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**Clinical, Immunological and Olfactory Aspects of
Sinusitis and Nasal Polyposis - with special reference to
patients with Cystic Fibrosis**

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ABSTRACT

Background: Opinions differ on how to treat the otorhinolaryngological manifestations (e.g. sinusitis, nasal polyps and hyposmia) of cystic fibrosis (CF), a recessive genetic disorder. This is the main reason why we decided to explore these questions, in an attempt to find reasonable grounds for a logical way of treating these disorders in patients with CF. The immunological impact that the normal microflora could exert in the upper respiratory tract have until recently been unknown. In parallel studies of mouth and pharynx mucosa, indigenous flora (*α-streptococcus*) have been used to inhibit pathological species involved in e.g. tonsillitis and otitis.

Aims: The aims of the work presented in this thesis were: *I.* To determine the frequency of sinusitis and nasal polyps within the paediatric population and relate this to bacteriology, in-patient treatment and correlation with possible parallel risk factors as cystic fibrosis. *2.* To compare the bacterial and inflammation status within the population of patients with CF (both children and adults) and to compare the frequency of nasal polyps and hyposmia/anosmia with the frequency of these findings present in the healthy population, highlighting how these affect the quality of life and the general health status in CF. *3.* To study how the normal bacterial flora in the upper airways affects nasal inflammation in the case of a monoinfection.

Methods: *I.* We have retrospectively analysed 13 years of data from an in-patient paediatric population for the frequency of sinusitis and nasal polyps. We have also examined bacteriology, treatment and risk factors. *II & IV.* We also studied the population of CF patients at their annual check-ups at the CF Centre of Karolinska University Hospital, Huddinge. A clinical endoscopic examination was carried out to determine the frequency of nasal polyps. The otolaryngological status was compared to the overall CF health status at the time of the examination. We carried out two studies of these patients: In the first study, a nasal lavage was carried out, revealing inflammation data of the upper airways (113 patients). In the second study, two different smell tests were carried out (122 patients). *III.* Germ-free rats were monoinfected with *Mycoplasma pulmonis* for 3 weeks. The T-cell population of the mucosa of the nasal cavity were compared with or without infection to determine the difference in immunological response depending on the normal nasal flora.

Results: *I.* Few of the in-patient paediatric patients with acute and chronic sinusitis needed surgical intervention. The risk factors, which include allergy, cilia dyskinesia and CF, were rare. Half of the paediatric population who underwent surgery for nasal polyps had CF. *II & IV.* The frequency of patients with nasal polyps in the CF population was 37-39%. The sense of smell of these patients was lower than in a healthy population when assessed by the butanol test and the identification tests. The prevalence of inflammation parameters such as IL-8 and lysozyme were elevated in nasal lavages. Other aspects of the overall health situation were not affected by the presence of nasal polyps or impairment in their sense of smell. *III.* The normal microbiota of the nasal cavity in rats modified the immunological response to the *Mycoplasma pulmonis* infection. TCRαβ+CD4+ T-cells were elevated both in the intraepithelial lining and in the lamina propria after three weeks of infection in GF rats.

Conclusions: In-patients with paediatric sinusitis required few surgical interventions within a 13-year period at our clinic. Risk factors for sinusitis were rare: one risk factor was CF. Half of the paediatric population that underwent surgery for nasal polyps were diagnosed with CF. Patients with CF had elevated levels of IL-8 and of lysozyme in nasal lavage. The frequency of nasal polyps was 39%, as determined by endoscopy. Patients with nasal polyps displayed an elevated risk of chronic colonisation of *Pseudomonas aeruginosa* in the lower airways, but their overall health situation was not otherwise impaired. The frequency of hyposmia and anosmia, as determined by a newly launched paediatric smell test in combination with other well-known olfaction tests (butanol and SOIT), was increased in a CF-population of 122 patients (aged 5-65 years). The subjective evaluation of the sense of smell differed markedly from the "objective" tests. No health parameters were correlated with the sense of smell in patients with CF. The normal microbiota of the nasal cavity modulated the mucosal T-cell response to a monoinfection with *Mycoplasma pulmonis*. An increased knowledge of the immunological influence of the normal microflora is important for developing strategies to inhibit pathological colonisation in the upper and lower airways in chronic diseases such as CF.

Key words: Nasal polyposis, sinusitis, cystic fibrosis, allergy, bacteria, in-patient, interleukin-8, muramidase, nasal lavage fluid, polymerase chain reaction, *Pseudomonas aeruginosa*, smell, olfaction, anosmia, hyposmia, olfactory threshold test, identification smell test, germ-free animal, T-cell receptor, normal microbiota.

*To
Eva
Erika and Linus*

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Manuscript

LIST OF ABBREVIATIONS

APC	antigen-presenting cell
CF	cystic fibrosis
CFU	colony-forming unit
CRP	C-reactive protein
CT	computed tomography
CV	conventional
DC	dendritic cell
ESS	endoscopic sinus surgery
FEV1	forced expiratory volume in 1 second
FVC	forced vital capacity
GF	germ-free
IEL	intraepithelial lymphocyte
IL	interleukin
LPL	lamina propria lymphocyte
mAbs	monoclonal antibodies
NALT	nasal-associated lymphoid tissue
PAMP	pathogen-associated molecular pattern
PRR	pattern recognition receptor
SOIT	The Scandinavian Odour Identification Test
SSIT-C	Swedish Smell Identification Test for Children
TCR	T-cell receptor
TLR	Toll-like receptor
T _{reg} cells	regulatory T cells
URI	upper respiratory infections
UPSIT	the University of Pennsylvania Smell Identification Test
WBC	white blood cell

Definitions:

Anosmia = inability to detect any qualitative olfactory sensation

Hyposmia = reduced sensitivity to all odours

Normosmia = normal sense of smell

BACKGROUND

1.1 INTRODUCTION

Bacterial inflammation has been studied for many years since it plays an important role in the development of sinusitis and nasal polyposis. These two diseases are closely related, and the underlying mechanisms are thought to be bacterial infection, bacterial colonisation and the subsequent reply of host factors connected to the inflammatory response. The prevalence and the clinical picture of these disorders vary considerably for different age groups. Furthermore clinical studies in the grown-up population have more often been carried out compared to the paediatric population, both in the case of sinusitis and in the case of nasal polyposis. As a result diagnosis and treatment are more clearly defined and have been more deeply evaluated in adults.

1.2 NASAL POLYPS

Nasal polyps are inflamed oedematous formations suspended from the middle and superior meatus in the nasal cavity. They generally emerge from different sinuses, mainly the ethmoidal sinus and the maxillary sinuses, but in some cases originate from the meatus media itself or the sphenoid sinus. One characteristic histopathological feature of these polyps is the relatively high content of eosinophils (1), and an overrepresentation of certain cytokines (1-3). Symptoms commonly associated with polyps are nasal blockage, nasal secretion and a lowered sense of smell. Nasal polyps increase the risk of intermittent sinusitis and sinus X-ray of the paranasal sinuses almost always show mucosal thickening. The aetiology is still unclear (4). Associations to non-allergic rhinitis, NSAID/ASA (nonsteroidal anti-inflammatory drug/ acetylsalicylic acid)-intolerance and non-allergic asthma are known.

1.2.1 Epidemiology

Nasal polyposis is a common disease in the adult population, with a prevalence of 2-4 % (5, 6). In patients with asthma, a prevalence of 7-15% has been recorded, and nasal polyps are found in 36% to 60 % of patients with NSAID intolerance (7-9). Nasal polyposis is associated with cystic fibrosis in the paediatric population.

1.2.2 Pathophysiology

The inflammatory basis of the polyp formation is thought to be the interactions of viral or bacterial infections and the host's defence (10). The complex underlying mechanism is mediated by inflammatory cells that act with the help of cytokines and granulocyte colony-stimulating factors (GM-CSF). Several explanations have been proposed for the mechanism of polyp formation in the mucosal lining and surrounding matrix (11, 12). Later studies have suggested an association between polyp formation and the presence of fungal antigens (13) or to the superantigen response to *Staphylococcus aureus* enterotoxin (14).

1.2.3 Diagnosis and Staging

A more detailed diagnosis can be achieved by endoscopy. Endoscopy reveal smaller polyps than anterior rhinoscopy can discover, and it provides better access to postoperatively altered sinus cavities. A commonly used grading scheme is: polyp score 0 = no polyps seen, 1 = polyps within meatus media above the lower edge of the middle turbinate, 2 = polyps below middle meatus but above the lower edge of the inferior turbinate, and 3 = polyps filling the whole nasal cavity (i.e. extending to the lower edge of the inferior turbinate or beyond) (15). A polyp hanging down from the superior meatus is graded as a grade 3 polyp in some scoring systems. A new sensitive method for polyp scoring is lateral imaging, grading the polyps in two dimensions (16).

1.2.4 Treatment

Treatment of choice of this chronic disease is a topical corticosteroid. Its efficacy is supported by several studies that have proved diminished polyp size and symptomatic relief (17, 18). The medication may reach the higher region of the nose more efficiently if the "head-down"-position is used when spraying or applying the nasal drops.

Oral corticosteroids can be used for shorter periods, and often have a more prompt effect on the polyp size and the symptoms (19). Certain contraindications to such systemic treatment have been established, including diabetes, osteoporosis, glaucoma, gastric ulcers.

Endoscopic sinus surgery (ESS) (including removal of polyps both in the nasal cavity and in the ethmoidal and maxillary sinuses) can be carried out in cases of extensive polyposis (4, 20). Surgery is further indicated if the polyps totally occlude the nasal cavity, since it reduces the nasal blockage more rapidly and offers the space needed for the topical medical treatments to reach their target area.

Endoscopic surgery has obvious effects on nasal blockage and nasal secretion, but the sense of smell is not always improved by the procedure (21).

Alternative medical treatments such as anti-leukotrienes and antifungal agents have been tried, but these are not yet established as a standard proceeding.

1.3 SINUSITIS (RHINOSINUSITIS)

The function of the paranasal sinuses is unclear. Nitric oxide is produced in the sinuses, which facilitates the oxygen exchange (optimizing ventilation/perfusion matching) in the lower airways (22). This is one of the possible explanation of the function of the sinuses. The high prevalence of sinusitis demands substantial medical resources in primary care. Spontaneous healing without treatment in the case of sinusitis is greater than 40% (23).

1.3.1 Epidemiology & Pathophysiology

It has been estimated that only 0.2–2% of viral URIs are complicated by bacterial rhinosinusitis (24). "Acute sinusitis" is a term often used to describe an ongoing infection in the paranasal sinuses that prevails for less than 8 weeks (12 weeks in children) and that occurs less than 5 times (6 times in children) per year.

When the osteomeatal complex is influenced by an upper respiratory tract infection, the ostium of the anterior sinuses will be affected resulting in a relative sinus hypoxia. This may lead to a sinus infection where the local host defence impairment decides the duration of the infection and the degree of the inflammatory response. Predisposing diseases in combination with microbial niches could enhance and prolong these infections.

1.3.2 Diagnosis and Treatment

Rhinosinusitis is defined as inflammation of the nose and the paranasal sinuses that results in two or more of the following symptoms:

- blockage/congestion
- reduction or loss of smell
- discharge: anterior/post-nasal drip
- facial pain/pressure

and either endoscopic signs of

- polyps or
- mucopurulent discharge from middle meatus or
- oedema/mucosal obstruction, primarily in middle meatus

or CT (computed tomography) changes

- mucosal changes within the osteomeatal complex and/or sinuses.

"Chronic sinusitis" is often used to describe cases in which the symptoms of sinusitis remain for more than 8 weeks (12 weeks in children) and cases in which more than 4 such episodes (more than 6 episodes in children) occur per year with persistent symptoms (25). A suggested terminology of intermittent or persistent sinusitis has been proposed on the basis of the duration of graded symptoms and signs.

It may be difficult to diagnose both acute and chronic infection of the sinuses in clinical practice. Certain symptoms (including facial pain, facial pressure, facial congestion, facial fullness, nasal obstruction, nasal blockage, nasal discharge, nasal purulence, post-nasal drip, hyposmia, anosmia and fever) indicate that the sinus cavity may be infected. Headache, dental pain and cough are less specific signs. The symptoms should be supported by objective findings seen at an anterior rhinoscopy, endoscopic examination or sinus X-ray for diagnose.

The symptoms and their severity that are required before a diagnosis of "sinusitis" can be made have been debated. In the paediatric population the symptoms may be blurred by a presence of an adenoid (in the nasopharyngeal space) in combination with the intermittent presence of the pre-school bacteria (*Streptococcus pneumoniae*, *Haemophilus influenza* and *Moraxella catarrhalis*) in the nasopharyngeal space (26). More clear clinical signs of purulence may be present in ethmoiditis with a swollen eyelid, which is more common in the youngest population of children. In the case of a

fulminant etmoiditis a supplementary CT could be demanded for revealing a developing orbital abscess.

The acute diagnose is based on the combination of certain symptoms that provide more clear evidence together with the status of the patient and sometimes supplemented with sinus X-ray. A common clinical situation is a prolonged common cold, which is complicated with raised levels of coloured and increasing amounts of secretion, in combination with facial pain and/or fever reaction.

Sinus X-ray and bacterial cultivation from the middle meatus is mostly limited to cases of recurrent or chronic infections that occur in spite of adequate treatment.

The drug of choice in the paediatric and the adult population suffering from sinusitis in Scandinavia has traditionally been penicillin V, while ampicillin or amoxicillin is recommended in the United States (27). Acute sinusitis is a self-limiting disease in the vast majority of cases, and this is the reason why conservative treatments such as saline irrigations and nasal decongestants are considered.

A broader spectrum of antibiotics should be applied if distinct symptoms are maintained or if the symptoms increase in spite of adequate antibiotic treatment.

The need for surgery in the younger population is often debated. Surgical techniques have developed and become more extensive over the years, and it has become a delicate task to decide whether one should expose children and youths to these procedures (28).

Sinus surgery may be the key to the prolonged effect of treatment in the adult population with recurrent or chronic sinusitis. Surgery is the most important ingredient of treatment in cases that involve, for example, mucocele.

The role of nasal corticosteroids in children with rhinosinusitis is not yet established, as study inclusion criteria vary (29). Efficacy parameters associated with asthma and allergy appears to be most improved (30).

1.4 CYSTIC FIBROSIS

This disorder is a common autosomal recessive genetic disorder in Caucasians. The familiar nature of CF was first described by Fanconi in 1936, and it includes thick mucus in the respiratory and gastrointestinal tract as a main ingredient ("mucoviscidosis"). The mean survival age in the 1960s was 10 years, while it had reached, with the help of intensified medical treatment, 35 years in the 1990s.

1.4.1 Epidemiology & Pathophysiology

The incidence in Sweden is one in 7,700, with a carrier frequency of the defective gene of one per 40. The mutations are situated in the long arm of chromosome 7 (31), the most common mutation being named $\Delta F508$. The protein encoded by the gene that is most commonly mutated is called the "cystic fibrosis transmembrane regulator" (CFTR), and it forms the major chloride channel expressed on the luminal surface of pancreatic and sweat gland cells, in submucosal glands and in the epithelial lining of the lower respiratory tract. In the exocrine glands the excretion of the chloride ion are inhibited leading to an uptake of sodium and water from the epithelial lining into the cell. This results in thicker more viscid mucus in the gastrointestinal tract and the airways. In the sweat gland the absorption of chloride ions are reduced followed by an increased excretion of sodium and chloride in the sweat. Diagnosis is mainly based on the abnormal electrolyte loss in a sweat chloride test. DNA genotyping allows the presence of one of the more than 1,200 currently identified mutations to be confirmed, thus verifying the diagnosis.

1.4.2 Aspects of airway involvement

Chronic pulmonary infections with progressive lung destruction are the most serious ingredient of the CF disease, and many of the established treatments are for this reason focused on this issue.

The frequent finding of nasal polyps and the recurrent or chronic tendency to sinusitis often lead to intermittent consultations with an ENT-doctor.

The incidence of nasal polyps in children with CF is 7% to 32% (32-34), and the incidence increases during adolescence until it reaches 44%-48% (35, 36) in adults. The use of an endoscopic examination has enabled a more accurate frequency of nasal

polyps to be determined. The major problems associated with polyps are nasal blockage (two thirds of affected patients) and rhinorrhea (34, 37).

The composition of inflammatory cells present in polyposis in CF differs from that found in histopathologic studies of non-CF polyposis, having a higher content of neutrophils (38). This phenomenon is thought to be triggered by the persistent sinusitis (*Pseudomonas aeruginosa* and *Staphylococcus aureus*) and these bacteria are also found in the lower respiratory tract (39).

A parallel chronic "sinopathy" is found, due to the impaired mucociliary flow in the sinuses (due to the viscid mucus and (in some case) a defect in the ciliar microtubulus (40)). Conventional sinus X-ray (41) and CT scans (42) show opacified sinuses in a high percentage of cases (90-100%). Clinical symptoms of sinusitis have been found in widely differing frequencies, from 11% to 94% in different studies (36, 42-46).

Medical (conservative) treatments are recommended in mild cases of nasal polyps and recurrent or chronic sinusitis. Polyp shrinkage can be achieved by using topical steroids (47). Surgery is indicated if obvious clinical symptoms are present (according to Ramsay et al. (48) in the case of sinusitis: a) chronic nasal obstruction with mouth breathing, b) chronic purulent draining nasal secretions unresponsive to medical treatment, and c) persistent headaches thought to be related to sinusitis - and in case of large nasal polyps). The main surgical technique currently used is endoscopic sinus surgery (ESS) (49, 50). However, patients have a relatively high risk of recurrence following this procedure (43, 49) ("50% risk either of their symptom returning to preoperative severity or of undergoing a second endoscopic sinus procedure, by 18 to 24 months of postoperative follow-up" according to Rowe-Jones et al. (51)) .

A complementary treatment in the case of obvious sinus problems is the use of intravenous antibiotics. Oral and/or local antibiotics can also be used (45).

1.5 NORMAL FLORA & IMMUNOLOGY

Various patterns of normal flora are found in the different regions of the upper respiratory tract. The commensal flora in the human nasopharynx is similar to the microbiota of the mouth and oropharynx. The amounts and the prevalence of various bacteria in the nasopharynx, including different species of streptococcus (i.e. *S. mitis*, *S. pneumonia*, *S. oralis*, and *S. viridans*), vary from one individual to the next, and depend on age (52). This area is colonized by potential pathogens during different periods of life. In childhood, *S. pneumonia*, *Haemophilus influenzae* and *Moraxella catarrhalis* are relatively frequent in the nasopharyngeal region (53), and levels of these potential pathogens are elevated in populations with, for instance, otitis media. Approximately 30% of adults harbour *Staphylococcus aureus* in the most anterior region of the nasal cavities. Bachert et al. (54) have suggested that an aberrant immune response of enterotoxin from *S. aureus* results in the formation of local IgE antibodies in association with nasal polyps. Increased numbers of *S. aureus* and other transient pathogens (such as *Pseudomonas* spp.) in the sinonasal region and lower respiratory tract may be associated in patients with CF with an increased prevalence of allergic eosinophilia (55). It is unclear whether the aberrant response patterns described above are the result of inappropriate immunoregulatory signals from the indigenous microbiota. A recent Danish study has shown that the normal flora of the human nasal cavity consists of *Corynebacterium*, *Aureobacterium*, *Rhodococcus* and staphylococci, including *S. epidermidis*, *S. capitis*, *S. hominis*, *S. haemolyticus*, *S. lugdunensis*, and *S. warneri* (56). This composition differs from the nasopharyngeal flora. Notably, the status and role of the commensal flora in the human nose and nasopharynx remain unknown in chronic or recurrent infection and inflammation.

Colonization of microbes in the nasal cavity and nasopharyngeal region is more prominent in rodents than it is in humans. The nose and the nasopharynx are exposed to the external environment and are thus in persistent contact with irritants and antigens, and it is probable that the immunological impact of the microflora in this region is an important homeostatic mechanism, similar to that in the gut (57-59).

A germ-free animal lacks contact with pathogenic bacteria and with the normal microflora (such as the microflora in the gastrointestinal tract, nose and mouth). Such animals thus develop a more immature

immune system with fewer submucosal antibody-producing plasma cells and fewer T lymphocytes (60). Lymph nodes and mucosal-associated lymphocyte tissue (such as Peyer's patches, bronchus-associated lymphocyte tissue and nasal-associated lymphocyte tissue) are less developed in these animals. The germ-free model has been successfully used to study the impact that the normal microflora in the gut has on its environment, and to study its immunological response.

Bacteria that are present in our normal flora have been used to exert pressure on pathological species and in this way diminish their ability to thrive in earlier studies in the region of the oropharynx (61-63). We believe that such a model can be applied to pathological bacteria in the airways. A recent study on patients with CF focused on characterising the bacterial flora/colonisation of the lower airways (64). This could become a good platform for a future use of indigenous flora in the upper airways/oral cavity to repress the pathological species in CF (e.g. *S. aureus*, *P. aeruginosa*, *B. cepacia* and *S. maltophilia*).

The understanding of the local immune-modulating effect of the normal microbiota may lead to future treatment strategies including the use of probiotics. The T-lymphocytes (CD4 (former T-helper cells) and CD8 (former T-suppressor cells)) plays a key role in the mucosal immune response evoked in the case of an antigen vs. host interaction.

1.6 OLFACTION

The most important function of the sense of smell in humans is to direct our attention toward environmental hazards (smoke and toxic fumes) or to positive sensations such as nutritious food products. Olfaction also plays an important role in interpersonal relations in various situations (65). Olfactory dysfunction leads to problems with for example cooking and appetite (66). The olfactory mucosa is located at the top of the nasal cavities on the superior and medial nasal conchae and on the superior part of the nasal septum. The sensation of smell is mediated through olfactory neurons, which pass the lamina cribrosa (a thin perforated bone plate at the top of the nasal cavity) up to the cranial nerve I (olfactory nerve).

1.6.1 Epidemiology & Pathophysiology

The incidence of impairment of olfactory function increases exponentially with age (67). An epidemiological Swedish study has shown that the incidence of olfactory disorders is 19.1% (13.3% hyposmia and 5.8% anosmia), with a higher incidence for aged persons, males and persons with nasal polyps (68). Common causes of a lowered sense of smell are nasal or sinus disease, head injury and previous upper respiratory infections (66, 69, 70).

The aetiologies of a lowered sense of smell can be divided into two main groups:

The "non-conductive disorders" depend on a sensorineural injury in the olfactory region or at a higher level. The nerve tissue may have been partially or totally damaged through head trauma, neurodegenerative diseases, exposure to toxic substances, medical treatment, endocrine/metabolic disorders, congenital disorders, idiopathic conditions, rare cases of CNS tumours or advancing age.

"Conductive disorders" arise from different blockages of the pathway of the odours to the olfactory neurons. Such disorders mainly depend on inflammatory conditions in the nasal cavities, such as nasal polyposis, chronic sinusitis, persistent non-allergic rhinitis, allergic inflammation and, in some cases, a preceding upper respiratory infection.

1.6.2 Tests of olfaction

Several tests are available for measuring olfaction. Tests that include a threshold test and a quality identification test (which are both classified as psychophysical tests) are most useful in clinical practice.

A. Threshold test

A threshold test measures the sensitivity of the olfaction system to low concentrations of odorants. The butanol test (n-butyl alcohol (1-butanol)) is regularly used in otorhinolaryngological practice world-wide. This test was introduced by Cain et al. (71) and is based on a procedure in which the lowest concentration of the odorant is presented at the same time as a parallel plastic bottle filled with distilled water. The subject is to identify the bottle that contains the odorant. The concentration at which the patient gives five correct answers in a row defines the lowest concentration of butanol (olfactory threshold) that he or she can detect. The strongest butanol mixture used is 4%, which is then diluted in

12 steps, where each step contains a third of the butanol amount of the previous bottle. This test is believed to be sensitive to variations in nasal mucosal swelling (72, 72).

B. Quality identification test

This test measures the ability to identify different odours that are represented in concentrations well above the normal threshold level.

The tests are presented as multiple-choice questions, where the odour-naming ability is measured. The most common identification test used is the University of Pennsylvania Smell Identification Test (UPSIT). This test consists of microcapsulated odours (74). Nordin et al. (75) have developed an odour identification test using odours that are familiar in the Scandinavian region. The test is designed to be used primarily by adults and teens (from the age of 14).

1.6.3 Treatment of olfactory dysfunction

Surgery

Endoscopic sinus surgery (ESS) can improve the sense of smell in patients with nasal polyposis or rhinosinusitis, although parallel medical treatment may have been the cause of the improvement in certain of the studies that have shown this (51, 76-78). Unilateral sinus surgery (ESS) in the case of nasal polyposis did not improve the sense of smell in a recent prospective randomised study (21). Indeed, all types of nasal surgery may impair olfactory function, due to injury to the delicate olfactory neuroepithelium itself or due to indirect disturbances (79).

Intranasal corticosteroids

Hyposmia was significantly reduced, both symptomatically and in olfactory testing (UPSIT), after six weeks of treatment with nasal corticosteroids for patients with perennial rhinitis (80).

Systemic corticosteroids

A distinct improvement in the sense of smell was achieved by short-term treatment with oral glucocorticoids (81, 82). Anosmia or hyposmia that did not respond to topical nasal steroids did respond to oral steroids (83), but the duration of symptom-relief is often relatively short. Systemic corticosteroids

may function by reducing the inflammatory swelling of the nasal mucosa, which increases the penetration of odours to the olfactory region, or they may act directly on the olfactory cell region.

2 AIMS OF THE STUDY

Paper I

The aim of this study was to obtain an insight into paediatric rhinosinusitis and paediatric nasal polyposis with respect to predisposing factors, clinical presentation and choice of treatment in an in-patient population.

Paper II

The aim of this study was to determine the frequency of nasal polyposis in CF patients attending the Stockholm CF Centre. Furthermore, the aim was to study the inflammatory status of the nose of these patients using nasal lavage. In addition, we wanted to relate the upper-airway findings to the total health situation of these patients, including their lower airway status, morbidity, and bacteriology. A further aim was to relate the findings to the genotype of these patients.

Paper III

The aim of this study was to investigate the impact that the normal microbiota exerts on the nasal T-cell response in rats (GF) inoculated with *Mycoplasma pulmonis*.

Paper IV

The aim of the present study was to illuminate the olfactory function in CF and relate the findings to the infectious and inflammatory status in the nose by performing an endoscopic evaluation. Additionally we wanted to correlate the upper-airway findings and the sense of smell with the total health situation of these patients (including lower airway status, morbidity, bacteriology and BMI) as well as to age, gender and genotype. Furthermore the aim was to evaluate if an endoscopic sinus surgery procedure — with the reduction of the nasal polyps and an additional opening procedure of the antral sinuses — in combination with a period of topical steroids or a treatment with only topical steroids had a positive effect on the sense of smell in patients with CF.

3 MATERIALS AND METHODS

3.1 STUDY GROUPS

Paper I

We studied retrospectively 213 children with sinusitis (aged 4 months to 15 years, mean 8.3 years) treated at the Department of Otorhinolaryngology (n=191) or the Department of Paediatrics (n=22) at Karolinska University Hospital, Huddinge, during the period 1980-1992. An additional 27 paediatric patients with nasal and/or choanal polyps were in-patients during the same period for elective surgery.

Paper II

All CF patients above the age of 2 years attending the Stockholm CF Centre, Karolinska University Hospital, Huddinge, were offered an examination of their upper respiratory tract at their yearly control examination in November 1994 to December 1996. 111 patients (aged 2 years to 57 years, mean 16.9 years) agreed to undergo the examination.

Paper III

20 AGUS rats (10 germ-free and 10 conventional, aged 12 weeks at entrance to the study) reared at the Department of Medical Microbial Ecology, Karolinska Institutet, Stockholm were studied before and after 3 weeks of monoinfection (five animals were studied in each group). The GF rats were kept in lightweight stainless steel isolators totally isolated from any microbe. Control animals (conventional=conv.) of the same strain were kept in a laboratory animal room and these animals were checked regularly (quarterly) to ensure that pathogenic microorganisms were not present.

Paper IV

122 CF patients older than 5 years of age (5 to 61 years, mean 19.3 years) participated in the study during the period 2000-2002. They were offered two different smell tests and an endoscopic examination of their upper respiratory tract at their yearly control at the Stockholm CF Centre. The patients completed a questionnaire regarding subjective aspects of nasal and olfactory function.

3.2 METHODS

Human studies

Nasal endoscopy (Papers II and IV)

CF patients were evaluated by nasal endoscopic examination after local anaesthesia and the application of a decongestant (nafazolin hydrochloride - lidocaine hydrochloride: 0.02% and 3.4 %, respectively). The occurrence of nasal polyposis, nasal congestion, secretion and redness were noted.

Polyp scoring (Papers II and IV)

The nasal polyps in CF patients were scored endoscopically:

Grade 0 – No polyps.

Grade 1 – The smallest size of polyps (concealed in middle meatus, not reaching the inferior edge of the middle turbinate).

Grade 2 – Polyp in the middle meatus, reaching the inferior edge of the middle turbinate.

Grade 3 – Nasal polyp extending into the nasal cavity below the edge of the middle turbinate, but not below the inferior edge of the inferior turbinate.

Grade 4 – Nasal polyp filling the nasal cavity.

Olfactory thresholds (Paper IV)

The olfactory threshold test as described by the Connecticut Chemosensory Clinical Research Centre was carried out with 1-butyl alcohol (= butanol). The highest aqueous concentration used was 4%, and this was placed into a squeeze plastic bottle. The solution was subsequently diluted by successive factors of three with deionised water, in 12 further steps.

The plastic bottle with a low concentration of butanol was compared with a bottle containing only deionised water. The patient squeezed the two bottles without knowing which of the bottles contained a substance. The threshold level for a patient was defined as the lowest concentration at which he or she successfully identified the bottle containing butanol five times in a row. The normal olfactory threshold for adults was taken to be dilution step 7 (71), while the normal olfactory threshold for children (ages under 14 years) was taken to be dilution step 6.

Identification smell tests (Papers II and IV)

The identification test used for patients older than 14 years of age was SOIT (Scandinavian Odour Identification Test) described by Nordin et al.(75).

Sixteen different odours that are familiar for Scandinavian people are presented in a multiple-choice manner, with 4 response alternatives available for each test stimulus. The levels for normosmia, hyposmia and anosmia depend on age and gender according to the following table (stating correct identifications).

A new identification test (the Swedish Smell Identification Test for Children = SSIT-C) was launched by Anna Hallberg at the AChemS XXIV-s meeting, April 2002, Sarasota, US. This test, as its name implies, is designed to be used with children (5-13 years of age) and contains 16 different odours that are well-known by children in Scandinavia and Western Europe. The children are initially given the opportunity to associate freely around the presented odour. The children then choose the image (one of three) that illustrates the presented odour. This smell test has been validated by a large group of healthy children, and levels of anosmia and hyposmia have been established (Hallberg, personal communication).

SOIT	Age		
	14-34	35-54	55-74
Normosmia	13-16	12-16	11-16
Hyposmia	10-12	9-11 / 10-11*	8-10
Anosmia (-4 SD)	≤9	≤8 / ≤9*	≤7

*Men/women (Nordin et al. 1998 (75))

Paper II describes experiments using a smell identification test from the University of Pennsylvania (UPSIT). The test contains forty different smells that are familiar to people in North America. The smells are presented in microcapsules – the patients scrape and sniff and then answer using multiple-choice alternatives (four alternatives for each odour). The intensities of the different smells are well above the sensitivity threshold level. Three of the smells presented in the test were unfamiliar to Scandinavians (pumpkin pie, root beer, and skunk), and the recommended levels for hyposmia and anosmia were for this reason lowered by three points each for this Swedish population we studied.

Nasal Lavage (Paper II)

Nasal lavage fluid was gathered by the following procedure: 3 ml of phosphate-buffered saline solution was poured into the antral part of the nose by means of a syringe with a cap on top to prevent the solution from leaking to the side. The solution was transferred twice (in and out) from the syringe into the cavity while the patients were leaning forward. The solution gathered was immediately spun at 340 g in a tabletop centrifuge (Sorvall RT 6000D; Kendro Laboratory Products; Newtown, CT) for 10 min. The supernatant was separated from the pellet (1 ml of the supernatant with some nasal secretion and nasal mucosal cells was collected). The pellet was then spun one more time (3,500 g for 2 min) (MSE MicroCentaur; Thistle Scientific Ltd; Uddingston, Glasgow, UK). All tubes were then immediately stored at -70°C. The presence of *P. aeruginosa* in the pellet was analysed by PCR, and by immunoassays for IL-5 and IL-8, while the lysozyme content of the supernatant was determined.

The pseudomonas PCR Test (Paper II)

Rapid PCR detection was performed using a primer pair based on the 16S ribosomal RNA sequence, following a method previously used by Karpati and Jonasson (84).

IL-5 and IL-8 (Paper II)

The levels of IL-8 and IL-5 in nasal fluid were measured after homogenizing the sample that consisted of a pellet and its overlying 1 ml of the supernatant. This method produces higher cytokine levels than assays based on supernatants. Measurements were made with commercially available sandwich enzyme immunoassays, following the recommended protocols of the manufacturer (R&D Systems; Abingdon, UK). Cytokine concentrations in lavage fluids were determined by comparing the experimental curve with a standard curve (obtained from positive controls) [$r > 0.98$ for all assays]. The specific enzyme-linked immunoabsorbent assay kits used were IL-8 (sensitivity better than 10 pg/ml; dynamic range 32 to 2,000 pg/ml) and IL-5 (sensitivity better than 3 pg/ml; dynamic range 7.8 to 500 pg/ml).

Lysozyme Test (Paper II)

Lysozyme is a marker for serous gland secretion in the nasal cavity, and it can be released from neutrophils. Increased lysozyme levels indicate an inflammatory status with the stimulation of serous gland secretion, the degranulation of neutrophils, and the destruction of epithelial cells. The lysozyme content

of nasal lavage was determined in a colorimetric assay slightly modified from the assay described by Ito et al. (85).

Genotype Analysis (Paper II and IV)

Genotype data were available for 41 patients with nasal polyposis and for 59 patients without polyposis. The genotype data could be compared with information concerning mutations that has been collected by Schaedel et al. (86). The following mutations were included: Δ -F508, Δ -F508 W79R, 3659delC, 394delTT, 297 3C A, S945 L, L206W, R553X, G551D, 2798 + 5G A, YJ09N, 711 + 3A G, D1152H, R75QN1088, R1162X, W1282X, G1244, and 2183AA G.

Spirometry (Papers II and IV)

Static and dynamic spirometric measurements were obtained from each patient older than 7 years. The functional residual capacity was determined by body plethysmography, while total lung capacity and residual volume were calculated. Vital capacity, FEV₁, FEV₁ as a percentage of vital capacity, peak respiratory flow, forced expiratory flow at 50% of FVC, and forced expiratory flow at 25% of FVC were all measured separately. All measurements were performed using a pulmonary function laboratory (Sensor Medics BV; Beilthaven, The Netherlands). All patients were coached by the same technicians and all patients were familiar with spirometric measurements.

Animal study

Immunofluorescent Staining (Paper III)

Lymphocyte cell surface markers were visualized in tissue sections by multicolour immunofluorescence, based on indirect staining procedures using combinations of primary unlabeled mAbs of different murine IgG subclasses (Table 1) and subclass-specific secondary antibody conjugates as described previously (87).

Microscopy and Cell Counting (Paper III)

The tissue sections were examined at a magnification of 400x in a Nikon microscope (Eclipse E800, Nikon, Tokyo, Japan) equipped with polychromatic filters for the selective observation of individual cells with regard to labelling by FITC, Cy2™ (green), Cy3™ (red) or AMCA (blue). A CCD video camera system (C5810, Hamamatsu Photonics, Hamamatsu, Japan) attached to the microscope was used to digitize fields for the computer analysis of multicolour images (Foto-station, Interfoto AS, Oslo, Norway).

Lamina propria lymphocytes (LPLs) and intra-epithelial lymphocytes (IELs) were identified using the appropriate cell surface markers, IELs being defined as cells with at least half of the surface profile located within the epithelium. The changes in the sizes of these cell populations were determined rela-

Table 1. Mouse monoclonal antibodies used as primary reagents

Clone	Rat specificity	Isotype	Working dilution	Source
Ox35	CD4	IgG2a	Supernatant: 1/4	MRC Cellular Immunology Unit, Oxford, UK
G28	CD8 α	IgG2a	Purified Ig: 1/50	BD Pharmingen, San Diego, CA
G4.18	CD3	IgG3	Purified Ig: 1/200	BD Pharmingen, San Diego, CA
R73	TCR $\alpha\beta$	IgG1	Supernatant: 1/4	T.Hünig, Würzburg, Germany
V65	TCR $\gamma\delta$	IgG1	Purified Ig: 1/50	BD Pharmingen, San Diego, CA
Ox34	CD2	IgG2	Supernatant: 1/10	MRC Cellular Immunology Unit, Oxford, UK
Ox33	B-cell LCA	IgG1	Supernatant: 1/4	MRC Cellular Immunology Unit, Oxford, UK
3.2.3	NKR-P1	IgG1	Ascitic fluid: 1/100	J.C. Hiserodt, Pittsburgh, PA

tive to the estimated length of the surface epithelium in a particular section (IELs and LPLs per mm epithelium). The proportions of TCR $\alpha\beta^+$ LPLs were determined by evaluating at least 200 cells for concomitant expression of a subset marker within at least half of the cross-sectional area of the nasal cavity (i.e. an area covering a region around one NALT structure and the respiratory epithelium of one of the two nasal cavities, including the olfactory epithelium in its superior region). IELs were rare, and thus counts from the whole cross-sectional area of the nasal cavity were accumulated, excluding intraepithelial cells of NALT regions. A median of 113 IELs were enumerated from a specimen (range 49-189 cells from a specimen).

The size of NALT was estimated by measuring the edges of the organized lymphoid aggregates that formed an approximately rectangular or triangular area, or a combination of a triangle stacked on a rectangle. Lymphoid cells within NALT itself could not be counted because they were too densely packed.

3.3 STATISTICAL METHODS

In all studies, values of $p < 0.05$ were considered to be significant.

Paper I

The data obtained were compared statistically using a χ^2 test (age and monthly incidence) and using Student's t test (prevalence relation to pre existing epidemiological data).

Paper II

The following statistical analyses were performed with statistical software (Statistica; StatSoft; Tulsa, OK): multilinear regression (age, lung function tests, some of the blood parameters); χ^2 test (genotype, sex, clinical status, some of the operations, tendency to allergy, acute otitis media and sinusitis, and ongoing symptoms such as snoring, rhinorrhea, mouth breathing, nasal blockage, and epistaxis); Whitney-Mann U test (some of the blood parameters, use of i.v. and oral antibiotics per year, age at diagnosis, concentrations of IL-8 and lysozyme in the lavage, smell test, and time from diagnosis to first positive cultivation of *P. aeruginosa* and *S. aureus*); Fisher's Exact Test (sinonasal surgery, otoscleritis, history of hyposmia, pancreatic insufficiency and continuous use of oral antibiotics);

logistic regression (earlier polypectomy, recurrent nasal blockage and secretion, antibodies against *P. aeruginosa* and *S. aureus*, and the number of patients with chronic colonization with *P. aeruginosa* and *S. aureus*); and log-rank test (chronic colonization with *P. aeruginosa* and *S. aureus*).

Paper III

The two-tailed Mann-Whitney U-test was used for the statistical analysis.

Paper IV

The following statistical analyses were performed with statistical software (Statistica; StatSoft; Tulsa, OK): multilinear regression (hyposmia and nasal polyps vs. age, lung function tests, and some of the blood parameters; persistent subjective hyposmia vs. age; both smell test vs. age; either of the smell tests vs. age); χ^2 test (polyps vs genotype, gender and the use of oral or intravenous antibiotics; hyposmia vs. genotype; anosmia vs. genotype); Fisher's Exact Test (age vs. persistent subjective hyposmia; anosmia and hyposmia vs. lowered weight (-2SD), nasal polyps vs. anosmia and persistent subjective hyposmia); Mann-Whitney U test (hyposmia and nasal polyps vs. use of intravenous and oral antibiotics per year), logistic regression (hyposmia vs. recent polypectomies, recent sinusitis, subjective (sense of) taste and the use of intravenous and oral antibiotics; nasal polyps vs. the frequency of hyposmia (objective and subjective), earlier recent polypectomies, recent sinusitis, aero allergies supported by objective test and subjective (sense of) taste; age group vs. the frequency of hyposmia (objective and subjective) and aero allergies supported by objective test; anosmia vs. ESS/polypectomies; persistent subjective hyposmia and objective hyposmia vs. age and genotype; identification test), and log-rank test (nasal polyps vs. accumulative frequency of chronic colonisation with *P. aeruginosa*).

4 RESULTS

Paper I

The total number of children studied was 219: 126 boys and 93 girls. The number of children who required hospital care for sinusitis amounted to 24 per 100,000 inhabitants (up to the age of 15) and year in this region during the period studied. The median length of stay in hospital was 2.5-3 days for all patients, while it was 4 days for the group with

chronic symptoms. The seasonal distribution of patients is shown in Figure 1 (of Paper I).

Group distribution

- I. 90 children were diagnosed to have a fulminant ethmoiditis with medial extension to engage periorbital tissues (group E) (age range was 4 months to 14 years (median 2 years, mean 3.9 years)).
- II. Chronic sinusitis (group C) was present in seven patients in the age range 8 to 15 years (median 12 years, mean 12.4 years).
- III. The remaining 122 patients was diagnosed to have other kinds of sinusitis (group O), such as acute or sub-acute pansinusitis, polysinusitis, frontal sinusitis or maxillary sinusitis (age range was 5 to 15 years (median 13 and mean 11.2 years)).

Blood tests

Fifty-eight patients, spread between the E group and the O group, had acute or sub-acute sinusitis. For patients for whom both WBC and CRP were analysed, these counts were elevated in 95% of cases.

Bacteriology

Nasopharyngeal cultures were obtained from 141 of the 219 children before any antibiotic treatment was initiated. Fifty-nine cultures proved negative for bacterial growth, showed normal respiratory flora, or only sparse colony growth. Among the recognized pathogens, *Haemophilus influenzae* was isolated from 49 patients, *Streptococcus pneumoniae* from 20 patients, *Moraxella (Branhamella) catarrhalis* from 20 patients, and strains from the haemolytic *Streptococci* group A or B from 10 patients. Anaerobic Gram-positive cocci were found in one patient. See Paper I for full details of the bacteriological findings.

Ophthalmology

Thirty-one of the 90 patients in the E group were seen by an ophthalmologist. Only three of these patients presented any significant disturbance in eye function. One patient (4 years of age) had a protrusion of the bulb and two (both 13 years old) had diplopia. In all three cases, the eye complications abated rapidly within 2-3 days with ampicillin-amoxycillin or cefuroxim medication, and surgery was not required.

Five of the 122 patients in the O group were seen by an ophthalmologist. In one case periorbital oedema lead to combination treatment with sinus surgery and intravenous antibiotics.

Medical therapy

In decreasing order, the most prescribed antibiotic drugs were: amoxycillin or ampicillin (54%), phenoxymethyl-penicillin or benzyl-penicillin (29%) and cefuroxim (8%). Alpha-adrenergic agonist (oxymethazoline or xylomethazoline) decongestants were routinely administered in the form of nasal drops or spray.

Surgery

Surgical intervention was considered necessary in only 12 of the 219 sinusitis patients. All seven patients in the C-group underwent surgery, but none of the children in the E-group. The chief intention of surgery was to improve sinus drainage and ventilation.

Two patients had previously undergone sinus surgery and two additional patients underwent surgery after the sinusitis episode due to subsequently diagnosed nasal polyposis.

Two of the 12 children treated by sinus surgery later had a recurrence of sinusitis: one needed a second polypectomy and the other was subjected to a Caldwell-Luc operation on the maxillary sinus. No other post-operative complications were recorded.

Possible predisposing conditions

A medical history of significant airway or immunological disorder other than sinusitis was recorded in 55 (25%) of the 219 patients. A history of (seasonal or perennial) allergic disease related to aero-allergens, mainly rhinitis and conjunctivitis, was recorded for 27 patients (12%). Seven children had a history of alimentary intolerance problems. Twenty-three children were subjected to skin prick testing and/or the radioallergosorbent test (RAST) because of suspected allergy, but no additional cases of allergy were diagnosed by these tests.

Furthermore, none of the 219 sinusitis patients was registered as having cystic fibrosis (CF) diagnosed up to 1994.

Children with nasal polyps

27 children (younger than 15 years of age) were operated on at the Department of Otorhinolaryngology, Huddinge University Hospital during the years 1980-1992 because of nasal polyposis (19 patients) or choanal polyps (8 patients).

Six of the 19 patients with nasal polyps had CF, two of whom had been admitted from other counties. The CF-patients who underwent surgery were younger (3 children of 3 years of age, and 3 further children aged 6, 7 and 10 years, respectively) than the other 13 children who underwent surgery for nasal polyps. These patients were aged from 9 years and upwards. Four patients (21%) in the non-CF polyposis group had a history of allergic disease, three of them suffering from asthma and one from rhinoconjunctivitis. Six of the 19 children had a relapse that required reoperation of their nasal polyposis during the period studied (re operated within 6 months to 10 years). Two of those were CF patients.

The eight children who suffered from antrochoanal polyps were aged 9 to 15 years. One of them had symptoms of a chronic sinusitis (this patient is included in the sinusitis study); these children were otherwise subjectively healthy.

Paper II

Paper II presents results from 111 of 150 CF patients older than 2 years of age that participated in the study. Half of the patients associated with the CF Centre came from the region around Stockholm.

Forty-four of the 111 CF patients (39%) had nasal polyps that were revealed by the endoscopic investigation. The vast majority showed polyposis of grade 1 (23%) or grade 2 (45%), i.e. polyps concealed in the meatus media. Only one third of the polyps were visible outside of the middle meatus of the nasal cavity. No large polyps were found. The mean age of subjects in the polyp group was 18.0 ± 8.8 years (range, 2 to 37 years; females outnumbering males by 26 to 18), compared to the mean age of 16.1 ± 12.4 years (range, 2 to 57 years; males outnumbering females by 35 to 32) in the non-polyp group. In the study described in Paper II, at least 23% of the patients with CF had undergone surgical intervention for nasal polyposis previously (and previous surgery on nasal polyps was more common in the polyp group) [$p < 0.0001$, log regression; odds ratio, 11.0].

Chronic colonization with *P. aeruginosa*, revealed either by consecutive sputum samples or by a high titre against *P. aeruginosa* anti-exotoxin A, was more prevalent in the patient group with polyps ($p < 0.0001$, log regression; odds ratio, 5.3), and this distinction became more clear during the follow-up period for 20 years ($p < 0.029$, log-rank test). Symptoms such as a tendency to recurrent nasal

blockage (46% in the polyp group and 22% in the non-polyp group; $p < 0.015$, log regression; odds ratio, 2.1) and nasal secretion (26% in the polyp group and 13% in the non-polyp group; $p < 0.04$, log regression; odds ratio, 4.5) were more prevalent in the polyp group. Thirty-five of the 111 patients were chronically infected with *P. aeruginosa* and 44 were infected with *S. aureus*. The mean duration of chronic colonization of *P. aeruginosa* was 10.7 ± 6.9 years in the polyp group and 5.8 ± 4.4 years in the non-polyp CF group. Chronic colonization was defined by culture findings in 18 of the 20 patients in the polyp CF group, by both culture and positive assay findings in a patient; and by a positive assay finding in a patient. All non-polyp CF patients had chronic colonization, as shown by culture findings. The pulmonary status in the CF group was expressed as a percentage of the predicted values. Mean FVC was $88.0\% \pm 20.7\%$ of the predicted value, and mean FEV1 was $77.3\% \pm 25.2\%$ of the predicted value (mean polyp-group FEV1, $75.1\% \pm 24.1\%$ of predicted ($n = 42$); mean non-polyp group FEV1, $78.9\% \pm 26.1\%$ of predicted ($n = 58$)).

The most common mutation in the CF group was the $\Delta F508$ mutation, which occurred in approximately 80% of cases (41% had $\Delta F508/\Delta F508$ and 40% had $\Delta F508$ in combination with other genotypes).

There were no differences between the polyp group and the non-polyp group concerning ongoing symptoms of rhinorrhea, nasal blockage, snoring, mouth breathing, epistaxis, or hyposmia. Nor were there any differences regarding nasal congestion, nasal secretion, mucosal rash, or size of the adenoid. Lung function capacity (FEV1 and FVC) did not differ between the groups, nor did the blood parameters (haemoglobin, blood cell counts, total IgE, ESR, CRP, albumin, haptoglobin, IgG, orosomucoid). There were no differences in atopy. There was no significant difference in the use of intravenous or oral antibiotics, and no difference in the expression of the CF transmembrane conductance regulator genotype.

The smell identification test revealed no difference between the two CF groups. However, the CF patients, with or without polyps, achieved a slightly lower score ($p < 0.04$, Mann-Whitney U test) than the healthy control group. The lysozyme test did not show any difference between the two CF groups. The lysozyme content in nasal lavage of patients in the polyp group did not differ between the group

with or without chronic colonization of *Pseudomonas* in the lower respiratory tract.

CF patients had significantly higher levels of lysozyme than healthy volunteers, indicating an elevated inflammatory status in the sinonasal tract ($p \leq 0.0001$, Mann-Whitney U test). The levels of IL-8 were equal in the two CF groups. The IL-8 content in nasal lavage from patients with polyps did not differ between the groups with and without chronic colonization of *Pseudomonas* in the lower respiratory tract. The lavage content of IL-8 was higher in the whole CF group than it was in the healthy volunteers ($p \leq 0.0001$, Mann-Whitney U test). IL 5 was not present in the nasal lavages. PCR of the lavages indicated that *P. aeruginosa* was not present.

Only 10 of the 44 patients with polyps attended the recommended follow-up examination after one month of treatment with topical steroids, and thus no conclusions can be drawn about the efficiency of this treatment. The travelling distances may explain to a certain extent the low compliance.

Paper III

Nasal T-Cell Phenotypes in Uninfected Conventional Rats

CD3⁺ LPLs were observed as single scattered cells or in small clusters located mainly in the subepithelial region. CD3⁺ IELs were distributed throughout the surface epithelium, occurring most frequently at the base of the nasal cavity. Both LPLs and IELs were scarce in the superior region of the cavities where the olfactory epithelium is located. Notably, nasal lymphocytes were mainly located adjacent to the NALT structures, and more than half of them were not CD3⁺ T cells, but NK cells (see below). Such site-specific differences in the distribution of T lymphocytes and other immune cells are present in the respiratory tract (related to the larynx) of rats (83).

Most (more than 90%) nasal CD3⁺ LPLs and IELs expressed TCR $\alpha\beta$, while only a few (approximately 5%) expressed TCR $\gamma\delta$. Surprisingly, most $\alpha\beta$ ⁺ T cells in the surface epithelium were CD4⁺ (median 65%), although a substantial fraction expressed CD8 (31%). As expected, most $\alpha\beta$ ⁺ T cells in the lamina propria were CD4⁺ (63%), while the proportion of CD8⁺ cells (21%) was somewhat lower than in the epithelium (Figures 1 and 2 in Paper III).

Nasal T-Cell Phenotypes and NALT Size are Influenced by the Normal Microbiota

The numbers of TCR $\alpha\beta$ ⁺ LPLs in nasal mucosa per mm of surface epithelium were quite similar in GF and in CV rats, whereas the corresponding IEL subset was more numerous in CV rats than in GF rats (see data in the next section). However, the percentage of TCR $\alpha\beta$ ⁺ LPLs that expressed CD4 (median for all rats, 54%) was significantly smaller in GF rats than in CV rats ($p < 0.01$). The percentage of the TCR $\alpha\beta$ ⁺ IELs that expressed CD4 in GF rats (49%) was also lower than it was in CV rats ($p < 0.01$) (Figure 1 from Paper III). Conversely, the proportion of TCR $\alpha\beta$ ⁺ LPLs that expressed CD8 (38%) in GF rats was significantly higher than it was in CV rats ($p < 0.001$). The fraction of TCR $\alpha\beta$ ⁺ IELs that expressed CD8 (42%) was also significantly higher ($p = 0.016$) in GF rats than in CV rats (Figure 2 in Paper III).

The estimated areas of NALT at corresponding section levels were only marginally, although significantly ($p < 0.01$), smaller in GF rats than in CV rats (Figure 3 in Paper III), and the lymphoid aggregates consisted of T cells (mainly CD4⁺) and primary B-cell follicles without recognizable germinal centres (Figures 4A and 4C in Paper III) in both types of rat. This observation agrees with previous work showing that the normal microflora of the nose provides insufficient immunostimulation to induce germinal centres in murine NALT (89).

Mycoplasma Pulmonis Infection Induces Expansion of NALT and Mucosal T Cells

The cross-sectional area of NALT increased approximately 2.6-fold in CV rats under the influence of mycoplasma infection ($p < 0.01$), and such NALT hypertrophy was greater in GF rats (5.2-fold, $p < 0.01$) (Figures 3 and 4B in Paper III). Thus, the calculated cross-sectional area of NALT became the same in CV rats and in GF rats when infected. Immuno-staining in both CV and GF animals showed that the mycoplasma-induced NALT hypertrophy included aggregated hyperplastic B-cell follicles with germinal centres rich in CD4⁺ T cells (see Figures 4B and 4D in Paper III), which were not present in uninfected rats (Figures 4A and 4C in Paper III). This pattern was easily distinguishable from the diffusely distributed B and T lymphocytes seen elsewhere in nasal mucosa.

The number of TCR $\alpha\beta$ ⁺ LPLs per mm surface epithelium was significantly higher after mycoplasma infection in nasal mucosa of both CV rats (from 7.2 to 10.8, $p=0.008$) and GF rats (from 7.1 to 12.9, $p=0.008$), and the same was true for TCR $\alpha\beta$ ⁺

IELs in GF rats (from 1.0 to 1.7, $p=0.02$), but not in CV rats (from 1.7 to 1.8, $p=0.31$). The numbers of TCR $\alpha\beta^+$ LPLs and IELs were the same in the two animal groups after infection. The number and the phenotype of the rare TCR $\gamma\delta^+$ T cells were not affected by the infection. These results agree with the T-cell distribution in rat laryngeal mucosa in CV rats following inhalation of heat-killed *Moraxella catarrhalis* (88).

***M. pulmonis* Infection Alters Nasal T-Cell Phenotypes in Germ-Free but not Conventional Rats**

Monoinfection with *M. pulmonis* induced a striking increase in the proportion of TCR $\alpha\beta^+$ CD4 $^+$ T cells in GF rats, both in the lamina propria ($p < 0.01$) and in the epithelium ($p < 0.01$), whereas the proportion did not change in CV rats (Figure 1). Conversely, the proportion of TCR $\alpha\beta^+$ CD8 $^+$ in both compartments decreased significantly in GF rats ($p < 0.01$) but remained virtually unchanged in CV rats (Figure 2 in Paper III). Notably, the proportion of the TCR $\alpha\beta^+$ LPLs that expressed CD4 before infection in the GF rats was significantly higher than it was in the CV rats ($p = 0.03$). This was also true for TCR $\alpha\beta^+$ IELs that expressed CD4 ($p < 0.01$) (Figure 1 in Paper III).

A Subset of Nasal Lymphocytes Expresses the NK-Cell Complex

Interestingly, a relatively large population of nasal CD8 $^+$ lymphocytes did not express TCR $\alpha\beta$ (Figure 4B in Paper III) or TCR $\gamma\delta$. The percentage of all CD8 $^+$ IELs that was TCR $\alpha\beta^+$ CD8 $^+$ was 53% in GF rats and 62% in CV rats, whereas in the lamina propria these fractions were 57% and 74%, respectively. Many of these cells co expressed the NK cell marker NKR-P1 (Figure 4E in Paper III), and were also CD2 $^+$; the phenotype of these cells led us to denote them as "NK cells". The proportion of cells in this subset was lower in CV rats after *M. pulmonis* infection, while it remained the same in GF animals.

Paper IV

Paper IV describes a study in which 76% (122/160) of the CF patients older than 5 years of age agreed to participate.

Olfactory sensitivity test measuring the concentration threshold for the detection of butanol showed that

the sense of smell was pathological in 64% of cases. (57% hyposmia and 7 % anosmia) while the smell identification tests showed olfactory disorders in 44%. The olfactory functions were further reduced by the presence of nasal polyps. Olfactory disorder according to the butanol test was found in 78% (58% hyposmia and anosmia 20%) of the polyp patients ($p=0.03$, OR 2.4 compared to the nonpolyp group). According to the identification test hyposmia or anosmia was found in 57% polyp patients ($p<0.004$, OR=2.9 compared to the nonpolyp group)

Hyposmia or anosmia according to both tests (i.e. the olfactory sensitivity test and the smell identification tests) was seen in 31% with an elevated risk, 48%, in the polyp group ($p<0.005$, OR=3.4). 74% had hyposmia or anosmia according to at least one of the tests with an elevated risk, 87%, in the polyp group ($p=0.03$, OR=2.8).

According to the questionnaire only 13% of the patients did experience any kind of lowered smell sensitivity on a continuous basis (with a higher frequency, 27 %, in the polyp group ($p=0.001$)). 75% of the patients, at the time of the examination, declared that their sense of smell was normal (56 % in the polyp group ($p<0.003$, OR=2.9)).

A higher proportion of the patients with a lowered sense of smell, according to the butanol test, had been operated with polypectomy or ESS (30%, $p=0.04$) or had experienced sinusitis (25%, $p<0.05$) compared to patients with a normal sense of smell.

The subjective estimation of reduced taste sensitivity was 12% with a higher frequency if parallel hyposmia was present (16%, $p=0.03$) or if nasal polyps were found (23%, $p=0.01$).

The frequency of nasal polyposis was estimated to 37% with the endoscope technique. Only 11% of the patients with nasal polyps declared continuous nasal blockage while 7% experienced continuous nasal secretion.

Figure 1 in Paper IV shows the difference in hyposmia in CF patients related to the presence of nasal polyposis (according to the smell tests and according to the subjective evaluation of the sense of smell). Figure 2 in Paper IV compares the frequencies of nasal polyposis and hyposmia as determined by the butanol test and the identification test in children with the frequencies in adults.

There were no tendencies of any elevated morbidity or pathological laboratory tests that correlated to the

prevalence of nasal polyps or the sense of smell. The spirometric values and genotype status were independent of the prevalence of polyps and the sense of smell. The BMI was not affected by the presence of lowered olfactory function.

There was no correlation between olfactory dysfunction or nasal polyposis and the use of oral or intravenous antibiotics.

The frequency of allergic reactions to aeroallergens (supported by any objective allergy test) was 35% with no differences between the polyp and non polyp group (31 vs. 37%), nor any difference between the adults compared to the children (37.5 vs. 32%). This is consistent with earlier observations stating no differences regarding allergic tendency between CF patients with and without nasal polyps (49, 50, 90).

26 of the patients had medium sized (\geq grade 2) polyps, or more, and were offered to participate in the randomised treatment of the study. 18 of these patients agreed to become treated according to the study trial.

Of the 10 patients treated with nasal steroids 8 were left to follow up (1 patient moved from the region and 1 patient experienced mild side-effects of the spray). Of the 8 patients operated on 7 were followed up (1 patient moved from the region). Five of seven patients in the operated group (combination treatment with endoscopic sinus operation and nasal steroid) were improved according to the butanol test while 6 of 7 were improved in at least one of the smell tests. In the group treated with nasal steroid five of eight were improved according to the butanol test while all of the patients were improved in at least one of the smell tests. In five of the eight patients' treated with nasal steroids the polyp size didn't shrink on either side in spite of 7 to 12 months of treatment (although one of the patients discontinued medication).

5 DISCUSSION

The number of in-patient treatment events during the period from 1980 to 1992 (24/100,000 children and year) indicate that considerably less than 0.5% of the population require hospital care due to sinusitis during childhood. We can conclude from this that the vast majority of children with uncomplicated sinus infections are adequately treated in a primary care setting. The recordings of body temperature and the results of laboratory tests in paediatric sinusitis

did not provide any important differential diagnostic or prognostic information. The level of CRP was the most indicative inflammatory parameter, most often significantly increased. This measurement was particularly coupled to the E (ethmoiditis) group. Our finding that aerobic bacteria dominate, and our finding of many negative cultures, are consistent with those of previous studies (91, 92).

X-ray imaging is a highly unselective method to diagnose therapy-requiring sinusitis in children, particularly in the youngest age group (93, 94). Sinus X-ray imaging (conventional or computed tomography) was performed in 163 (74 %) of the sinusitis children, and all of the images showed clearly sinus pathology. We did not study these findings further, as they were of little or no help in our therapeutic measures.

Predisposing diseases were of little significance in the work presented here (see Table 2 in Paper I). Previously reported allergic symptoms were not significantly higher among the sinusitis children than in the general population at this age in Northern Europe or North America (49, 91, 95-98), nor were positive results from allergy tests. Asthma (in the anamnesis) was not statistically over-represented in the sinusitis children (49, 91, 95, 99, 100).

The frequency of nasal polyposis found in the Stockholm CF Centre group (39%) is consistent with recent studies using endoscopic techniques for diagnosis, which show frequencies ranging from 32-56% (36, 42, 45). However, the nasal polyps in our study were smaller and produced less symptoms than those in some of the earlier studies. Fewer operations (polypectomy or sinus surgery) were carried out in this study than in another recent study (45). The milder form of nasal polyposis and lower frequency of operations in the sinonasal region in our study, compared with those found in earlier studies, may be partially explained by, for example, differences in access to specialised medical care and by socio-economic factors (101).

We have shown that early chronic colonization of *Pseudomonas* in the lower respiratory tract is correlated with the presence of nasal polyps. Three mechanisms could explain this observation: a mechanism supporting the hypothesis of a sino pulmonary reflex (which would lead to the colonizing of bacteria in the lower respiratory tract), a mechanism involving the spill-over of bacteria, or a mechanism based solely on an elevated inflammatory status in both the upper and the lower airways

due to the underlying disease. A naso-bronchial relation was postulated as early as 1919 by Sluder (102), and in 1925 by Gottlieb et al. (103). This mechanism has been discussed in the case of asthma (104, 105). The second mechanism may be spill-over of colonizing bacteria from the sinus to the lower airways. X-rays of the paranasal sinuses show opaque areas in 90-100% of the cases (32, 41, 43, 50, 106) (because of mucosal swelling and/or liquid in the paranasal sinuses), and both *P. aeruginosa* and *S. aureus* are commonly found in sinusitis (49, 107). These observations suggest that spill-over is occurring. On the other hand, Drake-Lee and Morgan (90) found no correlation between sinus washouts and the corresponding sputum samples.

Raj et al. (55) showed that chronic *Pseudomonas* colonization (in the lower respiratory tract) is associated with rhinitis and nasal polyposis. These authors were primarily interested in the development of allergy in the population in which colonization was chronic. They could not explain why polyps were associated with the chronic colonization, nor could they clarify the association between allergy and polyposis. In our study (Paper II), known allergic problems in patients with polyps (21%) did not exceed known allergic problems (33%) in the non-polyposis CF group.

We found a relatively high risk that polyps recur after simple polypectomy, as others have also found (34, 43, 107, 108). In cases with small-sized polyps with only mild or intermittent symptoms, medical treatment in the form of nasal steroids proved to be adequate and effective (47). Simple polypectomy has been widely used in cases of medium-sized to large-sized polyps, and has been successful in relieving the symptoms, but the risk of recurrence is very high. It has been suggested that endoscopic sinus surgery (ESS) in which the osteomeatal area (where the ethmoidal, maxillary and frontal sinuses enter the nasal cavity) is opened will reduce the risk of recurrence of both polyps and sinusitis (43, 49). Significant reduction of the symptoms related to polyposis and to sinusitis have been reported after surgery (37, 49, 109, 110). It is, however, unclear at what stage this procedure is appropriate. The risk of reduced and altered growth of the sinuses and the facial skeleton (in young patients) has been debated (111), but Senior et al. (28) found that sinus surgery in children was safe and without significant cosmetic sequelae. We conclude that the most effective treatment for sinus disease in CF is the combination of endoscopic surgery with serial antimicrobial lavage (ESSAL) described by Moss and King (45)

It is still unclear whether the intervention (ESS) improves the pulmonary status. A recent follow-up study did not show any reversibility in the progressive lung disease in CF patients after ESS (112). Some authors have advocated radical surgery before lung transplantation in CF-patients (113, 114). The Δ -F508/ Δ -F508 and the Δ -F508/G551D genotype are significantly more common among CF-patients with more severe polyposis (i.e. those that require surgical treatment) (115, 116). There was no difference in the genotypes of the CF groups (with and without polyps) in our study. This may be either an effect of the limited size of the material studied, or it may be an effect of a different tradition of medical treatment, which inhibits the development of polyps. Regular oral or i.v. antibiotic treatment as soon as the any signs of an infection are noticed may reduce the conditions that increase the risk for polyp growth. The regular mucolytic treatment also reduces the risk of chronic sinusitis, and thus stimulation of polyp formation. The sinuses of CF-patients quite often contain *P. aeruginosa* and *S. aureus* (117). Theories of an inflammatory mechanism for the occurrence of polyps have been proposed. Animal studies have shown that *P. aeruginosa* gives rise to a more severe infection (which increases the risk of polyp growth) (118). Mucolytic therapy may reduce polyp formation through its action on the viscous mucus in the sinuses.

The level of IL-8 in nasal lavages from CF patients was higher than that of the controls, but it was the same in the two CF groups. Earlier studies have shown an elevated IL-8 level in the bronchoalveolar lavage of CF-patients (119, 120), while the level of IL-8 in nasal lavage fluid was the same as that of healthy subjects (119).

The level of lysozyme in the nasal lavage showed that inflammation was higher in the CF patients than it was in the controls, but the levels were the same in the two CF groups. Lysozyme levels as a marker of serous secretion - are elevated in the tracheal mucosa from CF-patients (121). A certain leakage of lysozyme is to be expected from neutrophils involved in the bronchial airway pathology due to the colonization of *P. aeruginosa* in CF (122). However, no increased levels of lysozyme have previously been reported from nasal lavages of patients with cystic fibrosis (123). Elevated levels of lysozyme are present in the nasal lavage of patients in whom glandular secretion from the nose has been induced (124). The baseline secretion of lysozyme in patients with recurrent sinusitis is raised, while

the cholinergic response of these patients is lower, and has a lower secretion of lysozyme (125).

Our PCR analysis detected no *P. aeruginosa* RNA in the nasal lavage. There were, however, single findings of *Pseudomonas* detected from cultivation of nasopharyngeal swabs, and we suggest that the lavage technique is not suitable for detecting *Pseudomonas* species. The similarity of the markers in the nasal lavage from the two CF groups may be a result of the early stage of local disease as indicated by the limited size of the polyps.

Cultures from the nose and nasopharynx do not always reflect the species present in the paranasal sinuses (117). Furthermore, the nose may be a transient site for the *Pseudomonas* species.

The lung function test did not show any differences between the CF groups in spite of the difference in chronic colonization of *P. aeruginosa*. This agrees with previous findings that pulmonary function is not reduced in CF patients with polyps (116).

In our study 13 % of the CF patients reported decreased smell sensitivity on a continuous basis. At the time of the examination, 25 % declared that their sense of smell was decreased. This is higher than what has been reported for a general population. In an earlier American study the prevalence of self-reported olfactory problems was 1.4% in individuals older than 18 years of age, with a prevalence rate that increased exponentially with age (126). The subjective smell sensitivity in the CF patients was further reduced with age and the presence of nasal polyps but not altered by gender or genotype.

The butanol test and the smell identification tests revealed a surprisingly high prevalence of hyposmia and anosmia. The butanol test is more sensitive to mucosal swelling and therefore dependent on the inflammatory status including ongoing or previous infections. The identification test is additionally reflecting our odour memory and ability to discriminate between different odours. Certain odours in the test could be sensitive to differences in life-styles and preference of odorants. The variation in odour panoramas at home, in school and at work could alter with ageing and habits (teenagers of today are seldom familiar with odorants as e.g. camphor). Some of the odours in the SOIT test (peppermint, ammonia and vinegar) could furthermore contribute to some degree of trigeminal stimulations and be detected

by anosmics, which could bias the interpretation of the test (75).

Our findings that the smell tests revealed a higher prevalence of olfactory disorders than what the patients themselves reported is in agreement with an epidemiological Swedish study on randomly selected adults on whom an olfactory test was performed which showed a higher prevalence of olfactory disorders than the above mentioned American study. 13.3 % had hyposmia and 5.8% anosmia with an overweight for aged persons, males and persons with nasal polyps (68). In our study the CF patients showed a tendency to a slight reduction in olfactory function with age according to the butanol test. But when comparing the frequency of hyposmia in the younger population (5 to 16 years of age) with the older patients (17 to 61) we could not find any significant lowered values with the butanol test ($p=0.053$). No differences were seen by gender.

In our study the occurrence of hyposmia and anosmia was obvious with both the butanol threshold score and the two different smell identification tests used and the score were – within the CF group – even more reduced by the presence of nasal polyps. In a previous study (127) we did find a significant lowered sense of smell with the UPSIT (Univ. of Pennsylvania Smell Identification Test) compared to a healthy control group, but no further reduction because of nasal polyps. In a study by Aitiken et al. the UPSIT test showed an objective decrease in sensation of smell in 55% of the 49 examined CF patients. Patients with anosmia in the mentioned study were more likely to have had prior sinus surgery (128). In that study previous polypectomies and ESS were more frequently carried out in patients with hyposmia or anosmia. There is an obvious connection between the prevalence of sinusitis and nasal polyps and an olfactory disorder (128). In spite of adequate surgery on nasal polyps and sinus cavities the sense of smell could remain reduced (77, 128, 129). Additionally the risk of recurrence (of nasal polyps and sinus disease) is high after such operations, which could explain why the sense of smell could be affected in spite of recent surgery. Finally there are some studies pointing out the risks of affecting the olfactory region during the different sinonasal operations carried out (79, 130).

The noted reduction in the taste sensitivity in our study probably reflects the fact that complaints of taste loss usually reflect loss of smell function (74).

The more complex information around the "taste" of different food flavours are mediated through the olfactory region whereas the specific information given by the taste buds of the tongue are salt, sweet, sour and bitter. Earlier studies that separately tested the taste sensitivity of CF patients have shown normal values (131, 132).

In our study no correlation could be found between the genotype and the presence of polyps and/or a lowered sense of smell. A recent study did show a genotype-phenotype correlation (Δ -F508 homozygote) for the paranasal sinus diseases (nasal polyposis and chronic sinusitis) for patients with CF (115). Another previous study reported a correlation between nasal polyposis that required surgery and two specific genotypes: the Δ -F508/ Δ -F508 and the Δ -F508/G551D genotypes (116). In the latter study patients with CF and nasal polyps had better pulmonary function, better nutritional status, a higher frequency of *P. aeruginosa* colonisation, more office visits, more hospitalisations and a higher rate of acute exacerbations per year than did the comparison group in that study. However, in a recent study at our CF-centre (127) we were not able to point out any elevated risks for morbidity in patients with relatively mild nasal polyposis except for a long-term tendency to acquire chronic colonisation of *P. aeruginosa* in the lower airways earlier than other CF patients, but without any parallel risk of lowering the respiratory functional capacity. In the present study at the same centre we could not confirm the previous observation regarding the difference in chronic colonisation with *P. aeruginosa* of the CF patients with nasal polyps.

In the CF-population both nasal steroids (133) and endoscopic sinus surgery (37) have shown effects on nasal polyps. Earlier observations on idiopathic adult nasal polyposis did not find any positive effects on the sense of smell by such an operative procedure (21) while other studies in CF have shown beneficial results on the olfactory function with the use of endoscopic sinus surgery (134).

Surprisingly most CF-patients experienced their sense of smell not affected. This could depend on the fact that the patients become accustomed to a reduced level of olfactory sensation without any possibilities to compare their new olfactory level with their previous one. The most important function of the sense of smell in humans is to direct our attention toward environmental hazards (smoke and toxic fumes) or to positive sensations such as nutritious food products. In the mentioned situations a slight reduction in the olfactory function will not

make a difference as we are dealing with higher concentrations of odorants well over normal threshold. The greater discrepancy found in the CF patients may be due to this patient category partly ignoring the symptoms from the upper airways focusing as they are on the major problems from the lower airways (135).

Poor nutritional status in patients with CF is associated with increased mortality. As the sensation of smell is influencing our appetite a decreased sensation of smell may be associated with worse nutritional status in patients with CF. In the present study nutritional status was assessed by BMI. No association was found between sensation of smell (hyposmia or anosmia) and BMI. This is in agreement with observations by Aitken et al who could not show any association between sensation of smell and the nutritional status (128).

The tendency of increased olfactory disorders in the adult CF population (compared to the children) could depend on the increased frequency of polyps with age. The chronic sinopathy with the intermittent rhinosinusitis could be another source of local inflammation and mucosal swelling in this area that could affect the olfactory region. Another factor that might influence the olfactory epithelium with increasing age is the exposure of the potential neurotoxic drug tobramycin. This beta-lactam is given either intravenously or through inhalation. It is prescribed for *P. aeruginosa* colonisation of the lower airways nowadays. The general recommendation is that these inhalations should be performed with a mouth-piece instead of a face-mask in order to protect the olfactory region.

Corticosteroids may function by reducing the inflammatory swelling of the nasal mucosa, which increases the penetration of odours to the olfactory region, or they may function directly on the olfactory cells. Hyposmia in patients with perennial allergic rhinitis have been successfully treated with topical steroids, both symptomatically and in olfactory testing (UPSIT) (80). A more distinct improvement in the sense of smell could be achieved by short-term treatment with oral glucocorticoids (81, 82) but this is not appropriate treatment on a long-term basis. Anosmia or hyposmia not responding to topical nasal steroids did respond to oral steroids, but the duration of symptom-relief was relatively short according to Stevens et al.(83). The drop out from participating in the treatment part of our study could partly be explained by the mild symptoms that the CF-patients experienced in spite of the polyps. Because of the

limited size of the treatment group no significant conclusions could be made. For achieving a more definitive evaluation of the two applied treatments of this study a multicenter approach is required.

The results from the rat model described in Paper III show for the first time that a local immune-modulating effect of commensal bacterium in the phase of infection with an extracellular respiratory pathogen. Over time, *M. pulmonalis* is known to cause chronic inflammatory disease in the airways of rodents. We were not able to evaluate specific T cell responses in this *in situ* study, but we have shown that the mono-infection induced more severe perturbations of immunological variables in GF rats than in CV rats, by affecting, for example, both the number and proportion of CD4⁺ TCRαβ⁺ cells in the lamina propria and the surface epithelium. Thus, mucosal immune homeostasis was better maintained during the infection in the presence of the indigenous microbiota, a result that was also reflected by the presence of less relative hypertrophy of the local inductive NALT structures in CV rats than in GF rats.

Human coexistence with commensal bacteria in a mutually beneficial manner has developed over several million years of adaptation (58). The immunological hyporesponse to the indigenous flora may be a tolerance phenomenon (136), and may largely depend on regulatory T (T_{reg}) cells secreting transforming growth factor TGFβ and interleukin IL-10. These factors may suppress the immune responses. They inhibit both Th1- and Th2-dependent immunity by dampening both T cell-mediated and T cell-independent immunopathology (137, 138). There is currently a great deal of interest in the role of APCs in shaping the phenotypes of naive T cells during their initial priming, because differential expression of co-stimulatory molecules on both steady-state and activated dendritic cells (DCs) can play a decisive role. Thus, the function of DCs is modulated by pathogen-associated molecular patterns (PAMPs), which are sensed by pattern recognition receptors (PRRs) – many of which belong to the family of receptors known as “Toll-like receptors” (TLRs). The engagement of PRRs on DCs causes maturation, accompanied by the production of cytokines and the up-regulation or down-regulation of cell-surface molecules, according to strictly defined kinetic rules (139). Such signalling molecules will critically influence further induction of both innate and adaptive immunity.

Pathogens can quite early during an infection use their PAMPs to imprint their ‘signatures’ onto

subsequent immune responses. T_{reg} cells may also be directly affected by microbial products such as LPs through the TLRs that they express (140). It is important to remember that PRRs do not distinguish between pathogenic and commensal bacteria, which might seem incompatible with the mucosal homeostasis that normally exists. However, it cannot be excluded that the indigenous flora induces differential PRR signals that result in distinct molecular APC programmes (141). Non-pathogenic *Salmonella* strains are able to block the NF-κB transcription pathway in human gut epithelial cells *in vitro* and in this way reduce basolateral IL-8 secretion in response to pro-inflammatory stimuli, including apical infection with wild-type *S. typhimurium* (142). Another interesting observation is that the intestinal epithelium has inherent mechanisms to protect itself against activation from the luminal side, unless chemokines and pro-inflammatory cytokines are needed to defend the host against invading micro-organisms (143). Thus, mucosal epithelial cells possess sensing systems that allow the cells to discriminate between pathogenic and non-pathogenic bacteria, and these cells initiate an inflammatory reaction only when it is necessary to eliminate invading pathogens. The secretory immune system is part of this homeostatic mechanism, and specific dimeric IgA may, during polymeric Ig receptor-mediated epithelial transcytosis to the mucosal surface, prevent LP-induced NF-κB translocation and induction of pro-inflammatory cytokines (144).

Further work is needed to investigate whether the dampening effect of the normal microbiota on pathogen-induced immunological perturbations is mediated by T_{reg} cells or by the other homeostatic mechanisms that are described above. It has recently been shown in a rat model that the response to inhaled allergens in sensitized animals is regulated by bidirectional interactions between antigen-presenting DCs and memory T cells (145). The resident DCs rapidly matured *in situ* to potent APCs following exposure to aeroallergens. This points toward one mode of action for activated CD4⁺ T cells in the airway mucosa.

The commensal flora in the human nasopharynx is similar to the microbiota of the mouth and oropharynx, which has been more closely studied. The numbers and the prevalence of various bacteria in the nasopharynx, including different species of *Streptococcus* (i.e. *S. mitis*, *S. pneumoniae*, *S. orarilis*, and *S. viridans*) vary from one individual to the next and depend on age (52). This niche is

colonized with potential pathogens at different periods in life. In childhood, *S. pneumoniae*, *Hemophilus influenzae* and *Moraxella catarrhalis* are relatively frequent in the nasopharyngeal region, and the levels of these strains are elevated in populations with, for example, otitis media. Approximately 30% of adults harbour *Staphylococcus aureus* in the most anterior region of the nasal cavities. Bachert et al. (54) have suggested that an aberrant immune response on enterotoxin from *S. aureus* results in the formation of local IgE antibodies associated with nasal polyps. Increased numbers of *S. aureus* and other transient pathogens (such as *Pseudomonas* spp.) in the sinonasal region and lower respiratory tract in patients with cystic fibrosis may be associated with an increased prevalence of allergic eosinophilia in this population (55).

It is unclear whether the aberrant response patterns described above result from inappropriate immunoregulatory signals from the indigenous microbiota. A recent Danish study has shown that the normal flora of the human nasal cavity consists of *Corynebacterium*, *Aureobacterium*, *Rhodococcus* and staphylococci, including *S. epidermis*, *S. capitis*, *S. hominis*, *S. haemolyticus*, *S. lugdunensis*, and *S. warneri* (56). This composition differs totally from the nasopharyngeal flora. The status and the role of the commensal flora in the human nose and nasopharynx in chronic or recurrent infection and inflammation remain largely unknown.

The colonization of microbes in the nasal cavity and nasopharyngeal region of rodents is more pronounced than it is in humans. Further, rodents breathe mainly through the nose. This might explain the striking difference in pathogen-induced immunological perturbations between GF rats and CV rats. The nose and nasopharynx are quite exposed to the external environment, and are thus in persistent contact with irritants and antigens, and so the immunological impact of the microflora in this region is an important homeostatic mechanism possible similar to that known to exist in the gut (57, 59). Current development of strategies to colonize these niches with indigenous flora (for example α -streptococcus) could lead to treatments that through specific immunological mechanisms suppresses pathological bacteria in the corresponding region.

6 CONCLUSIONS

No particular predisposing condition was significantly over-represented among children requiring in-patient hospital care for sinusitis. Common childhood disorders such as acute otitis media, otitis media with effusion and subglottic laryngitis were no more common in the medical records of children requiring in patient care than in the general population. Improved preventive measures and early medical intervention may be the reason that these children are less likely to acquire a serious sinus infection.

Nasal polyps were found in 39% of the CF patients. Chronic colonization of *P. aeruginosa* in the lower respiratory tract of CF patients with nasal polyps was higher than it was in CF patients without polyps. Otherwise, non-severe nasal polyposis did not indicate raised lower respiratory tract morbidity in CF patients. The infectious and inflammatory parameters of the nasal lavage were similar for patients with nasal polyposis as for those without. Furthermore, the genotypes of patients with nasal polyps did not differ from those patients without polyps. Reduction in the frequency and the size of nasal polyps may be the result of intensive antibiotic and mucolytic treatment.

We have shown in a rat model (GF) that the normal microbiota modulates the response of various T-cell subsets (such as TCR $\alpha\beta$ ⁺CD4⁺ and CD8⁺ cells) during mono-infection with the pathogen *M. pulmonis*. This model may be further exploited to examine in more detail individual immunoregulatory network components engaged by commensal bacteria in the airways.

Hyposmia is frequent in the population of CF. The sense of smell was even more reduced by the presence of nasal polyps. The lowered sense of smell did not alter with age, allergy to aeroallergen, genotype or gender. The olfactory disorder did not alter the overall health status in this population and the majority of the patients were not affected or aware of their lowered sense of smell. To study operation of nasal polyps vs. nasal corticosteroids in CF requires a multicenter approach due to certain circumstances in recruiting the CF patients.

Together with threshold test (butanol) the newly presented smell test for children aged 5-13 years (SSIT-C) is a valuable complement in clinical evaluation of nasal and olfactory problems in this patient group.

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I

A 13-year report on childhood sinusitis: Clinical presentations, predisposing factors and possible means of prevention*

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SUMMARY

Two hundred and nineteen children with sinusitis treated as in-patients at Huddinge University Hospital during the period 1980-1992 have been reviewed. Epidemiological data, the clinical picture, treatment and complications are described. The prevalence of significant predisposing conditions (such as upper airway allergy, asthma, and immunoglobulin deficiency) has been estimated. Serious sinusitis complications are few, surgery is only rarely required, and previously-recognized important predisposing paediatric conditions are not significantly more common than in the general juvenile population. Improved medication and prevention may have reduced the incidence of serious sinus infections in risk groups today. Children with cystic fibrosis have been reviewed with regard to the necessity of both sinus and nasal polyp surgery. Aggressive medical therapy appears to have reduced their need for sinus surgery as well as polypectomy.

Key words: allergy, bacteria, cystic fibrosis, in-patient, nasal polyp

INTRODUCTION

It has been estimated that some 5-13% of the general population may have experienced sinusitis during childhood (Wald et al., 1991), but the exact incidence of acute or chronic sinusitis in children is not known. There is one obvious reason for this: diagnosis is usually based on a combination of signs and symptoms and sometimes also on plain radiographs (Cuyler and Monaghan, 1989), but only rarely confirmed by more sophisticated imaging techniques, histopathology, direct endoscopy or bacterial culture from the affected sinuses. Features generally considered to indicate acute sinusitis include cough (particularly nocturnal), nasal congestion and secretion, and fever (Kogutt and Shwachman, 1973). Headache and facial pain are symptoms less frequently seen in children than in adults. When the diagnosis of sinusitis is established in a child, symptoms often persist for more than the 7-10 day period, that is typical for uncomplicated upper airway infections (Fireman, 1992). The principal conditions which in the literature are regarded to predispose for or co-exist with sinusitis are: airway allergy (Kogutt and Shwachman, 1973; Crockett et al., 1987; Åberg et al., 1987; Rachelefsky et al., 1988; Savolainen, 1989; Fireman, 1990; Orobello et al., 1991; Furukawa et al., 1992; Rachelefsky et al.,

1992), asthma (Kogutt and Shwachman, 1973; Slavin, 1984; Crockett et al., 1987; Minor and Lockey, 1987; Orobello et al., 1991; Rachelefsky et al., 1992), immunodeficiency syndromes (Shapiro et al., 1991; Orobello et al., 1991; Rachelefsky et al., 1992) and cystic fibrosis (Shapiro et al., 1982; Stern et al., 1982; Reilly et al., 1985; Ceporo et al., 1987; Crockett et al., 1987; Cuyler and Monaghan, 1989; Drake-Lee and Morgan, 1989; Ramsay and Richardson, 1992).

The most severe cases of paediatric sinusitis seen at Huddinge University Hospital, i.e. those requiring in-patient hospital care, during the past 13 years have been reviewed and evaluated in this paper. This investigation gives a summary of the presently seen clinical presentations in this patient group at our hospital and updates the relative importance of certain conditions known to predispose for sinusitis in children.

MATERIAL AND METHODS

Two hundred and nineteen children with sinusitis (aged 4 months to 15 years) treated at the Department of Otorhinolaryngology (n=191) and the Department of Paediatrics (n=22) at Huddinge University Hospital during the period 1980-1992 were included in this retrospective study. The ENT specialist on

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duty judged whether they required in-patient hospital care, based upon the severity of symptoms from acute (pain, affected general condition, fever, oedema of eyelids or suspected orbital engagement), or chronic sinusitis (prolonged symptoms, requiring extended examination or surgery). The diagnosis – bacterial infection of the sinus mucous membrane – was based on disease history, clinical findings and, in the majority of patients, also on radiographs. In all cases, the diagnosis was confirmed by an experienced consultant.

The patients were divided into three major groups with regard to symptom duration and the severity of sinus infection: (1) acute fulminant ethmoiditis, with extension of the infection to the peri-orbita and/or the orbita (group E); (2) less severe forms of acute or subacute sinusitis (group O); and (3) chronic sinusitis, with symptoms persisting at least for three months before admission (group C).

For all patients the records were reviewed for previously or currently diagnosed or suspected allergic disease, asthma, cystic fibrosis, immunodeficiency syndrome, and ciliary dysfunction. Several clinical parameters were also taken into consideration: body temperature, erythrocyte sedimentation rate, C-reactive protein, white blood cell count, and bacterial culture from the nasopharynx by routine laboratory procedure at the time of admission. Drug therapy during hospitalization, the period of time with elevated temperature ($>38^{\circ}\text{C}$) after initiation of therapy, duration of hospitalization, any ophthalmological complications, and surgical intervention were also registered. In addition, patient data of all in-patient sinusitis children were checked with the 1994 accumulated register of cystic fibrosis (CF) patients at the Stockholm Cystic Fibrosis Centre (Department of Paediatrics, Huddinge University Hospital). This was done in order to reveal any patients who may have received the diagnosis of CF after their sinusitis episode.

In a parallel study, 28 children in the same age group who during 1980–1992 were treated as in-patients for nasal and choanal polyposis, were reviewed in a similar way for the prevalence of predisposing conditions. The data obtained were compared statistically with Chi-square test (age and monthly incidence) and with Student's *t*-test (prevalence relation to pre-existing epidemiological data).

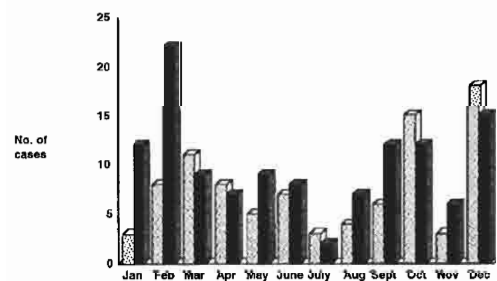


Figure 1. The cumulative monthly incidence of fulminant ethmoiditis (group E, spotted columns) or other acute sinusitis (group O, filled columns) during the period 1980–1992. There is a significant difference in monthly incidence within both groups (Chi-square test, $p < 0.001$).

RESULTS

Among the 219 children, boys outnumbered girls by 126 to 93. The age range was 4 months to 15 years (median: 11 years; mean: 8.3 years). The number of children who require hospital care due to sinusitis amounted to 24 per 100,000 inhabitants (up to the age of 15) annually in this region during the period studied. Median time in hospital was 2.5–3 days, for the chronic group 4 days. The seasonal distribution of patients is shown in Figure 1.

Group distribution

Ninety children suffered from acute ethmoiditis with extension to engage peri-orbital tissues (group E); age ranged from 4 months to 14 years (median: 2 years; mean: 3.9 years). Boys outnumbered girls by 50 to 40. Radiographic examination was performed in only 20 out of the 67 patients, aged ≤ 4 years.

Chronic sinusitis (group C) existed in seven patients, aged 8–15 years (median: 12 years; mean: 12.4 years). All of these children had suffered from recurrent or persistent sinusitis symptoms for more than 1 year or had pathological sinus X-ray findings recorded for more than 1 year.

The remaining 122 patients suffered from other kinds of sinusitis (group O), such as acute or sub-acute pansinusitis, polysinusitis, frontal sinusitis or maxillary sinusitis. Their age was 5–15 years (median: 13 years; mean: 11.2 years). Boys outnumbered girls by 71 to 51. Children with non-fulminant ethmoiditis were also included in this group.

A more detailed display of the age distribution in groups E and O is given in Figure 2.

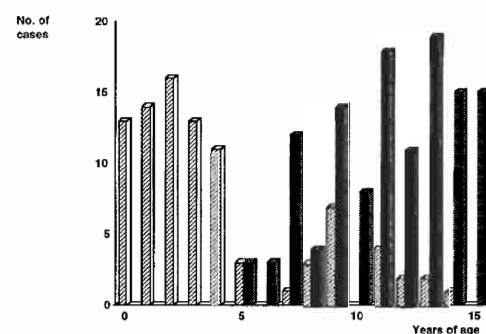


Figure 2. Age distribution of the children in group E (stribed columns) and group O (filled columns).

Body temperature

The mean body temperature upon admission to the hospital is given in Table 1. In group E, 74% of the children had a temperature of 38.0°C or higher, compared to 50% in group O. The mean duration of elevated temperature ($>38^{\circ}\text{C}$) during hospital care was 0.9 days, both in group E (range: 0–4.5 days) and group O (range: 0–12 days).

Blood tests

The mean erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), and white blood cell (WBC) count are shown in

Table 1. In group E, 65% had an increased ESR (>20 mm) and 86% had an increased CRP (>10 g/l). In group O, an increased ESR and CRP were found in 45% and 83%, respectively. In group E, 73% had an increased WBC count ($>10 \times 10^9$ cells/l) versus 41% in group O. In the 58 cases of acute or sub-acute sinusitis (groups E and O together) in which both WBC and CRP were analysed, either or both of the two tests showed elevated values in 95% of the cases.

Table 1. Clinical data recorded in the two acute sinusitis groups on admission to hospital, expressed as arithmetical means (range within parenthesis).

	group E	group O
age (years)	3.6 (4 months-14)	11.2 (5-15)
body temperature ($^{\circ}\text{C}$)	38.6 (36.7-40.3)	38.2 (36.6-41.0)
ESR (mm)	32 (2-210)	23 (1-94)
CRP (g/l)	66 (<10 -205)	53 (<10 -120)
WBC ($\times 10^9$ cells/l)	14.7 (3.8-37.5)	9.8 (2.4-26.0)

ESR: erythrocyte sedimentation rate; CRP: C-reactive protein; WBC: white blood cell count

Bacteriology

Nasopharyngeal cultures were set up from 141 of the 219 children before any antibiotic treatment was initiated. Fifty-nine cultures proved negative for bacterial growth, showed normal respiratory flora, or only sparse colony growth. Among the recognized pathogens, *Haemophilus influenzae* was isolated from 49 patients, *Streptococcus pneumoniae* from 20 patients, *Moraxella* (*Branhamella*) *catarrhalis* from 20 patients, and haemolytic *Streptococci* group A or B from 10 patients. Anaerobic Gram-positive cocci were found in one patient. The relative prevalences of bacterial pathogens are shown in Figure 3. In three patients, culture samples were obtained directly from the sinuses at surgery. *S. pneumoniae* was isolated in one case, *H. influenzae* in one, and no growth in one case.

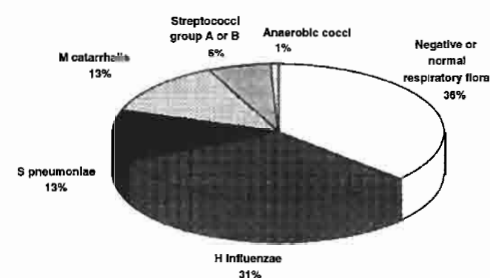


Figure 3. Bacteriological findings in nasopharyngeal cultures from 141 children with sinusitis, before any antibiotic therapy was instituted.

Ophthalmology

In group E, 31 of the 90 patients were seen by an ophthalmologist. Only three of these patients presented any significant disturbance in eye function. One patient (4 years of age) had a protrusion of the bulb and two patients (both 13 years old) had diplopia. In all three cases, the eye complications abated rapidly

within 2-3 days with ampicillin-amoxicillin or cefuroxime medication, and surgery was not required.

In group O, 5 of the 122 patients were seen by an ophthalmologist. One 13-year-old girl presented disturbance of the eye movement in the form of diplopia. Computerized tomography revealed right-sided frontal and ethmoidal sinusitis. Surgery in the form of external trepanation of the frontal sinus (preferred procedure at that time) and endoscopic ethmoidectomy was carried out and antibiotic treatment (ampicillin and chloramphenicol) was given as well. Her diplopia then resolved within 5 days, but some peri-orbital oedema persisted for weeks.

Medical therapy

In decreasing order, the antibiotic drugs prescribed for the children with acute sinusitis were: amoxicillin or ampicillin (54%), phenoxymethyl-penicillin or benzyl-penicillin (29%), cefuroxime (8%), trimethoprim-sulfamethoxazole (3%), erythromycin (2%), amoxicillin/chloramphenicol (2%), cefotaxime (1%), cefaclor (0.5%), and doxycycline (0.5%). The preference pattern for antibiotic use was not significantly changed over the study period. Routinely, α -adrenergic agonist (oxymetazoline or xylometazoline) decongestants were administered in the form of nasal drops or sprays, and children aged 9 years were also treated with cotton swabs soaked in naphazoline-lidocaine applied in the middle meatus, 2-3 times daily. Maxillary sinus irrigation was performed under local anaesthesia in 30 patients above the age of 9 years, by puncture of the medial sinus wall from the inferior meatus.

Surgery

In 12 out of 219 sinusitis patients, surgical intervention was considered necessary. All seven patients in group C were operated on, but none of the children in group E. The chief intention of surgery was to improve sinus drainage and ventilation by the functional approach. As stated above, one patient was operated on ophthalmological indication.

Besides sinus surgery, two patients also underwent polypectomy, one patient partial inferior turbinatectomy, and five patients adenoidectomy. Two patients had previously undergone sinus surgery and two additional patients were operated on after the sinusitis episode because of subsequently diagnosed nasal polyposis. Two of the 12 children treated with sinus surgery later had a recurrence of sinusitis: one needed a second polypectomy and the other was subjected to a Caldwell-Luc operation on the maxillary sinus. No other post-operative complications were recorded.

Possible predisposing conditions

A medical history of significant airway or immunological disorder other than sinusitis was recorded in 55 (25%) of the 219 patients. Table 2 displays the prevalence of such conditions in relation to previous studies.

A history of (seasonal or perennial) allergic disease related to aero-allergens was recorded in 27 patients (12%), mainly rhinitis or conjunctivitis. Seven children had a history of alimentary intolerance problems. Twenty-three children were subjected to

skin prick testing and/or RAST because of suspected allergy, but no additional cases of allergy were diagnosed by these tests. Table 2. Prevalence of conditions predisposing to sinusitis in this study, compared with findings in earlier studies.

	this study	earlier study	reference
allergy	12% ($p < 0.0005$)	25%*	Savolainen (1989)
asthma	5%		no data
allergy+asthma	13% ($p < 0.0005$)	38%	Kogutt and Shwachman (1973)
immunoglobulin deficiency	0.5% ($p < 0.005$)	5%	Kogutt and Shwachman (1973)
ciliary dyskinesia	0%		no data

*: young adults

Five children had a history of non-allergic non-infectious rhinitis, 10 children suffered from asthma, one from recurrent nasal polyposis (group C), one from antrochoanal polyp (group C), and 21 from recurrent acute otitis media with effusion (one of them in group C). Twenty-nine of the children stated they had previously suffered from recurrent acute otitis media, and two from chronic otitis media (one from group C). Fifteen children had previously been affected by recurrent acute subglottic laryngitis. One child had a diagnosed immunological disorder, dys-gammaglobulinaemia type I (low serum IgG and IgA). None of the 219 children had a CF or ciliary dyskinesia syndrome diagnosed. Furthermore, none of them was registered as having CF diagnosed up to 1994.

Nasal polyp study

Twenty-seven children (<15 years of age) were operated on at the Department of Otorhinolaryngology (Huddinge University Hospital) during the period 1980–1992, because of nasal polyposis (19 patients) and choanal polyps (8 patients).

Table 3. Prevalence of therapy requiring sinus and polyp disease in CF-children of this study, compared with data from earlier studies.

	this study	earlier study	reference
CF-children with severe sinusitis	0% ($p < 0.0005$)	11%	Ceporo et al. (1987)
sinus surgery in CF-children	0% ($p < 0.0005$)	10–20%	Ramsay and Richardson (1992)
polypectomy in CF-children	6% ($p < 0.005$)	19%	Stern et al. (1982)

Of the 19 patients with nasal polyps, six had CF, of whom two were admitted from other counties (Table 3). The CF-patients operated on were younger (three children aged 3 years, and the other three children aged 6, 7, and 10 years, respectively) than the other 13 children operated on for nasal polyps, who ranged in age from 9 years and upward. Nine patients in the non-CF polyposis group had a negative sweat test, and four patients had not been tested at that time. A history of allergic disease in the non-CF

polyposis group was found in four patients (21%), three of them suffering from asthma and one from rhinoconjunctivitis. Four polyposis patients had a history of recurrent secretory otitis media: two of perennial non-allergic rhinitis, one of chronic otitis media, and three had sinusitis problems (one with chronic sinusitis, and two with acute sinusitis, all three were included in the sinusitis study). Six out of 19 children had a relapse requiring re-operation of their nasal polyposis during the period studied (re-operated within 6 months to 10 years). Two of those were CF-patients.

The eight children who suffered from choanal polyposis were aged 9 to 15 years. One of them had a diagnosed chronic sinusitis (included in the sinusitis study); otherwise, these children were subjectively healthy.

DISCUSSION

The number of in-patient treatment events during these years (24 per 100,000 children annually) imply that by far <0.5% of the population require hospital care due to sinusitis during childhood, and hence the vast majority of children with uncomplicated sinus infections are adequately treated on an out-patient basis or by general practitioners.

The recordings of body temperature and laboratory tests did not provide any important differential diagnostic or prognostic information. CRP was found to be the most useful inflammatory parameter, since it was most often significantly increased and particularly reliable in group E. The high incidence of negative bacterial cultures may in part be the result of insufficient search for anaerobic bacteria (Brook, 1981), but the present finding of aerobic dominance and a high proportion of negative cultures is consistent with the majority of previous studies (Orobello et al., 1991; Wald, 1992). Sinus X-ray imaging (plain or computerized tomography) was performed in 163 (74%) of the sinusitis children and, naturally, all of them were indicative of sinus pathology. These findings were not further evaluated, as they were of little or no help in our group distribution of patients. Furthermore, roentgenography is a highly unselective method to diagnose therapy-requiring sinusitis in children, particularly in the youngest age group (Shopfner and Rossi, 1973; Lesserson et al., 1994). Neither reported allergic symptoms nor allergy tests indicated any significantly increased prevalence of allergy among sinusitis children compared to the general prevalence in this age group in Northern Europe or North America (Kogutt and Shwachman, 1973; Åberg et al., 1987; Crockett et al., 1987; Rachelefsky et al., 1988; Savolainen, 1989; Orobello et al., 1991; Furukawa et al., 1992; Rachelefsky et al., 1992); nor was asthma statistically overrepresented (Kogutt and Shwachman, 1973; Slavin, 1984; Crockett et al., 1987; Minor and Lockey, 1987; Orobello et al., 1991; Rachelefsky et al., 1992). In comparison to previously cited studies, recognized predisposing diseases seem to be less important in this material (see Table 2). Huddinge University Hospital is a well-established centre for the treatment of CF in the central region of Sweden, continuously monitoring about 65 juvenile CF-patients. Therefore, it was somewhat surprising that none of them needed hospital care or surgery because of sinusitis during the studied period. Nasal symptoms from polyps were significantly less

prevalent in CF-patients, and fewer of them underwent polypectomy than previous studies would indicate (Stern et al., 1982). Although this sample is limited in size and the study is affected with the inevitable weaknesses of retrospectivity, certain reflections can be made in the view of the advances of therapy for the airway diseases in question during recent decades. More potent anti-allergic drug therapy with fewer side effects and improved counselling to families with allergic children may have significantly reduced the risk of sinusitis among allergies. The fact that no child in group E needed surgical intervention should in part be credited to effective and appropriate antibiotic therapy, and in part also to the awareness of parents, as severe orbital complications could be avoided when there was no delay in instituting medical treatment.

Of particular interest are the findings concerning the children with CF. The need for surgical intervention in the sinuses of CF-children has been emphasized, particularly by certain American colleagues (Ramsay and Richardson, 1992). The 35 paediatric CF-patients living in Stockholm County (prevalence: 10 per 100,000) are given on average one specialist consultation every month. Bacterial cultures are routinely obtained from the nasopharyngeal cavity of infants and pre-school children and from sputum of older children, in order to chart bacterial colonization of the upper and lower airways. Whenever signs of incipient bacterial infection occur (such as cough, more viscous airway secretion, loss of appetite *et cetera*) antibiotic medication can be initiated, guided in part by the cultures. On average, these patients receive one course of antibiotic treatment of at least 10 days every 2 months. Furthermore, large oral doses of bromhexine as well as administration of salbutamol and acetylcysteine by inhalation reduce the complications of viscous mucus, combined with intensive physiotherapy. Vitamins A and E are administered after checking serum levels of retinol and tocopherol, and 85% of the CF-patients in Stockholm County receive continuous substitution with pancreatic enzymes. Their nutritional status is monitored equally careful. Otolaryngologists are invariably consulted whenever nasal secretion, congestion symptoms or suspicion of polyposis occur. The absence of serious sinus problems in CF-children must be considered as the commendable result of vigorous medical treatment and preventive measures instituted.

CONCLUSION

None of the predisposing conditions was significantly over-represented among the children requiring in-patient hospital care because of sinusitis. Common childhood disorders (such as acute otitis media, secretory otitis media and subglottic laryngitis) were also common in the medical records of the sinusitis children, but probably thanks to improved preventive measures and medical intervention are the previously recognized important predisposing diseases nowadays less inclined to become complicated by serious sinus infection.

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II

Nasal Polyps in Cystic Fibrosis*

Clinical Endoscopic Study With Nasal Lavage Fluid Analysis

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Study objectives: Nasal polyps frequently appear in patients with cystic fibrosis (CF). The aims of this study were to focus on what problems (symptoms, endoscopic findings, and laboratory correlates) nasal polyps cause the CF patient, and how these correlate to the total health situation of this patient group.

Patients and study design: The clinical histories, endoscopic investigations of the nasal cavity, and analyses of nasal lavage fluid of 44 patients with CF complicated with nasal polyposis have been compared with those of 67 CF control subjects. The patients were examined at annual control examinations (with pulmonary tests, working capacity, liver tests, and bacterial and blood tests) from 1995 to 1996 at Stockholm Cystic Fibrosis Center, Huddinge University Hospital. All patients were > 2 years of age. The endoscopic findings were related to the actual pulmonary function, inflammatory blood parameters, colonizing pathogens, antibodies (*Staphylococcus aureus* and *Pseudomonas aeruginosa*), and genotype.

Results: The patients with nasal polyps differed with respect to chronic colonization of *P aeruginosa* in sputum samples and had a higher occurrence of serum antibodies against the same species. The two groups did not differ in pulmonary functions, inflammatory parameters, or genotype. The polyps found were mainly small (within the meatus media) and gave no significant increase in ongoing clinical symptoms such as rhinorrhea, nasal obstruction, or hyposmia. Neither was any significantly marked finding concerning the nose (mucosal swellings, secretion, etc.) made in the polyp patients. The patients with CF scored slightly lower in a smell identification test in comparison with the healthy control group. The nasal lavage fluid was analyzed (in 93 of the 111 patients) for the occurrence of *P aeruginosa* (by polymerase-chain reaction [PCR]), interleukin [IL]-5, IL-8, and lysozyme. The lysozyme and IL-8 content was equal in the two CF groups but increased in comparison with the healthy control group. *P aeruginosa* was not detected with PCR in any nasal lavage fluid. No measurable levels of IL-5 in the nasal lavage were found.

Conclusions: There was a higher frequency of chronic colonization of *P aeruginosa* in the lower respiratory tract in patients with nasal polyps. Otherwise, nonsevere nasal polyposis was not an indicator of lower respiratory tract morbidity in CF patients. (CHEST 2002; 121:40–47)

Key words: cystic fibrosis; interleukin-8; mucinase; nasal lavage fluid; nasal polyps; polymerase chain reaction; *Pseudomonas aeruginosa*; smell

Abbreviations: CF = cystic fibrosis; CRP = C-reactive protein; ESR = erythrocyte sedimentation rate; ESS = endoscopic sinus surgery; IL = interleukin; PCR = polymerase chain reaction; RBB-R = Remazol brilliant blue R; UNG = uracil-N-glycosylase

Cystic fibrosis (CF) is the most common lethal autosomal recessive disease that affects white persons. It is caused by mutations in the CF trans-

membrane conductance regulator gene. This gene encodes for a protein that functions as a cyclic adenosine monophosphate-regulated chloride channel. Abnormal function of the channel results in

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aberrant conductance across the apical membrane of epithelial cells of ducts in a variety of organs (lung, pancreas, sweat gland, liver, nasal polyps, salivary glands, and colon). So far, > 800 mutations have been identified; the most common mutations in Sweden are Δ -F508, 394delTT, and 3659delC. The clinical manifestations of the disease include pancreatic enzyme deficiency with malabsorption; chronic progressive obstructive pulmonary disease; chronic pulmonary infection with *Staphylococcus aureus*, *Pseudomonas aeruginosa*, or both; and an abnormal electrolyte loss in sweat.

The otolaryngologic manifestations of CF include chronic sinusitis and nasal polyposis. Sinusitis based on various signs/symptoms has been reported in 11 to 94%.¹⁻⁶ Radiographic signs of sinopathy (opacifications and/or liquid levels) have been shown with plain radiographs in 92 to 100%^{2,7,8} and with CT in 100% of the CF patients investigated.^{9,10} Sinonasal disease may be the source of significant morbidity in the population of patients with CF.

The prevalence of nasal polyposis has been estimated at 7 to 56%,^{1,2,5-7,10-15} with the higher prevalence when the patient was examined with an endoscopic technique. Topical nasal steroids have been administered in the case of small polyps and usually resulted in some polyp shrinkage and reduction of the associated symptoms.^{7,13,16,17} Larger polyps usually demand polyp surgery (which is the second most common class of operations performed on CF patients).² Polypectomy is the simplest procedure and provides satisfactory relief of nasal obstructive symptoms, but symptomatic recurrence occurs in about 60% of patients within 18 months.^{2,18} A more aggressive procedure that is becoming widespread is endoscopic sinus surgery (ESS), which decreases the polyp recurrence and enhances the drainage of the paranasal sinuses. ESS has been well tolerated by CF patients and provides good symptomatic relief.¹⁹

One earlier study²⁰ reported a correlation between nasal polyposis that required surgery and two specific genotypes: the Δ -F508/ Δ -F508 and the Δ -F508/G551D genotypes. Another study¹⁵ showed a genotype-phenotype correlation (Δ -F508 homozygote) for the paranasal sinus diseases (nasal polyposis and chronic sinusitis) in patients with CF. The purpose of this study was to determine the frequency of nasal polyposis in CF patients attending the Stockholm CF Center, to relate the findings to the infectious and inflammatory status in the nose by studying the nasal lavage fluid, and to correlate the upper-airway findings to the total health situation of these patients, including lower airway status, morbidity, and bacteriology, as well as to the genotype.

MATERIALS AND METHODS

Patients

All CF patients, except those < 2 years old, attending the Stockholm CF Center, Huddinge Hospital, were offered an examination of their upper respiratory tract at their yearly control examination in November 1991 to December 1996. The annual control examination at the CF center includes pulmonary radiographs, lung function tests, working capacity test, α sound of the liver, inflammatory parameters (C-reactive protein [CRP], albumin, haptoglobin, α -acid glycoprotein, erythrocyte sedimentation rate [ESR] and WBC count), liver function tests, serum tocopherol, serum retinol, and cultivations and antibodies for *S aureus* and *P aeruginosa*.

The diagnosis of CF was based on repeated positive sweat test finding (> 60 mmol/L of chloride in an adequate sample of sweat). Repeated borderline measurements (40 to 60 mmol/L) were required in addition to clinical correlation or DNA genotyping for diagnosis.

The CF patients in the county of Stockholm are examined monthly; CF patients outside the county are examined at least yearly at the center. Bacterial samples are taken (nasopharynx or sputum) monthly. Signs of low-grade infection (ie, increased cough, changes in sputum, inadequate gain of weight/weight loss, lowered appetite, tiredness, worsening of symptoms at physical examination) are indications for antibiotic treatment. The above-mentioned signs usually correlate well with a slight rise in inflammatory markers. The choice of antibiotics depends on the result of the bacterial cultivation. The length of the course of antibiotics is usually 10 days. Patients with elevated staphylococcal antibodies (α toxin and teichoic acid) are receiving continuous flucloxacillin treatment. Otherwise, our center does not practice any prophylactic treatment. The patients are regularly treated with high oral doses of mucus-dissolving agents three times daily (bromhexin and acetylcysteine in double recommended doses). They inhale salbutamol (usually 2.5 mL [1 mg/mL]) and acetylcysteine (usually 2 mL [200 mg/mL]) bid or tid. Small children are recommended nightly mist-tent therapy. The patients are supplemented with E vitamins (100 to 400 mg/d depending on the level of α -tocopherol in blood sample) and A vitamin (5,000 to 7,500 IU/d) as well as pancreatic enzymes in the case of pancreatic insufficiency (85% of the CF patients).

Study Design

Clinical data on nasal symptoms were obtained by interviewing the patients and/or their parents. After the interview, endoscopic examination of the nasal cavity was carried out. The occurrence of nasal polyposis, nasal congestion, secretion, redness, and the presence of an adenoid were noted. The nasal polyps were graded according to their size, with grade 1 equal to the smallest size of polyps (concealed in middle meatus, not reaching the inferior edge of the middle turbinate). Grade 2 described a polyp in the middle meatus, reaching the inferior border of the middle turbinate. Grade 3 was equal to a nasal polyp extending into the nasal cavity below the edge of the middle turbinate but not below the inferior edge of the inferior turbinate, whereas a polyp filling up the nasal cavity was regarded as a grade 4 polyp. Nasal lavage fluid was gathered with the following procedure: 3 mL of phosphate-buffered saline solution was poured into the atral part of the nose by means of a syringe with a cap on top to prevent the solution from leaking aside. The solution was poured twice (in and out) from the syringe into the cavity while the patients were leaning forward. The gathered solution was immediately spun at 340g by a tabletop centrifuge (Sorvall RT 6000D; Kendro Laboratory Products; Newtown, CT) for 10 min. The

supernatant was separated from the "pellet" (equal to 1 mL of the supernatant with some nasal secretion and nasal mucosal cells). The pellet was then spun one more time (3,500g for 2 min) [MSE MicroCentaur; Thistle Scientific Ltd; Uddingston, Glasgow, UK]. All tubes were then immediately stored in a -70°C freezer. The pellet was analyzed for polymerase chain reaction (PCR)-detection of *P. aeruginosa*, immunoassays for interleukin (IL)-5 and IL-8, while the remaining supernatant was analyzed for lysozyme content. Patients > 14 years old evaluated their sense of smell with the Smell Identification Test (Sensonics; Haddon Heights, NJ).

The collected data from the otolaryngologic examination were compared with the data routinely gathered at the annual control examination at the Stockholm CF center with regard to pulmonary function, colonization of *S. aureus* and *P. aeruginosa* in sputum samples, and other blood parameters (ESR, WBC count, proteins signaling inflammation, total serum IgE, etc.). Chronic colonization of the above-mentioned bacteria was defined as six consecutive isolations separated by ≥ 1 month for *P. aeruginosa* and/or elevated level of antibodies (*P. aeruginosa* anti-exotoxin A).

The patients with polyps were offered treatment with a topical steroid (budesonide) in the form of nose spray (50 μg in both cavities bid). These patients were offered a follow-up examination 1 month later.

A sex-matched and age-matched control group consisting of 42 healthy volunteers was monitored during 1997 (20 male and 22 female subjects; mean \pm SD age, 18.79 ± 10.41 years; range, 4 to 44 years). This group underwent nasal lavage and the Smell Identification Test mentioned above. In this group, no airborne allergy was allowed, or any ongoing nasal symptoms (no nasal congestion, no ongoing infection in the nasal area). No earlier surgery in the nasal or sinus region was allowed or any ongoing nasal medical treatment.

Statistical Analysis

The following statistical analyses were performed with statistical software (Statistica; StatSoft; Tulsa, OK): multilinear regression (age, lung function tests, some of the blood parameters); χ^2 test (genotype, sex, clinical status, some of the operations, tendency to allergy, acute otitis media and sinusitis, and ongoing symptoms such as snoring, rhinorrhea, mouth breathing, nasal blockage, and epistaxis); Whitney-Mann U test (some of the blood parameters, use of IV and oral antibiotics per year, age at diagnosis, amounts of IL-8 and lysozyme in the lavage, smell test, and time from diagnosis to first positive cultivation of *P. aeruginosa* and *S. aureus*); Fisher's Exact Test (sinonasal surgery, otitis media, history of hyposmia, pancreas insufficiency and continuous per oral antibiotics); logistic regression (earlier polypectomy, recurrent nasal blockage and secretion, antibodies against *P. aeruginosa* and *S. aureus*, and number of patients with chronic colonization with *P. aeruginosa* and *S. aureus*); and log-rank test (chronic colonization of *P. aeruginosa* and *S. aureus*).

Ethical Permission

The Ethics Committee of Huddinge University Hospital approved the study. Consent from the patients or their parents was obtained before enrollment.

Methods

Lysozyme Test

Lysozyme is a marker for serous gland secretion in the nasal cavity but can also be released from neutrophils.²⁴ Increased lysozyme levels indicate an inflammatory status with stimulation

of serous gland secretion, degranulation of neutrophils, and destruction of epithelial cells. The lysozyme content of nasal lavage was determined in a colorimetric assay slightly modified from the assay described by Ito et al.²⁵

Materials: *Micrococcus luteus* (*Micrococcus lysodeikticus*) and purified lysozyme to be used as a standard were obtained from Sigma (St. Louis, MO). Remazol brilliant blue R (RBB-R) sodium salt, a reactive blue dye, was the product of ICN Pharmaceuticals (Cosa Mesa, CA).

RBB-R-Labeled *M. luteus*: Briefly, a solution of 200 mg of RBB-R in 20 mL distilled water was added to a suspension of 300 mg of *M. lysodeikticus* cells in 20 mL of distilled water at 50°C with stirring. During the following 30 min, 4 g of sodium sulfate was added to the mixture in several portions. A solution of 200 mg trisodium phosphate in 2 mL of distilled water was then added, and the mixture was stirred for an additional 30 min at 50°C . The reaction mixture was centrifuged (1,200g, 10 min), and the supernatant was discarded. The pellet of the labeled cells was suspended in 20 mL of 0.05 mol/L KH_2PO_4 -NaOH, pH 7.0 and repeatedly washed with the same buffer until the supernatant became colorless. Finally, the cells were washed with distilled water and then dried by lyophilization and stored in a -20°C freezer until use.

Lysis of Blue *M. lysodeikticus* by Lysozyme: Dry blue *M. lysodeikticus* (16 mg) was suspended in 10 mL of 0.05 mol/L KH_2PO_4 -NaOH, pH 7.0 and centrifuged once (1,200g, 40 min). The pellet was resuspended in 10 mL of the same buffer, mixed with a vortex mixer, and divided into 240- μL portions in 96-well microplates. After preincubation at 40°C , 40 μL of sample or lysozyme standard was added to each well. The mixture was incubated at 40°C for 15 to 20 h. The reaction was stopped by the addition of 5 μL of 1 mol/L NaOH to each well. The microplate was centrifuged for 15 min at room temperature (1,500 revolutions per min, 400g). One hundred microliters of the supernatant of each well was transferred to a new 96-well microplate. The absorbance was measured at 600 nm. The resulting level of lysozyme, as determined from the standard curve, was the mean value of two separate measurements. The range of the linear interval of the standard curve was 0.102 to 0.891.

Pseudomonas PCR Test

The rapid PCR detection was performed with a set comprising one primer pair based on 16S ribosomal RNA sequence according to a method previously used by Karpati and Jonasson.²⁴ The PCR was performed on a DNA Thermal Cycler (TC 2400; Perkin-Elmer; Wellesley, MA) using two primers selected from 16S ribosomal RNA sequences (GenBank; National Center for Biotechnology Information; Bethesda, MD). The oligonucleotides 5'-GTGCCCTGCCAGCCGCGTAAT-3' (bases 515-534) and 5'-TCCGCCCACTAAGATCTCAAG-3' (bases 841-860) amplify *P. aeruginosa* with high sensitivity, as previously described.²³ For *P. aeruginosa*, the PCR detected 5×10^2 bacteria per reaction and the sensitivity was 93% compared to culture, as reported in the same study. Positive controls (a reference strain of *P. aeruginosa*) were included in each run. Fifty-seven nasal lavages were tested, including all patients with chronic colonization with *P. aeruginosa* in their sputum samples. The reaction mix consisted of 10 mM Tris-HCl (pH 8.3); 50 mM KCl; 200 μM of deoxyadenosine triphosphate, deoxycytidine triphosphate, deoxyguanosine triphosphate, and 300 mM of deoxyuridine triphosphate; 3 mM of MgCl_2 ; Ultra DNA polymerase (Perkin-Elmer) 2.5 U; uracil-N-glycosylase (UNG) [AmplErase; Perkin-Elmer] 1 U; 20 pm of each primer; and 10 μL of target DNA (nasal lavage fluid or positive and negative controls prepared by heating at 95°C and sonication for 30 min) in a total

volume of 100 μ L. As a first step, to prevent carryover contamination, the reaction tubes were incubated at 50°C for 7 min to cleave contaminating amplicons. Heating at 95°C for 10 min to denature the UNG followed this procedure. Thereafter, 30 thermal cycles were performed: denaturation, 1 min at 94°C; primer annealing, 1 min at 55°C; and primer extension, 2 min at 72°C. After completion of the run, PCR products were soaked at 72°C before collection and stored at -20°C to prevent degradation by residual UNG activity. The amplicons were visualized by electrophoresis (1.5% agarose gel with 0.05% ethidium bromide in 0.5 mol/L Tris/ethylenediaminetetra-acetic acid buffer).

IL-5 and IL-8

The nasal fluid levels of IL-8 and IL-5 were measured after homogenizing the sample with a pellet and its overlying 1 mL of the supernatant. This method produces higher cytokine levels than assays based on supernatants.²⁴ Measurements were made with commercially available sandwich enzyme immunoassays according to the recommended protocols of the manufacturer (R&D Systems; Abingdon, UK). Cytokine concentrations in lavage fluids were quantified by comparison with a standard curve (acting as positive controls) ($r > 0.98$ for all assays). The specific enzyme-linked immunosorbent assay kits used were IL-8 (sensitivity, < 10 pg/mL; dynamic range, 32 to 2,000 pg/mL) and IL-5 (sensitivity, < 3 pg/mL; dynamic range, 7.8 to 500 pg/mL).

Spirometry

Static and dynamic spirometric measurements were obtained from each patient > 7 years of age. Functional residual capacity was determined by body plethysmography. Total lung capacity and residual volume were calculated. Vital capacity, FEV₁, FEV₁ as a percentage of vital capacity, peak respiratory flow, forced expiratory flow at 50% of FVC, and forced expiratory flow at 25% of FVC were measured separately. All measurements were performed using a pulmonary function laboratory (Sensor Medics BV; Beilthoven, the Netherlands). All patients were coached by the same technicians and were familiar with spirometric measurements.

Biochemical Analysis

Proteins signaling inflammation (CRP, albumin, haptoglobin, α -acid glycoprotein), ESR, hemoglobin, and WBC count were measured with routine methods.

Staphylococcal and *P aeruginosa* Antibody Assays

Antibodies to staphylococcal teichoic acid and α -toxin and *P aeruginosa* exotoxin A in serum were determined by enzyme-linked immunosorbent assay and interpreted by routine bacteriologic laboratory.^{25,26}

Genotype Analysis

Genotype data were available on 41 patients with nasal polyposis and 59 patients without polyposis described by Schaedel et al.²⁷ The following mutations were included: Δ -F508, Δ -F508-W79B, 3639delC, 394delTT, 297-3C-A, S945L, L206W, R553X, G551D, 2798 + 5G-A, Y109N, 711 + 3A-G, D1152H, R75Q-N108S, R1162X, W1282X, G1244, and 2183AA-G.

Smell Test

The Smell Identification Test²⁸ was used to analyze sense of smell. Forty different smells are presented in the form of

multiple-choice alternatives (four alternatives for each smell). The concentrations of the different smells are well over the threshold of identification level. As three of the smells presented in the test were typical of American countries (pumpkin pie, root beer, and skunk) the recommended levels for hyposmia and anosmia were lowered with three points each (in this Swedish population).

RESULTS

One hundred eleven of 150 CF patients > 2 years of age participated in the study. Half of the patients associated with the CF center came from the county of Stockholm, while the other half came from regions around Stockholm and from the northern part of Sweden. The loss of patients from the study could be explained partly by a tight schedule for the CF patients during their yearly control examination and partly by the voluntary design of the study. The latter factor may have increased the frequency of patients with polyps or nasal symptoms.

Forty-four of the 111 CF patients (39%) revealed nasal polyps by the endoscopic investigation. The vast majority showed polyposis of grade 1 (23%) and grade 2 (45%), ie, polyps concealed in the meatus media. Only one third of the polyps were visible outside the middle meatus of the nasal cavity. No large polyps were found. The mean age of subjects in the polyp group was 18.0 ± 8.8 years (range, 2 to 37 years; females outnumbering males by 26 to 18), compared to mean age of 16.1 ± 12.4 years (range, 2 to 57 years; males outnumbering females by 35 to 32) in the nonpolyp group. In the presented data, at least 23% of the patients with CF had undergone surgical intervention for nasal polyposis (and previous surgeries on nasal polyps were more common in the polyp group) [$p < 0.0001$, log regression; odds ratio, 11.0].

Chronic colonization with *P aeruginosa* revealed either by consecutive sputum sample or high titer against *P aeruginosa* anti-exotoxin A was more prevalent in the patient group with polyps ($p = 0.0001$, log regression; odds ratio, 5.3), clearly shown when plotting a follow-up period for 20 years ($p = 0.029$, log-rank test). Symptoms such as tendency to recurrent nasal blockage (46% in the polyp group vs 22% in the nonpolyp group; $p = 0.015$, logistic regression; odds ratio, 2.1) and nasal secretion (26% in the polyp group vs 13% in the nonpolyp group; $p = 0.04$, logistic regression; odds ratio, 4.5) were more prevalent in the polyp group.

Of the 111 patients, 35 patients were chronically infected with *P aeruginosa* and 44 with *S aureus*. The durations of chronic colonization of *P aeruginosa* were 10.7 ± 6.9 years in the polyp group and 5.8 ± 4.4 years in the nonpolyp CF group. Chronic colonization was defined by culture findings in 18 of

the 20 patients in the polyp CF group, by both culture and positive assay findings in 1 patient; and by a positive assay finding in 1 patient. All nonpolyp CF patients had chronic colonization defined by culture findings.

The pulmonary status in the CF group was expressed as a percentage of the predicted. Mean FVC was $88.0 \pm 20.7\%$ predicted, and mean FEV₁ was $77.3 \pm 25.2\%$ predicted (mean polyp-group FEV₁, $75.1 \pm 24.1\%$ predicted [$n = 42$]; mean nonpolyp-group FEV₁, $78.9 \pm 26.1\%$ predicted [$n = 58$]). The most common mutation in the CF group was the Δ -F508, occurring in approximately 80% (41% had Δ -F508/ Δ -F508 and 40% had Δ -F508 in a combination with other genotypes).

There were no differences between the polyp and nonpolyp groups concerning the ongoing symptoms of rhinorrhea, nasal blockage, snoring, mouth breathing, epistaxis, or hyposmia. No difference regarding nasal congestion, nasal secretion, mucosal rash, or size of the adenoid was seen. Lung function capacity (FEV₁ and FVC) did not differ between the groups, nor did the blood parameters (hemoglobin, blood cell counts, total IgE, ESR, CRP, albumin, haptoglobin, IgG, orosomucoid). There were no differences in atopy. There was no significant difference in the use of IV or oral antibiotics and no difference in the CF transmembrane conductance regulator genotype expression.

The Smell Identification Test revealed no difference between the two CF groups. However, in comparison with the healthy control group, CF patients with or without polyps presented a slightly reduced score ($p = 0.04$, Mann-Whitney U test).

The lysozyme test did not show any difference between the two CF groups. Within the polyp group, the lysozyme content in nasal lavage did not differ between the group with or without chronic colonization of *Pseudomonas* in the lower respiratory tract. In comparison with the healthy volunteers, CF patients had significantly elevated levels of lysozyme indicating an elevated inflammatory status in the sinonasal tract ($p < 0.0001$, Mann-Whitney U test). The levels of IL-8 were equal in the two CF groups. Within the polyp group, the IL-8 content in nasal lavage did not differ between the group with or without chronic colonization of *Pseudomonas* in the lower respiratory tract. We found an elevated lavage content of IL-8 in the whole CF group vs the healthy volunteers ($p < 0.0001$, Mann-Whitney U test). No detectable levels of IL-5 were found in the nasal lavages. *P. aeruginosa* was not identified in the lavages with PCR technique.

Only 10 of the 44 patients with polyps attended the recommended follow-up examination after 1 month of treatment with topical steroids. There-

fore, no conclusions can be made about the efficiency of this treatment. The traveling distances can partly explain the low compliance.

DISCUSSION

The frequency of nasal polyposis found in this CF group (39%) is consistent with studies^{1,4,6,10,12} using endoscopic techniques for diagnosis (with frequencies ranging from 32 to 56%). However, the nasal polyps in our study were smaller and produced fewer symptoms in comparison with some of the above-cited studies. The lack of parallel symptoms to the occurrence of polyposis and chronic sinusitis/"sinopathy" has earlier been described by Kennedy and Loury²⁰ in this patient group. The numbers of operations carried out (polypectomy or sinus surgery) were scarcer in this study in comparison with recent studies.¹ The milder form of nasal polyposis and lower frequency of surgeries in the sinonasal region in our study compared with earlier studies (carried out in other countries) could be explained partly by differences in health insurance²⁰ causing differences in access to intense medical care and other economic factors.

In this study, we found a correlation between early chronic colonization of *Pseudomonas* in the lower respiratory tract and nasal polyps. Three mechanisms could explain this phenomenon. A mechanism supporting the hypothesis of a sinopulmonary reflex (that could lead to the colonizing of bacteria in the lower respiratory tract), a mechanism with the spillover of bacteria, or just a mechanism based on an elevated inflammatory status in both the upper and the lower airways in a parallel manner due to the basic disease. A nasobronchial relation was postulated by Gottlieb³¹ in 1925 and by Sluder³² in 1919. Similarly, a more recent hypothesis³³⁻³⁵ has claimed that inflammatory mediators produced in the paranasal sinuses elicit bronchoconstriction, either by stimulating neural receptors in the nose, sinuses, or pharynx, or by dripping these mediators into the lower airway. This mechanism has been discussed in the case of asthma.^{36,37} The second mechanism could be spillover of colonizing bacteria from the sinus to the lower airways. As radiographs of the paranasal sinuses shows opaque areas in 90 to 100% (because of mucosal swelling and/or liquid in the paranasal sinuses)^{2,7-10} and as *P. aeruginosa* and *S. aureus* are commonly found in the case of sinusitis,^{17,18} the latter mechanism may be applicable. This mechanism is contradicted in a study by Drake-Lee and Morgan,³⁸ who found no correlation between sinus washouts and corresponding sputum samples.

Recently, Raj et al³⁹ showed an association be-

tween chronic *Pseudomonas* colonization (in the lower respiratory tract) and rhinitis and nasal polypoidosis. The main issue in this study was the development of allergy in the population with chronic colonization. The study could not point out why polyps were associated with the chronic colonization nor clarify the association between allergy and polypoidosis. In our study, known allergic problems in patients with polyps (21%) did not exceed known allergic problems (33%) in the nonpolypoidosis CF group.

Consistent with earlier studies,^{2,13,14,17,18} we found a relatively high risk of recurrence of polyps after simple polypectomy. In the case of small-sized polyps with only mild or intermittent symptoms from the nose and paranasal sinuses, medical treatment in the form of nasal steroids proved to be adequate and effective.¹⁶ Simple polypectomy has been widely used in the case of medium-sized to large-sized polyps and has been successful in relieving the associated symptoms, but the risk of recurrence is great. It has been stated that the ESS with an opening up of the osteomeatal area (where the ethmoidal, maxillary, and frontal sinuses enter the nasal cavity) should reduce the risk of recurrence of both polyps and sinusitis.^{2,16} Significant reduction of the polyposis-related and sinusitis-related symptoms has been seen after such surgery in earlier studies.^{18,19,40,41} It is, however, unclear at what stage this procedure is appropriate. The risk of reduced and altered growth of the sinuses and the facial skeleton (in young patients) has been debated,⁴² but Senior et al⁴³ found that sinus surgery in children was safe and without significant cosmetic sequelae. It is still unclear if the intervention (ESS) improves the pulmonary status. A recent follow-up study did not show any reversibility in the progressive lung disease in CF patients after ESS.⁴⁴ Radical surgery before the rare cases of lung transplantation in CF patients has been advocated.^{45,46} An even more effective treatment (in the cases of chronic sinusitis in CF) seems to be a combination of endoscopic surgery with serial antimicrobial lavage described by Moss and King.⁴

The $\Delta DF508/\Delta DF508$ and the $\Delta DF508/G551D$ genotypes have previously been shown to be significantly more common among the CF patients with more severe polyposis (ie, demanding surgical treatment).^{45,50} In our study, there was no difference in genotype between the CF groups (with and without polyps). This may be either an effect of the limited size of the material studied or an effect of a different tradition of medical treatment that could inhibit polyp development. Regular oral or IV antibiotic treatments as soon as there is the slightest sign of an upcoming infection will reduce the conditions that could increase the risk of polyp growth. The regular

mucolytic treatment also seems to reduce the risk of chronic sinusitis and thereby the stimulation of the polyp formation. We know from previous studies that the sinuses of the CF patients quite often contain *P aeruginosa* and *S aureus*.⁴⁷ Theories of an inflammatory mechanism for the occurrence of polyps have been stated. Observations in animal studies have pointed out *P aeruginosa* as a pathogen that gives rise to a more marked infection (increasing the risk of polyp growth).⁴⁸ Perhaps mucolytic therapy plays an important role in reducing the polyp formation due to its action on the viscous mucus in the sinuses? These factors may together diminish the differences between the polyp-prone patients vs the others.

IL-8 analyzed in the nasal lavage indicated an elevated degree of inflammation in the CF patient compared to the control subjects, but no differences were found between the two CF groups. Earlier studies have shown elevated IL-8 levels in the BAL of CF patients,^{49,50} while IL-8 in nasal lavage fluid did not differ compared with healthy subjects.⁴⁹ IL-5 in BAL in an earlier study⁵⁰ was lowered in comparison to asthmatic patients. Lysozyme analyzed in the nasal lavage indicated an elevated degree of inflammation or increased secretion in the CF patient compared to the control subjects, but no differences were found between the two CF groups. Lysozyme levels (as a marker of serous secretion) were elevated in tracheal mucosa from CF patients,⁵¹ while a certain lysozyme leakage was expected from neutrophils involved in the bronchial airway pathology due to the colonization of *P aeruginosa*.⁵² No increased levels of lysozymes have so far been reported from nasal lavages of patients with cystic fibrosis.²¹ Elevated levels of lysozyme have been reported in nasal lavage due to induced glandular secretion from the nose.⁵³ At the presence of recurrent sinusitis, the baseline secretion of lysozyme was enriched while these patients had a blunted cholinergic response with decreased secretion of lysozyme.⁵⁴ There were no positive PCR findings of *P aeruginosa* in the nasal lavage. There were, however, single findings of *Pseudomonas* caught with cultivation of nasopharyngeal swabs, which could indicate that the lavage technique was not relevant for catching the mentioned species. We know from earlier studies⁴⁷ that a culture from the nose and nasopharynx does not reflect the species revealed in the paranasal sinuses. Furthermore, the nose could only be a transient site of the *Pseudomonas* species. The lack of difference between the CF groups in the markers of the nasal lavage could be explained by the limited size of the polyps and of the study. The lung function test did not show any differences between the CF groups in spite of the difference in chronic colonization of *P*

aeruginosa. This is consistent with earlier findings that did not show reduced pulmonary function in patients with polyps.²⁰

CONCLUSION

Nasal polyps were found in 39% of the CF patients. There was a higher prevalence of chronic colonization of *P. aeruginosa* in the lower respiratory tract in patients with nasal polyps. Otherwise, non-severe nasal polyposis was not an indicator of lower respiratory tract morbidity in CF patients. There was no difference in the infectious or inflammatory parameters of the nasal lavage between the patients with or without nasal polyposis. No differences were found in the genotype between the patients with or without nasal polyps. Intensive antibiotic and mucolytic treatment seemed to reduce the frequency and size of nasal polyps.

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III

Immune Response to *Mycoplasma pulmonis* in Nasal Mucosa is Modulated by the Normal Microbiota

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The impact of commensal bacteria on lymphocyte responses in the upper airways was studied in rat nasal mucosa after infection with the pathogen *Mycoplasma pulmonis*. Phenotyping was performed in situ by paired immunofluorescence staining in germ-free (GF) and conventional (CV) rats before and three weeks after the mono-infection. Intraepithelial lymphocytes had expanded significantly in GF ($p = 0.02$) but not CV rats. Furthermore, a striking proportional increase of T-cell receptor (TCR) $\alpha\beta$ ⁺CD4⁺ cells was observed both in the lamina propria and epithelium of GF ($p < 0.01$) but not CV rats. Notably, in contrast to the pre-infection state, both mucosal compartments showed a percentage of TCR $\alpha\beta$ ⁺CD4⁺ cells that was significantly higher in GF ($p = 0.03$ – $p < 0.01$) than in CV rats following the mono-infection. In parallel, both compartments displayed a percentage of TCR $\alpha\beta$ ⁺CD8⁺ cells that was decreased in GF ($p < 0.01$) but not in CV rats. The small fraction of TCR $\delta\gamma$ ⁺ T cells observed (<5%) did not change quantitatively or phenotypically after infection. The size of organized nose-associated lymphoid tissue was, on average, increased 5.2-fold in GF rats vs. 2.6-fold in CV rats. Collectively, our results demonstrated that the normal microbiota modulated markedly the nasal immune response elicited by mono-infection with *M. pulmonis*.

T lymphocytes play an important role in the regulation of mucosal immune responses to exogenous antigens, but virtually nothing is known about the effect of the indigenous microbiota on such responses in the upper airways (1). Only few studies of the immunohistology of human nasal mucosa, with or without inflammatory conditions, have been carried out (2–4). Conversely, there are several reports documenting that commensal bacteria modulate mucosal immune responses in the gut (reviewed in Refs. 5–7). Germ-free (GF) animals (pathogen-free with no indigenous flora) are known to possess an immature immune system and have been used to study the impact of the normal

microbiota on mucosal immunity. Thus, in GF mice and rats, the absence of the stimulation exerted by commensal bacteria affects both the numbers and phenotypes of intestinal T lymphocytes (8, 9) as well as the development of gut-associated lymphoid tissue with its antigen-sampling M cells (10). Importantly, GF mice are defective with regard to induction and maintenance of oral tolerance and also recovery of oral tolerance after its abrogation by bacterial toxins (5–7, 11). It remains unknown whether this positive effect of the commensal flora is mediated through modulation of costimulatory molecules on antigen-presenting cells (APCs) or an effect on intestinal permeability. Notably, indigenous bacteria are important both to establish (12) and regulate (13) an appropriate epithelial barrier function.

Ichimya et al. (14) showed that fewer IgM⁺ B cells and CD4⁺ T cells were present in the upper respiratory tract of GF mice compared with specific pathogen-free conventional (CV) mice. IgG⁺ and IgA⁺ B cells as well as CD8⁺ T cells were rare and apparently unchanged. It was concluded that both B and T cells were attracted to the nasal mucosa in response to microbial stimuli from the commensal flora (14). In the rodent nasal cavity, lymphocytes are both diffusely distributed in the mucosa and aggregated in the paired lymphoid organs called nose-associated lymphoid tissue (NALT) (15), which shows similarities to the ileal Peyer's patches but differs with regard to organogenesis. Thus, Peyer's patches consist of organized T- and B-cell compartments at birth, although the development of germinal centers only takes place postnatally under the influence of the microflora. Conversely, NALT antigen cannot be detected in mice until shortly after birth (16), and germinal center formation is again dependent on exogenous stimuli (17). Functionally, rodent NALT may be equivalent to nasopharyngeal lymphoid tissue called Waldeyer's ring in humans, including the palatine tonsils and adenoids (18, 19), and human nasal mucosa may in addition contain a few isolated (solitary) lymphoid follicles (20). The CD4:CD8 T-cell ratio in rodent NALT has been

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Abbreviations: antigen-presenting cell, APC; conventional, CV; dendritic cell, DC; germ free, GF; intraepithelial lymphocyte, IEL; lamina propria lymphocyte, LPL; monoclonal antibodies mAb; nose-associated lymphoid tissue, NALT; natural killer, NK; natural killer receptor protein 1, NKR-1; pattern recognition receptor, PRR; T-cell receptor, TCR; regulatory T cells, T_{reg} cells.

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calculated to be 2.4, apparently having a larger T:B lymphocyte ratio than human Peyer's patches (18).

Mycoplasma pulmonis is an extracellular bacterial pathogen that attaches to rodent respiratory surface epithelium and, over time, causes a chronic mucosal inflammatory condition (21-23). Mycoplasmas induce specific humoral and cell-mediated immunity, as well as non-specific stimulatory and suppressive effects on the immune system (21). In the interactions between the bacteria and the host cells, some of the immunopathological events are more damaging than the direct toxic effects of the infecting organism. In the lower respiratory tract of CV rats, *M. pulmonis* infection has been shown to alter significantly lymphocyte populations in terms of numbers as well as subset distribution (24). The morphological and microbiological features of the nasal cavity of GF rats related to these pathogens have been described as "chronic respiratory disease" (23).

The aim of this work was to investigate the possible impact that the normal microbiota exerts on the nasal T-cell response in rats monoinoculated with *M. pulmonis*. For reasons explained previously, including the size of the experimental animals, we have found the rat model quite useful for studies of the effect of bacteria on the responsiveness in various mucosal immune compartments (10). Here, we observed numerical and phenotypic T-cell differences between GF and CV rats in both the epithelium and lamina propria of nasal mucosa, in addition to hypertrophy of NALT as a result of dramatic pathogen-induced T- and B-cell expansion. Our results documented that the indigenous microbiota exerts a modulating effect on airway immunity to an infectious agent, thus serving to maintain mucosal homeostasis.

Materials and Methods

Animals

Inbred GF and CV rats of the same AGUS strain were reared at the Department of Medical Microbial Ecology, Karolinska Institute, Stockholm, Sweden. The GF rats were kept in light-weight stainless steel isolators and monitored weekly for GF status. The CV rats were kept in a laboratory animal room and checked quarterly by the National Veterinary Institute (Uppsala, Sweden) by serology for the absence of pathogens. All animals were fed a steam-sterilized standard rat chow (R36, Lactamin, Vadstena, Sweden) and had free access to water. Artificial light was available between 6 a.m. and 6 p.m., the temperature was maintained at 24°C +/-

2 °C and the humidity at 55 +/- 10%. Permission for this study was obtained from the Ethics Committee at Huddinge Hospital.

Mycoplasma Cultivation and Inoculation

M. pulmonis (strain M 61/82) originally isolated from a rat with pneumonia, was obtained from The National Veterinary Institute (Uppsala, Sweden). After the second passage the bacteria were stored at -70°C. The medium used for mycoplasma cultivation was as described previously (25). Samples for two inoculations were prepared as follows: Thawed mycoplasma was cultured in broth for 2 days. The number of colony-forming units (CFU) was counted and the cultures were appropriately diluted in isotonic phosphate-buffered saline (PBS), pH 7.5. The first inoculate contained 2.8×10^9 CFU/ml and the second 5×10^7 CFU/ml. GF and CV rats received inoculates through the nasal cavity (drop infection) at 8 wk of age and were killed 3 wk later. Age- and sex-matched GF and CV control rats were analyzed in parallel to the inoculated animals. Each test group ($n = 5$) had a similar distribution of females (weight, 160-180 g) and males (weight, 200-220 g). There was no apparent weight difference between GF and CV rats before or after the experiments.

Sampling from nostrils and pharynx of inoculated rats was performed with cotton wire swabs immersed in 2.7 ml broth (amoxicillin, PBS, and horse serum) and left for 6 h at room temperature. Portions of this solution (0.01 ml) were then streaked onto agar plates with overlying broth. The plates were incubated for 7 days in air with 5% CO₂ at 37°C. Mycoplasma colonies were identified by indirect immunofluorescence with a primary antiserum against *M. pulmonis*. If colonies were not detected after one week, the plates were incubated for another 1-2 weeks followed by a new identification.

Tissue Processing

Rat skulls were dissected and the facial bone with nasal mucosa removed. The bony structures of the zygoma, maxillary region and the frontal bone were thinned out with a drill, which was also used to extract the two front teeth while the more posterior teeth were removed together with the tongue. The nasal cavities were filled with OCT compound (Tissue-Tek, Miles Laboratories, Elkhart, IN) and the specimens were immediately snap-frozen in liquid nitrogen. Serial sections were cut at 9 µm in a Leitz (Wetzlar, Germany) 1720 cryostat equipped with a Leitz, Wetzlar, Germany bone knife. The sectioning plane was perpendicular to the long axis, and the level behind the proximal end of alveoli of the frontal teeth was selected for optimal data

collection on the NALT structures as well as the nasal respiratory and olfactory epithelium. Sections were mounted on poly-L-lysine-coated glass slides, dried overnight at room temperature, and fixed in acetone for 10 min. The slides were then wrapped in foil and kept at -20°C until use.

Immunofluorescence Staining

Lymphocyte cell surface markers were visualized in tissue sections by multicolor immunofluorescence, which was based on indirect staining procedures with combinations of primary unlabeled mAbs of different murine IgG subclasses (Table 1) and subclass-specific secondary antibody conjugates as detailed previously (8). For analysis of CD4/CD8 α coexpression by subsets with different T-cell receptor (TCR), cryosections were incubated with IgG1 mAb against TCR $\alpha\beta$ (R73) or TCR $\delta\gamma$ (V65), together with IgG2a mAb against CD4 (OX35) or CD8 α (G28). Subsequently, bound mAb was identified with appropriately diluted Cy3TM-conjugated ('red') goat anti-mouse IgG1 and biotinylated goat anti-mouse IgG2a (both Southern Biotechnology, Birmingham, AL) mixed with 20% rat serum to remove cross-reactivity. A final incubation step included Cy2TM-conjugated ('green') streptavidin (Southern Biotechnology). The same procedure was employed when NKR-P1⁺ cells were analyzed for CD8 α or CD2 coexpression. When anti-CD3 (G4.18, mouse IgG3) was applied in combination with the anti-TCR mAbs (IgG1), the second incubation step included Cy3TM-conjugated goat anti-mouse IgG3 and biotinylated goat anti-mouse IgG1 (Southern Biotechnology)

absorbed with 20% rat serum, followed by Cy2TM-conjugated streptavidin as above. In most of the staining experiments, the epithelium was visualized by adding a rabbit antiserum (1/100) to cytokeratin (our laboratory) in the second step, followed by AMCA-conjugated ('blue') goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA).

To analyze the distribution of T and B cells in NALT, three cell surface markers were traced simultaneously. The first incubation step included a mixture of anti-CD3, anti-CD4 and anti-B-cell mAbs, followed by a mixture of subclass-specific goat anti-mouse IgG1, IgG2a and IgG3 conjugated with Cy3TM, FITC or biotin, respectively (Southern Biotechnology), and finally AMCA-conjugated streptavidin (Vector). The mixture of anti-CD3 and the anti-B-cell mAbs enabled a satisfactory mapping of the distribution of the T and B cells, but CD4 showed faint expression on dendritic cells and macrophages in addition to strong expression on T cells. Negative controls were obtained by substituting the primary reagents with irrelevant isotype- and concentration-matched mAbs. All antibody reagents were applied for 1 h at room temperature.

Microscopy and Cell Counting

The tissue sections were evaluated at 400 magnification in a Nikon microscope (Eclipse E800, Nikon, Tokyo, Japan) equipped with dichroic filters for selective observation of individual cells with regard to FITC and Cy2TM (green), Cy3TM (red) or AMCA (blue) emission. A CCD video camera system (C5810, Hamamatsu Photonics, Hamamatsu,

Table 1. Mouse monoclonal antibodies used as primary reagents

Clone	Rat specificity	Isotype	Working dilution	Source
Ox35	CD4	IgG2a	Supernatant: 1/4	MRC Cellular Immunology Unit, Oxford, UK
G28	CD8 α	IgG2a	Purified Ig: 1/50	BD Pharmingen, San Diego, CA
G4.18	CD3	IgG3	Purified Ig: 1/200	BD Pharmingen, San Diego, CA
R73	TCR $\alpha\beta$	IgG1	Supernatant: 1/4	T.Hünig, Würzburg, Germany
V65	TCR $\gamma\delta$	IgG1	Purified Ig: 1/50	BD Pharmingen, San Diego, CA
Ox34	CD2	IgG2	Supernatant: 1/10	MRC Cellular Immunology Unit, Oxford, UK
Ox33	B-cell LCA	IgG1	Supernatant: 1/4	MRC Cellular Immunology Unit, Oxford, UK
3.2.3	NKR-P1	IgG1	Ascitic fluid: 1/100	J.C. Hiserodt, Pittsburgh, PA

Definition of abbreviations: Ig, immunoglobulin; LCA, leukocyte common antigen; NKR-P1, natural killer receptor protein 1.

Japan) attached to the microscope was used to capture digitalized fields for computerized multicolor images (Foto-station, Interfoto AS, Oslo, Norway).

Lamina propria lymphocytes (LPLs) and intraepithelial lymphocytes (IELs) were recorded based on appropriate cell surface markers, the latter subset being defined as cells with at least half of the surface profile located within the epithelium. The numerical changes of these cell populations were determined in relation to the estimated length of the surface epithelium in the actual section (IELs and LPLs per mm epithelium). Proportions of TCR $\alpha\beta$ ⁺ LPLs were determined by evaluating at least 200 cells for concomitant expression of a subset marker within at least half of the cross-sectional area of the nasal cavity (i.e. covering a region around one NALT structure and the respiratory epithelium of one of the two nasal cavities, including the olfactory epithelium in its superior region). Because IELs were rare, counts from the whole cross-sectional area of the nasal cavity were accumulated, excluding intraepithelial cells of NALT regions. A median of 113 IELs were enumerated (range, 49-189 cells in each specimen).

The size of NALT was estimated by measuring the edges of the organized lymphoid aggregates which formed an approximately rectangular or triangular area, or the combination of a triangle stacked on a rectangle. Lymphoid cells within NALT itself were too densely packed for enumeration.

Statistics

The Mann-Whitney U-test (two-tailed) was used for the statistical analysis, and $p < 0.05$ was considered significant.

Results

Nasal T-Cell Phenotypes in Uninfected Conventional Rats

CD3⁺ LPLs were observed as single scattered cells or in small clusters located mainly in the subepithelial region. CD3⁺ IELs were distributed throughout the surface epithelium but occurred most frequently at the base of the nasal cavity. Both LPLs and IELs were scarce in the superior region of the cavities where the olfactory epithelium occurs. Such site-specific differences in the distribution of T lymphocytes and other immune cells has previously been described in the respiratory tract (related to the larynx) of rats (26). Notably, nasal lymphocytes were mainly located adjacent to the NALT structures, and more than half of them were not CD3⁺ T cells but rather natural killers (NK) cells (see below).

Most nasal CD3⁺ LPLs as well as IELs expressed TCR $\alpha\beta$ (> 90%), and only a few expressed TCR $\alpha\beta$ (~ 5%). Surprisingly, most $\alpha\beta$ ⁺ T cells in the surface epithelium were CD4⁺ (median, 65%), although a substantial fraction expressed CD8 (31%). As expected, most $\alpha\beta$ ⁺ T cells in the lamina propria were CD4⁺ (63%) while the proportion of CD8⁺ cells (21%) tended to be somewhat lower than in the epithelium (Figures 1 and 2).

Nasal T-Cell Phenotypes and NALT Size are Influenced by the Normal Microbiota

The number of TCR $\alpha\beta$ ⁺ LPLs in nasal mucosa estimated per mm of surface epithelium was quite similar in GF and CV rats, whereas the corresponding IEL subset was more numerous in the CV than GF condition (see data in next section). However, the percentage of TCR $\alpha\beta$ ⁺ LPLs that expressed CD4 (median, 54%) was significantly smaller in GF than CV animals ($p < 0.01$). The same was true ($p < 0.01$) for the percentage of the TCR $\alpha\beta$ ⁺ IELs that expressed CD4 in GF (49%) versus CV rats (Figure 1). Conversely, the proportion of TCR $\alpha\beta$ ⁺ LPLs that expressed CD8 (38%) in GF rats was significantly higher than in CV rats ($p < 0.001$). The fraction of TCR $\alpha\beta$ ⁺ IELs expressing CD8 (42%) was likewise significantly higher ($p = 0.016$) in GF than CV animals (Figure 2).

The estimated area of NALT at corresponding section levels was only marginally, although significantly ($p < 0.01$), smaller in GF than CV rats (Figure 3), and in both cases the lymphoid aggregates consisted of T cells (mainly CD4⁺) and primary B-cell follicles without recognizable germinal centers (Figures 4A and 4C). This observation accorded with the recent report that the normal microflora of the nose provides insufficient bacterial stimulation to induce germinal centers also in murine NALT (17), whereas the gut flora drives germinal-center formation in rat Peyer's patches (10).

Mycoplasma Pulmonis Infection Induces Expansion of NALT and Mucosal T Cells

The cross-sectional dimension of NALT increased approximately 2.6-fold in CV rats under the influence of mycoplasma infection ($p < 0.01$), and such NALT hypertrophy tended to be relatively more marked (5.2-fold, $p < 0.01$) in GF rats (Figures 3 and 4B). Thus, in the postinfection state there was no difference between CV and GF animals in the calculated section area of NALT. Immunostaining in both CV and GF animals showed that, compared with the uninfected state (Figures 4A and 4C), the mycoplasma-induced NALT hypertrophy included

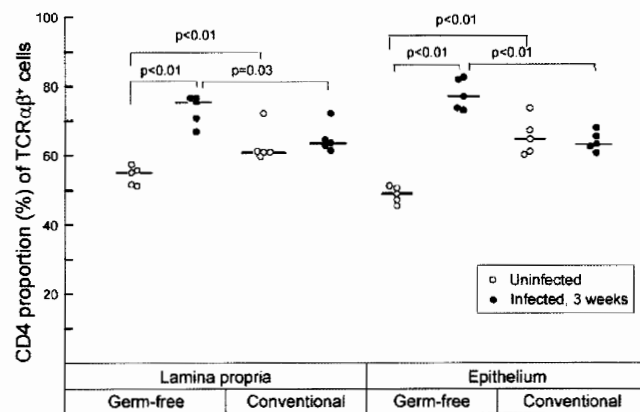


Figure 1. The percentage of CD4⁺ T cells of the total TCRαβ⁺ cell population in two tissue compartments of nasal mucosa before and after monoinfection with *M. pulmonis* in germ-free and conventional rats. Medians and significant differences are indicated.

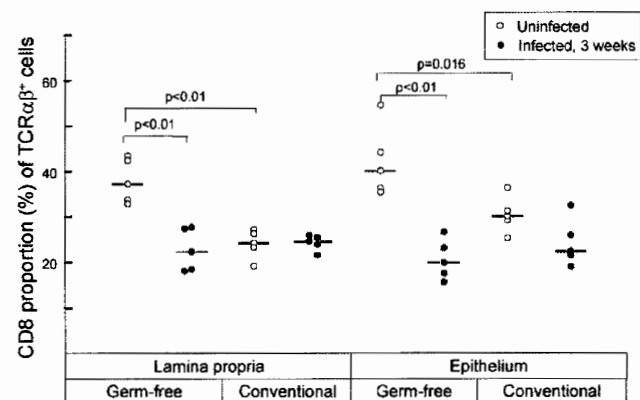


Figure 2. The percentage of CD8⁺ T cells of the total TCRαβ⁺ cell population in two tissue compartments of nasal mucosa before and after monoinfection with *M. pulmonis* in germ-free and conventional rats. Medians and significant differences are indicated.

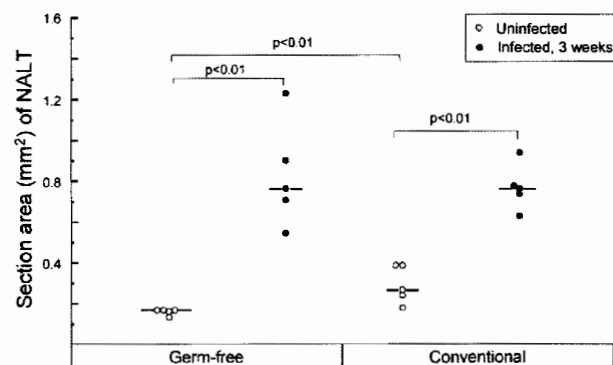


Figure 3. The section area size of organized nose-associated lymphoid tissue (NALT) before and after monoinfection with *M. pulmonis* in germ-free and conventional rats. Medians and significant differences are indicated.

aggregated hyperplastic B-cell follicles with germinal centres rich in CD4⁺ T cells (Figures 4B and 4D), which were easily distinguishable from the diffusely distributed B and T lymphocytes seen elsewhere in nasal mucosa.

A significant numerical elevation of TCR $\alpha\beta$ ⁺ LPLs per mm surface epithelium was seen after mycoplasma infection in nasal mucosa of both CV (from 7.2 to 10.8, $p=0.008$) and GF (from 7.1 to 12.9, $p=0.008$) rats, and the same was true for TCR $\alpha\beta$ ⁺ IELs in GF rats (from 1.0 to 1.7, $p=0.02$) but not in CV rats (from 1.7 to 1.8, $p=0.31$). In the postinfection state, the estimated number of TCR $\alpha\beta$ ⁺ LPLs and IELs did not differ between the two animal groups. The rare TCR $\delta\gamma$ ⁺ T cells were apparently not numerically or phenotypically affected by the infection. These results harmonized with the T-cell distribution observed in rat laryngeal mucosa of CV rats following inhalation of heat-killed *Moraxella catarrhalis* (26).

M. Pulmonis Infection Alters Nasal T-Cell Phenotypes in Germ-Free but not Conventional Rats

Monoinfection with *M. pulmonis* induced a striking proportional increase of TCR $\alpha\beta$ ⁺ CD4⁺ T cells in GF rats, both in the lamina propria ($p < 0.01$) and epithelium ($p < 0.01$), whereas this LPL and IEL subset ratio did not change in CV rats (Figure 1). Conversely, the percentage of TCR $\alpha\beta$ ⁺ CD8⁺ in both compartments was significantly decreased in GF ($p < 0.01$) but remained virtually unchanged in CV rats (Figure 2). Notably, in contrast to the pre-infection state, the infected rats showed a significantly higher proportion of the TCR $\alpha\beta$ ⁺ LPLs expressing CD4 in the GF than in the CV condition ($p = 0.03$). The same striking difference between the two conditions was observed for TCR $\alpha\beta$ ⁺ IELs expressing CD4 when comparing the pre-infection with the post-infection state ($p < 0.01$) (Figure 1).

A Subset of Nasal Lymphocytes Expresses the NK-Cell Complex

Interestingly, we found a relatively large population of nasal CD8⁺ lymphocytes with no detectable TCR $\alpha\beta$ (Figure 4B) or TCR $\delta\gamma$ (data not shown). The fraction of TCR $\alpha\beta$ ⁺ CD8⁺ of all CD8⁺ IELs was 53% in GF rats and 62% in CV rats, whereas in the lamina propria these fractions were 57% and 74%, respectively. Many of these cells coexpressed the NK cell marker NKR-P1 (Figure 4E) and were also CD2⁺; they could thus be phenotypically characterized as NK cells. In CV rats this subset tended to be decreased after *M. pulmonis* infection, while no difference was observed in GF animals (data not shown).

Discussion

This is apparently the first study to show a local immune-modulating effect of commensal bacteria in the face of infection with an extracellular respiratory pathogen. Over time, *M. pulmonis* is known to cause chronic inflammatory disease in the airways of rodents. Although specific T-cell responses could not be evaluated in this *in situ* study, we found that the monoinfection induced more severe perturbations of immunological variables in GF than CV rats, e.g. by affecting both the number and proportion of CD4⁺ TCR $\alpha\beta$ cells in the lamina propria and surface epithelium. Thus, mucosal immune homeostasis was better maintained during the infection in the presence of the indigenous microbiota, and this was also reflected by less hypertrophy in relative terms of the local inductive NALT structures in CV than GF rats.

The host's coexistence with commensal bacteria in a mutually beneficial manner has developed over several million years of adaptation (7). The immunological hypo-responsiveness to the indigenous flora is believed to reflect a tolerance phenomenon (27), and may largely depend on regulatory T (Treg) cells secreting transforming growth factor (TGF)- β and interleukin (IL)-10, which may suppress immune responses. These cytokines inhibit both Th1- and Th2-dependent immunity by dampening both T cell-mediated as well as T cell-independent immunopathology (28, 29). There is currently great interest in the role of APCs in shaping the phenotypes of naïve T cells during their initial priming because differential expression of co-stimulatory molecules on both steady-state and activated dendritic cells (DCs) can exert a decisive impact. Thus, the function of DCs is modulated by pathogen-associated molecular patterns (PAMPs), which are sensed by pattern recognition receptors (PRRs) – many of which belong to the so-called Toll-like receptors (TLRs). The engagement of PRRs on DCs causes maturation accompanied by production of cytokines and upregulation or downregulation of cell-surface molecules according to strictly defined kinetics (30). Such signalling molecules will critically influence further induction of both innate and adaptive immunity.

By means of their PAMPs, pathogens can quite early during an infection imprint their 'signatures' on subsequent immune responses. Treg cells may even be directly affected by microbial products such as LPS through the TLRs that they express (31). It is important to be aware of the fact that PRRs apparently do not distinguish between pathogenic and commensal bacteria, which might seem incompatible with the mucosal homeostasis that

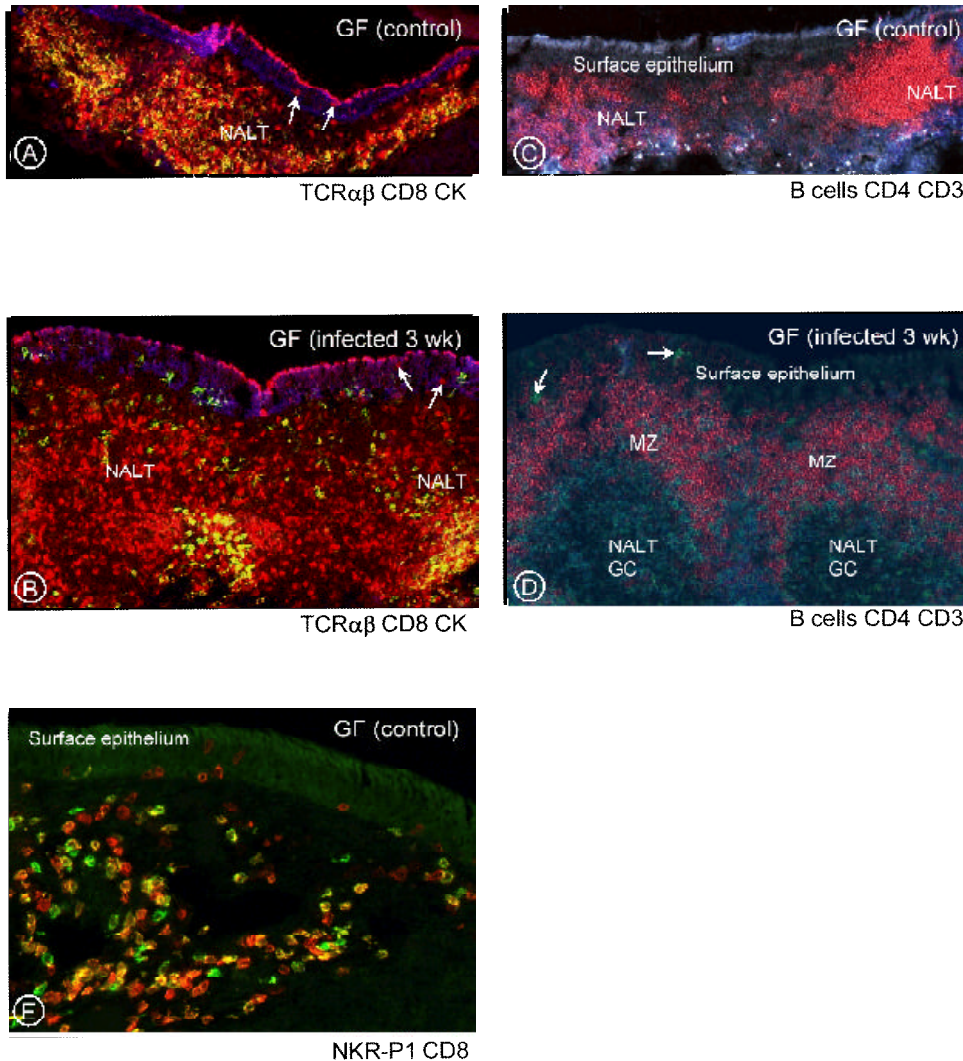


Figure 4. Multicolor immunofluorescence localization of T-cell subsets and B cells in the nose of germ-free (GF) control rats and GF rats monoinfected with *M. pulmonis* for 3 weeks. Tissue sections were immunostained for TCRαβ, CD3, CD4, CD8, NKR-P1 (NK-cell marker), B cells and epithelium (cytokeratin, CK) in different combinations (see color keys). A and B, In the controls, nose-associated lymphoid tissue (NALT) aggregates are much less prominent than after monoinfection for 3 weeks, when the dominance of CD4⁺ T cells with purely red color becomes even more apparent than before. Note that in both situations there are several purely green CD8⁺ cells (mostly with NK phenotype; see E), and these also occur in the epithelium after infection together with purely red (CD4⁺) intraepithelial T cells (arrows). The red apical staining in the epithelium is nonspecific. C and D, The hypertrophy of the NALT aggregates is largely caused by follicular hyperplasia of B cells with expanded germinal centres (GCs) and mantle zones (MZs), whereas in the control state the follicles are mainly of primary type without GCs. Note that the B-cell marker detected by mAb OX33 is downregulated on GC B cells. The T cells show abundantly blue-green color mix, verifying their preferential CD4 phenotype, particularly inside of GCs. The few purely green subepithelial cells (arrows in D) represent CD4⁺ macrophages or dendritic cells. E, Many of the CD8⁺ cells, both in the lamina propria and surface epithelium, express the NKR-P1 marker and therefore appear reddish or yellow, but there are also purely red NKR-P1⁺ cells without CD8 expression. Original magnification, A-D, x100; E, x200.

normally exists. However, it cannot be excluded that the indigenous flora may induce differential PRR signals resulting in distinct molecular APC programs (32). Notably, it has been reported that non-pathogenic *Salmonella* strains are able to block the NF- κ B transcription pathway in human gut epithelial cells in vitro and thereby reduce basolateral IL-8 secretion in response to proinflammatory stimuli, including apical infection with wild-type *Salmonella typhimurium* (13). It is also notable in this context that the intestinal epithelium appears to have inherent mechanisms to protect itself against activation from the luminal side unless production of chemokines and proinflammatory cytokines is needed in defense against invading microorganisms (33). Thus, mucosal epithelial cells apparently possess sensing systems that allow discrimination between patho-genic and non-pathogenic bacteria in order to initiate an inflammatory reaction only when elimination of invading pathogens is needed. The secretory immune system appears to be part of this homeostatic mechanism because specific dimeric IgA may, during polymeric Ig receptor-mediated epithelial trans-cytosis to the mucosal surface, prevent LPS-induced NF- κ B translocation and induction of proinflammatory cytokines (34).

Further work is needed to investigate whether the dampening effect of the normal microbiota on pathogen-induced immunological perturbations observed in our study was mediated by Treg cells or other homeostatic mechanisms alluded to above. Notably, it was recently shown in a rat model that the response to inhaled allergens in sensitized animals is regulated via bidirectional interactions between antigen-presenting DCs and memory T cells (35). Following aeroallergen challenge, the resident DCs rapidly matured *in situ* to potent APCs. This

model provided a plausible model for a regulatory role of activated CD4⁺ T cells in airway mucosa.

The commensal flora in the human nasopharynx is similar to the better known microbiota of the mouth and oropharynx. The numbers and prevalence of various bacteria in the nasopharynx, including different species of streptococcus (i.e. *S. mitis*, *S. pneumoniae*, *S. oralis*, and *S. viridans*) vary individually and appear to depend on age (36). During different periods in life, colonization with potential pathogens is known in the nasopharynx. In childhood, *S. pneumoniae*, *Hemophilus influenzae* and *Moraxella catharalis* are relatively frequent in the nasopharyngeal region and elevated in populations with for instance otitis media. Approximately 30% of adults harbor *Staphylococcus aureus* in the most anterior region of the nasal cavities. Bachert et al. (37) have suggested that an aberrant immune response of exotoxin from *S. aureus*

results in the formation of local IgE antibodies in association with nasal polyps. Increased numbers of *S. aureus* and other transient pathogens (i.e. *Pseudomonas* spp.) in the sinonasal region and lower respiratory tract in the case of cystic fibrosis could be associated with an increased prevalence of allergic eosinophilia in this population (38).

Whether the aberrant response patterns referred to above are explained by inappropriate immunoregulatory signals from the indigenous microbiota remains elusive. According to a recent

Danish study, the normal flora of the human nasal cavity consists of *Corynebacterium*, *Aureobacterium*, *Rhodococcus* and staphylococci, including *S. epidermidis*, *S. capitis*, *S. hominis*, *S. haemolyticus*, *S. lugdunensis*, and *S. warneri* (39). This composition differs totally from the nasopharyngeal flora. Notably, the status and role of the commensal flora in the human nose and nasopharynx remain unknown in chronic or recurrent infection and inflammation.

In the case of rodents, the colonization of microbes in the nasal cavity and nasopharyngeal region appears to be more prominent than in humans, and rats breathe mainly through the nose. This might explain that we achieved such a striking effect on pathogen-induced immunological perturbations when comparing GF with CV rats. The fact that the nose and nasopharynx are quite exposed to the external environment, being in persistent contact with irritants and antigens, makes it most likely that the immunological impact of the microflora in this region represents an important homeostatic mechanism similar to that known to exist in the gut (5-7). Thus, in our rat model we demonstrated that the normal microbiota modulates the response of various T-cell subsets (i.e. TCR $\alpha\beta$ ⁺ CD4⁺ and CD8⁺ cells) during monoinfection with the pathogen *M. pulmonis*. This model may be further exploited to dissect immunoregulatory network components engaged by commensal bacteria in the airways.

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IV

Significant hyposmia in Cystic Fibrosis

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Study objectives: The sense of smell is sensitive to inflammatory diseases in the upper respiratory airways for example rhinitis, sinusitis and nasal polyposis. All these diagnoses are frequent in cystic fibrosis (CF). The aims of this study were to examine the sense of smell in CF and relate the odour perception to age, gender and genotype, to endoscopic examination and the total health situation of this patient group/the severity of the disease (e.g. inflammatory parameters, bacterial examinations, spirometry) as well as to allergic status. Furthermore we wanted to evaluate whether treatment of diagnosed polyps would influence the sense of smell.

Patients and study design: The clinical histories, endoscopic investigations of the nasal cavity, and smell tests (Butanol test, SOIT and SSIT-C) of 45 patients with CF complicated with nasal polyposis were compared with those of 77 CF control subjects without polyps. The patients were examined at their annual control examinations from 2000 to 2002 at Stockholm Cystic Fibrosis Centre, Karolinska University Hospital Huddinge. All patients were ≥ 5 years of age. The smell tests were related to an endoscopic investigation of the upper airways, inflammatory blood parameters, colonizing pathogens, actual pulmonary function, BMI, number of antibiotic courses, as well as to age, gender and genotype.

Results: The CF patients showed hyposmia in 44% and 64% according to two different smell tests (identification and sensitivity threshold test). The sense of smell was even more reduced by the presence of nasal polyps. Subjective hyposmia was reported in 25%. The total health situation was not affected by the lowered olfactory function. There was no correlation between hyposmia and a lowered BMI.

Conclusions: This study shows that hyposmia is frequent in the population of CF. The sense of smell was even more reduced by the presence of nasal polyps. The lowered sense of smell did not alter with age, allergy to aeroallergen, genotype or gender. The total health situation was not affected by the lowered olfactory function. Too few of the patients with polyp size 2 or larger participated in the treatment trial of the study. For achieving a more definitive evaluation of the two applied treatments of this study a multicenter approach required.

The newly launched smell test for children aged 5 to 13 years (SSIT-C) is a valuable complement in increasing the understanding of nasal problems in this patient group.

Key words: cystic fibrosis; smell; nasal polyps; butanol test; identification test

Abbreviations: CF - cystic fibrosis; CRP - C-reactive protein; ESR - erythrocyte sedimentation rate; ESS - endoscopic sinus surgery; SOIT - The Scandinavian Odour Identification Test; UPSIT - The University of Pennsylvania Smell Identification Test; WBC - white blood cell; SSIT-C - Swedish Smell Identification Test for Children

INTRODUCTION

Little is known about the olfactory function in cystic fibrosis (CF), the most common lethal autosomal recessive disease that affects Caucasians. The disease is caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene. This gene encodes for a protein that functions as a cyclic adenosine monophosphate-regulated chloride channel.

Abnormal function of the channel results in aberrant conductance across the apical membrane of epithelial cells in a variety of organs — lung, pancreas, sweat gland, liver, nasal polyps, salivary glands, and colon. So far, >1200 mutations have been identified; the most common mutations in Sweden being Δ -F508,

394delTT, and 3659delC (1). The clinical manifestations of the disease include pancreatic enzyme deficiency with malabsorption; chronic progressive obstructive pulmonary disease; chronic pulmonary infection with *Staphylococcus aureus*, *Pseudomonas aeruginosa*, or both; and an abnormal high concentration of salt in sweat.

The otolaryngologic manifestations include chronic sinusitis, nasal polyposis and recurrent infectious rhinitis, all of which may affect the sense of smell. Radiological signs of sinopathy are seen in 92-100% (2-4) while clinical signs are reported in 11-94% (2, 5-7). Nasal polyps are seen in the nasal cavity to some degree in 39-56% (8-12) when examined with endoscopic technique.

The aim of the present study was to illuminate the olfactory function in CF and relate the findings to the infectious and inflammatory status in the nose by performing an endoscopic evaluation. Additionally we wanted to correlate the upper-airway findings and the sense of smell with the total health situation of these patients (including lower airway status, morbidity, bacteriology and BMI) as well as to age, gender and genotype. An attempt was done to recruit a patient material so that conclusions could be made regarding treatment strategies in patients with the combination of lowered sense of smell and nasal polyps. The question is if endoscopic sinus surgery procedure — with the reduction of the nasal polyps and an additional opening procedure of the antral sinuses — in combination with a period of topical steroids or a treatment with only topical steroids will have any positive effects on the sense of smell in patients with CF.

MATERIALS AND METHODS

Patients

160 CF patients ≥ 5 years old attending the Stockholm CF Centre, Karolinska University Hospital Huddinge (former Huddinge University Hospital), were offered to participate in the study in connection with their yearly control examination September 2000 to November 2002. The diagnosis of CF was based on repeated positive sweat test findings (> 60 mmol/L of chloride in an adequate sample of sweat) and clinical correlates.

Principles of treatment:

The CF patients in the county of Stockholm are examined monthly; the CF patients outside the county at least yearly at the centre (shared care with local monthly visits). Bacterial samples (nasopharyngeal swabs or sputum) are taken monthly. Signs of low-grade infection (i.e. increased cough, changes in sputum, inadequate gain of weight/weight loss, lowered appetite, tiredness, worsening of symptoms at physical examination) are indications for antibiotic treatment together with intensified physiotherapy. The above-mentioned signs usually correlate well with a slight rise in inflammatory markers. The choice of antibiotics depends on the result of the bacterial cultivation. The length of the course of antibiotics is usually 10 days. Patients with elevated staphylococcal antibodies (alphatoxin and teichoic acid) are receiving continuous flucloxacillin treatment. Otherwise, our centre does not

practice any prophylactic treatment. The patients are regularly treated with high oral doses of mucus-dissolving agents three times daily (bromhexin and acetylcysteine in double recommended doses). They inhale salbutamol (usually 2.5 mL [1 mg/mL]) and acetylcysteine (usually 2 mL [200 mg/mL]) bid or tid accompanied by individualised physiotherapy. Small children are recommended nightly mist tent therapy. The patients with pancreatic insufficiency are supplemented with pancreatic enzymes as well as with vitamin E (100 to 400 mg/d) and vitamin A (5,000 to 7,500 IU/d) depending on their serum levels.

The annual examination at the CF Centre includes pulmonary radiographs, lung function tests, working capacity test, ultrasound of the liver, inflammatory parameters (C-reactive protein (CRP), albumin, haptoglobin, α -acid - glycoprotein, erythrocyte sedimentation rate (ESR) and white blood cell (WBC) count), liver function tests, serum tocopherol, serum retinol, and cultivations of sputum or nasopharyngeal swabs and serum antibodies for *S. aureus* and *P. aeruginosa*.

Study design

A questionnaire was filled in by the patients. The questions aimed to score the subjective sense of smell (and taste) but reflected also other sinonasal problems. Clinical data on nasal symptoms were obtained by interviewing the patients and/or their parents.

An endoscopic examination of the nasal cavity was carried out after the patients had filled in the questionnaire and had been interviewed. The occurrence of nasal polyposis, nasal congestion, secretion and redness were noted. The nasal polyps were graded according to their size, with grade 1 equal to the smallest size of polyps (concealed in middle meatus, not reaching the inferior edge of the middle turbinate). Grade 2 described a polyp in the middle meatus, reaching the inferior border of the middle turbinate. Grade 3 was equal to a nasal polyp extending into the nasal cavity below the edge of the middle turbinate but not below the inferior edge of the inferior turbinate, whereas a polyp filling up the nasal cavity was regarded as a grade 4 polyp.

Two different olfactory tests were used for the two age groups. The teens (> 13 years of age) and the adults were tested with the threshold test (butanol test) and the sensitivity smell identification test Scandinavian Odor Identification Test (SOIT) while the children 5 to 13 years of age used the butanol

test and the newly presented identification test Swedish Smell Identification Test for Children (SSIT-C).

The collected data from the otolaryngologic examination were compared with the data routinely gathered at the annual control examination at the Stockholm CF centre with regard to inflammatory blood parameters (i.e. CRP, ESR, WBC count, proteins signalling inflammation) as well as to total serum IgE, pulmonary function, *S. aureus* and *P. aeruginosa* in sputum samples or nasopharyngeal swabs. An allergy test for aeroallergens, mainly Phadiatop® analysis (n=104), RAST (n=4) or skin prick test (n=1) was added to the annual examination. BMI (Body Mass index (kg/cm²)) data was collected and compared to age-dependant reference values (13).

The patients with nasal polyps \geq grade 2 were randomized into one of the following two treatments. One group was offered treatment with a topical steroid (Nasonex®) in the form of nose spray (50 µg in both cavities bid) for 7 months. The other group was offered the same nasal spray for 7 months. Beside, one month after the initiated treatment with the topical steroid spray an ordinary endoscopic sinus surgery was carried out in the latter group (the polyps were eliminated, a broad opening were established to the maxillary sinus, a complete anterior ethmoidectomy and a partial posterior ethmoidectomy were carried out).

The two groups of treatment were followed by examination after 12 months with questionnaire, nasal endoscopy and olfactory testing.

Statistical analysis

The following statistical analyses were performed with statistical software (Statistica; StatSoft; Tulsa, OK): multilinear regression [hyposmia and nasal polyps vs. age, lung function tests and some of the blood parameters; persistent subjective hyposmia vs. age; both smell test vs. age; either of the smell tests vs. age]; chi2test [polyps vs genotype, gender and the use of oral or intravenous antibiotics; hyposmia vs. genotype; anosmia vs. genotype]; Fishers exact [age vs. persistent subjective hyposmia; anosmia and hyposmia vs. lowered weight (-2SD); nasal polyps vs. anosmia and persistent subjective hyposmia]; Whitney-Mann U test [hyposmia and nasal polyps vs. use of IV and oral antibiotics per year]; logistic regression [hyposmia vs. recent polypectomies, recent sinusitis, subjective (sense of) taste and the use of intravenous and oral antibiotics; nasal polyps

vs. the frequency of hyposmia (objective and subjective), recent polypectomies, recent sinusitis, aero allergies supported by objective test and subjective (sense of) taste; age group vs. the frequency of hyposmia (objective and subjective) and aero allergies supported by objective test; anosmia vs. ESS/polypectomies; persistent subjective hyposmia and objective hyposmia vs. age and genotype; identificationtest]; and log-rank test [nasal polyps vs. accumulative frequency of chronic colonisation with *P. aeruginosa*].

Ethical permission

The Ethics Committee of the former Huddinge University Hospital (now Karolinska University Hospital Huddinge) approved the study. Consent from the patients or their parents were obtained before enrolment.

Methods

Questionnaire

The questionnaire contained questions regarding nasal symptoms, earlier operations, earlier treatments of the ENT-disorders, stated allergies and subjective evaluations of the sense of smell and taste.

Smell tests

The new identification test (SSIT-C) used for the children aged 5-13 years was presented by Anna Hallberg at the AChemS XXIV-s meeting, April 2002, Sarisota, US. This test contains 16 different odours well known by children in Scandinavia and Western Europe. Initially the children have an opportunity to make a free association around the presented odour. Secondly the children choose one of three presented images that illustrate the odour. This test was carried out by Anna Hallberg at the department of otorhinolaryngology or paediatrics before the endoscopic investigation of the nose was performed. The smell test has been validated and a large group of healthy children has set the levels of anosmia and hyposmia in the test (Hallberg, personal communication)

The identification test used for patients >13 years of age was SOIT, Scandinavian Odor Identification Test described by Nordin et al. (14). It consists of 16 odours (pine needle, peppermint, juniper, violet, anise, clove, vanilla, bitter almond, orange, cinnamon, lemon, lilac, vinegar, tar, ammonia, and apple) with 4 response alternatives for each of them.

The butanol test was used as a threshold test for children and adults (15, 16). In this test the sensi-

vity of the olfaction system to low concentrations of odorants is measured. The test is based on a procedure where the lowest concentration of the odour is presented and compared with a parallel plastic bottle filled with distilled water. In this blinded manoeuvre the patient should give five correct answers on a row to define what concentration of butanol that he or she could detect. The strongest butanol mixture is 4%, which then is diluted in 12 further steps, each step containing a third of the butanol amount compared to the previous bottle. This smell test is one of the most sensitive tests to variations in mucosal swellings in the nose.

Genotype Analysis

Genotype data were available on all the patients with and without nasal polyposis (1), the mutations being: Δ -F508, Δ -F508-W79R, 3659delC, 394delTT, 297-3C-A, S945 L, L206W, R553X, G551D, 2798 + 5G-A, YJ09N, 711 + 3A-G, D1152H, R75QN1088, R1162X, W1282X, G1244, and 2183AA-G.

Biochemical and immunological Analyses

White blood cell count (WBC), thrombocyte count, erythrocyte sedimentation rate, C-reactive protein (CRP), serum protein electrophoresis, and hemoglobin were analysed by routine laboratory methods. Antibodies to staphylococcal teichoic acid and alfa toxin and to *P. aeruginosa* exotoxin A in serum were determined by enzyme-linked immunosorbent assay (ELISA) at the bacteriological department (17, 18)

Phadiatop®

The concentration of serum IgE antibodies against 10 airborne allergens present in a solid phase was assayed with the Phadiatop test with the capsulated hydrophilic carrier method (Phadiatop/Cap system FEIA; Pharmacia Diagnostics AB). The results were expressed as positive (allergic) or negative (non-allergic). Other tests used to establish sensitization were RAST (Radio Allergo Sorbent Test) or a skin prick test. In RAST the results were expressed in RAST units per millilitre (U/ml, one RAST unit representing approximately 2.4 ng of specific IgE). Skin prick tests were performed against inhalant allergens. All skin prick tests were performed by an experienced nurse on the volar side of the lower arm, according to the manufacturer's instructions (ALK, Copenhagen, Denmark). The allergens included birch, timothy grass, cat, dog, horse, *Derмато-phagoides pteronyssinus* and *Cladosporium* species (Soluprick, 10 histamine-equivalent potency, ALK). Histamine chloride, 10 mg/mL, was used as a positive control, and the allergen diluent was used

as the negative control. Resulting wheal reactions were measured 15 minutes after puncture. Skin tests were considered valid only if the difference in wheal diameter between positive and negative controls was more than 3 mm.

Bacteriology

Sputum samples were analyzed using routine procedures in the bacteriological laboratory of Karolinska University Hospital Huddinge (19). Special attention was given to the following pathogens: *S. aureus*, *P. aeruginosa*, *Hemophilus influenza*, *Burkholderia cepacia* and *Stenotrophomonas maltophilia*. First, current and chronic colonisation were established. Chronic colonization of *P. aeruginosa* was defined as six consecutive isolations separated by > 1 month and/or elevated level of antibodies (*P. aeruginosa* anti-exotoxin A).

Spirometry

Static and dynamic spirometric measurements were obtained from each patient >7 years of age. Functional residual capacity was determined by body plethysmography. Total lung capacity and residual volume were calculated. Vital capacity, FEV1, FEV1 as a percentage of vital capacity, peak respiratory flow, forced expiratory flow at 50 of FVC, and forced expiratory flow at 25 of FVC were measured separately. All measurements were performed using a pulmonary function laboratory (Sensor Medics BV; Beilthaven, the Netherlands). All patients were coached by the same technicians and were familiar with spirometric measurements. The data were presented as percent predicted.

Antibiotic courses

The number of yearly oral and intravenous antibiotic courses was collected from the Stockholm CF Centre database

RESULTS

76% (122/160) of the CF patients \geq 5 years of age agreed to participate in the study.

Olfactory sensitivity test (i.e. absolute threshold for butanol) was pathological in 64% (57% hyposmia and 7% anosmia) while the smell identification tests showed olfactory disorders in 44%. The olfactory functions were further reduced by the presence of nasal polyps. Olfactory disorder according to the butanol test was found in 78% (58% hyposmia and

anosmia 20%) of the polyp patients ($p=0.03$, OR 2.4 compared to the nonpolyp group). According to the identification test hyposmia or anosmia was found in 57% polyp patients ($p<0.004$, OR=2.9 compared to the nonpolyp group).

Hyposmia or anosmia according to both tests (i.e. the olfactory sensitivity test and the smell identification tests) was seen in 31% with an elevated risk, 48%, in the polyp group ($p<0.005$, OR=3.4). 74% had hyposmia or anosmia according to at least one of the tests with an elevated risk, 87%, in the polyp group ($p=0.03$, OR=2.8).

According to the questionnaire only 13% of the patients did experience any kind of lowered smell sensitivity on a continuous basis (with a higher frequency, 27 %, in the polyp group ($p=0.001$)). 75% of the patients, at the time of the examination, declared that their sense of smell was normal (56 % in the polyp group ($p<0.003$, OR=2.9)).

A higher proportion of the patients with a lowered sense of smell, according to the butanol test, had been operated with polypectomy or ESS (30%, $p=0.04$) or had experienced sinusitis (25%, $p<0.05$) compared to patients with a normal sense of smell.

The subjective estimation of reduced taste sensitivity was 12% with a higher frequency if parallel hyposmia was present (16%, $p=0.03$) or if nasal polyps were found (23%, $p=0.01$).

The frequency of nasal polyposis was estimated to 37% with the endoscope technique. Only 11% of the patients with nasal polyps declared continuous nasal blockage while 7% experienced continuous nasal secretion.

(Figure 1 shows the difference in hyposmia in CF depending on the presence of nasal polyposis (according to the smell tests and according to the subjective evaluation of the sense of smell). Figure 2 shows the frequency of nasal polyposis and hyposmia according to butanol and identification tests in children vs. adults.)

There were no tendencies of any elevated morbidity or pathological laboratory tests that correlated to the prevalence of nasal polyps or the sense of smell. The spirometric values and genotype status were independent of the prevalence of polyps and the sense of smell. The BMI was not affected by the presence of lowered olfactory function.

There was no correlation between olfactory dysfunction or nasal polyposis and the use of oral or intravenous antibiotics.

Figure 1. Hyposmia (objective and subjective) compared to the endoscopical finding of nasal polyposis (mean-values stated; significant differences shown).

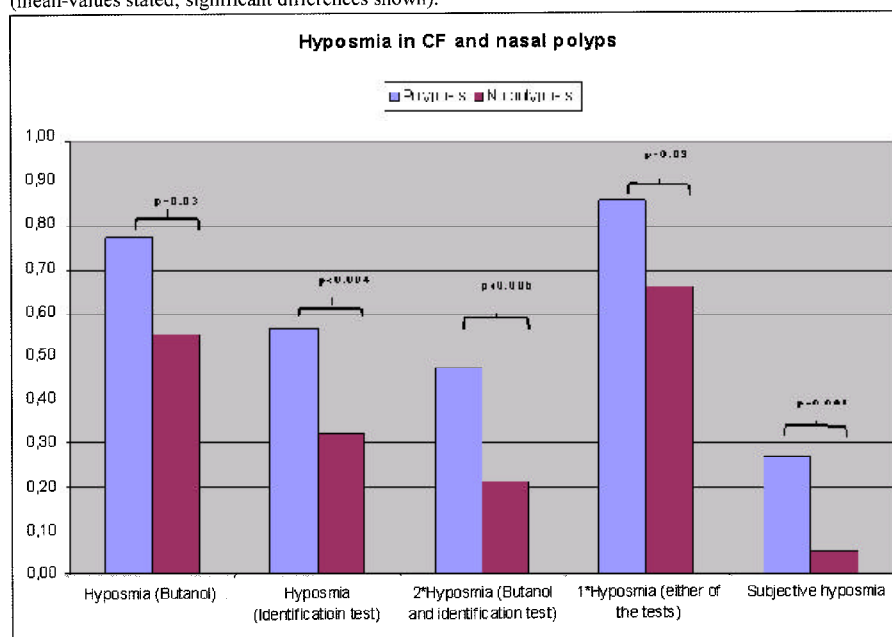
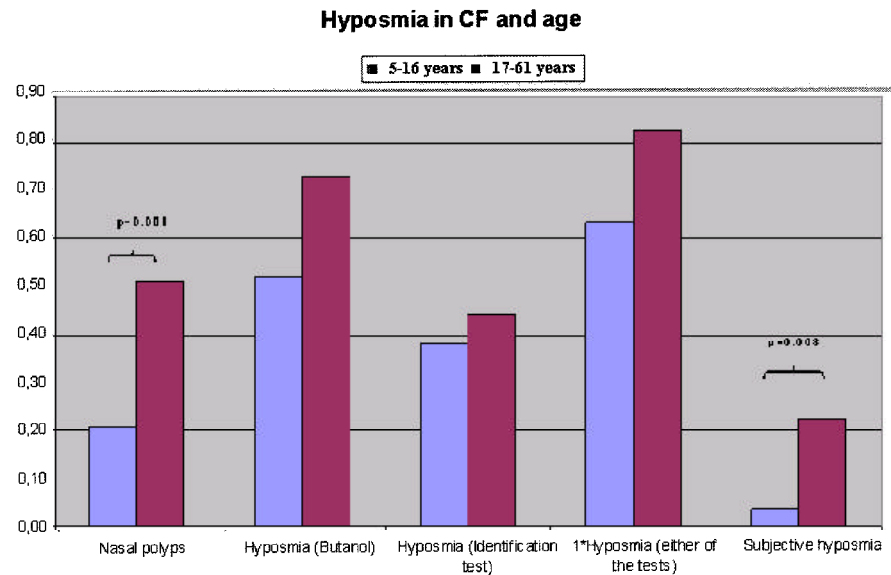


Figure 2. Hyposmia and the frequency of nasal polyps related to age (children and youths compared with



The frequency of allergic reactions to aeroallergens (supported by any objective allergy test) was 35% with no differences between the polyp and non polyp group (31 vs. 37%), nor any difference between the adults compared to the children (37.5 vs. 32%). This is consistent with earlier observations stating no differences regarding allergic tendency between patients with and without nasal polyps (20-22).

26 of the patients had medium sized (\geq grade 2) polyps, or more, and were offered to participate in the randomised treatment of the study. 18 of these patients were treated according to the study trial.

Of the 10 patients treated with nasal steroids 8 were left to follow up (1 patient moved from the region and 1 patient experienced local irritation of the spray). Of the 8 patients operated on 7 were followed up (1 patient moved from the region). Five of seven patients in the operated group (combination treatment with endoscopic sinus operation and nasal steroid) were improved according to the butanol test while 6 of 7 were improved in at least one of the smell tests. In the group treated with nasal steroid five of eight were improved according to the butanol test while all of the patients were improved in at least one of the smell tests. In five of the eight patients' treated with nasal steroids the polyp size didn't shrink on either side in spite of 7 to 12 months of treatment (although one of the patients discontinued medication).

DISCUSSION

In our study 13 % of the CF patients reported decreased smell sensitivity on a continuous basis. At the time of the examination, 25 % declared that their sense of smell was decreased. This is higher than what has been reported for a general population. In an earlier American study the prevalence of self-reported olfactory problems was 1.4% in individuals older than 18 years of age, with a prevalence rate that increased exponentially with age (23). The subjective smell sensitivity in the CF patients was further reduced with age and the presence of nasal polyps but not altered by gender or genotype.

The butanol test and the smell identification tests revealed a surprisingly high prevalence of hyposmia and anosmia. The butanol test is more sensitive to mucosal swelling and therefore dependant on the inflammatory status including ongoing or previous infections. The identification test is additionally reflecting our odour memory and ability to discriminate between different odours. Certain odours in the test could be sensitive to differences in life-styles and preference of odorants. The variation in odour panoramas at home, in school and at work could alter with ageing and habits (teenagers of today are seldom familiar with odorants as e.g. camphor). Some of the odours in the SOIT test (peppermint, ammonia and vinegar) could furthermore contribute to some degree of trigeminal

stimulations and be detected by anosmics, which could bias the interpretation of the test (14).

Our findings that the smell tests revealed a higher prevalence of olfactory disorders than what the patients themselves reported is in agreement with an epidemiological Swedish study on randomly selected adults on whom an olfactory test was performed which showed a higher prevalence of olfactory disorders than the above mentioned American study. 13.3 % had hyposmia and 5.8% anosmia with an overweight for aged persons, males and persons with nasal polyps (24). In our study the CF patients showed a tendency to a slight reduction in olfactory function with age according to the butanol test. But when comparing the frequency of hyposmia in the younger population (5 to 16 years of age) with the older patients (17 to 61) we could not find any significant lowered values with the butanol test ($p=0.053$). No differences were seen by gender.

In our study the occurrence of hyposmia and anosmia was obvious with both the butanol threshold score and the two different smell identification tests used and the score were – within the CF group – even more reduced by the presence of nasal polyps. In a previous study (8) we did find a significant lowered sense of smell with the UPSIT (Univ. of Pennsylvania Smell Identification Test) compared to a healthy control group, but no further reduction because of nasal polyps. In a study by Aitiken et al. the UPSIT test showed an objective decrease in sensation of smell in 55% of the 49 examined CF patients. Patients with anosmia in the mentioned study were more likely to have had prior sinus surgery (25). In that study previous polypectomies and ESS were more frequently carried out in patients with hyposmia or anosmia. There is an obvious connection between the prevalence of sinusitis and nasal polyps and an olfactory disorder (26). In spite of adequate surgery on nasal polyps and sinus cavities the sense of smell could remain reduced (26–28). Additionally the risk of recurrence (of nasal polyps and sinus disease) is high after such operations, which could explain why the sense of smell could be affected in spite of recent surgery. Finally there are some studies pointing out the risks of affecting the olfactory region during the different sinonasal operations carried out (26, 29).

The noted reduction in the taste sensitivity in our study probably reflects the fact that complaints of taste loss usually reflect loss of smell function (30). The more complex information around the "taste" of different food flavours are mediated through the olfactory region whereas the specific information

given by the taste buds of the tongue are salt, sweet, sour and bitter. Earlier studies that separately tested the taste sensitivity of CF patients have shown normal values (31, 32).

In our study no correlation could be found between the genotype and the presence of polyps and/or a lowered sense of smell. A recent study did show a genotype-phenotype correlation (Δ -F508 homozygote) for the paranasal sinus diseases (nasal polyposis and chronic sinusitis) for patients with CF (10). Another previous study reported a correlation between nasal polyposis that required surgery and two specific genotypes: the Δ -F508/ Δ -F508 and the Δ -F508/G551D genotypes (33). In the latter study patients with CF and nasal polyps had better pulmonary function, better nutritional status, a higher frequency of *P. aeruginosa* colonisation, more office visits, more hospitalisations and a higher rate of acute exacerbations per year than did the comparison group in that study. However, in a recent study at our CF-centre (8) we were not able to point out any elevated risks for morbidity in patients with relatively mild nasal polyposis except for a long-term tendency to acquire chronic colonisation of *P. aeruginosa* in the lower airways earlier than other CF patients, but without any parallel risk of lowering the respiratory functional capacity. In the present study at the same centre we could not confirm the previous observation regarding the difference in chronic colonisation with *P. aeruginosa* of the CF patients with nasal polyps.

In the CF-population both nasal steroids (34) and endoscopic sinus surgery (35) have shown effects on nasal polyps. Earlier observations on idiopathic adult nasal polyposis did not find any positive effects on the sense of smell by such an operative procedure (36) while other studies in CF have shown beneficial results on the olfactory function with the use of endoscopic sinus surgery (37).

Surprisingly most CF-patients experienced their sense of smell not affected. This could depend on the fact that the patients become accustomed to a reduced level of olfactory sensation without any possibilities to compare their new olfactory level with their previous one. The most important function of the sense of smell in humans is to direct our attention toward environmental hazards (smoke and toxic fumes) or to positive sensations such as nutritious food products. In the mentioned situations a slight reduction in the olfactory function will not make a difference as we are dealing with higher concentrations of odorants well over normal threshold. The greater discrepancy found in the CF

patients may be due to this patient category partly ignoring the symptoms from the upper airways focusing as they are on the major problems from the lower airways (38).

Poor nutritional status in patients with CF is associated with increased mortality. As the sensation of smell is influencing our appetite a decreased sensation of smell may be associated with worse nutritional status in patients with CF. In the present study nutritional status was assessed by BMI. No association was found between sensation of smell (hyposmia or anosmia) and BMI. This is in agreement with observations by Aitken et al who was unable to show any association between sensation of smell and the nutritional status (25).

The increased tendency of olfactory disorders in the adult CF population (compared to the children) could depend on the increased frequency of polyps with age. The chronic sinopathy with the intermittent or chronic rhinosinusitis could be another source of local inflammation and mucosal swelling in this area that could affect the olfactory region. Another factor that might influence the olfactory epithelium with increasing age is the exposure of the potential neurotoxic drug tobramycin. This beta-lactam is given either intravenously or through inhalation. It is prescribed for *P. aeruginosa* colonisation of the lower airways nowadays. The general recommendation is that these inhalations should be performed with a mouth-piece instead of a face-mask in order to protect the olfactory region.

Corticosteroids may function by reducing the inflammatory swelling of the nasal mucosa, which increases the penetration of odours to the olfactory region, or they may function by a direct effect on the olfactory cells. Hyposmia in patients with perennial rhinitis have been successfully treated with topical steroids, both symptomatically and in olfactory testing (UPSIT) (39). A more distinct improvement in the sense of smell could be achieved by short-term treatment with oral glucocorticoids (40, 41) but this is not appropriate treatment on a long-term basis. Anosmia or hyposmia not responding to topical nasal steroids did respond to oral steroids, but the duration of symptom-relief was relatively short according to Stevens et al.(42). The drop out from participating in the treatment part of our study could partly be explained by the mild symptoms that the CF-patients experienced in spite of the polyps. Because of the limited size of the treatment group no significant conclusions could be made. For achieving a more definitive evaluation

of the two applied treatments of this study a multicenter approach is required.

CONCLUSION

In conclusion this study shows that hyposmia is frequent in the population of CF. The sense of smell was even more reduced by the presence of nasal polyps. The lowered sense of smell did not alter with age, allergy to aeroallergen, genotype or gender. The olfactory disorder did not alter the overall health status in this population and the majority of the patients weren't affected or aware of their lowered sense of smell. Too few of the patients with polypsize 2 or larger polyps participated in the treatment trial of the study and therefore could no conclusions be made regarding what treatment to recommend.

The newly presented smell test for children aged 5-13 years (SSIT-C) is a valuable complement in the increased understanding of nasal and olfactory problems in this patient group.

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