsies probed for various cytokine mRNA by in situ hybridisation revealed a marked increase in inflammatory cells expressing mRNA for GM-CSF, IL-3 and TNF-alpha (Denburg, Gauldie et al. 1991; Groves, Allen et al. 1995; Hamilos, Leung et
These cytokines are known to promote eosinophil accumulation through the up-regulation of endothelial cell adhesion molecules, including vascular cell adhesion molecule-1, and to cause eosinophil activation and prolonged survival (GM-CSF, IL-3). Activated eosinophils in nasal polyps produce GM-CSF and TNF- alpha mRNA and eosinophils per se account to some extent for the presence of these cytokines in nasal polyp tissues. However, the driving force for eosinophil accumulation in chronic rhinosinusitis remains unknown. Various cellular elements such as constitutive cells (epithelial cells, fibroblasts, and mast cells) and monocytes produce cytokines within nasal polyps (Dolovich, Ohtoshi et al. 1990; Marini, Vittori et al. 1992; Finotto, Ohno et al. 1994). Epithelial and fibroblast explants produce abundant quantities of GM-CSF, IL-6 and IL-8 (Denburg, Gauldie et al. 1991; Marini, Vittori et al. 1992). These studies suggest that the epithelium and constitutive cells contribute significantly to chronic eosinophilic inflammation, possibly producing a vicious cycle in which eosinophil infiltration is triggered by abnormalities within the epithelium and perpetuated further by the damaging effects of eosinophil-derived proteins and mediators on the epithelium.

A role for T lymphocytes and T helper-2 cytokines in the development of chronic rhinosinusitis and nasal polyposis has not been clearly defined. Most studies have indicated that approximately 40% of patients with chronic hyperplastic rhinosinusitis with nasal polyposis have associated allergies (Slavin 1988; Hamilos, Leung et al. 1993; Hamilos, Leung et al. 1995). Nasal polyps from these patients show increased numbers of CD4+ T lymphocytes and increased numbers of inflammatory cells positive for IL-4 and IL-5 mRNA.
(Slavin 1988; Hamilos, Leung et al. 1993; Hamilos, Leung et al. 1995). The features of these polyps, combined with eosinophilia, mimic the late phase of allergic inflammation in the nasal mucosa (Durham, Ying et al. 1992). Recent work shows the presence of CD4+ lymphocytes, mainly in polyps, in the mucosa of patients with recurrent rhinosinusitis (Igarashi, Goldrich et al. 1995; Moss, Scott et al. 1996). However studies of polyps from non-allergic subjects have shown that the number of CD3+ T Lymphocytes and the number of IL-4 or IL-5 mRNA+ cells are not increased (Hamilos, Leung et al. 1993; Hamilos, Leung et al. 1995). Because eosinophilia is a prominent feature of both allergic and non-allergic nasal polyposis, these data suggest that a non-allergic mechanism exists for eosinophilia in chronic rhinosinusitis.

1.7.1 The Genetic Factors Associated with Sinus Disease

Although chronic sinus disease has been observed in multiple family members, the role of genetic factors in rhinosinusitis remains unclear. Two well-defined genetic disorders, cystic fibrosis and primary cilia dyskinesia (Kartagener's syndrome) are associated with persistent sinus disease. Cloning of the gene responsible for cystic fibrosis has provided new insights into electrolyte transport in respiratory epithelia. The cystic fibrosis transmembrane conductance regulator (CFTR) functions as a cyclic adenosine monophosphate activated chloride channel and as a regulator of two separate channels: one that conducts
chloride and one that conducts sodium. Mutations in the CFTR gene can affect one or both functions.

In patients with a classic form of cystic fibrosis, both functions are disrupted. Interestingly, some genetic defects cause subtle alterations in CFTR function. The resulting phenotype in patients carrying these mild mutations is attenuated; only one or two features of the disease are observed. Indeed, persons who have chronic sinus disease and elevated sweat chloride concentrations but no other symptoms of cystic fibrosis have been reported to carry mutations in each CFTR gene. Together these observations suggest that defects in CFTR and/or one of the proteins that interact with CFTR may account for a fraction of patients with persistent sinus disease.

Genetic tools are available to analyse the entire CFTR gene in select groups of patients. Screening of patients with sinus disease recalcitrant to medical and surgical therapy may provide insights into the frequency of this association. Future studies could involve other genes that encode components of apical membrane electrolyte transport in sinus epithelia.
1.7.2 The Environmental Factors Associated with Sinus Disease

No convincing evidence exists to support the role of environmental pollutants and toxicants in causing or prolonging rhinosinusitis. However, whereas most studies on this subject have focused on the potential carcinogenic effects of toxicants on the upper aero-digestive tract, a scant few have addressed their potential for causing sub-acute and chronic inflammatory disorders of the nasal passages. For example, ozone is known to cause increased respiratory symptoms by compromising the host defence mechanism and by disrupting cellular membranes through its powerful oxidising properties. In some cases an inhaled toxicant (such as CO$_2$, NO$_2$ and SO$_2$) is selectively absorbed by the nasal mucosa, giving rise to a concentrated effect of irritation and/or inflammation. This cellular damage can initiate a series of cascading events in the inflammatory process such as the release of inflammatory mediators, causing vascular extravasation of inflammatory cells and tissue oedema. Such events lead to nasal mucosal swelling, decreased air flow and the possibility of ostiomeatal complex obstruction.

Certain occupations may expose workers to more occupational hazards than others. These occupations include woodworking and carpentry, dye, paint, and solvent manufacturing, leather tanning, oil and gas distilleries, chemical plants, and hazardous waste disposal units. Host susceptibility may influence the inflammatory reaction to toxicant exposure, perhaps on a genetic basis. Interaction with or sensitisation by a pre-existing nasal condition such as allergic or vasomotor rhinitis may also potentiate the inflammatory reaction by an
environmental toxicant. A ‘susceptibility/exposure index’ would relate the factor of host susceptibility to the level and duration of exposure, an important epidemiologic evaluation. This type of potentiation is illustrated in studies of IgE responses induced by inhalation of diesel fuel particulates (Diaz Sanchez, Dotson et al. 1994).

1.8 Conditions Associated with Sinus Disease

1.8.1 Nasal Polyps and Rhinosinusitis

Nasal polyps represent the ultimate manifestation of chronic inflammation. Nasal polyps most commonly arise from the lateral wall of the nose, although the anterior ethmoids and other sinuses may also be the primary site of origin. This inflammatory entity differs distinctly from the normal nasal mucosa and is characterised histologically by (1) cystically dilated, inspissated mucous glands that are completely different than the seromucinous glands of the inferior or middle turbinates, (2) a large influx of inflammatory cells, with the eosinophil predominant; and (3) dedifferentiation of the epithelium, including basal cell hyperplasia, goblet cell hyperplasia, and squamous metaplasia.

Nasal polyposis is commonly associated with cystic fibrosis, but nasal polyps occur most frequently in chronic rhinosinusitis and occur universally in association with aspirin sensitivity. Inflammation in the polyp can cause dedifferentiation of the polyp epithelium, possibly leading to defective migration.
of the CFTR protein into the cell membrane (Waltner, Church et al. 1987). This phenomenon could alter sodium absorption and chloride secretion, which might lead to water retention - the pathophysiologic hallmarks of nasal polyposis. In non-CF nasal polyps, a defect in the apical sodium channel also exists, which is characterised by an increase in sodium absorption. This increased sodium absorption can be abrogated by amiloride, which might represent a new direction in the treatment of recurrent nasal polyposis. Thus, it is possible that polyps form because of inflammatory changes which result in abnormal electrolyte fluxes in the nasal epithelium.

1.8.2 Asthma and Rhinosinusitis

Rhinosinusitis frequently complicates asthma, and medical and/or surgical therapy for underlying rhinosinusitis can improve asthma. In addition, in some patients with chronic cough who are thought to have asthma, the cough is probably the result of rhinosinusitis (secondary to the associated postnasal drip). When the rhinosinusitis is treated, the cough is eliminated or significantly diminished. In a summary of experience with rhinosinusitis and its relationship to asthma, Adinoff and Cummings noted that active sinus intervention improved asthma, and also noted a trend (though not significant) toward better results of pulmonary function testing and reduced methacholine reactivity in the actively treated patients compared with the patients treated with placebo (Adinoff and Cummings 1989). In 1984 Rachelefsky et al, studied a non randomised group of
48 children with a mean age of 8.2 years who had moderate to severe asthma and almost daily wheezing for at least 7 months (Rachelefsky, Katz et al. 1984). With pharmacologic or surgical intervention for associated rhinosinusitis, 80% were able to discontinue their asthma medicine, 80% had normal findings on x ray films, and the majority of patients had normalised pulmonary function and could stop taking their asthma medications (Rachelefsky, Katz et al. 1984). Continued follow-up in these patients revealed that their asthma recurred when rhinosinusitis subsequently developed (Rachelefsky, Katz et al. 1984).

Appropriate surgical intervention for medically resistant rhinosinusitis has also been shown to benefit patients with coexistent asthma. This is also true both for bilateral intranasal sphenoidectomy and functional endoscopic sinus surgery in children and adults. The mechanism for rhinosinusitis-induced asthma is not known but several possibilities have been suggested, including damage from the eosinophil, a cell found in both sites, inflammation from mediators produced by the sinus mucosa and vagal neural reflexes.

1.8.3 Rhinitis and Rhinosinusitis

The division of chronic rhinosinusitis into allergy associated and non-allergy associated is primarily based on its association with allergic rhinitis, that is, the clinical symptomatology and the results of skin testing and/or serum-specific immunoglobulin (IgE) to the allergens producing the symptoms. Many studies have shown that the composition of the inflammatory substrate in chronic
rhinosinusitis is largely similar to that seen in allergic rhinitis and the late phase response to antigen challenge (Durham, Ying et al. 1992; Varney, Jacobson et al. 1992). Mononuclear cells consisting of T and B lymphocytes and activated eosinophils are prominent in the sinus mucosa of patients with chronic rhinosinusitis (Harlin, Ansel et al. 1988). Rachelefsky et al and Shapiro have described an increased incidence of rhinosinusitis defined by history, physical examination, and imaging criteria in children referred to the allergist-immunologist for evaluation of rhinitis (Rachelefsky, Katz et al. 1984; Shapiro 1985). In addition, skin testing proved that a large proportion of children in both groups were atopic. This supports a connection between allergic rhinitis and sinus disease. Among a group of children with recalcitrant rhinosinusitis who were evaluated for allergy and immunodeficiency, approximately 50% were atopic, again supporting the connection between allergic rhinitis and rhinosinusitis. In a sample of patients undergoing surgery for chronic sinus disease, only the group with extensive disease exhibited an association between chronic rhinosinusitis and allergy (Rachelefsky, Katz et al. 1984; Shapiro 1985).

1.9 Role of Infectious Agents in Acute Rhinosinusitis

Radiological evidence of rhinosinusitis is frequently found in patients who have a viral associated common cold (Gwaltney, Phillips et al. 1994). Viral rhinosinusitis and infundibulitis may be important predisposing factors for the development of rhinosinusitis in an estimated 0.5% to 2.5% of adult patients with
upper respiratory infections in whom secondary acute bacterial rhinosinusitis develops (Berg, Carenfelt et al. 1986). *Streptococcus pneumoniae* (*S. pneumoniae*) and *Haemophilus influenzae* (*H. influenzae*) cause more than half of the cases of acute bacterial rhinosinusitis in adults. Other streptococcal species, *Moraxella catarrhalis* (*M. catarrhalis*), *Staphylococcus aureus* (*S. aureus*) and mixtures of anaerobic bacteria also each cause a small proportion of cases. The role of bacterial infection in chronic sinus disease is less well defined. The role of normal flora in the genesis of pathologic sinus infection is poorly understood. Many of the same organisms are cultured from purulent secretions in patients with chronic rhinosinusitis with *H. influenzae* also being isolated (Klossek, Dubreuil et al. 1998). A further study of patients undergoing endoscopic sinus surgery for chronic rhinosinusitis failed to isolate significant amounts of 'pathogenic organisms', the authors postulating that chronic rhinosinusitis was not a bacterial disease but rather the result of chronic inflammation produced by a previous bacterial infection (Rontal, Bernstein et al. 1999). An additional finding was of a transient bacteraemia during surgery in 7% of cases, consistent with organisms from the operative site (Rontal, Bernstein et al. 1999).

1.9.1 Allergic Fungal Sinusitis

Fungal pathogens have continued to attract interest in the causation of rhinosinusitis. Allergic fungal sinusitis (AFS) is thought to involve a chronic non-invasive autoimmune reaction to fungal elements in immunocompetent patients.
Patients typically have a combination of asthma, atopy, nasal polyposis, and chronic refractory sinusitis unresponsive to medical management. There are often elevated levels of specific IgE and IgG to the responsible fungal antigen. Extensive sinus involvement and bony erosion are frequent, with the potential for extension to the orbit producing ophthalmic complications (Carter, Graham et al. 1999; Marple, Gibbs et al. 1999). Allergic fungal sinusitis is caused by a variety of fungi, and the mechanisms that initiate or perpetuate the disease remain unknown. Various serum markers, including total and fungal-specific IgE and serum mucin eosinophilic cationic protein have been investigated to predict disease recurrence with no conclusive correlation. Left untreated, allergic fungal sinusitis is associated with bone reabsorption and remodelling and may markedly distort the face. Possible progressive epithelial damage resulting from eosinophil influx associated with the presence of fungi and apparent allergic responses may initiate a self-perpetuating, cyclical inflammatory/immunologic response only temporarily suppressed by oral steroids. Current treatment requires corticosteroids and surgery, usually functional endoscopic sinus surgery, although we still do not know the optimal treatment program. The disease often progresses into the development of a polypoid sinus mucosa.
1.10 Pharmacotherapy

1.10.1 Antimicrobial Therapy for Rhinosinusitis in Adults

Antimicrobial therapy has been shown to reduce or eliminate bacteria in the maxillary sinus and to improve symptoms in acute community-acquired bacterial rhinosinusitis (Gwaltney 1996). However, because most cases of acute community-acquired rhinosinusitis have a viral component that also affects the clinical course (Gwaltney, Phillips et al. 1994), and because acute rhinosinusitis is usually a self-limited disease, defining the relative efficacy of various agents and regimens may be difficult. For patients with acute bacterial rhinosinusitis for whom organisms have been rather consistently identified, the antimicrobial selected should be effective against strains of *S. pneumoniae* with intermediate penicillin resistance, as well as beta lactamase producing strains of *H. influenzae* and *M. catarrhalis*. In chronic rhinosinusitis, bacteria are less consistently obtained from the sinuses and the complexity of the flora increases, with a shift toward multiple organisms, gram-negative organisms, *S. aureus*, and more antimicrobial resistance. In these patients antibiotic therapy should usually be based on culture and sensitivity results. In patients who have continued disease after sinus surgery, chronic *S. aureus* and *Pseudomonas aeruginosa* infections are a special problem. Often sinus CT (computed tomographic) examination of these patients shows osteitis as evidence of bone involvement as part of the disease process. Several factors affect the optimal duration of therapy for rhinosinusitis.
Ten to 14 days of antimicrobial therapy has repeatedly been shown to eradicate or markedly reduce bacteria in the sinus cavity in patients with acute rhinosinusitis and is the generally accepted duration of treatment (Gwaltney, Scheld et al. 1992). In patients with persistent or chronic sinus disease, there is little published information on which to base duration of treatment. Longer and repeated courses of antimicrobial therapy are usually given to these patients, but clinical response is often unsatisfactory.

1.10.2 Corticosteroids

The use of corticosteroids for rhinosinusitis remains controversial. The properties of corticosteroids that make them potentially useful include their ability to reduce mucosal swelling and thereby facilitate drainage of the sinuses, the reduction in tissue eosinophilia accompanying corticosteroid administration, and the proven efficacy of corticosteroids in shrinking nasal polyps. Moreover, topical corticosteroids have been proved effective and are widely accepted in treating allergic and non-allergic rhinitis. Thus, many clinicians believe that corticosteroids are essential in the treatment of all forms of rhinosinusitis; however, only scant controlled studies support this conjecture.

The few relevant studies of the use of corticosteroids in the treatment of rhinosinusitis can be summarised briefly. One study compared groups treated with either topical dexamethasone alone or dexamethasone combined with topical neomycin and patients treated with placebo. The patients in the dexamethasone
groups improved significantly compared with the patients who received a placebo (Sykes, Wilson et al. 1986). A second study showed equivalent results when resolution of abnormal radiographs in groups treated with antibiotics were compared with subjects treated with topical beclomethasone (Businco, Fiore et al. 1981). In a third study, adding topical flunisolide to antibiotic treatments improved global evaluations and possibly reduced exacerbations (Meltzer, Orgel et al. 1993). However, none of these studies unequivocally proves efficacy or justifies general advocacy of topical corticosteroids in rhinosinusitis. To determine the outcomes of the medical management of rhinosinusitis, large studies should compare antibiotic and topical corticosteroid treatment for patients with either chronic or recurrent symptoms.

Glucocorticoids are clearly the most potent agents available to relieve the symptoms of allergic rhinosinusitis. The molecular substrate of their action is to inhibit or decrease the allergen induced synthesis of Th2 cytokines (IL-4, IL-13) and MCP-3 and MCP-4 chemokines, as well as cells expressing receptors for IL-4, IL-5, and GM-CSF, particularly eosinophils (Al Ghamdi, Ghaffar et al. 1997; Christodouloupolos, Wright et al. 1999; Kondo, Nachtigal et al. 1999). An interesting finding is that corticosteroid treatment in allergic rhinosinusitis is associated with an up-regulation of Th1-type cytokines, particularly IFN-gamma, and IL-12. This suggests that one of the biologic effects of corticosteroids is to shift the T-cell differentiation from a Th2 to a predominant Th1 response.
1.10.3 Decongestants

Topical and systemic decongestant therapies have been recommended to adjunctively treat acute and chronic rhinosinusitis. Decongestants are vasoconstrictor agents that reduce the thickness of the nasal mucosa. They act on alpha adrenoreceptors or are involved in the release, re-uptake, or degradation of noradrenalin. No controlled studies document the beneficial effects of topical or systemic vasoconstrictors in rhinosinusitis.

1.10.4 Anti-leukotrienes

With the increasing recognition of the role of leukotrienes as important inflammatory mediators, novel therapies targeting leukotrienes have been developed. Pharmacologically, there are two ways of inhibiting the actions of leukotrienes: blocking the 5-lipoxygenase enzyme or its activating protein 5-lipoxygenase activating protein and thus inhibiting synthesis, or by specifically blocking the interaction of cysteinyi leukotrienes (CysLTs) with their receptors (CysLTR1) using competitive antagonists (Holgate, Bradding et al. 1996). Both of these types of compounds have been shown to inhibit eosinophil survival by inducing apoptosis (Lee, Robertson et al. 2000). Pathologically, cysteinyi leukotrienes contribute to changing airway function by contracting airway smooth muscle (Barnes, Piper et al. 1984), increasing vascular permeability (Drazen, Austen et al. 1980), stimulating mucous secretion and decreasing mucociliary
clearance (Marom, Shelhamer et al. 1982), and are capable of recruiting eosinophils into the airways (Munoz, Douglas et al. 1997). Studies have shown that cysteinyl leukotriene receptor antagonists such as Montelukast, through inhibition of leukotriene signalling via the CysLTR1, acts to down-regulated progenitor commitment to the eosinophil lineage and/or inhibit the maturation of existing, committed progenitors. Thus, Montelukast in addition to effects on mature eosinophils may exert an anti-inflammatory effect by inhibiting the differentiation of eosinophils (Crawford, Saito et al. 2002; Saito, Matsumoto et al. 2002).

1.10.5 Antihistamines

The use of antihistamines is a common treatment for atopic diseases (Simons and Simons 1994). It has been proposed that the new generation of antihistamines may regulate certain effector functions of eosinophils, which include the inhibition of eosinophil chemotaxis and recruitment into the airways after allergen exposure (Walsh 1994). One of these new antihistamines is Loratadine/Desloratadine. In recent studies, Desloratadine has been shown to act as a suppressor of differentiation of Eosinophil progenitors in vivo through mechanisms that may directly or indirectly relate to antagonism of H1 histamine receptors (Cyr, Baatjes et al. 2002).
1.11 Radiographic Imaging

CT is the radiographic modality of choice to examine the nasal cavity and paranasal sinuses. The coronal plane best simulates the endoscopic view, and slices taken at intervals of 3mm or less, provide the most accurate representation of the regional anatomy. Because CT is primarily performed to provide anatomic information, it should be performed when the patient's mucosal inflammation is under optimal control (Zinreich, Kennedy et al. 1987). Inflammatory mucosa obscures the fine bony detail, resulting in a more limited evaluation. Thus, the optimal information is achieved when the CT study is performed 4-6 weeks after the initiation of medical therapy.

1.12 Endoscopy

Nasal endoscopy has greatly improved our ability to diagnose nasal and sinus disease and assess the response to medical and surgical therapies. Patients with recalcitrant complaints referable to the nose or paranasal sinus are particularly good candidates for this procedure. Flexible or rigid endoscopy is typically performed after a careful history and physical examination. The technique of nasal endoscopy is well described (Kennedy, Zinreich et al. 1985).
The absolute indications for surgery in rhinosinusitis include the presence of brain abscess secondary to rhinosinusitis and malignant tumours within the sinus. However, the relative indications for surgery are more difficult to define. Chronic rhinosinusitis is the most common 'relative indication' for surgery for rhinosinusitis. In general, a thorough trial of medical therapy should be performed before surgical intervention in patients with chronic rhinosinusitis. Such medical therapy may include antibiotics, mucolytics, decongestants, steroids, and/or immunotherapy. However, a wide variety of factors will modify the timing of surgical therapy as well as the type and extent of surgical intervention. In the majority of patients who have chronic rhinosinusitis refractory to an adequate trial of medical therapy, surgery decreases symptoms and improves the quality of life.

Classic rhinosinusitis involves the maxillary sinus and the anterior ethmoid sinuses with the common symptoms of facial pain, rhinorrhoea, postnasal drip, anosmia, and dental pain. In this circumstance surgery is directed to the middle meatus and the infundibulum. The posterior sinuses composed of the posterior ethmoids and sphenoid, may present with a more complex set of complaints and change the progression to surgery. For example, sphenoid sinusitis may present with a higher prevalence of orbital problems, such as optic neuritis and visual loss. The frontal sinus may also provide its own set of medical and surgical challenges. A patient with forehead pain or oedema may need an immediate trial of oral and or intravenous antibiotics, with surgical intervention if
symptoms do not resolve. The reason for this aggressiveness in frontal sinusitis is to avoid intracranial infection or bony infection such as osteomyelitis.

The goals of surgery are to provide adequate sinus drainage and thereby reduce inflammation, and to provide access to the sinuses for appropriate biopsies, irrigation, and cultures. There is a strong trend toward meticulous preservation of the mucoperiosteum, not just within the maxillary sinus, but also within the ethmoid and sphenoid sinuses. This is based on the observation that regenerating respiratory epithelium has a markedly diminished ciliary density (Kennedy 1997). Surgery may also be used to aerate the sinuses, to remove foreign bodies and polyps, and to prevent or reduce the morbidity of orbital bony and intracranial complications. Revision surgery for residual or recurrent disease demands special attention. Noses and sinuses that have been previously manipulated may present unique diagnostic and surgical challenges. First, significant areas of scarring and bony re-growth that are not normally seen may be present. Second, a higher predominance of gram-negative and anaerobic organisms may be present. Indications for surgery in these patients may depend on a different natural history of the disease or a series of missed diagnoses and surgical problems.
1.14 Glycodelin Background

1.14.1 Lipocalins

The lipocalin superfamily consists of small extracellular ligand-carrying proteins with a common beta-sheet dominated three-dimensional structure. Lipocalins have well documented roles in cell proliferation and differentiation (Flower 1994). The three-dimensional structure of lipocalins is typically characterised by an eight-stranded anti-parallel beta-barrel (reminiscent of the calyx of a flower) that forms an internal pocket for binding small hydrophobic ligands (Flower 1996).

Conserved sequence motifs and higher order structural features place glycodelin into this family of proteins. Retinoids constitute potential ligands for several of the lipocalins, including retinol binding protein (RBP), beta-lactoglobulin, alpha-1-microglobulin, and purpurin, as well as for glycodelin (Flower 1996). In addition to binding small hydrophobic ligands internally, lipocalins are also known to engage specific cell surface receptors. The receptor for retinol-binding protein is best characterised. Bavik et al. cloned the cDNA of a 63-kDa RBP-binding membrane protein on bovine retinal pigment epithelial cells (Bavik, Levy et al. 1993). A specific receptor for odorant binding protein another lipocalin has also been identified on bovine nasal mucosa (Boudjelal, Sivaprasadara et al. 1996). Receptors for alpha-1-microglobulin have been
identified on mouse splenocytes, and receptors for beta-lactoglobulin found on calf intestine microvilli (Papiz, Sawyer et al. 1986; Babiker-Mohamed, Akerstrom et al. 1990).

The acute phase response includes a defined set of changes in plasma protein levels during inflammatory stimulation, induced mainly by the actions of cytokines, such as interleukin (IL)-6 on the synthesis of these plasma proteins. A subset of lipocalins, notably alpha-1-acid glycoprotein, alpha-1-microglobulin, and glycodelin, has been reported to be acute phase proteins and to exert significant immunomodulatory effects in vitro (Logdberg and Wester 2000). All three are encoded from the q32-34 region of chromosome 9 in the human genome, together with at least four other lipocalins (neutrophil gelatinase-associated lipocalin, complement factor gamma-subunit, tear prealbumin, and prostaglandin D synthase) that also may have anti-inflammatory and/or antimicrobial activity. It is likely that these proteins have evolved to be an integrated part of the body’s defence system as part of the extended cytokine network. Its members exert a regulatory, dampening influence on the inflammatory cascade, thereby protecting against tissue damage from excessive inflammation. The name ‘immunocalins’ has been assigned to this subfamily of lipocalins (Logdberg and Wester 2000). The fact that most major mammalian allergens are lipocalins may reflect this connection of lipocalins with the immune system.

Lipocalins have been used extensively as biochemical markers of disease. The clinical indications relate to almost any field of medicine, such as inflammatory disease, cancer, lipid disorders, liver and kidney function. Some of the more well-known lipocalins that have been used as markers of disease are
alpha-1-microglobulin (Fernandez-Luna, Mendez et al. 1989), alpha-1-glycoprotein (Louis, Belaiche et al. 1997) apolipoprotein D (Lea, Kvinnsland et al. 1987), and retinol binding protein (Corso, Serricchio et al. 1999).

1.14.2 Glycodelin

Glycodelin has been a subject of major interest recently. Glycodelin, also known as placental protein (PP 14) (Bohn, Kraus et al. 1982) or progesterone-associated endometrial protein (Kamarainen, Julkunen et al. 1991), is a glycoprotein with 17.5% carbohydrate. It is a 28kDa glycoprotein, containing 180 amino acids, encoded via a single gene located to chromosomal region 9q34 (Van Cong, Vaisse et al. 1991). Glycodelin was originally named after the placental tissue it was thought to derive from, but it was later shown to originate from associated endometrial tissue (Bohn, Kraus et al. 1982; Julkunen, Koistinen et al. 1986).

1.14.3 Glycodelin and the Uterine Tract

Glycodelin is the principle secretory phase product of endometrial epithelial cells. It increases during the first trimester of pregnancy and during the secretory phase of a normal menstrual cycle (Bohn, Kraus et al. 1982). The level of glycodelin is lowest in the follicular phase of the cycle. Glycodelin is secreted into the uterine cavity and has been recovered in uterine flushings, rising 6 days
after the LH peak (Li, Ling et al. 1993). On post-ovulatory day 4-5, significant
glycodelin secretion is associated with an increase in ovarian progesterone
secretion. This rise in glycodelin, during ovulatory cycles, is probably due to
progesterone (Julkunen, Koistinen et al. 1986). Glycodelin has been implicated,
among other proteins, as a facilitator of implantation (Clark, Oehninger et al.
1996). During the pregnancy that ensues, glycodelin levels in both decidualised
endometrium and amniotic fluid peak at 10-18 weeks (Julkunen, Rutanen et al.
1985).

1.14.4 Glycodelin Outside the Uterine Tract

Glycodelin has been identified in normal and malignant glandular
epithelium outside of the reproductive tract including breast tissue, hidradenoma,
parabronchial glands, sweat glands and pancreatic cystadenoma (Kamarainen,
Seppala et al. 1997). Glycodelin is expressed in human endometrial carcinoma
cells, human ovarian adenocarcinoma cells, human cervical epitheloid carcinoma
cells and human endometrial epithelial cells (Poddar, Kim et al. 1998). It is also
raised in plasma and tissues of patients with gynaecological malignancies
(Horowitz, Cho et al. 2001). Glycodelin is also reported in haematopoietic cells
of the megakaryocytic lineage (Morrow, Xiong et al. 1994).
1.14.5 Glycodelin and the Immune System

Reports have described several glycodelin-mediated immunoinhibitory functions, including inhibition of T-cell proliferation in allogeneic two-way mixed lymphocyte reactions (Pockley, Barrett et al. 1989; Morrow, Xiong et al. 1994), inhibition of the mitogenic responsiveness of lymphocytes to phytohaemagglutinin (Bolton, Pockley et al. 1987), inhibition of IL-1 beta and IL-2 cytokine production by immune cells (Pockley and Bolton 1990), and inhibition of natural killer cell function. (Okamoto, Uchida et al. 1991). Glycodelin has been shown to inhibit lymphocyte proliferation (Bolton, Pockley et al. 1987). Subsequent studies have noted an inhibitory effect of glycodelin on the synthesis of interleukins 1 and 2 and soluble interleukin 2 receptors by peripheral blood mononuclear cells, suggesting potential explanations for the anti-proliferative effect of glycodelin on lymphoid cells (Pockley and Bolton 1988; Pockley and Bolton 1989; Pockley and Bolton 1990). Studies by Okamoto et al. have shown that the addition of as little as 5μg of glycodelin to large granular lymphocytes suppressed their cytolytic action against K562 target cells without affecting the binding of lymphocytes to K562 cells (Okamoto, Uchida et al. 1991). In these experiments, extracts of human decidual tissue were added to mixed lymphocyte cultures (MLCs) and a linear relationship was observed between the quantity of glycodelin present and the inhibition of lymphocyte proliferation observed (Okamoto, Uchida et al. 1991). Moreover, anti-glycodelin antibodies added to the mixed lymphocyte cultures inhibited the anti-proliferative effect, verifying the functional link between glycodelin and anti-proliferative activity (Okamoto, Uchida et al. 1991).
Glycodelin dose-dependently increases IL-6 production by epithelial cells prepared from secretory endometrium, with stimulated levels reaching twice the basal values (Laird, Tuckerman et al. 1994).

The immunosuppressive role of glycodelin might be involved in the mechanisms underlying the protection of the human embryo or foetus from the maternal immune response (Clark, Oehninger et al. 1996). Studies have shown that umbilical cord endothelial cells have a high capacity for taking up and accumulating glycodelin from medium in which they are grown (Zhou, Ramachandran et al. 2000). The current literature suggests that glycodelin may have at least two major functions in pregnancy; (1) glycodelin may inhibit mammalian oocyte fertilisation by virtue of its carbohydrate residues, and (2) glycodelin may inhibit natural killer cell-mediated cytolysis of target cells (Morrow, Xiong et al. 1994; Morris, Dell et al. 1996).

The presence of mRNA encoding this potent immunoregulatory cytokine in normal megakaryocytes and platelets has been demonstrated (Morrow, Xiong et al. 1994). Thus a potentially significant connection between the coagulation and immune system may exist.
1.14.6 Glycodelin and T-Cell Inhibition

The appearance of pre G0/G1 cell population in peripheral mononuclear cells after treatment with glycodelin indicates that glycodelin induces apoptosis (Mukhopadhyay, Sundereshan et al. 2001). Similar studies in T-cell line cultures e.g. Jurket cells, MOLT-4 cells produced the same results (Mukhopadhyay, Sundereshan et al. 2001). Glycodelin induced apoptosis in both of the T cell lines. These results were confirmed by DNA fragmentation studies, and caspase 3 assays (Mukhopadhyay, Sundereshan et al. 2001).

Data suggests a direct interaction between Glycodelin and T-cells, placing this protein into the special class of immunoinhibitory proteins with direct T-cell effects (e.g. TGF beta, and IL-16) (Rachmilewitz, Riely et al. 1999). In addition to inhibiting lectin and allo-stimulated T-cell proliferation within peripheral blood mononuclear cells, glycodelin also inhibits T-cell proliferation triggered by other agents, including soluble anti-CD3 and the superantigen staphylococcal enterotoxin B (Rachmilewitz, Riely et al. 1999). It inhibits lectin-stimulated blast transformation of both CD4+ and CD8+ T-cell subsets within peripheral blood mononuclear cells to a similar degree (Rachmilewitz, Riely et al. 1999). Glycodelin inhibits the proliferation of T-cells and appears to abrogate T cell receptor mediated signalling without affecting distal signalling mechanisms (Rachmilewitz, Riely et al. 1999). Pregnancy has been associated with a number of alterations in the maternal immune status. T-cell mediated immunity is
generally decreased and there is evidence for a relative increase in Th2-associated immunity. It has been speculated that glycodelin in serum, present at immunomodulatory levels, may contribute to these immunological phenomena via its effects on T-cells (Rachmilewitz, Riely et al. 1999).

Rachmilewitz et al. showed that glycodelin mediates its anti-inflammatory activity by elevating the T-cell activation threshold, thereby rendering T-cells less sensitive to stimulation (Rachmilewitz, Riely et al. 2001). They went on to show that glycodelin targets early events during T cell receptor signal transduction and facilitates the dephosphorylation of T cell receptor-induced phosphoproteins, and in so doing, operates via a unique immunoregulatory mechanism, 'rheostatically' elevating T-cell receptor activation thresholds (Rachmilewitz, Riely et al. 2001). The tyrosine phosphatase receptor CD45 appears to be a critical mediator (Rachmilewitz, Borovsky et al. 2003). Glycodelin also translocates to antigen-presenting cell T- cell contact sites and its inhibitory activity depends upon its access to the triggered T cell receptor (Rachmilewitz, Borovsky et al. 2003). Thus, glycodelin negatively regulates T cell activation by diminishing T cell responses in the contact site at the time of T cell receptor triggering. This indicates that glycodelin may interfere with the organisation of the immune synapse (Rachmilewitz, Borovsky et al. 2002).
1.14.7 Glycodelin and B-Cells

Recent studies demonstrate that glycodelin has a negative role in B-cell antigen receptor-mediated activation of human B cells (Yaniv, Borovsky et al. 2003). However, while significant inhibition of B cell proliferation, IgM secretion and MHC (major histocompatibility class) up-regulation was observed in the presence of glycodelin, no inhibition of CD 69 and CD 86 was observed regardless of the extent of B-cell receptor (BCR) triggering (Yaniv, Borovsky et al. 2003). Thus, glycodelin seems to affect several B cell responses while not affecting others. Although it is not clear what the biological significance of this selective effect on the different B cell responses is, it implies that glycodelin may skew B cell response pattern and consequently the overall biological outcome (Yaniv, Borovsky et al. 2003). Glycodelin’s activity in B cells, in contrast to its effect on T cells, appears to be due to interference with late events in BCR signalling (Yaniv, Borovsky et al. 2003).
1.14.8 Glycodelin and Pregnancy

Glycodelin appears to have 'contraceptive properties' (Okamoto, Uchida et al. 1991). It inhibits, in a dose-dependent manner, the binding of human sperm to the zona pellucida (Oehninger, Coddington et al. 1995). The absence of glycodelin around ovulation may be important for successful fertilisation (Clark, Oehninger et al. 1996). Uterine flushings for glycodelin reveal increased glycodelin, in the uterine cavity, at the time of implantation (Li, Ling et al. 1993). These elevated glycodelin levels may protect the embryo, at the endometrial level, from natural killer cell destruction and facilitate the embryonic evasion of the maternal immune response (Julkunen, Rutanen et al. 1985). If conception ensues, glycodelin levels remain elevated.

1.14.9 Regulation of Glycodelin

The underlying mechanism regulating the synthesis of glycodelin has not been clarified. While hormones like progesterone might be important in regulating glycodelin production in reproductive tissues, its presence in non reproductive cells and tissues as well as its induction by phorbol myristate acetate (PMA) in K562 cells suggest that its synthesis may be regulated by other mediators (Morrow, Xiong et al. 1994). The induction of glycodelin protein gene expression in K562 cells by lysophosphatidic acid (LPA) has recently been reported (Ramachandran, Ramaswamy et al. 2002). This is a phospholipid that
mimics the action of phorbol myristate acetate. The action of phorbol myristate acetate on neutrophils leads to the activation of phospholipase D and the production of lysophosphatidic acid (Qi, Park et al. 1998). Furthermore, in vitro studies have suggested that regulation of glycodelin synthesis by progesterone might be complex as anti-progestins fail to prevent the induction of glycodelin (Taylor, Savouret et al. 1998).

1.14.10 Oxidation and Cancer

The involvement of an oxidative process in cancer has been long suspected. Oxidants are reported to induce a number of growth factors that are related to cell growth and angiogenesis (Khanna, Roy et al. 2001). A wide range of metabolic pathways that include activation of protein kinases, PPAR gamma (peroxisome proliferator-activated receptor gamma), cyclins, and other transcription factors has been implicated in the stimulation of cell growth by oxidants (Nagy, Tontonoz et al. 1998; Rahman, Anwar et al. 2000) (Brar, Kennedy et al. 2002). Oxidants induce a number of oncogenes associated with the cancer process (Kovacic and Jacintho 2001). Oxidants also induce a number of growth factors that are related to cell growth and angiogenesis. Induction of VEGF (vascular endothelial growth factor) by oxidants has been reported recently (Khanna, Roy et al. 2001). It has been hypothesised that that lysophosphatidic acid and oxidative stress might be involved in stimulating glycodelin production via the activation of protein kinase C, thus glycodelin may play a role in cancer.
development and progression by promoting angiogenesis (Ramachandran, Ramaswamy et al. 2002).

1.14.11 Glycodelin and Angiogenesis

Angiogenesis plays an important role in cancer promotion. Vascular endothelial growth factor (VEGF) is a potent angiogenic factor that might play a major role in the proliferation and migration of endothelial cells and neovascularisation (Ferrara 1995). Granulocyte-macrophage colony stimulating factor, interleukin-5, and other cytokines have been reported to induce the expression of VEGF (Horiuchi and Weller 1997; Kamarainen, Miettinen et al. 1998).

Glycodelin peptide can induce angiogenesis (in vitro) not only by enhancing migration and tube formation of endothelial cells directly but also by increasing the tyrosine kinase receptor Flt-1, in endothelial cells and increasing VEGF production in VEGF producing cells as a paracrine factor to induce angiogenesis (Song, Ramaswamy et al. 2001). This activity may be another important function of glycodelin in tumour development and embryogenesis (Song, Ramaswamy et al. 2001). A synthetic peptide mimic of glycodelin increases both the migration of human umbilical vein and arterial endothelial cells as well as capillary tube-like formation, both important components of angiogenesis (Song, Ramaswamy et al. 2001). Rapidly growing tumours and tissues may be producing glycodelin and thus promoting cell growth and the angiogenesis necessary to support this growth (Song, Ramaswamy et al. 2001).
Both benign and malignant gynaecological tumours produce elevated levels of plasma glycodelin and messenger RNA within their tissues (Horowitz, Cho et al. 2001). Using a polyclonal antiglycodelin peptide antibody (anti-Gp) generated against a synthetic glycodelin protein, it has been observed that levels of glycodelin, were increased in the plasma and blood vessels of the tumour in subjects with gynaecological malignancies (Horowitz, Cho et al. 2001). There are several studies showing increased glycodelin levels in endometrial, ovarian and breast cancers along with synovial sarcomas, pancreatic cystadenoma and trophoblastic tumours (Kamarainen, Leivo et al. 1996; Kamarainen, Halttunen et al. 1999). There are two lines of evidence suggesting that glycodelin is important in tumour development because of increased levels in the plasma of cancer subjects, increased localisation in the endothelium of the tumour blood vessels and in vitro inhibition of natural killer (NK) cell-mediated cytolysis (Okamoto, Uchida et al. 1991; Horowitz, Cho et al. 2001; Song, Ramaswamy et al. 2001). Transfection of glycodelin in MCF-7 breast carcinoma cells inhibits cell proliferation and induces apoptosis as well as differentiation (Kamarainen, Seppala et al. 1997; Kamarainen, Halttunen et al. 1999). This observation suggests a role for glycodelin in the growth regulation of glandular epithelium.

Studies with K562 human myelogenous leukaemia cell line, a known target for natural killer cells have shown that they have an inducible glycodelin gene (Horowitz, Cho et al. 2001). Hence, it is possible that plasma cells can play a crucial role in increasing glycodelin levels and promoting tumour survival. A
possible justification of increased plasma glycodelin levels in gynaecological cancers is that neoplastic cells are capable of producing glycodelin, which could give malignant cells a survival or growth advantage by suppressing NK cell activity (Horowitz, Cho et al. 2001). Alternatively cancer cells could release a tumour factor that either inhibits natural killer cell activity or induces glycodelin expression by surrounding cells. Since glycodelin has been shown to suppress NK cell activity, this may be a mechanism by which glycodelin can be utilised as a tumour marker (Horowitz, Cho et al. 2001). However, whether increased expression of glycodelin in tumours influences their capacity to escape immunosurveillance is still unclear.

1.14.13 Glycodelin in Bone Marrow

Glycodelin has previously been shown to be expressed in a megakaryocytic cell line (Morrow, Xiong et al. 1994). Its presence in human bone marrow cells has recently been described, along with the observation of a similar expression intensity of the antigen between healthy subjects and patients with myeloproliferative disorders (Zetterberg, Lundberg et al. 2003).
Glycodelin in the Respiratory Tract

Within the respiratory system, glycodelin expression has thus far been demonstrated in the bronchi in a very limited study examining expression patterns of glycodelin throughout the body (Seppala, Koistinen et al. 2001). Other lipocalins including retinoic binding protein and odorant binding proteins have been shown to be expressed in the nose (Logdberg and Wester 2000).

The presence of glycodelin expression in the nose and paranasal sinus mucosa has not been previously described. The hallmarks of inflammation within the nose and paranasal sinus are an inflammatory reaction, and the development of congested nasal mucosa with or without progression to nasal polyp formation. As glycodelin has been shown to have immunoinhibitory activities, along with angiogenic properties, and has been shown to play a role in inflammatory processes throughout the body, investigation of its role in inflammation of the paranasal sinuses is warranted.
CHAPTER 2

AIMS OF THIS THESIS
2.1 Aims of This Thesis

The aim of this study is to investigate and develop an understanding of the role of glycodelin, a novel immunomodulatory protein, in rhinosinusitis. We hypothesise that nasal inflammation leads to persistent nasal congestion and superinfection leading to the release of proteins called lipocalins specifically glycodelin. Glycodelin suppresses further T-cell activation and promotes angiogenesis leading to neovascularisation within the nasal mucosa. This can result in nasal mucosal inflammation and polyp formation. We set out to determine whether or not glycodelin is present in the nasal mucosa and in the serum of patients with and without symptoms of sinus disease.

We hypothesise that the production of glycodelin by peripheral white blood cells may prove a novel marker for nasal inflammation. A screening blood test, such as serum glycodelin estimation, which could indicate and differentiate between the different types of sinus disease (bacterial vs. non-bacterial), would be of great diagnostic benefit for health care professionals and promote cost-effective management. Novel therapeutic methods directed against glycodelin production by peripheral blood cells may play an important role in the management of chronic nasal disease and nasal polyposis. These experiments will enhance and extend our understanding of glycodelin’s role in the complex immune system dysfunction that characterises this disease.
The specific aims of this thesis are:

(1) To examine the gene expression of glycodelin in K562 cells (lymphoid precursor cell line), and the up regulation of this expression following exposure to various stimuli in vitro;

(2) To demonstrate the glycodelin gene expression in human peripheral white blood cells;

(3) To confirm glycodelin protein production (thus translation of the gene) by mononuclear cells of patients and normal controls;

(4) To confirm the presence of glycodelin in the nasal mucosa of patients with chronic rhinosinusitis and compare to normal subjects;

(5) To investigate glycodelin production in the serum of patients with chronic rhinosinusitis and nasal polyposis;

(6) To examine glycodelin protein levels in nasal washings and compare to serum levels in patients with reactive airway disease; and

(7) To examine the effect of various treatment modalities on glycodelin gene expression and glycodelin protein production in vitro.
CHAPTER 3

GLYCODELIN EXPRESSION IN VITRO
3.1 Introduction

Glycodelin has been identified in normal and malignant glandular epithelium outside of the reproductive tract including breast tissue, hidradenoma, parabronchial glands, sweat glands and pancreatic cystadenoma (Kamarainen, Seppala et al. 1997). It has also been reported that glycodelin is expressed in human endometrial carcinoma cells, human ovarian adenocarcinoma cells, human cervical epitheloid carcinoma cells and human endometrial epithelial cells (Poddar, Kim et al. 1998).

The K562 cell line is a progenitor cell line of lymphoid lineage with the ability to differentiate into monocytes and neutrophils under particular conditions. In vitro studies with the K562 human myelogenous leukaemia cell line have shown that these cells have an inducible glycodelin gene.

Allergen exposure is an important trigger of nasal inflammation in allergic rhinitis. Recently a systemic role in inflammatory disease has been suggested (Denburg, Sehmi et al. 2000; Togias 2000). In patients with allergic diseases, allergen provocation can activate a systemic response that provokes inflammatory cell production by the bone marrow (Denburg, Sehmi et al. 2000). Lymphoid cells may play a role in the systemic response to allergens. The effect of house dust mite antigen, a known common nasal allergen, on glycodelin expression in K562 cells has not previously been reported.
3.2 Aim

(1) To confirm the expression of glycodelin gene by K562 cells, and to confirm the promotion of glycodelin gene expression by phorbol myristate acetate (PMA), a plant mitogen and tumour promoter;

(2) To examine the effect of exposure to an allergen protein, house dust mite antigen on glycodelin gene expression in K562 cells.
3.3 Materials and Methods

3.3.1 Cell Culture

Materials

Growth media for K562 cells:

- RPMI 1640 (Mediatech, Herndon, VA, USA)  
  +L-glutamine 90%: 225ml

- Foetal Bovine Serum (FBS; Mediatech, Herndon, VA, USA) 10%:  
  25ml

- Penicillin/Streptomyacin 1%: 2.5ml

The above were combined under aseptic technique in the cell culture laboratory and stored at 4° Celsius.
Method

The human chronic myelogenous leukaemia cell line K562 (American Type Culture Collection (ATCC) Rockville, MD), was grown up to 75% confluence in RPMI 1640 medium with 10% foetal bovine serum. The cells were maintained at 37° C in a 5% CO₂ incubator.

3.3.2 Reverse Transcriptase- Polymerase Chain Reaction (RT-PCR) Analysis

Materials

For list of materials and detailed method see Appendix II (i).

Method

Total RNA was isolated from the cultured K562 cells using TRI reagent (Life Technologies, Gaithersburg, MD). Quality of RNA was checked, and 3.0μg was reverse transcribed. The product was PCR amplified using the following:

- 5'- AAGTTGGCAGGGACCTGGCCTC-3' (sense)

oligonucleotides for glycodeolin; and
• 5'-ACGGCACGGCTCTTCCATCTGTT-3' (antisense)

oligonucleotides for glycodelin.

Following this procedure, a product of 422 base pairs was obtained. Sample sizes and number of amplifications were optimised to produce measurements within a linear range. After an initial denaturation at 95°C for 1 minute, PCR was conducted as follows: denaturation at 95°C for 45 seconds, annealing at 60°C for 45 seconds, and extension at 72°C for 1 minute. This was repeated for 35 cycles followed by a final extension at 72°C for 10 minutes. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the internal control to substantiate equal amplification of the total RNA.

The GAPDH primer set was as follows:

• 5'-ACCACAGTCCATGCCATC-3' (sense); and

• 5'-ACGGCACGGCTCTTCCATCTGTT-3' (antisense).

This yielded a product of 451 base pairs. All PCR products were analysed on 2% agarose gel containing ethidium bromide.
3.3.3 Enzyme Linked Immunosorbant Assay (ELISA)

Materials

- The primary antibody used for glycodelin was anti-glycodelin antibody generated in chicken and affinity column purified (See Appendix 1). This was prepared previously in our laboratory (Poddar, Kim et al. 1998).

- The secondary antibody used was anti chick IgG raised in rabbit. This was conjugated with alkaline phosphatase.

- The antigen-antibody complex was visualised using para-nitrophenyl phosphate pNPP (Sigma), a chromagen in conjunction with alkaline phosphatase.

- A 1X solution of Phosphate buffered saline (PBS) was prepared by adding 8g of NaCl, 0.2g of KCl, 1.44g of Na$_2$HPO$_4$, and 0.24g KH$_2$PO$_4$. The solution was made up to a pH of 7.6. This was then made up to 1000ml with deionised water.

- Bovine serum albumin (BSA)(Sigma-Aldrich, St. Louis, MO, USA): 0.1% solution was prepared.

- A 96 well ELISA plate was utilised.
Method:

Glycodelin peptide was used to quantify the glycodelin present.

Glycodelin peptide standards in the following concentrations were prepared with deionised water, by serial dilution:

- 1μg; • 7.812ng;
- 500ng; • 3.6ng;
- 250ng; • 1.8ng;
- 125ng; • 0.9ng;
- 62.5ng; • 0.45ng;
- 31.25ng; • 0.225ng;
- 15.625ng; • 0.112ng

Amniotic fluid at 13 weeks was prepared in the following dilutions with phosphate buffered saline:

- 1:1000;
- 1:5000;
- 1:25,000;
- 1:50,000;
- 1:100,000.
The cells were pelleted, and the serum withdrawn, lyophilised and re-suspended in phosphate buffered saline. 100μl was added in triplicate wells of the ELISA plate and incubated overnight at 37°C. The next day the plates were washed 3 times in phosphate buffered saline solution (PBS) and blocking was done at room temperature for 1 hour using 0.1% BSA (bovine serum albumin). Wells were washed with PBS (x 6) and incubated for 2 hours at 37°C with 100μl of affinity column purified chicken anti-glycodelin antibody raised in this laboratory (Poddar, Kim et al. 1998) at a dilution of 1: 200. Following this the wells were washed with PBS (x3), blocked with 0.1% BSA for 1 hour at room temperature, then washed with PBS (x3). A secondary antibody (rabbit anti-chicken IgG conjugated with alkaline phosphatase) was added at a dilution of 1:30,000 in 0.1% BSA, and incubated for 2 hours at 37°C. The wells were washed again with PBS (x3) and the substrate p-nitro phenyl phosphate was added. The optical density was measured using a micro titre plate reader set to 405nm with wavelength correction at 570 nm to correct optical imperfections in the plate. Measurements were taken after 30, 60 and 90 minutes. The amount of glycodelin was quantified using glycodelin peptide and amniotic fluid standard curves. The entire experiment was repeated three times.
3.4 Statistical Analysis

Inferential statistics were performed using Analysis of the variance of the mean (ANOVA). Significance at P<0.05 is assumed throughout. Tukey simultaneous tests were performed and pairwise comparisons were made between treatment doses. Statistical analysis was conducted using Minitab statistical software for windows.

3.5 Glycodelin Gene Expression and Upregulation by Phorbol Myristate Acetate (PMA)

The cells were cultured to 75% confluence, serum deprived for a period of 8 hours, then transferred to a 12 well treatment plate. The concentration of cells was no less than 1x10^6 cells per well. Half of the cells were exposed to phorbol myristate acetate (PMA) while the other half was untreated, for a period of 24 hours. The cells were then harvested, pelleted via centrifugation at 200g x 5 minutes, the supernatant removed, and RNA isolation and PCR for the glycodelin gene performed on the cell pellets. The glycodelin protein present in the supernatant was analysed using the ELISA technique as described.
3.5.1 Results

Agarose gel electrophoresis of PCR products confirmed glycodelin gene expression in K562 cells and in K562 cells treated with phorbol myristate acetate (PMA), a plant mitogen and tumour promoter. Glycodelin bands are seen at 422kb on gel electrophoresis (Figure 3.1 (a, b)). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the internal control to substantiate equal amplification of the total RNA. The GAPDH bands are seen at 451kb on gel electrophoresis (Figure 3.2 (a, b)). Densitometric analysis of the electrophoresis gels shows upregulation of the glycodelin gene expression on exposure of the K562 cells to PMA (Figure 3.3). This up regulation was reflected on examination of the protein level in cell supernatant using the ELISA technique (Figure 3.4).
Figure 3.1 Glycodelin Gene Expression

(a)

Gel electrophoresis with glycodelin bands seen at 422kb. Glycodelin gene expression is shown to be present in both the K562 cells (lanes 1-3), and those K562 cells treated with 1nM PMA (lanes 3-6)

(b)
Figure 3.2 GAPDH Gene Expression

(a) Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the internal control to substantiate equal amplification of the total RNA. GAPDH bands are seen at 451kb.

(b)
Figure 3.3 (a)

*Densitometric analysis of the glycodelin gel shows glycodelin gene upregulation in K562 cells when phorbol myristate acetate is added (p=0.02). Densitometric analysis was performed on the triplicate samples in each group. The experiment was repeated three times.*
Analysis of variance (ANOVA) of the densitometry readings in the three groups, control, K562 cells, and K562 cells activated with 1nM PMA suggests a significant upregulation in glycodelin gene expression ($p=0.02$). There is no evidence of dose and date interaction ($p=0.15$). Thus the effect of glycodelin gene expression appears similar for each date.
Figure 3.4

The optical densities of the cell supernatants were calculated following ELISA. The glycodelin concentration was obtained and plotted here in graph form. There is a trend towards up regulation of glycodelin protein production following treatment of cells with PMA. However, our sample size did not have sufficient power to detect statistical significance (p=0.27).
3.6 Glycodelin Gene Expression in K562 Cells Following Exposure to an Allergen Protein, House Dust Mite Antigen

In order to determine the effects of allergen proteins on the immune system, K562 cells (lymphoid precursors) were cultured to 75% confluence. The cells were then treated with media containing varying concentrations of the dust mite antigen for a 24-hour period. The starting cell count concentration was approximately $1 \times 10^6$ per ml prior to starting the experiments. Parallel cultures were exposed to phorbol myristate acetate (PMA - known from section 3.3 to induce glycodelin production in K562 cells) as a positive control; cultures with no added reagents were also analysed. After 24 hours the cells were harvested, pelleted via centrifugation at 200g x 5 minutes, the supernatant removed and analysed for the presence of glycodelin using the ELISA technique as previously described.

3.6.1 Results

The results from the antigen challenge assay of K562 cells show a dose-dependent increase in the amounts of glycodelin produced in the presence of dust mite antigen (Figure 3.5). There is a statistically significant, dose-dependent increase in glycodelin production induced when compared to baseline levels (*$p<0.05$, **$p<0.01$).
Figure 3.5:

Production of glycodelin by K562 cells after allergen challenge. K562 cells in culture were exposed to 5, 50, 100, and 500 μg of dust mite antigen suspension. PMA – a compound known to induce glycodelin production was included as a positive control. There is a statistically significant, dose-dependent increase in glycodelin production induced when compared to baseline levels (* p<0.05, ** p<0.01).
3.7 Discussion

To date, glycodegin’s role has been studied extensively in pregnancy (Seppala, Koistinen et al. 1997; Seppala 1999). It is a potent suppressor of Th2 cell function and serves a critical role in protecting the foetus from the maternal immune system. Outside of the female reproductive tract, glycodegin expression has been shown in other “glandular” tissues such as the breast, as well as the male genital system (Koistinen, Koistinen et al. 1997; Kamarainen, Halttunen et al. 1999; Keil, Husen et al. 1999).

Our in vitro studies have shown that K562 cells in the lymphoid lineage are capable of expressing the glycodegin gene. This glycodegin gene expression is up regulated in the presence of PMA a known tumour promoter. This experiment shows that glycodegin may be produced in a similar fashion in response to nasal allergen exposure such as dust mite antigen, suggesting that glycodegin production is increased early in the inflammatory cascade.

Recently a systemic role in inflammatory disease has been suggested. In patients with allergic diseases, allergen provocation can activate a systemic response that provokes inflammatory cell production by the bone marrow. After release and differentiation of progenitor cells, eosinophils, basophils and mast cells are typically recruited to tissues in atopic individuals thus there is a systemic response between the target organ and the bone marrow/ peripheral circulation (Denburg, Sehmi et al. 2000). Braunstahl et al showed that the number of blood eosinophils and the concentration of serum-interleukin 5 (IL 5) increased in allergic subjects after natural and experimental allergen exposure (Braunstahl,
Kleinjan et al. 2000). Thus, Braunstahl et al. provide evidence to support the theory of allergic rhinitis as a component of a systemic inflammatory response (Braunstahl, Kleinjan et al. 2000; Braunstahl, Overbeek et al. 2001; Marcucci, Sensi et al. 2001). In seasonal allergic rhinitis, the temporal association of the development of upper airway symptoms with the seasonal elevation of circulating specific IgE antibodies against the culprit allergen offers further evidence that the systemic manifestations are part of the pathophysiologic factors of the disease.

Lymphoid precursors not only generate pro-inflammatory cytokines in the presence of allergens but also release molecules such as glycodelin whose putative role is to regulate the inflammatory cascade. Thus, glycodelin may play a role in the inflammatory reaction during nasal allergy. Previous studies have shown that glycodelin is an acute phase protein, which is produced in response to bacterial infection and/or chronic inflammation (Logdberg and Wester 2000). Once produced glycodelin may act to dampen the inflammatory cascade via its immunoinhibitory properties. This may be an attempt by the host to control and temper the cycle of T-cell mediated inflammation and tissue destruction.
CHAPTER 4

GLYCODELIN EXPRESSION IN
PERIPHERAL WHITE BLOOD CELLS (IN VIVO)
4.1 Introduction

Glycodelin expression has been reported in haematopoietic cells of the megakaryocytic lineage and was shown to have potent suppressive activity in mixed lymphocytes and cytolytic action in natural killer cells (Bolton, Pockley et al. 1987; Pockley, Barrett et al. 1989; Okamoto, Uchida et al. 1991; Morrow, Xiong et al. 1994). Its presence in human bone marrow cells has recently been described, along with the observation of a similar expression intensity of the antigen between healthy subjects and patients with myeloproliferative disorders (Zetterberg, Lundberg et al. 2003).

Interestingly, both benign and malignant gynaecological tumours produce elevated levels of plasma glycodelin and messenger RNA within their tissues (Horowitz, Cho et al. 2001). This suggests that rapidly growing tumours and tissues may be producing glycodelin and thus promoting cell growth and the angiogenesis necessary to support this growth.

Until now, the properties of glycodelin and its regulation have been mainly studied in K562 cells, a progenitor cell line of lymphoid lineage with the ability to differentiate into monocytes and neutrophils under particular conditions. However, the in vivo expression of glycodelin in circulating lymphocytes has not been determined. The presence of glycodelin in peripheral blood cells would suggest that those cells may play a role in reproduction and in other tissues in the body where glycodelin activity has been noted.
4.2 **Aim**

(1) To determine if circulating leukocytes in normal subjects express the glycodelin gene; and

(2) To determine if this expression translates to glycodelin protein production within the cytoplasm of circulating leukocytes.

4.3 **Materials and Methods**

4.3.1 **Cell culture**

**Materials**

Growth media for K562 cells:

- RPMI with L-glutamine (Mediatech, Herndon, VA, USA) 90%: 225ml
- Foetal Bovine Serum (FBS) (Mediatech, Herndon, VA, USA) 10%: 25ml
- Penicillin/Streptomycin 1%: 2.5ml

The above were combined under aseptic technique in the cell culture laboratory and stored at 4º Celsius.
Method

The human chronic myelogenous leukaemia cell line K562 (American Type Culture Collection (ATCC) Rockville, MD), were grown up to 75% confluence in RPMI 1640 medium with 10% foetal bovine serum. The cells were maintained at 37°C in a 5% CO₂ incubator.
4.3.2 Isolation of Monocytes (Buffy Coat Layer)

Figure 4.1

Diagrammatic representation of Buffy coat isolation process.
Materials

- Lithium/sodium heparin containing vials for blood sample collection;

- Ficoll-hypaque gradient: Histopaque 1077 (Sigma Diagnostics, St Louis, MO, USA): containing Polysucrose 5.7g/dl, Sodium diatrizoate 9.0g/dl. Aseptically filtered;

- Hanks buffered saline solution (Mediatech, Herndon, VA, USA); and

- Phosphate buffered saline: A 1x solution of Phosphate buffered saline (PBS) was prepared by adding 8g of NaCl, 0.2g of KCl, 1.44g of Na$_2$HPO$_4$, and 0.24g KH$_2$PO$_4$. The solution was made up to a pH of 7.6. This was made up to 1000ml with deionised water.

Method

Approximately 20 ml of peripheral blood was drawn from:

(a) Three healthy male and three healthy female volunteers; and

(b) 34 patients with sinus disease
The blood samples were collected by venepuncture into heparinised ‘vacutainer’ tubes. Blood samples from the female volunteers were taken both midway during the menstrual cycle and at the end of the menstrual cycle. A gradient of 20ml of histopaque 1077 (Sigma, St. Louis, MO, USA) was prepared and the 20 ml of blood layered onto this. The gradient was centrifuged at 400xg for 30 minutes at room temperature. Following centrifugation the cells layered as seen in figure 4.1. The plasma layer was carefully aspirated to within 0.5cm of the opaque interface containing the mononuclear cells. This layer was discarded. The buffy coat layer containing monocytes was collected and cells were washed three times with Hanks buffered saline solution (HBSS) and pelleted by centrifugation at 250xg for 10 minutes. After washing the pellet was resuspended and cells were counted and viability assessed with trypan blue dye. The cells were then stored in 0.5ml Hanks Buffered Saline solution at -70° Celsius.

4.3.3 RNA isolation

For a detailed description of materials see Appendix II (i)

Method

White blood cells (buffy coat) were lysed in 1ml TRI reagent by repetitive pipetting and stored at -70°C overnight. The samples were then incubated at room temperature for 30 minutes. 0.2ml Chloroform was added to each sample,
which was shaken vigorously for 15 seconds and then incubated at room temperature for 2 minutes. Samples were then centrifuged at 9000rpm for 15 minutes at 2-8°C. Following centrifugation there were three distinct layers- the RNA being found in the aqueous layer. The aqueous layer was removed and placed in a clean 15ml tube. 0.5ml isopropyl alcohol was added to each sample and the samples were incubated at room temperature for 10 minutes. The samples were then centrifuged at 9000rpm for 15 minutes. The supernatant was removed and the remaining RNA pellet washed with 1ml 75% ethanol. The samples were centrifuged at 14000rpm for 5 minutes, the supernatant removed and the RNA pellet allowed to air dry for 45 minutes. Using spectrophotometry the RNA concentration and the RNA/DNA ratio were obtained.

4.3.4 Reverse transcriptase- polymerase chain reaction (RT-PCR) analysis

The quality of the isolated mRNA was checked, and 3.0μg was reverse transcribed. The product was PCR amplified using:

- 5’- AAGTTGGCAGGGACCTGGCACTC-3’ (sense) oligonucleotides for glycodelin; and

- 5’-ACGGCACGGCTCTTCCATCTGTT-3’ (antisense) oligonucleotides for glycodelin.
A product of 422 base pairs was obtained via these procedures. Sample sizes and number of amplifications were optimised to produce measurements within a linear range. After an initial denaturation at 95°C for 1 minute, PCR was conducted as follows: denaturation at 95°C for 45 seconds, annealing at 60°C for 45 seconds, and extension at 72°C for 1 minute. This was repeated for 35 cycles followed by a final extension at 72°C for 10 minutes. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the internal control to substantiate equal amplification of the total RNA. The GAPDH primer set was as follows:

- 5'-ACCACAGTCCATGCCATC-3' (sense); and

- 5'-ACGGCACGGCTCTTCCATCTGTT-3' (antisense).

These procedures yielded a product of 451 base pairs. All PCR products were analysed on a 2% agarose gel containing ethidium bromide.
4.3.5 Immunocytochemistry:

Materials:

- The primary antibody used for glycodelin was anti-glycodelin antibody generated in chicken and affinity column purified. This was prepared previously in our laboratory (Poddar, Kim et al. 1998).

- The secondary antibody used was anti chick IgG raised in rabbit. This was conjugated with alkaline phosphatase.

- The antigen-antibody complex was visualised using Fast Red (Sigma), a chromagen in conjunction with alkaline phosphatase substrate naphthol phosphate (Sigma).

- Cytospin apparatus

- 0.1% PBS

- 3% Bovine serum albumin (Sigma-Aldrich, St.Louis, MO): 3g of Bovine serum albumin was added to 100mls PBS to make a 3%BSA solution.
• 4% Paraformaldehyde: 100mls PBS added to 4g paraformaldehyde to make a 4% solution.

• Haematoxylin

• Permount (Fisher-Scientific, Hampton, NH, USA)

• 100mls 1x PBS was added to 900mls deionised water to make 0.1x PBS

• Acid alcohol solution was obtained by mixing 0.5% HCL in 70% ethanol.

**Method**

100μl aliquot of buffy coat sample was diluted 1:3 in Hanks buffered saline solution. 100μl was placed on cytospin slide (poly-L-lysine coated) and spun for 5 minutes at 800 rpm. The slides were fixed in 4% paraformaldehyde for 20 minutes. Circles were drawn around the cells with a pap pen, being careful that the cells did not dry out. The slides were washed (x3) in PBS and incubated for 2 hours at room temperature in a humidity chamber with a 1:200 dilution of purified chicken anti-glycodelin peptide antibody (Poddar, Kim et al. 1998). Bovine serum albumin (3%) was used as a negative control. After washing 3 times with PBS the cells were incubated for 2 hours at room temperature with
secondary antibody (antichicken immunoglobulin G at a 1:200 dilution in 3% BSA) conjugated with alkaline phosphatase. Cells were washed 3 times with PBS and Fast Red was added as a chromagen in conjunction with alkaline phosphatase substrate naphthol phosphate (Sigma) at room temperature. Following this, the slides were washed in deionised water three times and counterstained lightly with haematoxylin for 10-20 seconds. Slides were immersed briefly in acid alcohol to clear any non-nuclear staining. Finally, the slides were allowed to dry thoroughly and mounted using Permount® and a coverslip before viewing. Haematoxylin stains all nuclei blue whilst the cytoplasm stains pink with anti-glycodelin antibody. The immunostaining was visualised under the microscope and the results were analysed.

4.4 Statistical Analysis

Inferential statistics were performed using the T test for independent samples. Significance at P<0.05 is assumed throughout. Statistical analysis was conducted using the Minitab 14 statistical software for windows.
4.5 Results

To determine the presence of glycodelin in normal lymphocytes, PCR analysis was done on samples from 3 males and 3 females. The experiment was repeated in triplicate. We then examined for glycodelin gene expression in theuffy coat of our experimental patient group (See Chapter 6). This group of patients had attended the ENT department in Emory with symptoms of sinus disease. Glycodelin gene expression was observed in the human mononuclear cells isolated from all patient samples, all healthy volunteer samples, as well as in the control cell line (K562) (Figure 4.2, 4.3). Densitometry was performed on the PCR gels in order to compare and quantify the glycodelin gene expression between the experimental groups. Statistical analysis was performed using a two-sample T-Test (Figure 4.4). There was no significant difference in glycodelin gene expression found between male and female subjects (P-value = 0.564). No statistical difference in glycodelin gene expression was found between those female subjects midway through the menstrual cycle and at the end of the menstrual cycle.

On immunocytostaining peripheral mononuclear cells are seen to stain positive with antiglycodelin antibody in patients and healthy subjects (Figure 4.5).

The presence of glycodelin protein within the monocytes is demonstrated by the pink stain seen in the cytoplasm and around the nucleus of these cells. No staining was observed in the negative control (Figure 4.5a).
Figure 4.2:

PCR analysis of male and female normal subjects. A) Glycodelin gene expression is illustrated at 422kb (PCR markers at 500kb and 300kb. B) GAPDH gene expression (internal control) illustrated at 451kb.

Lane 1-3 = male subjects, lane 4-6 = female subjects mid menstrual cycle, lane 7-9 = female subjects end menstrual cycle, lane 10 = K562 cells.
Figure 4.3: PCR gels of patients with sinus disease

(a) glycodelin gene expression in patient group 3-18

(b) glycodelin gene expression in patient group 19-29, 35-37

(c) glycodelin gene expression in patient group 27-34
(d) **glycodelin gene expression in patient group 35-40**

<table>
<thead>
<tr>
<th>PCR marker</th>
<th>35</th>
<th>36</th>
<th>37</th>
<th>38</th>
<th>39</th>
<th>40</th>
</tr>
</thead>
</table>

(e) **Gap gene expression in patient group 3-20**

<table>
<thead>
<tr>
<th>PCR marker</th>
<th>3a</th>
<th>3b</th>
<th>7</th>
<th>9</th>
<th>10</th>
<th>13</th>
<th>14</th>
<th>15</th>
<th>16</th>
<th>17</th>
<th>18</th>
<th>19</th>
<th>20</th>
</tr>
</thead>
</table>

(f) **Gap gene expression in patient group 21-33**

<table>
<thead>
<tr>
<th>PCR marker</th>
<th>21</th>
<th>22</th>
<th>23</th>
<th>24</th>
<th>25</th>
<th>26</th>
<th>27</th>
<th>28</th>
<th>29</th>
<th>30</th>
<th>31</th>
<th>32</th>
<th>33</th>
</tr>
</thead>
</table>
(g) *Gap gene expression in patient group 35-40*
Figure 4.4  Densitometric analysis of glycodeolin gene expression in patient population

Box plots of densitometry findings from glycodeolin PCR analysis in female and male subjects. The mean glycodeolin concentration in females was 0.768ng/ml (SEM 0.16) and in males was 0.63ng/ml (SEM 0.17). At the 95% confidence level there was no significant difference in glycodeolin gene expression between these two groups (p=0.564).
Figure 4.5

*Immunocytostaining of peripheral mononuclear cells with antiglycodelin antibody illustrating the presence of glycodelin within the cytoplasm of the cells. The pink appearance of the antiglycodelin antibody stain is seen within the cytoplasm of the mononuclear cells (see black arrows). A) negative control B) positive staining x 100, C) positive staining x 40.*
4.6 Discussion

This study demonstrates that peripheral white blood cells express messenger RNA for the glycodeiin gene. White blood cells were also seen to cross react with specific antibodies raised against the glycodeiin protein (antiglycodeiin antibody), the identity of which has been previously validated in our laboratory (Poddar, Kim et al. 1998).

What is the significance of this finding? To date, glycodeiin has been looked at from two perspectives. In vivo studies identified gene expression in specific tissue such as endometrial tissue and placental tissue. In vitro studies focused on K562 cells, which are lymphoid precursor cells with the ability to differentiate into monocytes and neutrophils under particular conditions. We have now demonstrated that the glycodeiin gene is present in human peripheral mononuclear cells and that this is translated into the production of the glycodeiin protein, which is present in the cytoplasm of these cells. The presence of glycodeiin within human peripheral leukocytes may be important in a number of ways. Previous studies have shown that glycodeiin is produced in elevated levels in normal pregnancy where it is generated in high quantities by the decidua. The level is highest in the amniotic fluid in the early 2nd trimester. Many tumours also show increased glycodeiin gene expression and glycodeiin protein in plasma. Leukocytes produce cytokines and regulatory factors that are part of both normal tissue growth and development as well as tumourigenesis and the inflammatory process. Our results showing the presence of glycodeiin in peripheral blood monocytes suggest additional mechanisms by which these cells, and the peripheral
blood system may contribute to tumour development, placental tissue growth, and inflammatory processes.

Glycodelin is known to have homology to beta lactoglobulin. However the antibodies used in our study did not cross-react with bovine beta lactoglobulin in previous studies; thus the antiglycodelin antibodies used in this study are specific for glycodelin (Kim, Ramachandran et al. 2000).

Factors affecting glycodelin gene expression are poorly understood. However hormones (e.g. progesterone), oxidised lipids and lysophosphatidic acid can stimulate glycodelin production (Ramachandran, Ramaswamy et al. 2002). In early pregnancy the elevated levels of progesterone may trigger the chemotraction of leukocytes, which we have shown to express the gene for glycodelin. These leukocytes could then play a role in foetal growth and placental development, through their expression of glycodelin. A similar process could also occur in tumourigenesis. For example, increased lysophosphatidic acid has been noted in a number of cancers including ovarian cancer. These findings would indicate that increased oestrogenic and progesterogenic hormones as well as lysophosphatidic acid would be conducive to recruitment of monocytes and the promotion of angiogenesis. If this is the case, treatment of malignancies might be influenced not only by target-directed surgery or chemotherapy but also by reducing chemotaxis and the expression of genes such as glycodelin that may promote angiogenesis and/ or tumour formation. It would be of interest to determine whether peripheral blood glycodelin gene expression is altered under conditions where both normal and abnormal growth is present.
In summary, the underlying mechanism regulating the syntheses of glycodelein has not been clarified. While hormones like progesterone might be important in regulating glycodelein production in reproductive tissues, its presence in non-reproductive cells and tissues as well as its induction by phorbol myristate acetate in K562 cells suggest that its synthesis may be regulated by other mediators (Bolton, Pockley et al. 1987). The presence of glycodelein in peripheral white blood cells, and its ability to stimulate angiogenesis and act as an immunosuppressive agent could be an important indicator of the role of peripheral leukocytes in various disease states.
CHAPTER 5

GLYCODELIN EXPRESSION IN NASAL MUCOSA
5.1 Introduction

Chronic rhinosinusitis is a chronic inflammatory process that leads to repeated bacterial infections within the paranasal sinuses. Many prior studies have shown elevations in the pro-inflammatory cytokines, especially those expressed by T_{H2}-cells (T helper 2) (Davidsson, Danielsen et al. 1996; Jyonouchi, Sun et al. 2001). These helper T-cell induced cytokines, lead to eosinophil and neutrophil chemotaxis (Cara, Negrao-Correa et al. 2000), which in turn induce more tissue destruction and inflammation (Sobol, Fukakusa et al. 2003). This cyclical inflammatory cascade then leads to bacterial infections due to ostial obstruction, mucus stasis and impaired mucociliary clearance (Lanza and Kennedy 1997). Left unchecked and uncontrolled, the process may lead to formation of inflammatory polyps in the nose, which in turn lead to further ostial obstruction and infections.

Regardless of what the inciting event(s) may be, histologically, chronic rhinosinusitis is characterized by the presence of inflammatory cells, tissue oedema and fibrosis, and in the case of polyp formation, neo-vascularisation and angiogenesis (Jyonouchi, Sun et al. 1999; Ramadan, Meek et al. 2002). Paradoxically, for tumour growth, a localised immunosuppression (Hakim 1978; Dorsch and Cook 1983) is necessary in addition to neo-vascularisation (Song, Ramaswamy et al. 2001).

The aim of this experiment was to characterise the expression pattern of a novel molecule glycodelin that has putative roles in all the aforementioned biochemical processes. Our hypothesis was based on existing data that glycodelin
is a potent suppressor of $T_h^2$ cells (Rachmilewitz, Riely et al. 1999; Logdberg and Wester 2000), and can induce angiogenesis via the VEGF pathway. Given that immunomodulation and angiogenesis are the hallmarks of chronic rhinosinusitis, we examined the expression pattern of glycodel in the nose, in order to further understand glycodelin’s role in the pathogenesis of chronic rhinosinusitis.

5.2 Aim

To examine the expression pattern of glycodelin in nasal and sinus mucosa

5.3 Materials and Methods

Tissue samples

- Archived sinus mucosa tissue from twenty patients having undergone endoscopic sinus surgery were used for immunohistochemical studies;

- Tissues from an additional ten patients without chronic rhinosinusitis having undergone endonasal surgeries were used as controls.
5.3.1 Immunohistochemistry

Materials

- The primary antibody used for glycodelin was anti-glycodelin antibody generated in chicken and affinity column purified. This was prepared previously in our laboratory (Poddar, Kim et al. 1998);

- The secondary antibody used was anti chick IgG raised in rabbit. This was conjugated with horseradish peroxidase (HRP);

- The antigen-antibody complex was visualised using a chromagen 3,3' diaminobenzidine tetrahydrochloride (DAB; Sigma, St. Louis, MO);

- 1% Bovine serum albumin (Sigma-Aldrich, St. Louis, MO);

- Haematoxylin;

- 1x PBS.

Method

Five-micron sections of archived tissue were cut on a vibratome and mounted onto poly-L-lysine coated slides. The tissues were deparaffinised as
follows: immersion in Xylene for 30 minutes, divided into two 15-minute periods, following this, slides were immersed in absolute alcohol for 5 minutes (x 2 changes), 90% alcohol for 3 minutes, 80% alcohol for 3 minutes and 70% alcohol for 3 minutes.

Slides were then rinsed in running water and then washed using phosphate buffered saline (x3) for 2 minutes each. After rinsing in PBS, the sections were incubated in 1% bovine serum albumin for two hours to act as a protein block. An anti-glycodelin antibody raised in chicken diluted 1:200 in the same bovine serum albumin was placed on the slides. After overnight incubation, the sections were then washed three times in PBS, and incubated with an HRP-conjugated anti-chicken secondary antibody diluted 1:1000 in bovine serum albumin. Following this, the sections were incubated with 3,3’-diaminobenzidine tetrahydrochloride (DAB; Sigma, St. Louis, MO) for 10 minutes at room temperature. The sections were counterstained with haematoxylin to clarify the histology. The sections were then dehydrated, cover slipped and analysed. Adjacent sections were processed without the primary antibody as control to confirm the specificity of the immunohistochemistry.

5.4 Results

These immunohistochemical studies showed glycodelin to be expressed in nasal tissues, primarily in the epithelium and the mucus-producing glands (Fig. 5.1A-C). This pattern was observed in all patient samples. However, the staining did appear to be more robust in patients with chronic rhinosinusitis, although this
was not quantified. It was felt that this would not be a fair assessment since the tissues were archived and the tissue processing initially would have been variable. In patients with chronic rhinosinusitis, glycodelin expression was seen in the endothelial cells of the vasculature - a pattern not seen in control patient tissue samples (Fig. 5.1 E and F). Representative sections from tissues processed without the primary antibody are shown (Fig. 5.1D and F), thus confirming that the staining seen is specific for glycodelin.
Figure 5.1 Expression patterns of glycodeolin in the nasal mucosa.

Figure 5.1 (a)

* A low-power image showing glycodeolin expression in the nasal epithelium and the vasculature (see black arrows) in a patient with chronic rhinosinusitis. 
Figure 5.1 (b)

A higher power image showing expression in the vascular endothelium (long arrow) and in the cells surrounding a venous lake (short arrow).
A high-magnification image of glycodelin expression in the submucosa showing expression in the mucus glands (see black arrows).
An adjacent section to that in 5.1 (c), processed without primary antibody showing no positive staining thus demonstrating the specificity of the antibody.
Image from the nasal mucosa of a patient without chronic rhinosinusitis shows glycodelin expression in the glandular tissues and the epithelium (arrowhead) but no expression in the vasculature (see black arrow).
Adjacent section to that in 5.1 (e) again processed without primary antibody showing no glycodelin staining. The arrowhead points to the epithelium.
5.5 Discussion

This is the first study to demonstrate the presence of glycodelin in nasal and paranasal sinus tissues. Glycodelin, an acute phase protein of the lipocalin family, is released in response to bacterial infection and inflammation. It is a potent suppressor of $T_{h2}$ cell function and serves a critical role in protecting the foetus from the maternal immune system. In addition to its immunosuppressive functions, glycodelin also has angiogenic properties, which are mediated via vascular endothelial growth factor (VEGF) (Song, Ramaswamy et al. 2001).

This experiment demonstrates that the glycodelin protein is present in the endothelial tissue and mucous producing glands of sinus mucosa. Although there is baseline expression of glycodelin in nasal tissues, in patients with chronic rhinosinusitis, there appears to be up-regulation of glycodelin expression by endothelial cells in the vasculature.

Based on these studies and glycodelin’s known roles, it appears that glycodelin may play a role in the pathogenesis of chronic rhinosinusitis. Nasal inflammation, in the form of allergen exposure, for example, leads to T-cell activation, which in turn releases cytokines and leads to eosinophil chemotaxis. Persistent tissue inflammation leads to persistent nasal congestion and infection within the paranasal sinuses. Bacterial infection and/or chronic inflammation lead to tissue destruction, which in turn induces glycodelin production. This may be an attempt by the host to control and temper the cycle of T-cell mediated inflammation and tissue destruction.
Glycodelin, expression also leads to VEGF production, angiogenesis and polyp formation. Given glycodelin’s potent immunosuppressive abilities, and in particular its effect on $T_h2$ cells, targeting the immunosuppressive abilities at the expense of its angiogenic properties could have a potential role in the treatment of chronic rhino sinusitis.
CHAPTER 6

SERUM GLYCODELIN LEVELS IN RHINOSINUSITIS AND NASAL POLYPOSIS
6.1 Introduction

At its broadest level, rhinosinusitis can be defined as mucosal inflammation of the nasal cavity and paranasal sinuses. It is a common co-morbid condition in patients with atopic disorders or asthma. It is also prevalent in patients with autoimmune disorders (e.g. Wegener’s granulomatosis) and in those with cystic fibrosis, which is the most common genetically inherited lethal mutation in the Caucasian population. Symptoms associated with sinusitis include congestion, rhinorrhoea, facial pain and pressure, postnasal discharge, and occasionally, olfactory disturbances. The aetiologies of the inflammation can be broadly classified into infectious factors (bacteria, viruses), and patient factors (cystic fibrosis, HIV, atopy) (Kennedy 2000).

Costs associated with the diagnosis and management of rhinosinusitis run into the billions of dollars, primarily due to the antibiotics used to treat the condition and time lost from work and school (Moss and Parsons 1986). The most significant challenge in the management of rhinosinusitis is accurately diagnosing when a bacterial infection is present and using antibiotics appropriately, since the symptoms associated with bacterial and non-bacterial rhinosinusitis overlap considerably.

The pathophysiology of rhinosinusitis is well described. Inflammation of the nasal mucosa leads to obstruction of the sinus outflow tracts and stasis of secretions. This in turn may lead to a bacterial infection. However, symptoms associated with the disease including headache, congestion, rhinorrhoea, and postnasal discharge, are not specific for bacterial infections; in fact, patients with
allergic rhinitis or viral upper respiratory tract infections (URIs) have similar symptoms. Non-specific symptomatology leads to inappropriate and unnecessary usage of antibiotics, which not only contributes to the overall costs associated with the disease, but also compounds the problem of emerging bacterial resistance.

Chronic infections or inflammation often lead to the formation of nasal polyps, which in turn induce further obstruction of the ostia, inflammation and infection. Bacterial rhinosinusitis requires antibiotic therapy. An objective test to differentiate between bacterial rhinosinusitis with or without nasal polyps and allergic rhinitis or viral upper respiratory tract infections, would be an invaluable tool for the family physician.

In the previous chapter the presence of glycodelin, a novel immunomodulatory molecule in the nasal mucosa was demonstrated. In the nose, glycodelin is present in the seromucinous glands and the epithelium.

Given this finding, the aim of this study was to investigate whether this elevation in nasal endothelial expression observed locally was also reflected in the peripheral blood by a raised serum glycodelin level.

6.2 Aim

To investigate whether the elevation of glycodelin expression observed locally in nasal mucosa is reflected in the peripheral blood by a raised serum glycodelin level.
6.3 Materials and Method

Patient Sample Collection

Patients referred to the Otorhinolaryngology clinic at Emory University Hospital for evaluation of their nasal and sinus problems were included in this study. After obtaining consent, a detailed history, including recent antibiotic and steroid use was taken, and all patients underwent nasal endoscopy. Patient symptoms were recorded and divided into major and minor criteria (Rhinosinusitis Taskforce Committee guidelines 1996 (Lanza and Kennedy 1997)(Table 1)).

The patients chief complaints were tabulated along with the endoscopic findings. The nasal mucosa was assessed for congestion, purulence, polyps, debris and scarring. Findings were then recorded and blood was drawn for glycodelin assay. The purpose of this was to correlate endoscopic findings with serum glycodelin levels. Sinus computer tomography (CT) scans were obtained for review. Patients were considered to have “severe” nasal polyps when the polyps extended inferior to the middle turbinate into the nasal cavity.
Table 6.1

<table>
<thead>
<tr>
<th>Major Criteria</th>
<th>Minor Criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nasal congestion</td>
<td>Cough</td>
</tr>
<tr>
<td>Post nasal drip/ Rhinorrhoea</td>
<td>Headache</td>
</tr>
<tr>
<td>Facial pain/ Pressure</td>
<td>Halitosis/ Dental pain</td>
</tr>
<tr>
<td>Fever</td>
<td>Ear Pain/ Pressure</td>
</tr>
<tr>
<td>Olfactory disturbance</td>
<td>Fatigue</td>
</tr>
</tbody>
</table>

Table 6.1

*Major and minor criteria for the diagnosis of rhinosinusitis (Rhinosinusitis Task Force Committee, 1996) (Lanza and Kennedy 1997; Logdberg and Wester 2000). A diagnosis of sinusitis is considered “likely” if there are two or more major factors with two or more minor factors. The diagnosis is considered to be “possible” with one major factor or two minor factors.*
6.3.1 Enzyme-linked Immunosorbant Assay (ELISA)

Materials:

- The primary antibody used for glycodelin was anti glycodelin antibody generated in chicken and affinity column purified. This was prepared previously in our laboratory (Poddar, Kim et al. 1998);

- The secondary antibody used was anti chick IgG raised in rabbit. This was conjugated with alkaline phosphatase;

- The antigen-antibody complex was visualised using para-nitrophenyl phosphate pNPP (Sigma), a chromagen in conjunction with alkaline phosphatase;

- Phosphate buffered saline (PBS): 1x solution was prepared as previously described;

- 96 well ELISA plate;

- Bovine serum albumin (BSA: Sigma-Aldrich, St.Louis, MO): 0.1% solution was prepared.
Method:

Approximately 10ml of venous blood was collected from the patients after obtaining their consent by venepuncture into heparinised ‘vacutainer’ tubes. After centrifugation at 400 x g for 20 minutes, the plasma was removed and stored at -70°C. The Emory University Human Investigations Committee approved both the collection of the blood samples from patients and the experimental protocol.

Glycodelin peptide was used to quantify the glycodelin present. Glycodelin peptide standards in the following concentrations were prepared with deionised water by serial dilutions:

- 1ug;
- 500ng;
- 250ng;
- 125ng;
- 62.5ng;
- 31.25ng;
- 15.625ng;
- 7.812ng;
- 3.6ng;
- 1.8ng;
- 0.9ng;
- 0.45ng;
- 0.225ng; and
- 0.112ng.
Amniotic fluid at 13 weeks was prepared in the following dilutions with phosphate buffered saline:

- 1:1000;
- 1:5000;
- 1:25,000;
- 1:50,000; and
- 1:100,000.

Plasma samples were diluted 1:10 in a buffered saline solution (PBS). 100 μl was added in triplicate wells of the ELISA plate and incubated overnight at 37°C. The next day the plates were washed 6 times in phosphate buffered saline solution (PBS) and blocking was done at room temperature for 1 hour using 0.1% BSA (bovine serum albumin). Wells were washed with PBS (x6) and incubated for 2 hours at 37°C with 100 μl of affinity column purified chicken anti-glycodelin antibody raised in this laboratory (Poddar, Kim et al. 1998) at a dilution of 1:50. Following this the wells were washed with PBS (x6), blocked with 0.1% BSA for 1 hour at room temperature, then washed with PBS (x6). A secondary antibody (rabbit antichicken IgG conjugated with alkaline phosphatase) was added at a dilution of 1:30,000 in 0.1% BSA, and incubated for 2 hours at 37°C. The wells were washed again with PBS (x6) and the substrate p-nitro phenyl phosphate was added. The optical density was measured using a microtitre plate reader set to 405 nm with wavelength correction at 570 nm to correct optical imperfections in the plate. Readings were taken after 30, 60 and 90 minutes. The amount of glycodelin was quantified using glycodelin peptide and amniotic fluid standard curves.
<table>
<thead>
<tr>
<th>No.</th>
<th>Complaint:</th>
<th>Physical findings:</th>
<th>Intervention</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Rhinorrhoea, post nasal drip</td>
<td>Right nasal polyps, mucosal hypertrophy, watery discharge</td>
<td>None</td>
</tr>
<tr>
<td>2</td>
<td>Congestion, post nasal drip, facial pain</td>
<td>Bilateral nasal polyps, purulent discharge</td>
<td>Antibiotics</td>
</tr>
<tr>
<td>3</td>
<td>Recurrent sinusitis, congestion, rhinorrhoea, post nasal drip</td>
<td>Bilateral nasal polyps, mucosal hypertrophy, watery discharge, scarring</td>
<td>Sinus surgery</td>
</tr>
<tr>
<td>4</td>
<td>Congestion, snoring, recurrent sinusitis</td>
<td>Mucosal hypertrophy, purulent discharge</td>
<td>None</td>
</tr>
<tr>
<td>5</td>
<td>Right facial pain, congestion, rhinorrhoea</td>
<td>Right ethmoidal polyps, purulent discharge</td>
<td>None</td>
</tr>
<tr>
<td>6</td>
<td>Anosmia, congestion, rhinorrhoea</td>
<td>Bilateral nasal polyps, mucosal hypertrophy, watery discharge, scarring</td>
<td>Topical steroid, Antihistamine</td>
</tr>
<tr>
<td>7</td>
<td>Congestion, rhinitis</td>
<td>Mucosal hypertrophy, septal deviation.</td>
<td>Antihistamine</td>
</tr>
<tr>
<td>8</td>
<td>Rhinorrhoea, headache, facial pressure</td>
<td>Crusting, purulent discharge, mucosal hypertrophy</td>
<td>Sinus surgery</td>
</tr>
<tr>
<td>9</td>
<td>Congestion, facial pressure, post nasal drip</td>
<td>Bilateral mucosal hypertrophy, purulent discharge, maxillary polyps</td>
<td>Antihistamine</td>
</tr>
<tr>
<td>10</td>
<td>Headache, recurrent sinusitis</td>
<td>Septal deviation, mild congestion</td>
<td>Topical steroid</td>
</tr>
<tr>
<td></td>
<td>Symptom(s)</td>
<td>Diagnosis(s)</td>
<td>Treatment(s)</td>
</tr>
<tr>
<td>---</td>
<td>------------------------------------</td>
<td>-------------------------------------------------</td>
<td>-----------------------</td>
</tr>
<tr>
<td>11</td>
<td>Congestion, recurrent sinusitis</td>
<td>Bilateral ethmoidal polyps, mucosal hypertrophy</td>
<td>Topical steroid</td>
</tr>
<tr>
<td>12</td>
<td>Congestion, rhinorrhoea, snoring</td>
<td>Mucosal hypertrophy, septal deviation</td>
<td>Topical steroid</td>
</tr>
<tr>
<td>13</td>
<td>Congestion, recurrent sinusitis</td>
<td>Bilateral ethmoidal polyps, mucosal hypertrophy, watery discharge</td>
<td>Oral steroid</td>
</tr>
<tr>
<td>14</td>
<td>Congestion, facial pressure, rhinorrhoea</td>
<td>Bilateral ethmoidal polyps, thick secretions</td>
<td>Oral steroid</td>
</tr>
<tr>
<td>15</td>
<td>Congestion</td>
<td>Mild ethmoid polyps, debris</td>
<td>Sinus surgery</td>
</tr>
<tr>
<td>16</td>
<td>Congestion</td>
<td>Mucosal hypertrophy, clear discharge</td>
<td>Oral steroid therapy</td>
</tr>
<tr>
<td>17</td>
<td>Congestion</td>
<td>Bilateral ethmoid polyps, mucosal hypertrophy, watery drainage</td>
<td>Oral steroid therapy</td>
</tr>
<tr>
<td>18</td>
<td>Headache, post nasal drip</td>
<td>Normal mucosa, septal deviation</td>
<td>None</td>
</tr>
<tr>
<td>19</td>
<td>Headache, post nasal drip</td>
<td>Mucosal hypertrophy, clear discharge</td>
<td>Topical steroid</td>
</tr>
<tr>
<td>20</td>
<td>Headache, congestion</td>
<td>Mucosal hypertrophy</td>
<td>Topical steroid</td>
</tr>
<tr>
<td>21</td>
<td>Anosmia, congestion, rhinorrhoea</td>
<td>Bilateral nasal polyps, thick secretions, septal deviation, mucosal hypertrophy</td>
<td>Antihistamine</td>
</tr>
<tr>
<td>22</td>
<td>Congestion, proptosis, rhinorrhoea</td>
<td>Bilateral nasal polyps, thick secretions, mucosal hypertrophy</td>
<td>Oral steroid therapy</td>
</tr>
<tr>
<td>23</td>
<td>Congestion, rhinorrhoea</td>
<td>Mucosal hypertrophy, purulent discharge</td>
<td>Topical steroid</td>
</tr>
<tr>
<td></td>
<td>Description</td>
<td>Diagnosis</td>
<td>Treatment</td>
</tr>
<tr>
<td>---</td>
<td>--------------------------------------</td>
<td>-----------------------------------------------</td>
<td>---------------------</td>
</tr>
<tr>
<td>24</td>
<td>Congestion, rhinorrhoea, snoring</td>
<td>Mucosal hypertrophy, purulent discharge</td>
<td>Topical steroid</td>
</tr>
<tr>
<td>25</td>
<td>Congestion</td>
<td>Right nasal polyps, thick discharge, septal deviation, mucosal hypertrophy</td>
<td>Antihistamine</td>
</tr>
<tr>
<td>26</td>
<td>Congestion</td>
<td>Nasal polyps, thick discharge, mucosal hypertrophy</td>
<td>Oral steroid therapy</td>
</tr>
<tr>
<td>27</td>
<td>Anosmia, congestion</td>
<td>Bilateral ethmoid polyps, thick discharge, mucosal hypertrophy</td>
<td>Antihistamine</td>
</tr>
<tr>
<td>28</td>
<td>Congestion, anosmia</td>
<td>Bilateral ethmoid polyps, thick discharge, mucosal hypertrophy</td>
<td>Oral steroid therapy</td>
</tr>
<tr>
<td>29</td>
<td>Congestion, post nasal drip</td>
<td>Mucosal hypertrophy, purulent discharge</td>
<td>Sinus surgery</td>
</tr>
<tr>
<td>30</td>
<td>Congestion, post nasal drip</td>
<td>Mucosal hypertrophy, scarring</td>
<td>Sinus surgery</td>
</tr>
<tr>
<td>31</td>
<td>Congestion</td>
<td>Mucosal hypertrophy</td>
<td>Topical steroid</td>
</tr>
<tr>
<td>32</td>
<td>Congestion, post nasal drip</td>
<td>Mucosal hypertrophy, nasal septal deviation</td>
<td>Topical steroid</td>
</tr>
</tbody>
</table>

**Table 6.2**

*This table illustrates the patients’ principal complaints and their clinical findings on examination, and whether or not the patients had been previously treated for their symptoms.*
6.4 **Statistical Analysis**

Descriptive statistics in the form of percentages and their standard deviations are presented. Inferential statistics were performed using the T test for independent samples. Significance at P<0.05 is assumed throughout. All statistical analysis was conducted using the Statistical Software Program (SPSS, Chicago IL) for Windows (version 10).

6.5 **Results**

Thirty peripheral blood samples were collected for examination from patients with nasal and sinus complaints. There were 18 female patients and 12 male patients, with patients ranging in age from 15 years to 65 years. Blood samples from healthy volunteers were collected for comparison. There were eight males and 5 females in this group, ranging in age from 24-49 years, all with no history of sinus disease or allergy related symptoms.

The spectrum of sinus disease included patients with allergic rhinitis, Samter’s triad, asthma, allergic fungal sinusitis (AFS), and acute as well as chronic rhinosinusitis. The patient’s symptoms, findings on nasal endoscopy, and sinus CT (computed tomographic) results, were compared and despite significant variability in severity of disease between patients, the symptoms were remarkably similar reinforcing the fact that bacterial rhinosinusitis is extremely difficult to diagnose by symptoms alone. CT scans were positive for sinus disease (Lund and
McKay score > 2) in all but one patient who had minimal disease; thus CT scan did not distinguish those patients with severe polyposis and/or purulence.

The optical densities and corresponding glycodelin levels in the patients' serum were tabulated and findings are plotted in a dot plot (Figure 6.1 (a)). The glycodelin levels in the patients' serum were also compared to that of the normal subject controls (Figure 6.1 (b)). Next, the glycodelin levels were correlated with the endoscopic findings. Our results indicate that in patients with severe “nasal” polyposis (polyps extended inferior to the middle turbinate into the nasal cavity in contrast to polyps confined to the ethmoid sinus) and/or purulent nasal discharge, serum glycodelin levels were highest (Figure 6.1 (c)). The mean concentration in those patients with severe polyposis and/or purulence was 40.4ng/ml (standard deviation (S.D) 19.1) while in patients with no polyps/purulence it was 18.61ng/ml (SD 4.97). There was a significant difference between the glycodelin concentrations in these two groups (p<0.001).

In addition, patients who had been previously treated with oral steroid therapy and/or antibiotics were found to have lower levels of serum glycodelin, indicating that serum glycodelin levels may also decrease following therapy for sinus disease (Figure 6.1 (d)). Glycodelin concentration of 23.7ng/ml (SD 12.9) compared with 33.2ng/ml (SD 19.2) (p=0.133, NS). It was noted that there was one patient in the steroid treatment group who had a notably high serum glycodelin concentration. It is possible that the duration since commencement of steroid therapy may have been less than in the other patients; thus a response had not yet occurred. Alternatively this could represent a patient specific response to steroid therapy. Further analysis of this group of patients without the outlier was performed. The
mean concentration in those who had been treated with oral steroid therapy was 19.28ng/ml (SD 3.18) compared with those who were not treated with steroids 33.2ng/ml (SD 19.2). At the 95% confidence interval there was a significant difference found between these two groups (p = 0.00233) (Figure 6.1 (e)).

The interexperimental variability was very low; on repeated testing the same group of patients with high glycodelin levels were identified. The glycodelin concentration was compared to nasal endoscopy as a predictor of the presence of nasal polyps and/or purulence. At a cut off point of 21.5ng/ml, the sensitivity was found to be 82.35% and the specificity 92.31%.
Figure 6.1

The glycodelin concentrations of male and female patients and normal subjects are illustrated in this dot plot. The mean glycodelin concentration in female patients was 30.4 ng/ml (SD 18.2), while in males was 28.7 (SD 15).
Figure 6.1 (b)

The mean glycodelin concentration in the normal subjects group was 28.2ng/ml (SD 18.2) and in the patient group was 30.9ng/ml (SD 9.14). At the 95% confidence interval there was no significant difference between these two groups (p=0.521).
Figure 6.1 (c) Severe Polyposis/Purulence on Examination

The glycodelin concentrations in those patients with severe nasal polyps and nasal purulence are compared to those with no nasal polyps/purulence on endoscopy. The mean concentration in those patients with severe polyposis and/or purulence was 40.4 ($SD$ 19.1) while in patients with no polyps/purulence was 18.6 ($SD$ 4.97) At the 95% confidence interval there is a significant difference between these two groups. ($p<0.001$).
Figure 6.1 (d)  
Patients Treated With Systemic Steroids vs. No Previous Treatment

The glycodelin concentrations in those patients who had been treated with systemic steroids prior to endoscopy, is compared to those who had not been so treated. The mean concentration in those who had been treated with oral steroid therapy was 23.7ng/ml (SD 12.9) compared with those who were not treated with steroids 33.2ng/ml (SD 19.2). At the 95% confidence interval there was no significant difference found between these two groups (p= 0.133).
Further analysis of this group of patients without the outlier was performed. The mean concentration in those who had been treated with oral steroid therapy was 19.28ng/ml (SD 3.18) compared with those who were not treated with steroids 33.2ng/ml (SD 19.2). At the 95% confidence interval there was a significant difference found between these two groups (p= 0.00233).
6.6 Discussion

A major weakness of symptom-based diagnosis of chronic rhinosinusitis is that the diagnostic symptoms only hint (likely vs. possible) at the diagnosis of bacterial vs. non-bacterial rhinosinusitis. Overlap of symptoms between acute bacterial rhinosinusitis, chronic rhinosinusitis, allergic rhinitis or viral upper respiratory tract infections leads to significant over-diagnosis of chronic rhinosinusitis and inappropriate usage of antibiotics as previously mentioned.

The role of sinus radiographs (CT scans or plain films) has been evaluated; these modalities also do not differentiate between bacterial and non-bacterial rhinosinusitis (Gwaltney, Phillips et al. 1994). Nasal endoscopy is useful, especially when active purulent drainage is seen, but is usually a tool that is available only to the trained otolaryngologist (Stankiewicz and Chow 2002). There have been attempts to identify a subgroup of patient who require antibiotic therapy using the screening blood test – C reactive protein (Bjerrum, Gahrn-Hansen et al. 2004). A raised C reactive protein level is an indicator of bacterial infection and the implementation of the test in general practice has been shown to lead to a reduction in antibiotic prescribing in patients with rhinosinusitis (Bjerrum, Gahrn-Hansen et al. 2004). However there are drawbacks associated with using an elevated C reactive protein level as an indicator for bacterial rhinosinusitis. C reactive protein is indeed raised in sinusitis due to Streptococcus pyogenes and Streptococcus pneumonia. Unfortunately, using C reactive protein levels, Haemophilus influenzae and Branhamella catarrhalis positive sinusitis (common causative pathogens) cannot be distinguished from a purely viral disease (Savolainen, Jousimies-Somer et al. 1997). It has been reported that a history of
purulent nasal discharge, and signs of pus in the nasal cavity, are better criteria than radiography or C reactive protein level, for selecting those patients who will benefit from antibiotic treatment (Young, Bucher et al. 2003). Thus a screening blood test, such as serum glycodelin estimation, which could indicate the presence of nasal polyposis and purulence, or differentiate between the different types of sinus disease, would be of great diagnostic benefit for primary health care professionals and promote cost-effective management. We found that above a cut-off point of 21.5ng/ml serum glycodelin was sensitive for the presence of severe polyposis/purulence in 82.35% patients.

A confounding factor was that a few of our normal patients also exhibited high levels of glycodelin. There are a number of factors, which may affect the baseline glycodelin level. These include hormonal influence, the presence of an unidentified tumour, or any oxidising stress such as a coexisting inflammatory or infective condition (Ramachandran, Rong et al. 2003). Women may have cyclical variations in serum glycodelin levels; the control women in our study group with high glycodelin levels may have been in mid-cycle. We did not pursue a follow up glycodelin assay at menstruation to determine whether these women had lower levels of glycodelin. The cut off point of 21.5ng/ml was chosen in reference to the group of patients in our pilot study. However, as glycodelin levels may be affected by age and gender, in order to determine a cut off point for serum glycodelin as a screening test, a larger trial looking at the above criteria along with other factors that might affect glycodelin levels would be necessary.

Glycodelin levels were also obtained from patients who had undergone recent surgical and/or medical therapy for their chronic sinusitis. Although their
clinical histories (e.g. AFS or Samter's triad asthma) would predict nasal polyps and therefore high serum glycodelin levels, our findings were otherwise. This data is significant for two reasons. Firstly, glycodelin levels decrease after medical and/or surgical treatment. A long-term follow-up study is underway to determine whether glycodelin levels in this cohort of patients, increases when their polyps recur. Secondly, serum glycodelin levels correlate with severity of disease and more importantly, with current endoscopic findings. In other words, serum glycodelin levels seem to reflect the degree and severity of nasal pathology and disease.

6.7 Conclusion

We have shown that glycodelin is elevated in the serum of those patients with sinusitis, who on examination are found to have severe nasal polyposis and purulent sinus secretions. Primary care physicians do not have recourse to nasal endoscopy. Performing a CT scan on each patient with sinus symptoms is an expensive exercise. Specialist opinion from an otolaryngologist is not always readily available. Therefore, in addition to careful history and physical examination, a glycodelin assay could help the primary care physician to diagnose those patients with severe polyposis and/or purulence. For the otolaryngologist or allergist/pulmonologist who treats patients with more severe and indolent disease, our data indicate that glycodelin levels could provide an objective assessment of the severity of disease at a given time-point.
CHAPTER 7

GLYCODELIN AND REACTIVE AIRWAY DISEASE
7.1 Introduction

Although assessment of reactive airway disease involves evaluating clinical history and pulmonary testing, a sensitive objective and non-invasive marker of asthma activity may be useful. Based upon the known immunosuppressive properties of glycodelin and its demonstrated presence in vascular endothelium, mucus-producing glands and the respiratory tract including the bronchi, (Seppala, Koistinen et al. 2001) we hypothesised that a glycodelin assay may indicate airway inflammation and that those levels may correlate with lung function in asthmatics.

Ground ozone exposure levels are directly proportional with air-pollutants and volatile organic compounds content of air. Over the past two decades, there has been increasing interest in studies of air pollution and its effects on human health and there is considerable evidence that asthmatic persons are at increased risk of developing asthma exacerbations with exposure to ozone and other environmental chemicals and inhalable particulate matter. In view of this, the effect of ozone exposure on glycodelin protein production is of interest.

7.2 Aim:

1) To evaluate a glycodelin assay as a marker of inflammation in the respiratory tract; and

2) To examine if glycodelin levels correlate with lung function in asthmatics
7.3 Materials and Method:

7.3.1 Patient Sample Collection

A prospective cross-sectional pilot study was performed in asthmatics and healthy controls. Patients attending the respiratory service at Emory University Hospital with a diagnosis of asthma were included in the study. All those included in the study were over 18 years of age and resident in Atlanta, Georgia, USA. Patients with a history of emphysema or chronic bronchitis, concurrent pregnancy or those patients actively taking antioxidant supplements such as vitamin C or E were excluded from the study. A group of healthy subjects were recruited as controls. These subjects were non-smokers, who had no history of chronic pulmonary disease and had normal spirometry testing. They were excluded from the study if they had any history of atopy or allergic rhinitis.

Patients were divided into those with an exacerbation of asthma and those with stable asthma. Those with an exacerbation of asthma were those patients who had shortness of breath, cough, wheeze, chest congestion or decreased peak flow reading less than 80% of base or FEV1 ≤ 70% predicted. From this group those who were smokers, or had severe dyspnoea, or had been treated with intravenous or oral steroids within two weeks of testing were excluded. Those with stable asthma were patients with pulmonary function tests showing obstruction and reversibility. They had not been treated with oral steroids in the preceding two weeks. They had no asthma instability in that they had not required
increased beta-agonist therapy, step-up therapy over the preceding 4 weeks. Patients were excluded from this group if they were smokers, had severe dyspnoea or had been treated with intravenous or oral steroids in the preceding two weeks.

After obtaining consent, a detailed history was taken including a symptom profile. An “ozone exposure dose,” reflecting ambient ozone exposure, was calculated based on the location in which the subject lived and on their activity level. Each patient underwent spirometry testing and blood was drawn for serum IgE testing, full blood count with differential and serum glycodelin assay. A nasal saline lavage sample was obtained for each study participant. An enzyme-linked immunosorbant assay (ELISA) for glycodelin in nasal saline lavage fluid and serum plasma was performed. 24 & 48-hour air quality indices were obtained from the city of Atlanta records and 24 & 48-hour ozone exposure doses were calculated for each subject (Table I). The antioxidant glutathione was measured in the nasal lavage samples (GSH/prot, ng/mL).

The Emory University Human Investigations Committee approved both the collection of the blood samples from patients and the experimental protocol.
7.3.2 Enzyme Linked Immunosorbant Assay (ELISA)

Materials

- The primary antibody used for glycodelin was anti glycodelin antibody generated in chicken and affinity column purified. This was prepared previously in our laboratory (Poddar, Kim et al. 1998).

- The secondary antibody used was anti chick IgG raised in rabbit. This was conjugated with alkaline phosphatase.

- The antigen-antibody complex was visualised using para-nitrophenyl phosphate pNPP (Sigma), a chromagen in conjunction with alkaline phosphatase.

- Phosphate buffered saline (PBS): 1x solution of Phosphate buffered saline (PBS) was prepared by adding 8g of NaCl, 0.2g of KCl, 1.44g of Na₂HPO₄, and 0.24g KH₂PO₄. The solution was made up to a pH of 7.6. This was made up to 1000mls in distilled water.

- 96 well ELISA plate
• Bovine serum albumin (BSA; Sigma-Aldrich, St.Louis, MO): 0.1g of Bovine serum albumin was added to 100mls PBS to make a 0.1% solution

Method

Following consent, approximately 10ml of venous blood was collected from the patients and healthy controls by venepuncture into heparinised ‘vacutainer’ tubes. After centrifugation at 400 x g for 20mins, the plasma was removed and stored at −70°C.

Nasal lavage was obtained as previously described using a disposable syringe filled with sterile, normal saline solution at room temperature. This device was used five times in each nostril while occluding the other nostril; then the subject was instructed to exhale through the lavaged side into a specimen cup and the returned fluid pooled.
Glycodelin peptide was used to quantify the glycodelin present. Glycodelin peptide standards in the following concentrations were prepared with deionised water by serial dilutions:

- 1ug;
- 500ng;
- 250ng;
- 125ng;
- 62.5ng;
- 31.25ng;
- 15.625ng; and
- 7.812ng;
- 3.6ng;
- 1.8ng;
- 0.9ng;
- 0.45ng;
- 0.225ng; and
- 0.112ng.

Amniotic fluid at 13 weeks was prepared in the following dilutions with phosphate buffered saline:

- 1:1000;
- 1:5000;
- 1:25,000;
- 1:50,000;
- 1:100,000.
Plasma samples were diluted 1:10 in a buffered saline solution (PBS). 100μl was added in triplicate wells of the ELISA plate and incubated overnight at 37°C. The next day the plates were washed 6 times in phosphate buffered saline solution (PBS) and blocking was done at room temperature for 1 hour using 0.1% BSA (bovine serum albumin). Wells were washed with PBS (x 6) and incubated for 2 hours at 37°C with 100μl of affinity column purified chicken anti-glycodelin antibody raised in this laboratory (Poddar, Kim et al. 1998) at a dilution of 1:50. Following this the wells were washed with PBS (x6), blocked with 0.1% BSA for 1 hour at room temperature, and then washed with PBS (x6). A secondary antibody (rabbit antichicken IgG conjugated with alkaline phosphatase) was added at a dilution of 1:30,000 in 0.1% BSA, and incubated for 2 hours at 37°C. The wells were washed again with PBS (x6) and the substrate p-nitro phenyl phosphate was added. The optical density was measured using a micro titre plate reader set to 405nm with wavelength correction at 570 nm to correct optical imperfections in the plate. Measurements were taken after 30, 60 and 90 minutes. The amount of glycodelin was quantified using glycodelin peptide and amniotic fluid standard curves. The entire experiment was repeated three times.
Table 7.1. Ozone Exposure Dosage Calculations

<table>
<thead>
<tr>
<th>Activity</th>
<th>Formula</th>
</tr>
</thead>
<tbody>
<tr>
<td>Outdoors at rest</td>
<td>( [O_3] \times \text{time (h)} \times 1 )</td>
</tr>
<tr>
<td>Outdoors with mild exercise</td>
<td>( [O_3] \times \text{time (h)} \times 2 )</td>
</tr>
<tr>
<td>Outdoors with moderate/severe exercise</td>
<td>( [O_3] \times \text{time (h)} \times 3 )</td>
</tr>
<tr>
<td>Indoors (air conditioning) at rest</td>
<td>( [O_3] \times \text{time (h)} \times 0.3 )</td>
</tr>
<tr>
<td>Indoors (air conditioning) with mild exercise</td>
<td>( [O_3] \times \text{time (h)} \times 0.3 )</td>
</tr>
<tr>
<td>Indoors (air conditioning) with moderate exercise</td>
<td>( [O_3] \times \text{time (h)} \times 0.8 )</td>
</tr>
<tr>
<td>Unaccounted hours weighted as indoors at rest</td>
<td>( [O_3] \times \text{time (h)} \times 0.3 )</td>
</tr>
</tbody>
</table>

Table 7.1

The local ozone concentration (\([O_3]\) in ppb) was assessed for each patient according to their zip code, during the morning, afternoon, evening and nighttime of an average day. The ‘ozone dose’ was calculated as the sum of pollutant doses over each 24-hour period. The hour average ozone dose was obtained by dividing the ‘ozone dose’ by 24 hours.
7.3.3 Ozone Exposure Calculation

The ozone exposure for metropolitan Atlanta (Georgia, USA) is recorded by the city of Atlanta, and levels can be obtained according to the zip code area in which the study candidate lives. The levels are recorded during morning, afternoon, evening and nighttime. An “ozone exposure dose,” reflecting ambient ozone exposure, was calculated based on location and activity level. Ozone exposure is higher outdoors than indoors and higher during exercise than at rest. Ozone exposure is higher indoors when air conditioning systems are in operation, than when there is no air conditioning operational. The ozone exposure dosage was calculated as in Table 7.1. The ozone dose was calculated as the sum of pollutant doses over each 24-hour period and the hour-average ozone dose obtained by dividing the ‘Ozone dose’ by 24 hours.

7.4 Statistical Analysis

T-tests were used to detect differences in demographics, spirometry, and glycodelin levels between groups. Correlations between nasal and plasma glycodelin levels with lung function, ozone exposure doses, and antioxidant levels were analysed using Pearson’s Correlation Coefficients. Statistical analysis was conducted using the Statistical Software Program (SPSS, Chicago IL) for Windows (version 10).
7.5 Results

The study was carried out between June and September 2003. 12 asthmatics and 5 healthy controls were enrolled in the study ranging in age from 22-70 years in age. The unstable asthma group had a percentage predicted FEV1 (forced expiratory volume in 1 second) of 66% +/- 0.16 and FVC (forced vital capacity) ratio of 0.60 +/- 0.11, while the stable group had a percentage predicted FEV1 of 82% +/- 0.18 and FVC ratio of 0.78 +/- 0.1 (Table 7.2). Healthy controls had a percentage predicted FEV1 of 88% +/- 0.9 and a FVC ratio of 0.82 +/- 0.3.

A significant difference was found in the level of blood eosinophilia in unstable asthmatics versus stable asthmatics: 9 ± 6 % (unstable asthmatics) versus 2 ± 2 % (stable asthmatics), (p=0.07). Among all patients, plasma glycodelin levels correlated with leukocytosis (R= 0.64, p=0.04). Among all patients, a positive correlation was found between plasma glycodelin and serum IgE levels (R= 0.59, p=0.05).

No difference in 48 hr ozone dose, 24 hr ozone dose, 48 hr peak air quality index score, and 24 hr peak air quality index score was seen across the groups: healthy controls versus asthmatics, unstable asthmatics versus stable asthmatics and unstable asthmatics versus healthy controls. A positive correlation was found between 24-hr ozone exposure dose and glycodelin levels (both nasal & plasma) in all patients (R>0.60, p<0.05) (Figure 7.1). Nasal lavage and plasma glycodelin levels significantly correlate with nasal lavage levels of the antioxidant glutathione (GSH/prot, ng/mL) (R= 0.90, p< 0.01).
testing showed no significant difference in plasma or nasal lavage
glycodelin in healthy controls versus all asthmatics, stable asthmatics versus
unstable asthmatics and healthy controls versus unstable asthmatics.

Higher plasma glycodelin levels correlated with better lung function
among asthmatics tested (p=0.023) (Figure 7.2). Preliminary results demonstrated
a trend toward an inverse correlation between plasma glycodelin and FEV1/FVC
ratio among the 5 unstable asthmatics (R= -0.93, p=0.07) (Figure 7.3).
Table 7.2. Pulmonary function in study groups

<table>
<thead>
<tr>
<th></th>
<th>FEV1</th>
<th>FVC Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unstable asthma</td>
<td>66% +/-0.16</td>
<td>0.60 +/- 0.11</td>
</tr>
<tr>
<td>Stable asthma</td>
<td>82% +/-0.18</td>
<td>0.78 +/- 0.1</td>
</tr>
<tr>
<td>Healthy control</td>
<td>88% +/-0.9</td>
<td>0.82 +/- 0.3</td>
</tr>
</tbody>
</table>

The unstable asthma group had a percentage predicted FEV1 of 66% +/-0.16 and FVC ratio of 0.60 +/- 0.11. The stable group had a percentage predicted FEV1 of 82% +/-0.18 and FVC ratio of 0.78 +/- 0.1. Healthy controls had a percentage predicted FEV1 of 88% +/-0.9 and FVC ratio of 0.82 +/- 0.3
In all asthmatic patients tested, nasal lavage glycodelin levels were lower when air pollution in the previous 24-48 hrs was worse.
Higher plasma glycodelin levels correlated with better lung function among asthmatics tested (p=0.023).
Figure 7.3

In patients experiencing asthma worsening or instability there was an inverse correlation between plasma glycodelin and FEV1/FVC ratio among the 5 unstable asthmatics (R= -0.93, p=0.07), thus lower nasal lavage glycodelin levels correlate with better lung function.
7.6 Discussion

The respiratory epithelium is buffered by a thin layer of respiratory tract lining fluid, which is rich in antioxidant defenses. Markers of inflammation and oxidative stress have been identified within the respiratory tract lining fluid, which represent the intensity of the ongoing pathological processes within the lungs. These can be obtained noninvasively via nasal lavage, or exhaled breath concentrate and then measured appropriately. We used nasal lavage to sample upper airway respiratory tract lining fluid as it represented a relatively non-invasive procedure and has been proven to be a reliable method to study acute inflammatory response to inhaled pollutants and reflect the acute inflammatory effect of pollutants such as ozone in the lower lung (Graham and Koren 1990; Koren, Hatch et al. 1990).

The prevalence of allergic respiratory diseases such as bronchial asthma has increased in recent years, especially in industrialised countries. A change in the genetic predisposition is an unlikely cause of the increase in allergic diseases because genetic changes in a population require several generations. Consequently, this increase may be explained by changes in environmental factors, including indoor and outdoor air pollution. Over the past two decades, there has been increasing interest in studies of air pollution and its effects on human health. Although the role played by outdoor pollutants in allergic sensitisation of the airways has yet to be clarified, a body of evidence suggests that urbanisation, with its high levels of vehicle emissions, and a westernised lifestyle are linked to the rising frequency of respiratory allergic diseases observed
in most industrialised countries, and there is considerable evidence that asthmatic persons are at increased risk of developing asthma exacerbations with exposure to ozone, nitrogen dioxide, sulphur dioxide and inhalable particulate matter.

Ozone is considered by far the most irritant gas to humans, with effects seen even at extremely low concentrations. Ozone is known to cause increased respiratory symptoms by compromising the host defence mechanism and by disrupting cellular membranes through its powerful oxidising properties (Blomberg 2000). This cellular damage can initiate a series of cascading events in the inflammatory process such as the release of inflammatory mediators, causing vascular extravasation of inflammatory cells and tissue oedema (Diaz Sanchez, Dotson et al. 1994). Inflammation and oxidative stress are involved in the pathogenesis of a variety of pulmonary diseases (Bascom, Bromberg et al. 1996). Oxidative stress mediated by reactive oxygen species is recognized to contribute significantly to the inflammatory process of bronchial asthma (Bascom, Bromberg et al. 1996). These species are released into the airway by activated inflammatory cells such as leukocytes. In controlled human studies, mild atopic asthmatics exposed to short-term peak ambient levels of ozone experience an acute inflammatory response in the lower airways and a decrement in lung function (Pryor 1991; Mudway and Kelly 2000). Healthy individuals who exercise heavily for brief periods (1 to 2 hours) may experience respiratory symptoms at levels exceeding the Hong Kong Air Quality Objective of 240 micrograms per cubic metre. They may also experience these symptoms at a lower concentration for longer exposure (6 to 8 hours) during moderate exercise. Individuals with sensitive respiratory systems (such as with asthma or respiratory disease) are more
susceptible to the effects of ozone (D'Amato, Liccardi et al. 2005). In our experimental group we did not find a significant difference in ozone exposure dose across all test groups. Thus we did not show that those patients with the most active respiratory disease were also those exposed to highest levels of environmental pollution. It is likely that a larger sample size would be necessary to show such a difference. However we did find a positive correlation between 24-hr ozone exposure dose and glycodelin levels (both nasal & plasma) in all patients (R>0.60, p<0.05). Thus glycodelin levels along with other inflammatory markers are raised in response to ozone exposure and high oxidant stress.

Inflammatory states like asthma, chronic obstructive lung disease (COPD) and parenchymal lung disorders have been shown to lead to serious disturbances in the oxidant/antioxidant balance of the lung with consequent oxidant mediated cell injury. In our study we found that nasal lavage and plasma glycodelin levels significantly correlated with nasal lavage levels of the antioxidant glutathione (GSH/prot, ng/mL) (R= 0.90, p< 0.01) a protective antioxidant which is upregulated in the respiratory tract in response to oxidative stress and is another marker of airway inflammation. Plasma glycodelin levels appear to mirror other signs of inflammation and atopy, such as white blood cell, and serum IgE levels. We found a raised serum eosinophil level as expected in those patients with unstable asthma and among all patients, plasma glycodelin levels correlated with both leukocytosis serum IgE levels, again markers of inflammation in the respiratory tract. We did not find a significant difference in plasma or nasal lavage glycodelin levels between our patients and normal groups again this is likely due to our small sample size. However there does appear to be a trend
toward an inverse relationship between plasma glycodelin and lung function in the unstable asthmatics group. Plasma glycodelin levels appear to correlate with airflow limitation in unstable asthmatics. Thus those patients with most active disease and poorest lung function had higher levels of glycodelin recorded. This may represent a systemic response with increased glycodelin production in an attempt to dampen this inflammatory response within the airway.

We conclude that assays for glycodelin may be useful markers of atopy, ambient oxidant exposure, and lower airway inflammation in asthma. There are some obvious limitations to this pilot study, sample size was small, and the effect of intercurrent medication usage and compliance with medical therapy was not assessed. Also the ‘Ozone Dose’ tool used in this study has not yet been fully validated. A larger population based study would be required in order to establish ‘normal’ levels of glycodelin in airway secretions, and levels in subjects exposed to high levels of oxidative stress, and then to compare this to patients with known airway inflammatory diseases.
CHAPTER 8

EFFECT OF SINUSITIS MEDICATIONS
ON GLYCODELIN EXPRESSION IN VITRO
8.1 Introduction

Glucocorticoids are widely used as immunosuppressive and anti-inflammatory agents in the therapy of many diseases and in transplantation. Moreover, glucocorticoids are hormones naturally released during the course of an immune response due to the existence of interactions between the immune and neuroendocrine systems (Besedovsky, Sorkin et al. 1975). This production of glucocorticoids during an immune response is regarded as a critical step in the regulation of the magnitude of the response, preventing potential damage to the host (Munck, Guyre et al. 1984). The immune and inflammatory responses are affected in many different ways by glucocorticoids. The down-regulation of the expression of many cytokine genes is one of the most important glucocorticoid actions (Gillis, Crabtree et al. 1979; Snyder and Unanue 1982; Arya, Wong-Staal et al. 1984; Culpepper and Lee 1985; Beutler, Krochin et al. 1986; Waage, Slupphaug et al. 1990; Wu, Fargeas et al. 1991). In contrast to the negative effects on cytokine production, some authors have shown that glucocorticoids induce the expression of several cytokine receptors (Fernandez-Ruiz, Rebollo et al. 1989; Hawrylowicz, Guida et al. 1994). It has been suggested that many of the anti-inflammatory effects of glucocorticoids are due to their ability to induce the expression of lipocortin, initially identified by its inhibitory action on phospholipase A2 (PLA2) (Flower and Blackwell 1979). Reported effects of glucocorticoid on T-cell activation are inhibitory (Gillis, Crabtree et al. 1979). These observations suggest that the overall effect of glucocorticoids,
therapeutically as well as physiologically, depends on both positive and negative actions on multiple systems and cells.

Antihistamines are commonly used in the treatment of atopic diseases (Simons and Simons 1994). It has been proposed that the new generation of antihistamines may regulate certain effector functions of eosinophils, which include the inhibition of eosinophil chemotaxis and recruitment into the airways after allergen exposure (Walsh 1994). Desloratadine, one of these new antihistamines, in recent studies has been shown to act as a suppressor of differentiation of eosinophil progenitors in vivo through mechanisms that may directly or indirectly relate to antagonism of H₁ histamine receptors (Cyr, Baatjes et al. 2002). All of the new second-generation antihistamines are efficacious in controlling symptoms of allergic rhinitis (i.e., sneezing, rhinorrhea, itching) but do not significantly improve nasal congestion. For this reason, some second-generation antihistamines are available as combination preparations containing a decongestant. In a multicentric, randomized, double blind, placebo-controlled, parallel-group study, physicians found that patients receiving loratadine were significantly improved compared to placebo patients. Glucocorticoid therapy is commonly employed in the treatment of chronic sinus disease. Antihistamine therapy is often employed in those patients who appear to have some allergic element to their disease. The effects of these medications on the action of the novel protein glycodelin have not previously been examined. The aim of this study was to examine the effect on the protein glycodelin of treatment with two separate medications in vitro.
8.2 Dexamethasone

Glucocorticoids are adrenocortical steroids, both naturally occurring and synthetic, which are readily absorbed from the gastrointestinal tract. Dexamethasone, a synthetic adrenocortical steroid, has a molecular weight of 392.47 and it is designated chemically as 9-fluoro-11β, 17,21-trihydroxy-16α-methylpregna-1,4-diene-3,20-dione. Its empirical formula is C_{22}H_{29}FO_{5}. At equipotent anti-inflammatory doses, dexamethasone almost completely lacks the sodium-retaining property of hydrocortisone and closely related derivatives of hydrocortisone. Dexamethasone is primarily used for its potent anti-inflammatory effects in disorders of many organ systems. Glucocorticoids cause profound and varied metabolic effects. In addition they modify the body's immune responses to diverse stimuli.

8.3 Loratadine

Loratadine has a molecular weight of 382.89 and empirical formula of C_{22}H_{23}ClN_{2}O_{2}. Its chemical name is ethyl 4-(8-chloro-5,6-dihydro-11H-benzo[5,6]cyclohepta[1,2-b]pyridine-11-ylidene)-1-piperidinecarboxylate. Loratadine is a long-acting tricyclic antihistamine with selective peripheral histamine H_{1}-receptor antagonistic activity. Human histamine skin wheal studies following single and repeated 10mg oral doses of loratadine have shown that the drug exhibits an antihistaminic effect beginning within 1 to 3 hours, reaching a
maximum at 8 to 12 hours, and lasting in excess of 24 hours (Roman, Kassem et al. 1986). There was no evidence of tolerance to this effect after 28 days of dosing with loratadine (Roman, Kassem et al. 1986). Loratadine was rapidly absorbed following oral administration of a 10mg tablet, once daily for 10 days to healthy adult volunteers with times to maximum concentration of 1.3 hours for loratadine and 2.5 hours for its major active metabolite, descarboethoxyloratadine. In vitro studies with human liver microsomes indicate that loratadine is metabolized to descarboethoxyloratadine predominantly by cytochrome P450 3A4 and to a lesser extent by cytochrome P450 2D6 (Yumibe, Huie et al. 1996).

8.4 Aim:

To examine the effect on glycolelin gene expression, and glycolelin protein production in K562 (lymphoid precursor cells) of treatment with the following:

1) Steroid therapy
2) Anti-histamine therapy
8.5 Materials and Methods

Materials

8.5.1 Cell Culture

Growth media for K562 cells:

- RPMI with L-glutamine (Mediatech, Herndon, VA, USA) 90%: 225ml
- Fetal Bovine Serum (FBS) (Mediatech, Herndon, VA, USA) 10%: 25ml
- Penicillin/Streptomycin 1%: 2.5ml

The above were combined under aseptic technique in the cell culture laboratory and stored at 4° Celsius.
8.5.2 Phorbol Myristate Acetate (PMA)

Phorbol myristate acetate (PMA), a tumour promoter has been reported to induce glycolelin gene expression in K562 cells (Morrow, Xiong et al. 1994). PMA, a plant mitogen, does not occur in mammalian tissues but its actions are mimicked in mammalian tissues by lysosphatidic acid (LPA), a bioactive phospholipid which exhibits pleomorphic functions in multiple cell lineages (Gaits, Fourcade et al. 1997).

8.5.3 Preparation of Phorbol 12-Myristate 13-Acetate (PMA)

FW: 616.84
Formula: C_{36}H_{56}O_{8}

A molar solution of PMA was prepared by adding Ethanol to PMA powder. This was further diluted with serum free media to provide a 1nM solution.

8.5.4 Cell Culture Method

The human chronic myelogenous leukemia cell line K562 (American Type Culture Collection (ATCC) Rockville, MD), was grown up to 75% confluence in RPMI 1640 medium with 10% fetal bovine serum. The cells were maintained at 37°C in a 5% CO₂ incubator. Once cells had reached 75% confluence, they were washed with Hanks buffered saline solution (HBSS) and serum deprived overnight.
8.6 Preparation of Dexamethasone Treatment Media

FW 392.5, 1M = 392.5g/L

2.4g dexamethasone powder was diluted with 1ml 100% Ethanol i.e. 2.4mg/ml. This volume was brought up to 10mls in serum free media, i.e. 2.4mg/10mls or 240μg/ml. Final concentrations of 20μg, 10μg, 5μg, 1μg and 0.1μg/ml were used. A control media containing ethanol was also prepared (1ml of 100% Ethanol was made up to 10mls in serum free media). The same volume was added as in the highest concentration of dexamethasone (i.e. 41.6μl).

Treatment dosages were extrapolated from the treatment dosages in patients by estimating the concentration of drug in the blood following administration.

8.7 Cell Treatment with Dexamethasone

Cells were plated in 12 well plates at a concentration of no less than 1x10^6 cells per well. All cells were activated with 1nM phorbol myristate acetate, apart from a control well containing only K562 cells. The cells were then treated with varying concentrations of dexamethasone, each concentration being added in triplicate (Figure 8.1). Cells were treated for 24 hours and then collected via centrifugation at 2000g x 5minutes. The supernatant was removed for further examination using the ELISA technique, while the RNA was isolated from the cells for RT-PCR analysis.
Figure 8.1  Plan for cell treatment with Dexamethasone

Figure 8.1  K562 cells were plated in 12 well plates at 1x10^6 cells per well. The cells were activated with 1nM PMA. Control wells contained K562 cells alone. Varying concentrations of Dexamethasone were added in triplicate as above.

8.8 Preparation of Loratadine
Loratadine powder is soluble at a concentration of 26mg/ml in DMSO. 10mgs loratadine powder was added to 384.6μl DMSO. The solution was alliquoted into 10 samples each containing 1mg and each was further diluted 1:10 in serum free media, to provide a final stock solution of 100μg per aliquot. Final concentrations of 20μg/ml, 10μg/ml, 2μg/ml, 1μg/ml and 0.5μg/ml were used. A control media containing DMSO was also prepared. 38.5μl DMSO was diluted with 346.5ul serum free media.

Treatment dosages were extrapolated from the treatment dosages in patients by estimating the concentration of drug in the blood following administration.

8.9 Cell treatment with Loratadine

Cells were plated in 12 well plates at a concentration of no less than 1x10^6 cells per well. All cells were activated with 1nM phorbol myristate acetate, apart from a control well containing only K562 cells. The cells were then treated with varying concentrations of Loratadine, each concentration being added in triplicate (Figure 8.2). Cells were treated for 24 hours and then collected via centrifugation at 2000g x 5minutes. The supernatant was removed for further examination using the ELISA technique, while the RNA was isolated from the cells for RT-PCR analysis.
Figure 8.2 Plan for cell treatment with Loratadine

(a) K562 cells were plated in 12 well plates at 1x10^6 cells per well. The cells were activated with 1nM PMA. Control wells contained K562 cells alone. Varying concentrations of Loratadine were added in triplicate as above.

(b) The cells were activated with 1nM PMA. Control wells contained K562 cells alone. Varying concentrations of Loratadine were added in triplicate as above.
8.10  Polymerase Chain Reaction (PCR)

Materials

RNA extraction kit:  Invitrogen Corp. (Carlsbad, CA, USA)

Method

8.10.1 Reverse Transcriptase- Polymerase Chain Reaction (RT-PCR)

Analysis.

The cell pellet was treated with cell lysis buffer, and an Invitrogen kit used to isolate RNA. After cell lysis the sample was transferred to a homogeniser and centrifuged at 12,000g for 2 minutes at 25° Celsius. 300μl of ethanol was added to each volume of cell homogenate, mixed well and then transferred to a RNA spin cartridge, centrifuged at 12,000g for 2 minutes, washed with ‘wash buffer I’ and ‘wash buffer II’, prior to collection of the RNA with 30μl of RNAase-free water. Quality of RNA was checked, and 3.0μg was reverse transcribed. The product was PCR amplified using:

- 5’- AAGTTGGCAGGGACCTGGCACTC-3’ (sense); and
- 5’-ACGGCACGGCTCTTCCATCTGTT-3’. (antisense)

oligonucleotides for glycodelin.
Following this procedure a product of 422 base pairs was obtained. Sample sizes and number of amplifications were optimised to produce measurements within a linear range. After an initial denaturation at 95°C for 1 minute, PCR was conducted as follows: denaturation at 95°C for 45 seconds, annealing at 60°C for 45 seconds, and extension at 72°C for 1 minute. This was repeated for 35 cycles followed by a final extension at 72°C for 10 minutes. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the internal control to substantiate equal amplification of the total RNA. The GAPDH primer set was as follows: 5’-ACCACAGTCCATGCCATC-3’ (sense) and 5’-ACGGCACGGCTCTTCCATCTGTT-3’ (antisense), yielding a product of 451 base pairs. All PCR products were analysed on 2% agarose gel containing ethidium bromide.

8.11 Enzyme Linked Immunosorbant Assay (ELISA)

Materials

- The primary antibody used for glycodelin was anti-glycodelin antibody generated in chicken and affinity column purified. This was prepared previously in our laboratory (Poddar, Kim et al. 1998).

- The secondary antibody used was anti chick IgG raised in rabbit. This was conjugated with alkaline phosphatase.
• The antigen-antibody complex was visualised using para-nitrophenyl phosphate pNPP (Sigma), a chromagen in conjunction with alkaline phosphatase.

• Phosphate buffered saline (PBS): 1x solution of Phosphate buffered saline was prepared by adding 8g of NaCl, 0.2g of KCl, 1.44g of Na₂HPO₄ and 0.24g KH₂PO₄. The solution was made up to a pH of 7.6. This was made up to 1000mls in distilled water.

• 96 well ELISA plate

• Bovine serum albumin (BSA): 0.1g of Bovine serum albumin was added to 100mls PBS to make a 0.1% solution

Method

Glycodelin peptide was used to quantify the glycodelin present. Glycodelin peptide standards in the following concentrations were prepared with deionised water 1ug, 500ng, 250ng, 125ng, 62.5ng, 31.25ng, 15.625ng, 7.812ng, 3.6ng, 1.8ng, 0.9ng, 0.45ng, 0.225ng, and 0.112ng by serial dilutions. Amniotic
fluid at 13 weeks was prepared in the following dilutions with phosphate buffered saline. 1:1000; 1:5000; 1:25,000; 1:50,000; 1:100,000.

100µl of the cell supernatant was added in triplicate wells of the ELISA plate and incubated overnight at 37°C. The next day the plates were washed 6 times in phosphate buffered saline solution (PBS) and blocking was done at room temperature for 1 hour using 0.1% BSA (bovine serum albumin). Wells were washed with PBS (x 6) and incubated for 2 hours at 37°C with 100µl of affinity column purified chicken anti-glycodelin antibody raised in this laboratory (Poddar, Kim et al. 1998) at a dilution of 1:50. Following this the wells were washed with PBS (x 6), blocked with 0.1% BSA for 1 hour at room temperature, and then washed with PBS (x 6). A secondary antibody (rabbit anti-chicken IgG conjugated with alkaline phosphatase) was added at a dilution of 1:30,000 in 0.1% BSA, and incubated for 2 hours at 37°C. The wells were washed again with PBS (x 6) and the substrate p-nitro phenyl phosphate was added. The optical density was measured using a micro titre plate reader set to 405nm with wavelength correction at 570 nm to correct optical imperfections in the plate. Readings were taken after 30, 60 and 90 minutes. The amount of glycodelin was quantified using glycodelin peptide and amniotic fluid standard curves.
8.12 Statistical Analysis

Inferential statistics were performed using Analysis of the variance of the mean (ANOVA). Significance at \( P<0.05 \) is assumed throughout. Tukey simultaneous tests were performed and pairwise comparisons were made between treatment doses. Statistical analysis was conducted using Minitab 14 statistical software for windows.

8.13 Results

Following treatment of the K562 cells for 24 hours with varying concentrations of dexamethasone glycodelin gene expression as shown in Figure 8.3 (1-4), was measured on densitometry. The findings are illustrated in Figure 8.4 (1,2). On statistical analysis using analysis of variance of the means no significant dose response was seen following treatment with the various concentrations of dexamethasone (p=NS). There was a trend towards decreased glycodelin gene expression at the higher doses of dexamethasone while glycodelin gene expression was increased at lower doses of dexamethasone. These findings were confirmed on analysis of the cell culture supernatant using the ELISA method (Figure 8.5 (1,2)). Again on ELISA it was shown that there was increased production of the glycodelin protein when treated with lower doses.
of dexamethasone and a reversal of these findings at higher treatment doses with resulting decrease in glycodelin protein production (p= 0.485).

Following treatment of the K562 cells for 24 hours with varying concentrations of Loratadine, glycodelin gene expression as shown in Figure 8.6 (1,2), was measured on densitometry. The findings are illustrated in Figure 8.7 (1,2). Treatment with Loratadine showed a dose dependent decrease in glycodelin gene expression, with glycodelin gene expression found to be lowest at the lower doses of Loratadine treatment (p=0.000). These findings were confirmed on analysis of the cell culture supernatant using the ELISA method. Tukey simultaneous tests were then performed in order to establish at which doses of Loratadine a significant dose effect was seen. Pairwise comparisons were made between each dose. A significant reduction in glycodelin expression was seen in K562 cells treated with 2μg, 1μg and 0.5μg Loratadine. These findings were confirmed on analysis of the cell culture supernatant using the ELISA method.
Figure 8.3.1 Glycodelin expression in K562 cells after treatment with Dexamethasone

Figure 8.3.1

Lane 1-3: K562 cells + 1nM PMA + control (ethanol), lane 4-6: K562 cells + 1nM PMA + 10µg Dexamethasone, lane 7-9: K562 cells + 1nM PMA + 5µg Dexamethasone, lane 10-12: K562 cells + 1nM PMA + 1µg Dexamethasone

Figure 8.3.2 GAPDH expression in K562 cells after treatment with Dexamethasone

Figure 8.3.2

Lane 1-3: K562 cells + 1nM PMA + control (ethanol), lane 4-6: K562 cells + 1nM PMA + 10µg Dexamethasone, lane 7-9: K562 cells + 1nM PMA + 5µg Dexamethasone, lane 10-12: K562 cells + 1nM PMA + 1µg Dexamethasone
Figure 8.3.3 Glycodelin expression in K562 cells after treatment with Dexamethasone

Lane 1-3: K562 cells, Lane 4-6: K562 cells + 1nM PMA, lane 7-9: K562 cells + 1nM PMA + 20μg Dexamethasone, lane 10-12: K562 cells + 1nM PMA + 10μg Dexamethasone, lane 13-15: K562 cells + 1nM PMA + 5μg Dexamethasone, lane 16-18: K562 cells + 1nM PMA + 1μg Dexamethasone, lane 19-21: K562 cells + 1nM PMA + control (ethanol)
Figure 8.3.4 GAPDH expression in K562 cells after treatment with Dexamethasone

Lane 1-3= K562 cells, Lane 4-6=K562 cells+ 1nM PMA, lane 7-9=K562 cells + 1nM PMA +20μg Dexamethasone, lane 10-12=K562 cells + 1nM PMA +10μg Dexamethasone, lane 13-15=K562 cells +1nM PMA+5μg Dexamethasone, lane 16-18= K562 cells + 1nM PMA+1μg Dexamethasone, lane 19-21= K562 cells + 1nM PMA + control (ethanol)
Figure 8.4.1

Densitometric analysis of PCR gels following treatment of K562 cells with varying concentrations of dexamethasone.
Analysis of the variance of the treatment of K562 cells with various doses of dexamethasone failed to show a significant dose response ($P=0.265$). There was however a significant date effect which can only be explained by interexperimental variation and protocol error. The readings follow a more consistent pattern for the second analysis and are therefore felt to be more accurate. (Subjects 1-6 above = treatment groups)
Figure 8.5.1

On ELISA examination of cell culture supernatant after treatment of K562 cells with varying concentrations of Dexamethasone, glycodelin protein production was reduced by high doses of Dexamethasone, while glycodelin protein levels were highest at lower doses of glycodelin (p=NS).
Figure 8.5.2

On analysis of our ELISA readings no significant dose response for dexamethasone was exhibited (p=0.485). Again a significant date effect was seen giving weight to the argument that there was experimental error involved in the first set of readings. (Subjects 1-6 above = treatment groups).
Figure 8.6.1 Glycodelin expression in K562 cells after treatment with Loratadine

Lane 1-3: K562 cells, Lane 4-6: K562 cells + 1nM PMA, lane 7-9: K562 cells + 1nM PMA +20μg/ml Loratadine, lane 10-12: K562 cells + 1nM PMA+10μg/ml Loratadine, lane 13-15: K562 cells + 1nM PMA+2μg/ml Loratadine, lane 16-18: K562 cells + 1nM PMA+1μg/ml Loratadine, lane 19-21: K562 cells + 1nM PMA+0.5μg/ml Loratadine, lane 22-23: K562 cells + 1nM PMA+DMSO (control).
Figure 8.6.2 GAPDH expression in K562 cells after treatment with Loratadine

Lane 1-3: K562 cells, Lane 4-6: K562 cells + 1nM PMA, lane 7-9: K562 cells + 1nM PMA +20µg/ml Loratadine, lane 10-12: K562 cells + 1nM PMA+10µg/ml Loratadine, lane 13-15: K562 cells + 1nM PMA+2µg/ml Loratadine, lane 16-18: K562 cells + 1nM PMA+1µg/ml Loratadine, lane 19-21: K562 cells + 1nM PMA+0.5µg/ml Loratadine, lane 22-23: K562 cells + 1nM PMA+DMSO (control).
Figure 8.7.1

Densitometric analysis of PCR gels following treatment of K562 cells with varying concentrations of Loratadine.
Analysis of the variance of treatment of K562 cells with various doses of Loratadine was performed. A significant dose effect was seen ($p=0.000$). No significant date effect was seen; therefore our results were reproducible on repeated experimentation. Tukey simultaneous tests were then performed in order to establish at which doses of Loratadine a significant dose effect was seen. Pairwise comparisons were made between each dose. A significant reduction in glycodelin expression was seen in K562 cells treated with 2µg, 1µg and 0.5µg Loratadine. (Groups 1-8 above = treatment groups).
8.14 Discussion

The immune and inflammatory responses are affected in many different ways by glucocorticoids. The production of glucocorticoids during an immune response is regarded as a critical step in the regulation of the magnitude of the response, preventing potential damage to the host (Munck, Guyre et al. 1984). The down-regulation of the expression of many cytokine genes is one of the most important glucocorticoid actions (Gillis, Crabtree et al. 1979; Snyder and Unanue 1982; Arya, Wong-Staal et al. 1984; Culpepper and Lee 1985; Beutler, Krochin et al. 1986; Waage, Slupphaug et al. 1990; Wu, Fargeas et al. 1991). In contrast to the negative effects on cytokine production, some authors have shown that glucocorticoids induce the expression of several cytokine receptors (Fernandez-Ruiz, Rebollo et al. 1989; Hawrylowicz, Guida et al. 1994). Reported effects of glucocorticoid on T-cell activation are inhibitory (Gillis, Crabtree et al. 1979). These observations suggest that the overall effect of glucocorticoids, therapeutically as well as physiologically, depends on both positive and negative actions on multiple systems and cells.

Glucocorticoids are the most potent agents available to relieve the symptoms of allergic rhinosinusitis (Mygind 1993; Scadding, Lund et al. 1995). The molecular substrate of their action is to inhibit or decrease the allergen-induced synthesis of Th2 cytokines (IL-4, IL-13) and MCP-3 and MCP-4 chemokines, as well as cells expressing receptors for IL-4, IL-5 and GM-CSF, particularly eosinophils (Al Ghamdi, Ghaffar et al. 1997; Christodouloupolous, Wright et al. 1999; Kondo, Nachtigal et al. 1999). An interesting finding is that
corticosteroid treatment in allergic sinusitis is associated with an up-regulation of Th1-type cytokines, particularly IFN-gamma, and IL-12. This suggests that one of the biologic effects of corticosteroids is to shift the T-cell differentiation from a Th2 to a predominant Th1 response (Rousset, Robert et al. 1991; Wright, Christodoulopoulos et al. 1999).

Reports have described several glycodelin mediated immunoinhibitory functions, including inhibition of T-cell proliferation, inhibition of Th2 cells, and inhibition of natural killer cell function (Pockley, Barrett et al. 1989; Morrow, Xiong et al. 1994). Studies have also noted an inhibitory effect of Glycodelin on the synthesis of interleukins 1 and 2 (IL 1, IL2) (Pockley and Bolton 1990). During the inflammatory cascade seen in sinus disease, and nasal allergy, Glycodelin may be produced in high amounts in order to halt the inflammatory response within the sinus. The effects of medications used in the treatment of sinusitis on glycodelin levels have not been previously studied.

Following treatment of K562 cells with dexamethasone, glycodelin gene expression was measured using RT-PCR analysis, and glycodelin protein production was measured on ELISA. Analysis of our findings showed no significant dose response following treatment with the various concentrations of dexamethasone ($p=0.265$). There was however a significant date effect which can only be explained by interexperimental variation and protocol error. The readings follow a more consistent pattern for the second analysis and are therefore felt to be more accurate. There was a trend towards decreased glycodelin gene expression at the higher doses of dexamethasone (which were consistent with therapeutic doses in vivo) and then a reversal of this finding at the lower treatment
doses. However in this study the power of the sample was not sufficient to show a statistically significant response.

Previously in Chapter 6 our findings following examination of glycodelin levels in patients treated with steroid therapy for sinus disease were outlined. In our study group of patients with sinus disease a cohort had been treated with steroid therapy. We found a significantly lower level of serum glycodelin in those patients treated with steroids at p value < 0.05.

Glycodelin levels appear to be diminished by therapeutic doses of steroid treatment in vitro and in vivo. We would suggest that glycodelin itself has immunoinhibitory and anti inflammatory properties. If glycodelin levels are diminished by dexamethasone therapy, then steroid interferes with this natural pathway, causing its inflammatory result mainly through its action of inhibiting or decreasing the allergen induced synthesis of Th2 cytokines (IL4, IL 13) and MCP 3 and MCP 4 chemokines.

Glycodelin’s immunoinhibitory function acts via Inhibition of T-cell proliferation, Th2 cells, IL-1β and IL-2 cytokine, and natural killer cell function. Steroid therapy does not appear to have a synergistic effect on this pathway.

Antihistamines are commonly used in the treatment of atopic diseases (Simons and Simons 1994). Antihistamine therapy is often employed for those patients with sinus disease who appear to have some allergic element to their disease. Loratadine in addition to standard therapy was found to improve the control of some symptoms of sinusitis (Braun, Alabert et al. 1997). Desloratadine one of the new antihistamines, in recent studies has been shown to act as a suppressor of differentiation of eosinophil progenitors in vivo through
mechanisms that may directly or indirectly relate to antagonism of H₁ histamine receptors (Cyr, Baatjes et al. 2002).

Following treatment of K562 cells with loratadine, glycodelin gene expression was measured using RT-PCR analysis, and glycodelin protein production was measured on ELISA. Treatment with Loratadine showed a dose dependent decrease in glycodelin gene expression, with glycodelin gene expression found to be lowest at the lower doses of Loratadine treatment (p=0.000) while at higher doses of loratadine, glycodelin levels were highest. A significant reduction in glycodelin expression was seen in K562 cells treated with 2μg, 1μg and 0.5μg loratadine. No significant date effect was seen; therefore our results were reproducible on repeated experimentation. Thus it appears that at lower doses loratadine inhibits glycodelin gene expression. These findings were again reflected in the glycodelin protein production by these cells on ELISA. Thus it is possible that loratadine may be involved in stimulating this anti inflammatory protein. Further studies are necessary in order to identify the pathway via which this effect is exerted. The doses at which this affect may be seen would appear to be above the recommended therapeutic doses.
CHAPTER 9

DISCUSSION
Rhinosinusitis has been understood as inflammation of the paranasal mucous membranes due to bacterial infection, which might be based on impaired ventilation and drainage of the sinuses due to either viral infection in acute sinusitis or obstruction of the ostiomeatal complex in chronic rhinosinusitis (Stammberger 1986; Baraniuk 1994; Gwaltney 1994). Understanding the pathomechanisms of inflammation, especially in chronic rhinosinusitis and nasal polyposis, seems to be crucial for further success in disease treatment. Ostial blockage leads to a local hypoxic environment in the sinuses, resulting in the accumulation of secretions and inflammatory products. This creates a vicious cycle of inflammation and trapping of secretions, thereby providing an ideal medium for bacterial growth. In the majority of cases, the aetiology of mucosal inflammation is either allergic or viral. The inflammatory mediators chiefly implicated in sinusitis are cytokines and chemokines. Eventually the inflammatory cycle becomes self-perpetuating and leads to long term epithelial thickening and polypoid changes in the sinus mucosa, goblet cell hyperplasia, sub-epithelial fibrosis, and persistent inflammation (Wellicome, Thornhill et al. 1990; Groves, Ross et al. 1992; Bochner, Klunk et al. 1995). There is much interest at present in the inflammatory process which results in the symptoms and signs of chronic rhinosinusitis. The factors that lead to the initial insult in the lateral wall of the nose are not known but could be bacteria, viruses, allergens, or pollutants. There is little consensus as to the triggering causes of the inflammatory cascade seen in chronic rhinosinusitis, the exact cytokines and chemokines involved in the process and how these chemokines and cytokines could be targeted in order to interrupt this inflammatory cycle.
As mentioned previously, glycdelin is a member of the lipocalin family of proteins (Logberg and Wester 2000). These proteins are released in response to acute phase proteins, which are in turn synthesised in response to bacterial infections and/or inflammation. It is likely that these proteins have evolved to be an integrated part of the body's defense system as part of the extended cytokine network. Its members exert a regulatory, dampening influence on the inflammatory cascade, thereby protecting against tissue damage from excessive inflammation. Glycdelin has several immunoinhibitory functions, including inhibition of T-cell proliferation, inhibition of IL-1beta and IL-2 cytokine production by immune cells, (Pockley and Bolton 1990) and inhibition of natural killer cell function (Okamoto, Uchida et al. 1991). It has been shown to inhibit lymphocyte proliferation (Bolton, Pockley et al. 1987). Data suggests a direct interaction between glycdelin and T-cells, placing this protein into the special class of immunoinhibitory proteins with direct T-cell effects (e.g. TGF beta, and IL-16). Glycdelin has a negative role in B-cell antigen receptor-mediated activation of human B cells. Pro-inflammatory cytokines especially in the Th2 pathway have been implicated in the pathogenesis of allergic rhinitis and bacterial sinusitis (Durham, Till et al. 2000; Burton, Papalia et al. 2002). Glycdelin is known to suppress T-cell activity thus may be produced in an attempt to suppress these pro-inflammatory cytokines and interrupt the process of sinus inflammation. Angiogenesis is required in the process of new tissue growth such as in the development of nasal polyposis. Glycdelin is known to have pro-angiogenic effects (Bolton, Pockley et al. 1987; Pockley, Barrett et al. 1989; Okamoto, Uchida et al. 1991; Song, Ramaswamy et al. 2001). Factors affecting glycdelin
gene expression are poorly understood. However hormones (e.g. progesterone), oxidised lipids and lysophosphatidic acid can stimulate glycodelin (Ramachandran, Ramaswamy et al. 2002). While hormones like progesterone might be important in regulating glycodelin production in reproductive tissues, its presence in non-reproductive cells and tissues as well as its induction by phorbol myristate acetate in K562 cells suggest that its synthesis may be regulated by other mediators (Bolton, Pockley et al. 1987).

Glycodelin is produced in response to bacterial infection and tissue inflammation; both conditions which are present in rhinosinusitis. Glycodelin then may cause inhibition of the immune response including a direct inhibition of T cells, inhibition of interleukin 1 beta and interleukin 2 and natural killer cells. Glycodelin may also trigger nasal polyp formation via promotion of angiogenesis within the sinus mucosa. Thus we hypothesise that this protein may play a role in the inflammatory process of sinusitis.

Initially we set out to confirm that glycodelin gene expression could be demonstrated in a lymphocyte precursor cell line (K562 cell line). We showed in chapter 3 that this was indeed the case using polymerase chain reaction and immunocytochemistry techniques. Our next experiment aimed to demonstrate that peripheral white blood cells in vivo express mRNA for the glycodelin gene and on immunocytochemical staining that these white blood cells would cross-react with specific antibodies raised against the glycodelin protein thus confirming the production of the glycodelin protein by these cells. The presence of glycodelin within human peripheral leukocytes may be important in a number of ways. It suggests a systemic involvement in inflammatory conditions in which
glycodelin levels have been identified and suggests an additional mechanism by which these cells may contribute to neovascularisation and angiogenesis. In early pregnancy the elevated levels of progesterone may trigger the chemotraction of leukocytes, which we have shown to express the gene for glycodelin. These leukocytes could then play a role in foetal growth and placental development, through their expression of glycodelin. A similar process could also occur in tumourigenesis. If this is the case, treatment of malignancies might be influenced not only by target-directed surgery or chemotherapy but also by reducing chemotaxis and the expression of genes such as glycodelin that may promote angiogenesis and/or tumour formation. Thus the presence of glycodelin in peripheral white blood cells, and its ability to stimulate angiogenesis and act as an immunosuppressive agent could be an important indicator of the role of peripheral leukocytes in various disease states.

Chronic nasal inflammation has previously been proposed to be a component of a systemic disease. Denburg et al, suggested that the bone marrow is an ongoing source of chronic, systemic inflammation that seeds atopic tissues with cells that are capable of maturing into the effector cells of allergy (Denburg, Dolovich et al. 1989). Therapies directed at the bone marrow need to be considered in the treatment of chronic allergic inflammation. Such therapies may include inhaled corticosteroids or may, more specifically, target bone marrow through the antagonism of haemopoietic cytokine receptors for factors such as IL-5 or through anti-IgE therapy, which could theoretically interfere with maturation of eosinophil/basophil progenitors. A corollary of these observations is that allergic inflammation involves a systemic process in which the bone marrow
actively contributes to maintain and sustain disease and symptoms. The chief goal of therapy in these conditions may well be to give sufficient anti-inflammatory and haemopoietic-modulating therapy to achieve attenuation of the bone marrow contribution to the development of allergic inflammation (Denburg, Sehmi et al. 2000). Glycodelin could be a linking factor between chronic rhinosinusitis locally confined to the paranasal sinusus, and chronic rhinosinusitis as a component of a systemic inflammatory response.

Having illustrated the presence of glycodelin in peripheral white blood cells we then examined tissue from the nasal cavity and sinuses of patients with and without sinus disease. Outside of the female reproductive tract where glycodelin has been well demonstrated, glycodelin expression has been shown in other “glandular” tissues such as breast, as well as the male genital system (Koistinen, Koistinen et al. 1997; Kamarainen, Halttunen et al. 1999; Keil, Husen et al. 1999). Glycodelin has been identified in normal and malignant glandular epithelium including hidradenoma, parabronchial glands, sweat glands and pancreatic cystadenoma (Kamarainen, Seppala et al. 1997). Glycodelin is also reported in haematopoietic cells of the megakaryocytic lineage (Morrow, Xiong et al. 1994). This is the first study to demonstrate the presence of glycodelin in nasal and paranasal sinus tissues. In this experiment it was shown that the glycodelin protein is present in the endothelial tissue and mucous producing glands of sinus mucosa. Although there is baseline expression of glycodelin in nasal tissues, in patients with chronic rhinosinusitis, there is up-regulation of glycodelin expression by endothelial cells in the vasculature.
Having shown this local up regulation of glycodelin in sinus mucosa in patients with sinus disease and that glycodelin was expressed by peripheral white blood cells, we set out to examine whether the elevation in nasal endothelial glycodelin expression observed locally was reflected in the peripheral blood, by a raised serum glycodelin level in patients with sinus disease. In Chapter 6 the findings are described. Results indicated that in patients with severe 'nasal' polyposis and /or purulent nasal discharge, serum glycodelin levels were highest. There was a significant difference between the glycodelin concentrations between those with such findings and those patients with no evidence of polyposis or purulent drainage. In addition, patients who had been previously treated with oral steroid therapy and /or antibiotics were found to have lower levels of serum glycodelin, indicating that serum glycodelin levels may also decrease following therapy for sinus disease.

Lipocalins have been used extensively as biochemical markers of disease. The clinical indications relate to almost any field of medicine, such as inflammatory disease, cancer, lipid disorders, liver and kidney function. We suggest that glycodelin may be a useful marker in sinus disease particularly in identifying those patients with severe active disease. The most commonly used lipocalin markers are alpha-1-glycoprotein, alpha-1-microglobulin, apolipoprotein D, retinol binding protein and prostaglandin D synthase (Xu and Venge 2000). Alpha-1-microglobulin is eliminated by glomerular filtration in the kidney. The filtered protein is broken down by renal tubuli thus levels in normal urine are very low. In conditions with disturbances in the tubular function, reabsorption of alpha-1-microglobulin is reduced and increased amounts will be found in the
urine. This protein is stable in the urine at different pHs and is therefore a suitable clinical marker of tubular injury. In malignant melanoma the levels of alpha-1-microglobulin in the serum shows a relationship to the clinical stage of the tumour (Fernandez-Luna, Mendez et al. 1989). Alpha-1-glycoprotein has been used as a marker in patients with inflammatory disease and in particular in patients with rheumatoid and inflammatory bowel disease (Gross, Andus et al. 1992). In patients with Crohn’s disease raised levels of this protein predicted the relapses of disease (Louis, Belaiche et al. 1997). The protein level was also a useful clinical tool in the guidance of corticosteroid treatment (Kjeldsen, Lauritsen et al. 1997).

Apolipoprotein D, which is expressed in a subset of breast carcinomas, has been proposed as a tumour marker and prognostic indicator for breast cancer progression (Lea, Kvinnsland et al. 1987). Apo D was also detected in gynaecomastia and a high percentage of male breast carcinomas (Serra Díaz, Vizoso et al. 1999). The measurement of urinary retinol binding protein has been shown to be a sensitive marker for renal tubular damage and dysfunction (Corso, Serricchio et al. 1999). Glycodelin may also be a useful indicator of inflammation within the nose and paranasal sinuses particularly in identifying those patients with severe polyposis and purulence. In our group of patients the glycodelin concentration was compared to nasal endoscopy as a predictor of the presence of nasal polyps and/or purulence. We found that above a cutoff point of 21.5ng/ml serum glycodelin was sensitive for the presence of severe polyposis / purulence in 82.35% patients. A major weakness of symptom-based diagnosis of chronic rhinosinusitis, which is currently, the standard used in practice, is that the diagnostic symptoms only hint (likely vs. possible) at the diagnosis of bacterial
vs. non-bacterial rhinosinusitis. Overlap of symptoms between acute bacterial rhinosinusitis (ABRS), chronic rhinosinusitis, allergic rhinitis, or viral upper respiratory tract infections leads to significant over-diagnosis of chronic rhinosinusitis and inappropriate usage of antibiotics. Nasal endoscopy is useful in making a diagnosis, especially when active purulent drainage is seen, but is usually a tool that is available only to the trained otolaryngologist (Stankiewicz and Chow 2002). Thus a screening blood test, such as serum glycodelin estimation, which could indicate the presence of nasal polyposis and purulence, or differentiate between the different types of sinus disease, would be of great diagnostic benefit for primary health care professionals and promote cost-effective management. Serum glycodelin levels correlate with severity of disease and more importantly, with current endoscopic findings. In other words, serum glycodelin levels seem to reflect the degree and severity of nasal pathology and disease. Therefore, in addition to careful history and physical examination, a glycodelin assay could help the primary care physician to diagnose those patients with severe polyposis and/or purulence. Potential benefits both in terms of reduced costs as well as reduced antibiotic resistance are significant. For the otolaryngologist or allergist/pulmonologist who treats patients with more severe and indolent disease, our data indicate that glycodelin levels could provide an objective assessment of the severity of disease at a given time-point.

A non-invasive marker of asthma activity may also be a useful tool. Based upon the known actions of glycodelin and its demonstrated presence in vascular endothelium and the respiratory tract, we hypothesised that a glycodelin assay may serve as a marker for inflammation in the airway, and that
levels of glycodelin in the serum may correlate with lung function in asthmatics. A thin layer of respiratory tract lining fluid buffers the respiratory epithelium, which is rich in antioxidant defenses. Markers of inflammation and oxidative stress have been identified within the respiratory tract lining fluid, which represent the intensity of the ongoing pathological processes within the lungs. The prevalence of allergic respiratory diseases such as bronchial asthma has increased in recent years, especially in industrialised countries. Although the role played by outdoor pollutants in allergic sensitisation of the airways has yet to be clarified, a body of evidence suggests that urbanisation, with its high levels of vehicle emissions, and a westernised lifestyle are linked to the rising frequency of respiratory allergic diseases observed in most industrialised countries, and there is considerable evidence that asthmatic persons are at increased risk of developing asthma exacerbations with exposure to ozone, nitrogen dioxide, sulphur dioxide and inhalable particulate matter. Ozone is known to cause increased respiratory symptoms by compromising the host defence mechanism and by the release of inflammatory mediators, causing vascular extravasation of inflammatory cells and tissue oedema (Diaz Sanchez, Dotson et al. 1994). Individuals with sensitive respiratory systems (such as with asthma or respiratory disease) are more susceptible to the effects of ozone (D'Amato, Liccardi et al. 2005). We found a positive correlation between 24-hr ozone exposure dose and glycodelin levels (both nasal & plasma) in the patients in our study illustrating that glycodelin levels along with other inflammatory markers are raised in response to ozone exposure and high oxidant stress. In our study we found that nasal lavage and plasma glycodelin levels significantly correlated with nasal lavage levels of the
antioxidant glutathione (GSH/prot, ng/mL) a protective antioxidant that is upregulated in the respiratory tract in response to oxidative stress and is another marker of airway inflammation. Plasma glycodelin levels appear to mirror other signs of inflammation and atopy, such as white blood cell, and serum IgE levels. Among all patients, plasma glycodelin levels correlated with both leukocytosis and serum IgE levels, other markers of inflammation within the respiratory tract. We did not find a significant difference in plasma or nasal lavage glycodelin levels between our patients and normal groups again this is likely due to our small sample size. However there does appear to be a trend toward an inverse relationship between plasma glycodelin and lung function in the unstable asthmatics group. Plasma glycodelin levels appear to correlate with airflow limitation in unstable asthmatics. Thus those patients with most active disease and poorest lung function had higher levels of glycodelin recorded. This may represent a systemic response with increased glycodelin production in an attempt to dampen this inflammatory response within the airway. We conclude that assays for glycodelin may be useful markers of atopy, ambient oxidant exposure, and lower airway inflammation in asthma.

We proceeded to examine the effect on glycodelin gene expression in vitro (using K562 cell line), of treatment with two separate treatment modalities commonly utilised in chronic rhinosinusitis, Dexamethasone and Loratadine. The former is a steroid medication while the latter is an antihistamine. Following treatment of the K562 cells for 24 hours with varying concentrations of Dexamethasone, no significant dose response was seen. There was a trend towards decreased glycodelin gene expression at the higher doses and then a
reversal of this finding at the lower doses. However in this study the power of the sample was not sufficient to show a statistically significant response. In our patient group we found that those patients with sinus disease who had been treated with steroid therapy had a significantly lower level of serum glycodelin. Thus glycodelin levels appear to be diminished by therapeutic doses of steroid treatment both in vitro and in vivo. Glycodelin's immunoinhibitory function acts via inhibition of T-cell proliferation, T_{h2} cells, IL-1β and IL-2 cytokine, and natural killer cell function. If glycodelin levels are diminished by dexamethasone therapy, then steroid therapy does not appear to have a synergistic effect on this pathway.

Following treatment of the K562 cells for 24 hours with varying concentrations of Loratadine, glycodelin gene expression was again measured. Treatment with Loratadine showed a dose dependent decrease in glycodelin gene expression, with glycodelin gene expression found to be lowest at the lower doses of Loratadine treatment. A significant reduction in glycodelin expression was seen in K562 cells treated with 2ug, 1ug and 0.5ug Loratadine. Thus it appears that at lower doses Loratadine inhibits glycodelin gene expression. Thus it is possible that loratadine may be involved in stimulating this anti inflammatory protein. The mechanism of this interaction has not been delineated and further studies are necessary in order to identify the pathway via which this effect is exerted.

Based on our findings and glycodelin's known roles, it appears that glycodelin may play a role in the pathogenesis of chronic rhinosinusitis and glycodelin levels may correlate with severity of sinus disease. Nasal
inflammation, in the form of allergen exposure, for example, leads to T-cell activation, which in turn releases cytokines and leads to eosinophil chemotaxis. Persistent tissue inflammation leads to persistent nasal congestion and infection within the paranasal sinuses. Bacterial infection and/or chronic inflammation lead to tissue destruction, which in turn induces glycodelin production. This may be an attempt by the host to control and temper the cycle of T-cell mediated inflammation and tissue destruction. This hypothesis is outlined in schematic form in Figure 9.1. Proinflammatory cytokines especially in the Th2 pathway have been implicated in the pathogenesis of allergic rhinitis and bacterial sinusitis. Glycodelin suppresses T-cell activity. Glycodelin, expression also leads to VEGF production, and angiogenesis. This glycodelin release could possibly lead to nasal polyp formation since tumour growth of any kind requires angiogenesis and neovascularisation. Given glycodelin’s potent immunosuppressive abilities, especially of Th2 cells, targeting the immunosuppressive abilities at the expense of its angiogenic properties could have a potential role in the treatment of sinusitis in the future.
Figure 9.1

Putative role of glycodelin in suppression of T-cell activation, and angiogenesis leading to polyp formation.
To summarise we feel that this novel immunomodulatory protein, glycodelin, may play an important role in the pathogenesis of chronic rhinosinusitis. Glycodelin has previously been identified in the uterine tract where its immunoinhibitory and angiogenic functions have been documented. We have shown the presence of this protein in peripheral white blood cells, in nasal tissues and in the serum of patients with sinus disease. Such molecules may be a target for therapy in the future. This is the first known immunoinhibitory cytokine type protein identified in sinusitis. Further evaluation is warranted in order to determine baseline glycodelin levels in the population as a whole, and to further delineate factors, which might affect the level of this protein in the blood serum. A large formal study should be undertaken in order to assess the usefulness of a glycodelin serum assay in determining the severity of sinus disease, and possibly in differentiating the type of disease process, and better defining a patient population which would most benefit from antibiotic therapy.
APPENDICES
APPENDIX I

Appendix I (i)  Generation of Glycodelin Antibody

Appendix I (ii)  Purification of the Polyclonal Antibody

Appendix I (iii)  Specificity of Purified Antiglycodelin Antibody Tested
APPENDIX 1

I (i) Generation of Glycodelin Antibody

Of 169 amino acids of glycodelin protein, a 15-AA peptide (2HN-KVLGEKTENPKKKFK-COOH) was chosen as the antigen because of the high number of lysine residues present within the peptide. Because the immunogen is a small peptide, a carrier protein, keyhole limpet haemocyanin, was conjugated to the peptide and using standard procedures, an antibody was generated in chicken. Antisera was characterised by Western blot, immunocytochemistry, and enzyme linked immunosorbent assay (ELISA) (Poddar, Kim et al. 1998).

I (ii) Purification of the Polyclonal Antibody

The polyclonal antibody was affinity column purified using the 13 AA peptide as the ligand in a HiTrap NHS-activated sepharose high performance column (Amersham Pharmacia Biotech Company, Piscataway, NJ, USA). The purity of the antisera was confirmed by Western blotting. The purified antibody did not cross react even with a closely related antigen such as beta-lactoglobulin.
I (iii) Specificity of Purified Antiglycodelin Antibody Tested

A standard curve using recombinant glycodelin was obtained for quantification. The range of the assay was from 5ng-2500ng. The minimum detectable quantity of glycodelin was 5ng. Glycodelin is abundant in amniotic fluid during the first few weeks of pregnancy. ELISA with various dilutions of amniotic fluid using antiglycodelin antisera was performed. A dilution-dependant change in glycodelin level was consistently observed. The assay showed an interassay variation of 7.92%, while the intra-assay variation was 6.82%.
APPENDIX II

Appendix II (i) RNA Isolation and RT-PCR Technique

Appendix II (ii) Buffy Coat Isolation

Appendix II (iii) Immunostaining of Buffy Coat Smears

Appendix II (iv) Enzyme Linked Immunosorbant Assay

Appendix II (v) Immunohistochemistry

Appendix II (vi) Cell Culture
APPENDIX II

Appendix II (i) RNA Isolation and RT-PCR Technique

Appendix II (i) a: RNA Isolation

Materials:

Tri reagent (Sigma-Aldrich, St.Louis, MO, USA)
Chloroform
Isopropyl alcohol
Ethanol
DEPC water (diethylpyrocarbonate)

Method:

The cell pellet was lysed in 1ml TRI reagent by repetitive pipetting and stored at −70°C overnight. The samples were then incubated at room temperature for 30 minutes. 0.2ml Chloroform was added to each sample, which was then shaken vigorously for 15 seconds and then incubated at room temperature for 2 minutes. Samples were then centrifuged at 9000rpm for 15 minutes at 2-8°C. Following centrifugation there were three distinct layers obtained, the RNA being found in the aqueous layer. The aqueous layer was removed and placed in a clean 15ml tube. 0.5ml isopropyl alcohol was added to each sample and then samples
were incubated at room temperature for 10 minutes. The samples were then centrifuged at 9000rpm for 15 minutes. The supernatant was removed and the remaining RNA pellet washed with 1ml 75% ethanol. The samples were centrifuged at 14000rpm for 5 minutes, the supernatant was removed and the RNA pellet allowed to air dry for 45 minutes. Using spectrophotometry the RNA concentration, and the RNA/DNA ratio were obtained.

The mRNA volume was calculated from spectrophotometer readings as follows:

\[
\frac{\text{MW RNA} \times \text{Dilution factor} \times \text{Absorbtion saturation at 260}\mu g/\mu l}{1000}
\]

i.e \[\frac{40 \times 200 \times \text{Sample}}{1000} = \text{Concentration of RNA } \mu g/\mu l\]

The volume containing 3 \(\mu g/\mu l\) RNA was calculated:

\[\frac{3}{\text{Concentration}} = \text{amount of mRNA to add}\]

The volume of water required was calculated:

\[3\mu l \text{ minus (volume of RNA)}\]
Appendix II (i) b: Polymerase Chain Reaction (PCR):

**Materials** (Applied Biosystems, Foster City, CA, USA)

- RNAase inhibitor
- MgCl2
- PCR buffer x10
- Gene Amp DNTPS: dATP, dCTP, dGTP, dTTP
- Reverse transcriptase
- Oligo d(T) 16 primers
- Taq polymerase
- Glycodelin Primers: 5’- AAGTTGGCAGGGACCTGGCACTC
  3’- ACGGCACGGCTCTTCCATCTGT (Sigma Genosys, Tx, USA)
- GAPDH primers (Sigma Genosys, Tx, USA)
Method:

RT reaction: (Reverse Transcription)

The Master Mix was prepared in the following proportions:

<table>
<thead>
<tr>
<th>Sample</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>RNAase inhibitor</td>
<td>1μl</td>
</tr>
<tr>
<td>MgCl2</td>
<td>4μl</td>
</tr>
<tr>
<td>PCR bufferx10</td>
<td>2μl</td>
</tr>
<tr>
<td>dNTP</td>
<td>8μl</td>
</tr>
<tr>
<td>Oligo</td>
<td>1μl</td>
</tr>
<tr>
<td>Reverse transcriptase</td>
<td>1μl</td>
</tr>
<tr>
<td>3g RNA</td>
<td></td>
</tr>
</tbody>
</table>

The correct H20 volume was placed in each PCR tube (see previous calculations). The correct volume of RNA from each sample was added. 17μl of master mix was added to each PCR tube to bring total content of each tube to 20μl. The samples were run on the reverse transcriptase programme on the PCR machine.
PCR Reaction:

Glycodelin master mix and control (GAPDH) master mix were prepared in the following proportions:

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 sample</td>
<td></td>
</tr>
<tr>
<td>H2O</td>
<td>33.5µl</td>
</tr>
<tr>
<td>MgCl2</td>
<td>4µl</td>
</tr>
<tr>
<td>PCR bufferx10</td>
<td>4µl</td>
</tr>
<tr>
<td>DNTP</td>
<td>5µl</td>
</tr>
<tr>
<td>Primers glycodelin 3’</td>
<td>1µl</td>
</tr>
<tr>
<td>glycodelin 5’</td>
<td>1µl</td>
</tr>
<tr>
<td>Taq polymerase</td>
<td>0.25µl</td>
</tr>
</tbody>
</table>

Glycodelin Primers: 5’- AAGTTGGCAGGGACCTGGCACTC 3’- ACGGCACGGCTCTTCCCATCTGTT
38μl of master mix were added to 2 μl of RT-product and run on the glycodelin PCR programme as follows:

a. denaturation 95°C X 1 min
b. 95°C X 45s
c. 60°C X 45s
d. 72°C X 1min
b-d X 35 cycles
e. 72°C X 10mins

GAPDH master mix was prepared as follows and run on standard GAPDH PCR programme.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>H2O</td>
<td>33.5μl</td>
</tr>
<tr>
<td>MgCl2</td>
<td>2μl</td>
</tr>
<tr>
<td>PCR Buffer x10</td>
<td>4μl</td>
</tr>
<tr>
<td>dNTP</td>
<td>5μl</td>
</tr>
<tr>
<td>Primer GAP 3’</td>
<td>1μl</td>
</tr>
<tr>
<td>Primer GAP 5’</td>
<td>1μl</td>
</tr>
<tr>
<td>Taq Polymerase</td>
<td>0.25μl</td>
</tr>
</tbody>
</table>

The PCR products were analysed by electrophoresis on a 2% agarose gel stained with ethidium bromide.
Appendix II (i) c: Gel Electrophoresis:

Materials

Gel tray and electrophoresis rig

Combs x2

TBE Buffer: A 5x solution of Tris-borate buffer (TBE) was prepared by adding 27.5g of Sodium Borate, 54g of Tris base, and 20ml 0.5M EDTA (pH 8.0). The solution was made up to a pH of 7.5-7.8. 100mls of 5x solution was then added to 900ml of deionised water to give 0.5x solution.

Agarose 1-2g for 1-2%

Ethidium Bromide 1.5μl

Loading dye X6:

Parrafin film

PCR Marker (Promega, Madison, WI)

Method:

2g Agarose was added to 100mls O.5 X TBE. This was microwaved for 90 seconds, 1.5μl ethidium bromide was added, the gel mixture was poured in to the electrophoresis tray and allowed to solidify for 30 minutes. The lanes were then loaded with 2μl loading dye, and 8μl PCR product. The loading process was
repeated for PCR markers. The gel was immersed in 0.5 X TBE in the electrophoresis rig and run at 120 Volts for 40 minutes.

The gels were then viewed and analysed using Kodak ID 3.6 Imaging analysis software (Kodak EDAS 290) (Eastman Kodak Company, Rochester, NY, USA).
APPENDIX II (ii) Buffy Coat Isolation:

Materials:

Histopaque: Histopaque 1077 (Sigma Diagnostics). Polysucrose, 5.7g/dl, and sodium diatrizoate, 9.0g/dl. Aseptically filtered.

1X PBS: This was prepared by adding 8g NaCl, 0.2g KCl, 1.44g Na2HP04, 0.24g KH₂PO₄ to 800mls distilled H₂O, then made up to 1000ml

Hanks Buffered Saline solution (HBSS) (Mediatech, Inc, Herndon VA)

Method:

Blood samples were collected in lithium/sodium heparin containing ‘vacutainers’. The Histopaque was brought to room temperature. 3 ml of blood was carefully layered onto 3ml Histopaque. Each sample was centrifuged at 1800rpm x 30 minutes at room temperature over the Ficoll–Hypaque gradient. Three distinct layers were obtained. The top layer – plasma was removed to within 0.5cm of interface and transferred for storage at −70° Celcius. The opaque interface containing the ‘buffy coat’ was removed and washed with Hanks Buffered Saline solution, by centrifugation at 1600rpm x 10min, 3 cycles in total. The ‘buffy coat’ pellet was then stored in 0.5ml Hanks Buffered Saline solution at −70° Celcius.
APPENDIX II (iii) Immunostaining of Buffy Coat Smears

Materials:

Cytospin slides

4% paraformaldehyde: 100mls of 1X PBS was added to 4g paraformaldehyde under the fume hood. The mixture was placed on a hot plate/stirrer until the cloudy appearance of the mixture turned clear.

HBSS

1X PBS,

0.1X PBS

Primary antibody

Secondary antibody

3% BSA

Fast red

Haematoxylin

Coverslips

Permount (Fisher Scientific, Hampton NH)
Method:

A 100μl aliquot of 'buffy coat' sample was gently mixed with 250μl HBSS. 200μl of this mixture were placed on a slide and placed in Cytospin for 5 minutes at 800rpm. The slide was then fixed with 4% Paraformaldehyde for 15 minutes before washing with 1 X PBS. The cells were incubated for 2 hours at room temperature with a 1:200 dilution of purified chicken anti-glycodelin peptide antibody in 3% BSA. Primary antibody was omitted for the negative control (3% BSA only). The slides were incubated for 2 hours at room temperature over a water-bath. After they were washed three times with 0.1 X PBS, cells were incubated for 2 hours at room temperature with secondary antibody (antichicken immunoglobulin G conjugated with alkaline phosphatase) at 1:200 in 3% BSA. Cells were washed with PBS three times and fast red was added as a chromagen in conjunction with alkaline phosphatase-substrate naphthol phosphate (Sigma) for 10-15 minutes. The slides were immersed in Haematoxylin X 30-60 seconds. The immunostaining was visualized under the microscope at X 40 and X 100 and the results were analysed.
Haematoxylin and Eosin (H&E) staining:

The slides were washed in tap water and placed in Haematoxylin for 10 minutes. They were then de-stained with a mixture of methanol and acetic acid and washed three times with tap water. They were placed in lithium carbonate for 2 minutes before washing again three times with tap water. They were then placed in Eosin for 5 minutes before being washed in 70% Ethanol for 30 seconds. The slides were allowed to dry before being mounted with Permount and coverslips.
APPENDIX II (iv) Enzyme Linked Immunosorbant Assay

Materials:

Primary antibody: anti glycodelin antibody generated in chicken

Secondary antibody: anti chick IgG raised in rabbit conjugated with alkaline phosphatase.

Para-nitro-phenyl phosphate (pNPP (Sigma))

96 well ELISA plate

Bovine serum albumin (BSA; Sigma-Aldrich, St.Louis, MO): 0.1% solution was prepared in PBS

Glycodelin peptide

Method

Glycodelin peptide (Applied Biosystems, USA) was used to quantify the glycodelin present. Glycodelin peptide standards in the following concentrations were prepared with deionised water 1ug, 500ng, 250ng, 125ng, 62.5ng, 31.25ng,
15.625ng, 7.812ng, 3.6ng, 1.8ng, 0.9ng, 0.45ng, 0.225ng and 0.112ng by serial
dilutions. Amniotic fluid at 13 weeks was prepared in the following dilutions with
phosphate buffered saline 1:1000; 1:5000; 1:25,000; 1:50,000; 1:100,000.

Plasma samples were diluted 1:10 in a buffered saline solution (PBS).
100μl was added in triplicate wells of the ELISA plate and incubated overnight at
37°C. The next day the plates were washed 6 times in phosphate buffered saline
solution (PBS) and blocking was done at room temperature for 1 hour using 0.1%
BSA (bovine serum albumin). Wells were washed with PBS (x 6) and incubated
for 2 hours at 37°C with 100μl of affinity column purified chicken anti-glycodelin
antibody raised in this laboratory (Poddar, Kim et al. 1998) at a dilution of 1:50.
Following this the wells were washed with PBS (x6), blocked with 0.1% BSA for
1 hour at room temperature, then washed with PBS (x6). A secondary antibody
(rabbit antichicken IgG conjugated with alkaline phosphatase) was added at a
dilution of 1:30,000 in 0.1% BSA, and incubated for 2 hours at 37°C. The wells
were washed again with PBS (x6) and the substrate p-nitrophenyl phosphate was
added. The optical density was measured at 405nm after 30, 60 and 90 minutes.
APPENDIX II (v) Immunohistochemistry

**Materials:**

1X PBS

**Formal Sucrose:** This was prepared by adding Paraformaldehyde (0.1g/ml) (40g) 400ml (4% (w/v)), 75g Sucrose (7.5% (w/v)), 25ml of 10MM Sodium Phosphate buffer (0.4M), 5ml of 2mM EDTA (0.4M) pH 7.4, 0.2ml of 20uM Butylated hydroxytoluene (BHT)(100mM) and making up to 1000ml with distilled H₂O

**Fast Red:** 1mg Fast red powder in 1ml Napthol Tris solution, 4mg Naphthol-AS-BI, 0.4ml Dimethylformide 19.6ml 1X TBS, pH 8.2

**Primary antibody:** chicken anti-Gp antibody

**Secondary antibody:** Rabbit antichicken IgG
Method:

Five micron sections of archived tissue were cut on a vibratome and mounted onto poly-L-lysine coated slides. The tissues were deparaffinised as follows: immersion in Xylene for 30 minutes, divided into two 15-minute periods, following this, slides were immersed in absolute alcohol for 5 minutes (x 2 changes), 90% alcohol for 3 minutes, 80% alcohol for 3 minutes and 70% alcohol for 3 minutes. Slides were then rinsed in running water and then washed using phosphate buffered saline (x3) for 2 minutes each. After rising in PBS, the sections were incubated in 1% bovine serum albumin for two hours to act as a protein block. An anti-glycodelin antibody raised in chicken diluted 1:200 in the same bovine serum albumin was placed on the slides. After overnight incubation, the sections were then washed three times in PBS, and incubated with an HRP-conjugated anti-chicken secondary antibody diluted 1:1000 in bovine serum albumin. Following this, the sections were incubated with 3,3’diaminobenzidine tetrahydrochloride (DAB; Sigma, St. Louis, MO) for 10 minutes at room temperature. The sections were counterstained with haematoxylin to clarify the histology. The sections were then dehydrated, coverslipped and analysed. Adjacent sections were processed without the primary antibody as control to confirm the specificity of the immunohistochemistry.
APPENDIX II (vi) Cell Culture

Materials:

Growth media for K562 cells:

- RPMI with L-glutamine (Mediatech, Herndon, VA, USA) 90% 225ml
- Fetal Bovine Serum (FBS) (Mediatech, Herndon, VA, USA) 10% 25ml
- Penicillin/Streptomycin 1% 2.5ml

The above were combined under aseptic technique in the cell culture laboratory and stored at 4°C Celsius.

Method:

The human chronic myelogenous leukemia cell line K562 (American Type Culture Collection (ATCC) Rockville, MD), were grown up to 75% confluence in RPMI 1640 medium with 10% fetal bovine serum. The cells were maintained at 37°C in a 5% CO₂ incubator.


"The Retinol Pigment Epithelial Membrane Receptor for Plasma Retinol Binding 
Protein. Isolation and CDna Cloning of the 63-Kda Protein." 

"Current Estimates from the 1993 National Health Interview Survey. National 
Center for Health Statistics." 
Vital Health Stat 10: 190.

"Occurrence of Asymptomatic Sinusitis in Common Cold and Other Acute ENT - 
Infections." 
Rhinology 24: 223-225.

"Changes in Blood Hormone Levels During the Immune Response." 

BEUTLER, P., N. KROCHIN, I. W. MILSARK, C. LEUDKE and A. CERAMI 
(1986). 
"Control of Cachectin (Tumour Necrosis Factor) Synthesis: Mechanisms of 
Endotoxin Resistance." 
Science 232: 977-980.

"C-Reactive Protein Measurement in General Practice May Lead to Lower 
Antibiotic Prescribing for Sinusitis." 
Br J General Practice 54(506): 659-662.

"Airway Inflammatory and Antioxidant Responses to Oxidative and Particulate 
Air Pollutants- Experimental Exposure Studies in Humans." 


"Monocyte Chemotactic Proteins in Allergen-Induced Inflammation in the Nasal Mucosa: Effect of Topical Corticosteroids."
J Allergy Clin Immunol 103: 1036-1044.

"A Role for Glycoconjugates in Human Development: The Human Feto-Embryonic Defence System Hypothesis."
Hum Reprod 11: 467-473.

"Assessment of Renal Function in Patients with Multiple Myeloma: The Role of Urinary Proteins."

"Effects of the Cysteinyl Leukotriene Receptor Antagonist, Montelukast, on Eosinophil Differentiation in an Experimental Mouse Model of Allergic Rhinitis."

"Regulation of IL-3 Expression by Glucocorticoids in Cloned Murine T Lymphocytes."

"The Effect of Desloratadine on Eosinophil/Basophil Progenitors and Other Inflammatory Markers in Seasonal Allergic Rhinitis: A Placebo-Controlled Randomized Study."
J Allergy Clin Immunol 109(suppl 1): s117.


255


"Glucocorticoid Induced Inhibition of T-Cell Growth Factor Production. I. The 
Effect on Mitogen-Induced Lymphocyte Proliferation."

"Biomarkers of Inflammation in Ozone-Exposed Humans. Comparison of the 
Nasal and Bronchoalveolar Lavage."

"Evidence for Continuous Stimulation of Interleukin-6 Production in Crohn's 
Disease."
Gastroenterology 102(2): 514-519.

GROVES, R. W., M. H. ALLEN, E. L. ROSS, J. N. BARKER and D. M. 
"Tumor Necrosis Factor Alpha Is Pro-Inflammatory in Normal Human Skin and 
Modulates Cutaneous Adhesion Molecule Expression."

GROVES, R. W., E. ROSS, J. N. BARKER, J. S. ROSS, R. D. CAMP and D. M. 
"Effect of in Vivo Interleukin-1 on Adhesion Molecule Expression in Normal 
Human Skin."

"Microbiology of Sinusitis."

"Computed Tomographic Study of the Common Cold."


"Mechanisms of Carcinogenesis: Focus on Oxidative Stress and Electron Transfer."  
Curr Med Chem. 8: 773-796.

"Stimulation of Human Endometrial Epithelial Interleukin 6 Production by Interleukin 1 and Placental Protein 14."  
Hum Reprod 9: 1339-1343.

"Adult Rhinosinusitis Defined."  

"Progesterone-Binding Cyst Protein in Human Breast Tumor Cytosol."  

"Leukotriene Receptor Antagonists and Synthesis Inhibitors Reverse Survival in Eosinophils of Asthmatic Individuals."  
Am J Respir Crit Care Med 161: 1881-1886.

"Concentration of Endometrial Pp14 in Uterine Flushings Throughout the Menstrual Cycle in Normal, Fertile Women."  

"Immunocalins: A Lipocalin Subfamily That Modulates Immune and Inflammatory Responses."  
"A High Serum Concentration of Interleukin-6 Is Predictive of Relapse in Quiescent Crohn's Disease."

"Eosinophil Cationic Protein and Specific IgE in Serum and Nasal Mucosa of Patients with Grass-Pollen Allergic Rhinitis and Asthma."
Allergy 56: 231-236.

"Expression of the Potent Inflammatory Cytokines, Granulocyte-Macrophage Colony-Stimulating Factor and Interleukin-6 and Interleukin-8 in Bronchial Epithelial Cells of Patients with Asthma."
J Allergy Clin Immunol 89: 1001-1009.

"Slow-Reacting Substances, Leukotrienes C4 and D4, Increase the Release of Mucus from Human Airways in Vitro."

"Allergic Fungal Sinusitis Induced Visual Loss."

"Trends in Antimicrobial Drug Prescribing among Office-Based Physicians in the United States."

"Intranasal Flunisolide Spray as an Adjunct to Oral Antibiotic Therapy for Sinusitis."


"Suppression by Human Placental Protein 14 of Natural Killer Cell Activity."

"The Structure of Beta-Lactoglobulin and Its Similarity to Plasma Retinol Binding Protein."

"Placental Protein 14(Pp14) Content and Immunosuppressive Activity of Human Cervical Mucus."

"Effect of Decidual Placental Protein 14 on Interleukin-2 Lymphocyte Interactions."

"Placental Protein 14(Pp14) Inhibits the Synthesis of Interleukin-2 and the Release of Soluble Interleukin-2 Receptors from Phytohaemagglutinin-Stimulated Lymphocytes."

"The Effect of Human Placental Protein 14 (Pp 14) on the Production of Interleukin-1 from Mitogenically Stimulated Mononuclear Cell Cultures."


**Blood** 98(13): 3727-3732.

**Cellular Immunology** 191(1): 26-33.

**Am J Physiology Cell Physiol** 279: C906-C914.


**J Allergy Clin Immunol** 103: 408-414.
Ann Allergy 57(4): 253-256.


ROUSSET, F., J. ROBERT, M. ANDARY and E. AL (1991). "Shifts in Interleukin-4 and Interferon-Gamma Production by T Cells of Patients with Elevated Serum IgE Levels and the Modulatory Effects of These Lymphokines on Spontaneous IgE Synthesis." 


Acta Otolaryngol suppl 529(144-147).