Non-allergic non-infectious perennial rhinitis.
Pathogenesis and treatment.

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Niet-allergische niet-infectieuze chronische rhinitis.
Pathogenese en behandeling.

Proefschrift

TER VERKRIJGING VAN DE GRAAD VAN DOCTOR AAN DE
ERASMUS UNIVERSITEIT ROTTERDAM OP GEZAG VAN DE
RECTOR MAGNIFICUS

PROF. DR. P.W.C. AKKERMANS M.A.

EN VOLGENS BESLUIT VAN HET COLLEGE VOOR PROMOTIES.
DE OPENBARE VERDEDIGING ZAL PLAATSVINDEN OP

VRIJDAG 23 OKTOBER 1998 OM 15.45 UUR

DOOR

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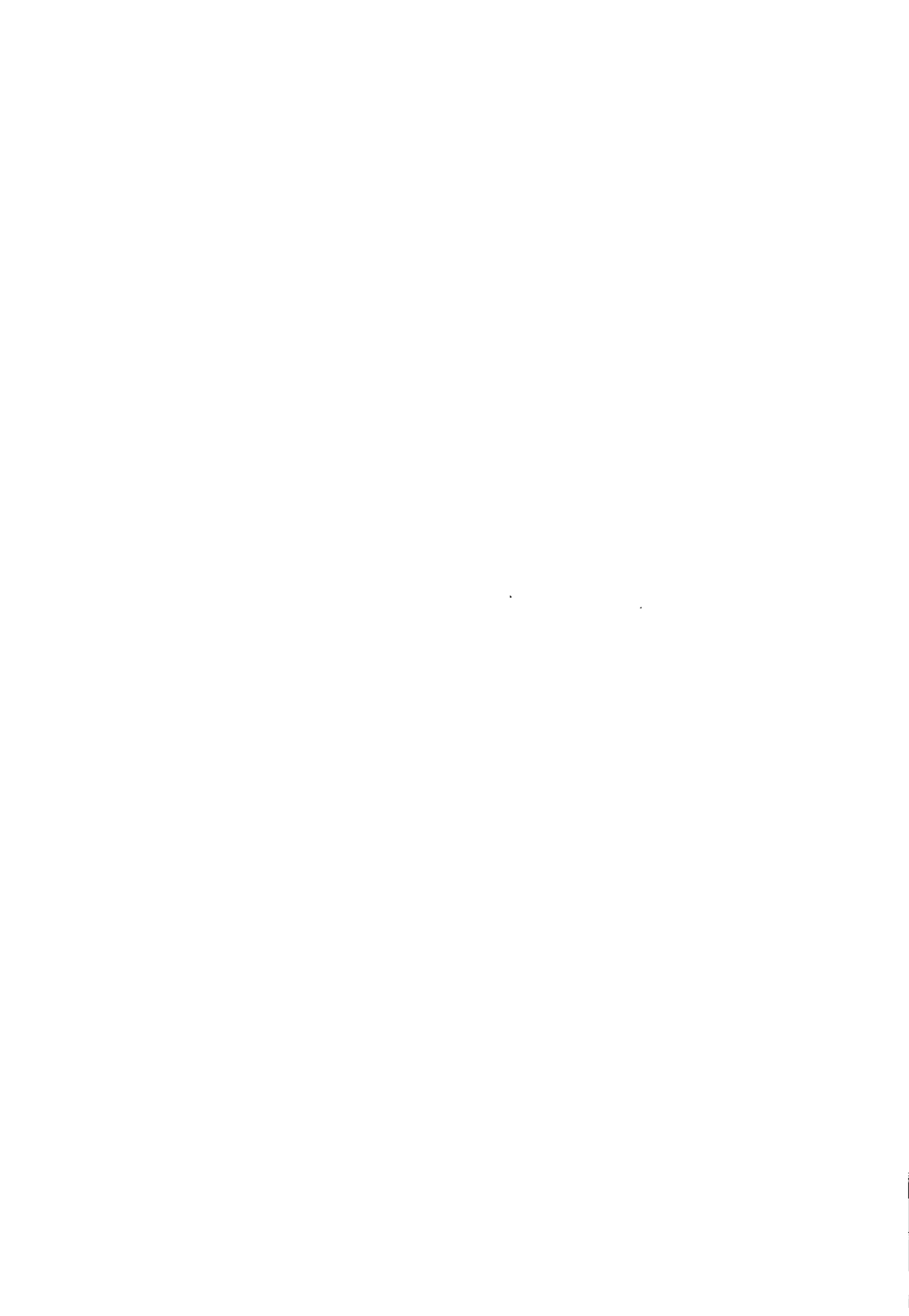   Dr. R. Gerth van Wijk

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INTRODUCTION

Rhinitis is a very common disorder. Most people suffer from an infectious rhinitis at least once a year. The symptoms usually disappear within a week. The patients with chronic rhinitis pose a much greater problem. At least 10 % of the general population are affected by a chronic allergic or non-allergic non-infectious rhinitis (1). The impact of the nasal complaints such as in rhinitis is often underestimated. Bousquet and Juniper demonstrated that the impact of the disease on the quality of life is greater in rhinitis than in asthma patients (2-4). There is no generally accepted system for the definition, classification and terminology of rhinitis (5). A distinction can be made between rhinitis of known and unknown etiology. Known causes for rhinitis can be subdivided in *mechanical factors* (e.g. septal deviation, foreign body,), *infections* (viral, bacterial, fungal), *miscellaneous causes* (e.g. rhinitis medicamentosa, pregnancy, cystic fibrosis) and *allergy*. Syndromes of unknown etiology include non-allergic non-infectious perennial rhinitis (NANIPER), nasal polyposis and non-allergic rhinitis with eosinophilia (NARES).

The subject of this thesis is the pathogenesis and treatment of NANIPER. As this term suggests the disorder is diagnosed through the exclusion of the known causes for rhinitis. Available studies are often difficult to compare. Different authors use different methods to exclude "the known causes". The patients are sometimes presented in a study as NANIPER patients without further specification. The way in which an allergic pathogenesis is excluded varies from skin prick tests, serum testing for specific IgE, total IgE, nasal provocation tests or a combination of these methods. To exclude infection some authors rely on the history (chronicity of the illness, lack of purulent secretions and or the classic symptoms of acute rhinosinusitis), some rely on laboratory parameters (sedimentation rate, white blood cell count, nasal smears), others use negative radiological findings (normal sinus X-ray or CAT-scan), all with or without the use of a nasal symptom score.

Studying the pathophysiology and treatment of NANIPER , we have to consider the nature of the diagnosis by exclusion, the variability of "patient phenotype" reported in the literature, and the dearth of "hard data". A clear definition of, and a lucid method for the selection of NANIPER patients are needed. Even then we will inevitably be confronted with various nosologic entities all presenting as NANIPER. Unfortunately these difficulties have precluded many investigators from studying the *pathophysiology* of this disorder. On the other hand, the prevalence and the impact of this disorder on the quality of life have stimulated researchers to investigate *treatment modalities.*

DEFINITION

The working definition of NANIPER as suggested by Mygind (1) is as follows:

"Patients with NANIPER suffer from chronic symptoms such as nasal congestion, rhinorrhoea, posterior nasal drainage, sneezing, itching and occassionaly pain over the sinuses. The etiology is unknown, and the diagnosis of this disorder is made by exclusion"

NANIPER, or vasomotor rhinitis, can be subcategorized according to the presence of nasal eosinophilia. Patients with a nasal smear showing more than 25% eosinophils are classified as non-allergic rhinitis with eosinophilia syndrome (NARES) (6).

NANIPER has to be distinguished from hyperreactivity. Nasal hyperreactivity or hyperresponsiveness refers to an increased sensitivity to non-specific stimuli or irritants. Nasal symptoms, related to hyperreactivity (sneezes, rhinorrhea and/or nasal blockage) occur on exposure to daily-life stimuli such as dust particles, change of temperature, tobacco smoke, perfumes and paint smells (7). Hyperreactivity can be observed in allergic rhinitis, infectious rhinitis and NANIPER.

EPIDEMIOLOGY

General population prevalence
Non-allergic non-infectious rhinitis is a common disorder (5). Its exact point prevalence is unknown. Malm reported (Rome, ERS 1992) a prevalence of 5-10% of NANIPER in Malmo City, suggesting the same general population prevalence as allergic rhinitis.

General practitioner prevalence
Crobach studied 365 patients suffering from rhinitis, in a general practice; 20 to 60% were diagnosed as NANIPER (8).

Specialist prevalence (ENT, allergy)
Annually an average of 350 patients (1995-1997) are referred to the outpatient clinic of the department of Otorhinolaryngology of the University Hospital Dijkzigt suffering from either allergic rhinitis (50%) or NANIPER (50%). In the department of Allergy in the same hospital 2661 patients suffering from rhinitis were examined in a period of four year. The percentage of non-infectious non-allergic rhinitis was 14% (380). NANIPER is reported to account for 30% to 70% of cases of chronic perennial rhinitis (9). Settipane reported a prevalence of 61% for NANIPER in a group of chronic perennial non-allergic rhinitis patients. In the same group

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31% of patients suffered from NARES (10). This agrees with a report by Mullarky who reported NANIPER to be almost twice as common as NARES (11).

Jessen published on the natural course of NANIPER and reported a spontaneous disappearance in 20% and a spontaneous improvement of nasal complaints in 36% of patients over a ten-year period (12).

PATHOGENESIS

Proposed pathophysiologic mechanisms for non-allergic rhinitis include the possibility of a chronic inflammatory disorder of antigenic or neurogenic nature, as well as the possibility of a functional neuronal disorder.

Neurogenic aspects of the pathogenesis of NANIPER.

Parasympathetic/sympathetic system imbalance.

In 1959 Malcomson stated that NANIPER was caused by *autonomic imbalance* (13). Normally, base line sympathetic tone provides constant alpha and beta adrenergic receptor stimulation (table 2). The marked alpha-1 predominance in nasal blood vessels leads to vasoconstriction (14). Underactivity of the sympathetic nervous system leads to nasal obstruction (15).

Parasympathetic effects on blood vessels are minimal under basal conditions. Stimulation of cholinergic nerves leads to hypersecretion and dilation of mainly resistance vessels (nasal blood flow) and to some extent capacitance vessels (nasal patency). Overactivity of the parasympathetic system leads to rhinorrhea (15). However, van Megen, in a group of 4 patients, was unable to show significant differences in alpha-2, alpha-1 and beta-adrenoreceptors between controls and vasomotor rhinitis patients (16).

Parasympathetic system	Sympathetic system	Peptidergic system
Acetylcholine VIP PHI	(nor)-adrenaline NPY	Substance P CGRP NKA GRP
Secretion Vasodilatation	Vasoconstriction	Vasodilatation Increased permeability Exocrine secretion

Table 2. The nerve system of the nose: transmitters and effects.

Non-adrenergic-non-cholinergic system; peptidergic system .

Wolf suggested that NANIPER could be the result of an "over-active" non-adrenergic non-cholinergic system (table 2) (17). Stimulation of sensory neurons results in sensory nasal

changes, rhinorrhoea (18), nasal blockage and sneezing. Sensory neural stimulation may produce these effects either through a central neural reflex, associated with efferent parasympathetic neurotransmission, or via anti-dromic release of neuropeptides from sensory neurons (figure 1) (19, 20). This hypothesis was corroborated by the findings of Lacroix, who reported an increased concentration of neuropeptides in a group of chronic non-allergic rhinitis patients (21), improvement of symptoms by local treatment of capsaicin giving a 50% reduction in CGRP-Li content in nasal biopsies (22), and a correlation between symptom intensity and CGRP-Li concentration in nasal mucosa (23).

Hyper- or dys-esthesia at CNS level.

Sanico has suggested that it is reasonable to raise the possibility of sensory imbalance that is characterized by dys- or hyper-esthesia at the CNS level (25).

Antigenic

Local occult allergy

Another theory concerning the pathogenesis of NANIPER includes a local, occult allergy (10). The diagnosis of NANIPER is made by exclusion and an allergy test is not 100% sensitive. Moreover systemic manifestations, such as a positive skin prick test or RAST, of atopic disease might be missed because the nose is a small shock organ. However, one has to consider the suggested NANIPER population prevalence of 5-10%.

Food allergy

Food allergy is also considered as a potential pathophysiological factor in NANIPER. In adults, if rhinitis is the only manifestation, food allergy is not very likely (4).

Non-allergic rhinitis with eosinophilia syndrome

It seems reasonable to suggest that NARES is a pathophysiological entity differing from NANIPER because of its association with nasal polyposis and good response to local steroid therapy (11, 26).
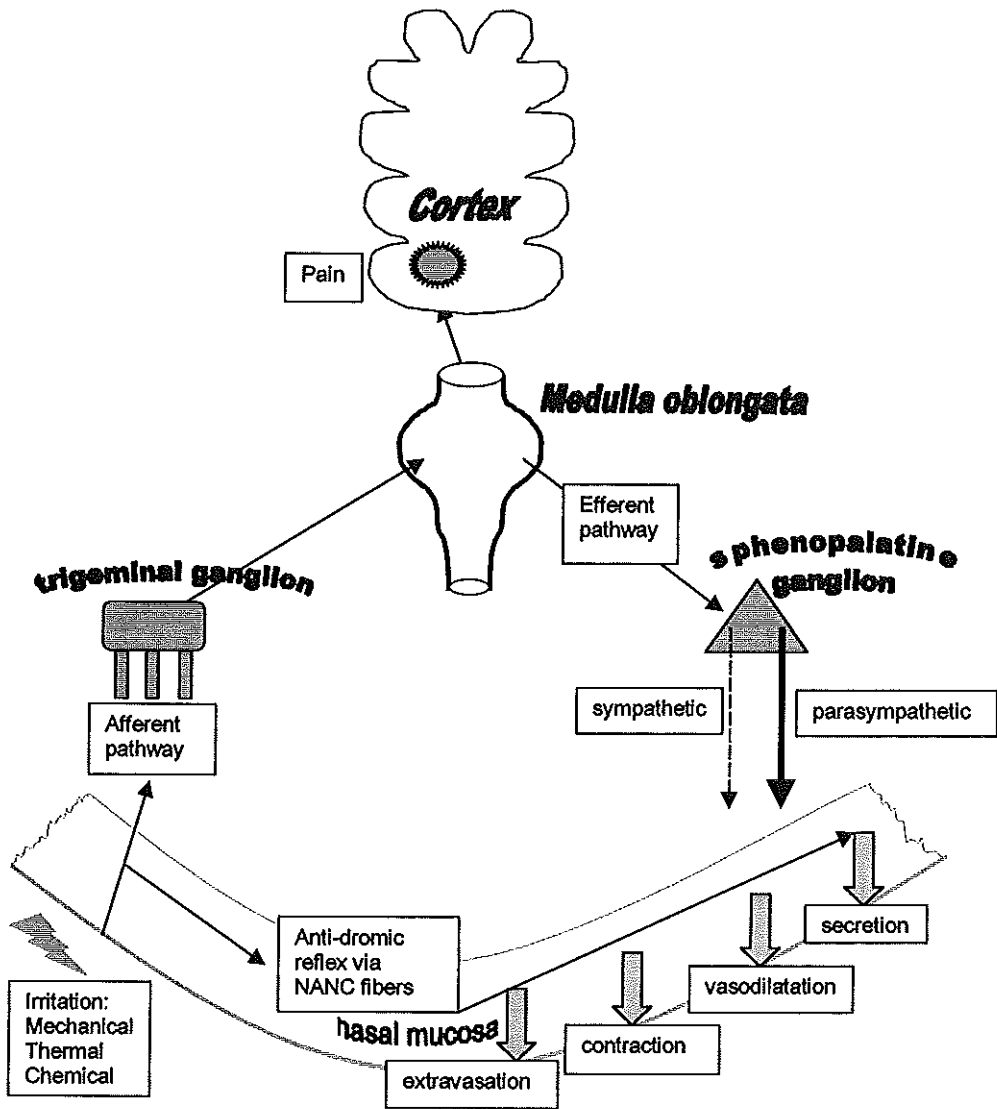
Figure 1.   Simplified scheme of autonomic and peptidergic innervation of the nasal mucosa.
Irritation initiates an afferent signal via the maxillary nerve and trigeminal ganglion into the medulla oblongata, where it is relayed to the cortex. An efferent signal is generated to the preganglionic parasymphathetic neurons which results in a signal to the sphenopalatine ganglion cells. The final result is a strong parasympathetic signal into the nasal mucosa which gives rise to increased secretion and vasodilatation. The initial irritation also induces the release of mediators (neuropeptides) from sensory nerves in the nasal mucosa which results in increased vasodilatation, vascular permeability and secretion (24). This is known as the anti-dromic reflex. In NANIPER patients the last mechanism may be „overheated".

TREATMENT MODALITIES

Therapy for NANIPER is symptomatic and includes pharmacotherapy and surgery.

Pharmacotherapy in NANIPER
Topical sympathicomimetica (xylometazoline e.g.) provide relief only for a short period after which rhinitis medicamentosa will coexist with the original disease (27).
Systemic symphaticomimetic decongestants are not allowed in the Netherlands and seem to have many side effects (28).
Patients who complain of excessive rhinorrhea are successfully treated with the local anticholinergic agent ipratropium bromide (Atrovent) (29-38).
The first studies showing efficacy of topical steroids in NANIPER were performed in the late seventies and in the beginning of the eighties (9, 38-41). Recent studies using fluticasone propionate aqueous nasal spray for the treatment of NANIPER have shown an efficacy comparable to the efficacy of topical steroids in allergic rhinitis (42, 43). Philips, in 1995, stated however, that although some clinical efficacy has been demonstrated in non-allergic non-infectious perennial rhinitis (NANIPER), these agents often do not provide the same relief as they do in allergic rhinitis (44).
Capsaicin is the pungent agent in red peppers. Its mode of action is well documented in rodents, where it affects mainly the thin unmyelinated sensory nerve fibers. It causes initial stimulation (with release of endogenous neuropeptides), followed by desensitization to capsaicin and other sensory stimuli (45). With higher doses long term functional or even morphological ablation of the thin sensory neurons occur (46). Several not placebo controlled studies have been published showing that capsaicin desensitization might be an important therapeutic modality in NANIPER (22, 47-49).

Surgery in NANIPER
Surgical procedures for NANIPER aim to either modify the size of the inferior turbinate or to derive the nasal mucosa of its autonomic supply. The surgical scalpel, chemical sclerosing solutions, electrocautery, cryosurgery, snake venom and laser surgery have all been reported to diminish obstruction complaints (50-64). The duration of effectiveness is 6 months to several years (14). The aim of this therapy is to diminish hyperfunction of glandular and vascular elements while preventing the kind of destruction that impairs normal mucosal functions such as humidification, mucosal transport and nasal passage. Adhesions, atrophic rhinitis and even blindness have been described as complications of the former therapies.
Golding-Wood described the effect of vidian neurectomy (65, 66). This procedure is effective in relieving excessive secretion but not the obstruction. Both parasympathetic and sympathetic fibers are interrupted. The net effect is anticholinergic. Grote concluded that

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vidian neurectomy was not the panacea it was claimed to be, since renervation would occur (67). This was corroborated by several authors (56, 57).

STUDY DESIGN; OBJECTIVES

This study focuses on answering the following questions:
1. Can we accurately define, select, and study a group of NANIPER patients?
2. Is NANIPER a chronic inflammatory disorder, and if so, are inflammatory cells involved in the pathogenesis of NANIPER?
3. Are local steroids effective in NANIPER? Can we explain this effect? Can we discern subgroups according to the response to steroids?
4. Is local capsaicin effective in NANIPER? What could be the working mechanism?

In chapter 2 the selection criteria for NANIPER are formulated. Using nasal biopsies, the involvement of inflammatory effector cells, the possibility of local allergy, and neurogenically induced mast cell degranulation are studied. Nasal brushes are taken to establish the NANIPER/NARES relation.

In chapter 3 the patient and control group are extended and, using nasal biopsies, immunocompetent regulatory cells are studied.

Chapter 4 describes the effect of fluticasone propionate aqueous nasal spray on nasal complaint scores and cellular infiltrates in NANIPER patients.

Chapter 5 deals with the effect of capsaicin on nasal complaints and inflammatory mediators in NANIPER patients.

Chapter 6 describes the effect of capsaicin on inflammatory cells in the nasal mucosa and nerve tissue.

In chapter 7 the experiments are summarized. The role of immunocompetent cells in NANIPER are discussed and the therapies are evaluated.

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Chapter 2

Mast Cells, Eosinophils and IgE-positive Cells in the Nasal Mucosa of Patients with Vasomotor Rhinitis.
An Immunohistochemical Study

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SUMMARY

Forty patients suffering of NANIPER were carefully selected on the basis of inclusion and exclusion criteria proposed by Mygind and Weeke. Nasal biopsy specimens were taken in the patient group as well as a group of ten controls. Brush cytology was also taken in the NANIPER group. Inflammatory cells were identified and counted in the nasal mucosa with the use of immunohistochemical techniques and a panel of monoclonal antibodies. Eosinophils were studied with the use of BMKI3, EG2, and Giemsa. Mast cells were studied with anti-chymase (B7), anti-tryptase (G3) and toluidine blue. Sections were stained with IgE as well. There was no significant difference in the number of eosinophils, mast cells and IgE-positive cells between the two groups. Additionally in contrast with other reports, in sections that were double-stained with anti-chymase and anti-tryptase, single chymase positive cells were found.

INTRODUCTION

Rhinitis is subdivided into a number of different entities, one of which is non-allergic non-infectious perennial rhinitis (NANIPER) (12). This term usually describes a chronic type of rhinitis with nasal congestion, rhinorrhoea and sneezing for which no plausible explanation can be found. In 1981 NANIPER was subcategorized based on nasal eosinophilia and the term "NARES" (non-allergic rhinitis with eosinophilia syndrome) was introduced (7).

By its nature as a diagnosis by exclusion, NANIPER represents a heterogeneous group of pathophysiological conditions. Faced with this group of patients with non-atopic nasal complaints, we excluded all patients with systemic, medical, and anatomical disorders that could explain complaints of rhinorrhea, sneezing, and nasal obstruction. This group with unexplainable nasal complaints was then homogenized on the basis of a daily record chart on which patients had to reach a minimum symptom score. The minimum was set using as a basis the definition of rhinitis put forward by Mygind and Wihl (25) in 1985. In affected patients periods of nasal discharge, sneezing and congestion had to persist for an average of at least 30 minutes to 1 hour per day.

Since NANIPER, according to prevailing theory, is thought to be the result of a neurogenic disorder (8), various authors have focussed on the neurogenic system by studying, for example, nasal mucosal innervation or neuropeptide distribution (26). Others have examined functional aspects using such provocational agents as histamine and metacholine (5), neuropeptides (26), and non-pharmacological agents such as cold, dry air (23), saline solutions and iso-osmolar ultrasound mist. Different therapeutic regimens including such treatments as topical steroids (18), ipratropium bromide (1), and various surgical interventions (9, 16, 19) have been studied as well.

In the present study we investigated the cellular infiltrates in the nasal mucosa of patients with a known NANIPER. To our knowledge this subject has not been addressed before. It is well known that in atopic rhinitis, for example, cells such as eosinophils, mast cells, antigen presenting cells and T-cells present in the nasal mucosa are involved in the pathogenesis and sustaining of this disorder (4.11). To study the cellular infiltrates in the nasal mucosa we took brush samples from NANIPER patients and biopsy samples from both NANIPER patients and controls. Biopsies were then studied for the presence, localization, and activation state of eosinophils as well as the occurrence and localization of mast cells and other surface IgE-positive cells.

MATERIALS AND METHODS

Patients were studied from 1988 to 1992 in the outpatient ENT Department of Leyenburg Hospital in The Hague, the Netherlands. Patients were admitted to the study if they had a history of nasal complaints such as nasal obstruction, sneezing and rhinorrhea for a period of more than 1 year and these symptoms could not be attributed to an atopic rhinitis, nasal or paranasal sinus infection, anatomical disorders affecting nasal function, pregnancy or lactation and/or systemic disorders (Table 1).

Inclusion criteria
- Age between 16 and 65 years
- Negative skin prick test and negative RAST score
- Symptoms for more than 1 year
- A cumulative score of 5 or more for the following nasal parameters:
  blockage, clear discharge and sneezing for at least 7 days during a period of 14 days.

Exclusion criteria
- The use of systemic or inhaled corticosteroids within the previous month
- The use of inhaled sodium cromoglycate or nedocromil sodium within the previous month
- The use of astemizole within the previous month
- Inability of the patient to stop taking therapy affecting nasal function
- A serious and/or unstable disease
- Nasal surgery within the previous 3 months
- Significant anatomical abnormalities affecting nasal function
- Nasal polyps of a history of nasal polyps
- Nasal or paranasal sinus infection
- Abnormal sinus X-ray
- Pregnancy or lactation
- Abnormal laboratory results for:
  blood: Na, K, Ca, total protein, albumin, urea creatinine, bilirubin, alkaline, phosphatase, aspartate aminotransferase, alanine aminotransferase, gamma glutamyl transpeptidase, hemoglobin, red blood cell count, plasma cell volume, mean corpuscular volume, platelets, total white blood cell count, neutrophils, lymphocytes, monocytes, eosinophils, basophils
  urine: blood, protein, glucose
- Abnormal findings at physical examination

Table 1 Criteria for the selection of patients with non-allergic non-infectious perennial rhinitis

Patients with nasal polyps were also excluded since they may belong to a different pathophysiological group and their polyps may contribute to a higher symptom score for nasal blockage and/or rhinorrhea. Of those individuals selected, 155 patients scored their nasal complaints for a period of 2 weeks, using our daily record their nasal complaints for a period of 2 weeks using our daily record nasal complaints for a period of 2 weeks using our daily record chart (DRC) (Table 2). The duration of complaints during the day was then used as the prime criterion for further study (25). Patients had to have a cumulative score of 5 or more for the following nasal parameters: blockage, clear nasal discharge, and sneezing for at least 7 days eligible for our study and participated under conditions of informed consent.

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Possible scores on the daily record chart	
Nasal blockage:	0 = absent
(not being able to breathe freely through the nose)	1 = between 0-1h per half day
Clear nasal discharge: (runny nose)	2 = between 1-2 h per half day
	3 = more than 2 h per half day
Sneezing	0 = absent
Coughing	1 = less than 5 periods per half day
	2 = between 5-10 periods per half day
	3 = more than 10 periods per half day
Mucus production:	0 = absent
(yellow, green or brown)	1 = present

Table 2. Scheme of the daily record chart for defining nasal symptoms in patients with NANIPER (VMR).

A nasal brush sample for cytology and a mucosal biopsy specimen were taken from all 40 patients. Ten healthy volunteers without nasal complaints or nasal abnormalities on ENT examination and a negative skin prick test were biopsied once and tissue specimens used as controls.

Nasal biopsies

At the time of biopsy, all patients had nasal complaints, as confirmed by their DRCs. After randomization of the biopsy side, specimens of nasal mucosa were taken from the lower edge of the inferior turbinate, about 2 cm posterior to the front edge, using a Gerritsma forceps with a cup diameter of 2.5 mm (3).

Local anesthesia was obtained by placing a cotton-wool carrier with 50 mg cocaine and one drop of adrenaline (1:1000) under the inferior turbinate but without touching the biopsy site. The specimens were embedded in Tissue-Tek II O.C.T. compound and frozen immediately.

Nasal brush cytology

Contralateral to the biopsy side a nasal brush sample was taken from the middle nasal fossa using the Gynobrush (Medeco, Eindhoven, The Netherlands). This is a modification of the technique advocated by Pipkorn (10). In our experience, this brush is less painful than the Rhinobrush (also Medeco). The brush was immediately placed in RPMI. Within 3 days cytospin preparations were made and cells were stained with Giemsa and toluidine blue.

STAINING PROCEDURES

Eosinophils

The monoclonal antibodies (mAb) BMK13, EG2 and Giemsa (Table 3) were used together with the immuno-alkaline phosphatase, anti-alkaline phosphatase (APAAP) method. BMK13 is a mAb against major basic protein and is reported to stain 95-97% of all eosinophils (13). EG2 is a mAb against eosinophilic cationic protein and stains the activated eosinophils (22).

Sections of nasal mucosa were cut 6 micrometer thickness on a cryostat (Jung Frigocut 2800E/20/40), transferred to poly-L-lysine-coated microscope slides, dried and fixed in acetone for 10 min at 20 degrees C. They were next rinsed in phosphate-buffered saline (PBS, pH 7.2), placed in a half-automatic stainer (Sequenza, Shandon), incubated with 2% bovine serum albumin in PBS for 10 min and incubated with normal rabbit serum (CLB, Amsterdam, the Netherlands) for 10 min. Following this the slides were incubated with the mAb for 30 min at 20 degrees C, rinsed in PBS and TRIS buffer (pH 8.0), and incubated for 30 min with a new fuchsin substrate (Chroma, Kongen, Germany). Finally, sections were rinsed with distilled water, counterstained with Mayer's hematoxylin, and mounted in glycerin-gelatin. Control staining was performed by substitution with PBS and incubation with an irrelevant mAb of the same subclass.

Antibody	Titer	Specificity	Source
BMK13	1:200	MBP	Sanbio, Uden, NL
EG2	1:40	ECP	Pharmacia, Woerden, NL
Anti-IgE	1:250	IgE	Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, NL
B7	1:100	Chymase	Chemicon, Temecula, California, USA
G3	1:250	Tryptase	Chemicon, Temecula, California, USA
Histochemical dyes			Source
Giemsa			Merck, Amsterdam, NL
Toluidine blue			BDH, Dorset , UK

Table 3. Monoclonal antibodies and histochemical dyes used to study mucosal biopsies in patients with NANIPER (VMR) and controls. (MBP: major basic protein, ECP: eosinophilic cationic protein)

Mast cells

Toluidine blue, an aniline dye, stains mast cells metachromatically. Tissue sections were stained with toluidine blue at pH 0.5 for 5 min and counts were performed immediately (4). The mAbs anti-chymase (B7) and anti-tryptase (G3) are mast cell specific (6). To check our atopic screening, the biopsy material was also stained with anti-IgE, since atopic patients usually have a large number of IgE-positive cells present in biopsy sections (4). For staining with anti-IgE, G3 and B7 (Table 3) supersensitive AP was used (BioGenex AZ000UM). This protocol followed the APAAP protocol up to the first PBS rinse. Sections were then incubated with

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normal goat serum (CLB, Amsterdam, the Netherlands) for 10 min and then for 60 min with the mAb. The sections were rinsed with PBS for 5 min and successively linked with biotinylated anti-mouse serum for 30 min, rinsed with PBS for 5 min and labeled with streptavidin-AP (ssAP) for 30 min. They were next rinsed in PBS for 5 min and TRIS buffer (pH 8.0) for 5 min and then incubated for 30 min with new fuchsin, after which the protocol again conformed to the APAAP protocol. Furthermore, for a general evaluation and control counting of eosinophils, hematoxylin-eosin (HE) and Giemsa staining were performed.

Double staining of mast cells was achieved by a 60 min. incubation with biotinylated anti-chymase, linked with anti-biotine alkaline-phosphatase for 30 min. After a 60 min. incubation with anti-tryptase, linking is performed for 30 min. with rabbit anti-mouse peroxidase and then a 30 min. labeling is performed with peroxidase anti-peroxidase after which samples were incubated with fast blue and AEC substrate. Slides were mounted in glycerin.

LIGHT MICROSCOPIC EVALUATION

Stained cells were counted in two sections of each biopsy specimen. The epithelium and lamina propria were evaluated separately. The total surface area of a section and its main parts (i.e. the epithelium and lamina propria) were estimated with the use of the Kontron Image Analysis System Videoplan. The number of cells/mm^2 was calculated for the epithelium and the lamina propria.

STATISTICAL ANALYSIS

The Mann-Whitney U-test was used to compare the differences in cell counts between the groups. A P value < 0.05 was considered to indicate a significant difference.

RESULTS

Biopsy specimens
The sections of nasal mucosa had an average surface area of 1.5 mm^2 and were generally of good quality. All but two biopsy specimens were evaluated. One exclusion was made because of artifact resulting from defrosting of the specimen and the other specimen was displaced. The mAb-APAAP and the mAb-ssAP staining showed red cells against a blue counterstained background. After toluidine-blue staining, mast cells could easily be identified by their dark-violet, metachromatic granules against a background of faintly stained tissue.

Eosinophils

The numbers of Giemsa-positive, BMK 13-positive and EG2-positive cells/mm^2 are shown in Table 4. Virtually no eosinophils were present in the lamina propria and the epithelium of both groups and any differences between the two groups were not statistically significant.

Mast cells and other IgE-positive cells

The numbers of toluidine-blue-positive, G3-positive, B7-positive and anti-IgE positive cells/mm^2 are shown in table 4. The number of B7-positive cells tended to be higher than the number of G3-positive cells in both patients and controls. Both tended to be higher than the number of toluidine-blue-positive cells. There were no significant differences between the two groups as to cell numbers in the epithelium and lamina propria for the various staining methods. Biopsy specimens from 2 of 40 patients showed substantial numbers of eosinophils, mast cells, and IgE-positive cells.

Eosinophils	Patients (n=40) Median (range)	Controls (n=10) Median (range)	P-value
Epithelium			
BMK13	0 (0-281)	0 (0-53)	Not significant
EG2	0 (0-128)	0 (0-0)	Not significant
Giemsa	0 (0-209)	0 (0-9)	Not significant
Lamina propria			
BMK13	0 (0-138)	0 (0-6)	Not significant
EG2	0 (0282)	0 (0-0)	Not significant
Giemsa	0 (0-124)	0 (0-1)	Not significant

Mast cells and IgE+ cells	Patients (n=40) Median (range)	Controls (n=10) Median (range)	P-value
Epithelium			
Toluidine blue	0 (0-138)	0 (0-4)	Not significant
Anti-tryptase	0 (0-282)	0 (0-29)	Not significant
Anti-chymase	0 (0-40)	0 (0-3)	Not significant
Anti-IgE	0 (0-480)	0 (0-198)	Not significant
Lamina propria			
Toluidine blue	22 (0-101)	17 (9-51)	Not significant
Anti-tryptase	74 (1-162)	70 (9-96)	Not significant
Anti-chymase	75 (25-238)	54 (24-83)	Not significant
Anti-IgE	21 (0-338)	7 (0-152)	Not significant

Table 4. Medain and range (-) of numbers of eosinophils , mast cells and other IgE+ cells in the nasal mucosa of patients with NANIPER (VMR) and controls.

Brush material

A total of 500 cells were counted per cytospin. Toluidine-blue-positive cells were found in just one cytopsin (50 toluidine-blue-positive cells per 500 counted cells). Eosinophils were not found.

DISCUSSION

We selected 155 patients with a history of nasal complaints for which no explanation could be found. However, only 40 of these patients satisfied our condition for inclusion in our study of nasal complaints for more than 1 h a day. That patients often overestimate nasal complaints underscores the importance of the use of DRCs to characterize patients objectively. The duration of complaints was used as our prime criterion. It is also common knowledge that subjective complaint scores in which the intensity of the complaints are graded can be influenced by a patient's state of mind. On a bad day, patients suffer more. Moreover, it is easier for a patient to score the duration of the complaints than to grade intensity. Biopsies from two patients with NANIPER had substantial numbers of IgE-positive cells, mast cells, and eosinophils. As a consequence, we feel that these patients were indeed allergic in spite of their negative allergy tests. The data of these two patients were included since they satisfied our overall inclusion criteria.

BRUSH CYTOLOGY

NANIPER patients with prominent nasal eosinophilia of 20% or more are subcategorized as NARES patients. In general the NARES group comprises 10 - 13% of the NANIPER patients (14). Contrary to expectations, none of our patients showed eosinophilia in cytospin preparations of brush samples. This might be explained by the eosinophilic subgroup being more susceptible to therapy (20). The use of nasal corticosteroids has gained increasing acceptance by the general practitioner during the last decade. It is thus possible that most NARES patients are less symptomatic with nasal corticosteroid spray and are therefore not referred to the ENT surgeon for further care. This hypothesis is supported by the finding in our group of patients that congestion was the primary symptom. In NARES patients sneezing and clear rhinorrhea are the main symptoms. Moreover, although in the eosinophilic subgroup polyps are seen clinically in 30% of cases, polyps were an exclusion criterion in our series.

BIOPSY SPECIMEN

Eosinophils
Since virtually no eosinophils were found with Giemsa or BMK13, not to mention activated eosinophils (i.e., EG2-positive cells), in biopsy specimens from our patients and controls, we currently believe that these cells are not important in the pathogenesis of NANIPER, excluding NARES.

Mast cells

Anti-tryptase is reported to stain all mast cells while some of these tryptase-positive cells are also positive for chymase (6). Contrary to our expectation, the number of chymase-positive cells were not lower than the number of tryptase-positive cells in our present study. Additionally, in sections that were double-stained with anti-chymase anti-tryptase, single chymase-positive cells were found (figure 1: page 102). We think that this is the result of our fixation method. While other authors all use Carnoy's solution for mast cell fixation, we fixed our sections with acetone. We then found fixation with Carnoy's solution drastically reduced the numbers of chymase-positive and toluidine-blue-positive cells, whereas the number of tryptase-positive cells was only slightly reduced. We therefore prefer acetone fixation.

Apart from the two biopsies that were previously mentioned, virtually no mast cells were found in the nasal epithelium from controls and patients. As reported by Okuda et al. (17) the predominant metachromatic cell in the lamina propria of the nasal mucosa is the mast cell, the basophilic neutrophil that can also be stained with toluidine blue, is generally not present in the lamina propria of the nasal mucosa. This is in accordance with our findings.

To our surprise we found no evidence for mast cell involvement in NANIPER. In allergic rhinitis cross-linking of IgE on the membrane of the mast cells and the subsequent release of mast cell mediators (degranulation) is thought to contribute substantially to the complaints associated with rhinitis. We detected no specific IgE (by RAST and skin-prick test) and there was no significant difference in the number of IgE-positive cells between our NANIPER patients and the controls. Apart from the two biopsies with substantial numbers of eosinophils, mast cells and anti-IgE positive cells, no evidence for degranulation as a results of IgE cross-linking was found. Separate studies have shown that neuropeptides have been found to induce degranulation in mast cells (2). Support for neuropeptide involvements in NANIPER has been found by investigations demonstrating a reduction in nasal complaints associated with a depletion of sensory neuropeptides by prior treatment with capsaicin (21). A recent study by Lacroix's group (11) also found an increased concentration of sensory neuropeptides in the nasal mucosa of patients with chronic non-allergic rhinitis.

One could hypothesize that an increased neuropeptide content could induce an ongoing degranulation of mast cells and thus a release of mast cell mediators, resulting in a possible decrease in mast cell numbers. However, a reduction in mast cell numbers was not found in our group of patients. Further evaluation of mast cell degranulation by electron microscopy in studies is in progress. In the near future we will report on the involvement of T-cell subsets, and other inflammatory cells, including cytokine production.

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Chapter 3

Inflammatory Cells are not Involved in Non-Allergic Non-Infectious Perennial Rhinitis.

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SUMMARY

Mucosal inflammatory cell densities are correlated with nasal complaints in NANIPER. Some authors suggest inflammation of neurogenic or immunogenic nature as underlying disorder for NANIPER. We examined whether inflammatory cells are involved in the pathogenesis of NANIPER. Nasal biopsies were taken of sixty-five patients with significant nasal complaints and twenty controls without nasal complaints. Inflammatory cells were quantified, using monoclonal antibodies directed against lymphocytes, antigen presenting cells, eosinophils, mast cells, macrophages and monocytes. No significant differences were found, for any cell, between patients and controls. We conclude that inflammatory cells are not involved in NANIPER.

INTRODUCTION

Non-allergic non-infectious perennial rhinitis, henceforth referred to as NANIPER, is a diagnosis by exclusion. This disorder probably represents a heterogeneous group of pathophysiological conditions. Proposed mechanisms include a chronic inflammatory disorder of antigenic or neurogenic nature.

Neurogenic

Wolf suggested that NANIPER could be the result of an "over-active" non-adrenergic non-cholinergic system (1). Stimulation of sensory neurons results in sensory nasal changes, rhinorrhea (2), nasal blockage and sneezing. Sensory neural stimulation may produce these effects either through a central neural reflex, associated with efferent parasympathetic neurotransmission, or via anti-dromic release of neuropeptides from sensory neurons (3). This hypothesis was corroborated by the findings of Lacroix who reported an increased concentration of neuropeptides in a group of chronic non-allergic rhinitis patients (4), improvement of symptoms by local treatment of capsaicin giving a 50% reduction in CGRP-li content in nasal biopsies (5), and a correlation between symptoms intensity and CGRP-Li concentration in nasal mucosa (6).

An increase of proinflammatory neuropeptides could result in a stimulation of T-cell proliferation, stimulation of mast cells, macrophages and eosinophils, and chemoattraction of eosinophils and neutrophils (7). Substance P is able to increase the percentage of neutrophils recovered from nasal lavage (8). Capsaicin, a specific activator of sensory nerve endings, induces a neurogenic inflammation, with an influx of inflammatory cells in nasal lavage after a single provocation (9). In 1990, Moneret- Vautrin (10) suggested that non-allergic rhinitis with eosinophilia syndrome (NARES), a subgroup of vasomotor rhinitis (11), originates as a neurogenic inflammation.

Antigenic

Another theory concerning the pathogenesis of NANIPER is that of a local, occult allergy (12). The diagnosis of NANIPER is made by exclusion. An allergy test is not 100% sensitive and systemic manifestations, such as a positive skin prick test or RAST,. of atopic disease might be missed because the nose is a small shock organ. In seasonal or perennial allergic rhinitis increased numbers of inflammatory cells, such as e.g. Langerhans cells, IgE positive cells, and eosinophils, can be found in the nasal mucosa as a sign of inflammation (13-15).

In order to determine whether there is an increase of inflammatory cells in the nasal mucosa of NANIPER patients we performed a nasal biopsy study in 65 NANIPER patients and 20 healthy controls.

MATERIALS AND METHODS

Patients and controls
The selection of patients has been described before (20). In short the 65 patients (male/female 32/33); mean age of 34 years (17-62 years), 20 non-smokers, 16 ex-smokers (had not smoked for more than 1 year) and 29 current smokers, 1 Oriental, 56 Caucasian, 6 Asian, and 1 African. They all had negative skin prick test and RAST. All patients had complaints of nasal obstruction, and/or rhinorrhea and/or sneezing for more than 1 hour per day for at least 5 days during a period of 14 days.
The controls consisting of twenty healthy volunteers (male/female 11/9); mean age 36 years (18-62), 9 non-smokers, 2 ex-smokers, 9 current smokers, 16 Caucasian, 3 Oriental, and 1 Asian, without nasal complaints or nasal abnormalities on ENT-examination, a negative skin prick test for the common inhalation allergens (16) and a negative Phadiatop (Pharmacia, Uppsala, Sweden). Patients and controls were biopsied once. Procedures were approved by the local Medical Ethics committees.

Nasal biopsies
At the time of the biopsy, all patients had nasal complaints, as confirmed by their daily record charts. After randomization of the biopsy side, specimen of nasal mucosa were taken from the lower edge of the inferior turbinate, about 2 cm posterior to the front edge, using a Gerritsma forceps with a cup diameter of 2.5 mm. (17). Local anesthesia was obtained by placing a cotton-wool carrier with 50 mg of cocaine and one drop of adrenaline (1:1000) under the inferior turbinate without touching the biopsy site. The specimen were embedded in Tissue-Tek II O.C.T. compound and frozen immediately.

Staining procedures
Monoclonal antibodies (mAb) directed against CD1, CD3, CD4, CD8, CD14, CD68, CD25, chymase, tryptase, IgE, and BMK13 (table 1) were used together with the super sensitive

on a cryostat (Jung Frigocut 2800E/20/40), transferred to poly-L-lysine-coated microscope slides, dried, and fixed in acetone for 10 min at room temperature (RT). They were then rinsed in phosphate-buffered saline (PBS, pH 7.2), placed in a half-automatic stainer (Sequenza, Shandon), incubated with 2 % bovine serum albumin in PBS for 10 min and incubated with normal goat serum (CLB, Amsterdam, The Netherlands) for 10 min. Following this the slides were incubated with the mAb for 30 min at RT.

Antibody	Specificity	Titer	Source
CD1	OKT6	1:100	Dept. Immunology, Erasmus University, Rotterdam, The Netherlands (NL)
CD3	leu4	1:25	BDH, Dorset, UK
CD4	leu3	1:50	
CD8	leu2	1:100	
CD25	IL2-r	1:150	
B7	Chymase	1:100	Chemicon, Temecula, Calif, USA
G3	Tryptase	1:250	
BMK13	MBP	1:200	Sanbio, Uden, NL
CD14	mon/1	1:20	Central laboratory of the Netherlands Red Cross Blood
anti-IgE	IgE	1:250	Transfusion service (CLB), Amsterdam, NL
CD68	KI-M6	1:50	Behring, Marburg, Germany

Table 1. Monoclonal antibodies used to study mucosal biopsies in patients with NANIPER and controls.

The sections were then rinsed again in PBS for 5 min and incubated for 30 min with a biotinylated goat anti-mouse (1:50) immunoglobulin antiserum, rinsed successively in PBS, incubated with strept Avidin AP (1:50) (Biogenics, Klinipath, Duiven, The Netherlands) for 30 min at RT, rinsed in PBS and TRIS buffer (pH 8.0), and incubated for 30 min with a new fuchsin substrate (Chroma, Kongen, Germany). Finally, sections were rinsed with distilled water, counterstained with Gills hematoxylin and mounted in glycerin-gelatin. Control staining was performed by substitution with PBS and incubation with an irrelevant mAb of the same subclass.

Light-microscopic evaluation
Stained cells were counted in two sections of each biopsy specimen. The epithelium and lamina propria were evaluated separately. The total surface area of a section and its main parts (i.e. the epithelium and the lamina propria) were estimated with the use of the Kontron Image Analysis System Videoplan. The number of cells/mm^2 was calculated for the epithelium and the lamina propria.

Statistical analysis.
The non-parametric Mann-Whitney U-test was used to compare the differences in cell counts between the groups. A p-value < 0.05 was considered to indicate a significant difference.
The mean difference between patients and controls is considered significant if it exceeds twice its standard error. To determine whether the groups in this study were large enough to validate our conclusions (to avoid a type 2 statistical error). The standard error of the mean

difference after Ln-transformation of the cell counts was determined in order to compensate for the skewness of the cell counts to justify parametric testing. The antilog of twice this difference gives the smallest ratio between the geometric means (estimation of the median if the variables have a normal distribution after Ln transformation) of both groups that would be significant at the 5% level in our study. For instance, if the geometric mean of the control group were 10 and the calculated ratio 2.6, the geometric mean of the patient group must exceed 26 in order to reach significance in the measurements. All analysis were performed on a personal computer using statistical software (SPSS 6.0.1 for Windows).

RESULTS

Biopsy specimen

The sections of the nasal mucosa had an average surface area of 1.6 mm^2 and usually showed a lining of ciliated columnar epithelium with or without goblet cells and/ or partially stratified cuboidal epithelium. The lamina propria consisted usually of a looser subepithelial cell-rich layer with most of the mucous glands and a deeper collagenous cell-poor layer. All sections were sufficiently deep to assess both layers. The sections were generally of good quality. Two biopsy specimen could not be evaluated (18). The mAb-ss-AP staining showed red cells against a blue counterstained background. Biopsy specimens from 2 of the 65 patients showed substantial numbers of eosinophils, langerhans cells, mast cells and IgE-positive cells.

T-lymphocytes

These small round cells were abundantly present in the epithelium as well as in the lamina propria. Sometimes, clusters of T-cells (500-1000 cells) were found in the lamina propria. The occurrence of these clusters did not differ between the groups.

The number of CD3, CD4 (figure 6, page 107: G, H), CD8 (figure 6, page 107: E, F), and CD25 positive cells/mm^2 are shown in table 2. As can be seen, hardly any IL-2 receptor (CD25) positive cells were found in either layer of the nasal mucosa. If there were any differences between the two groups at all, they were not statistically significant. The calculated ratios indicating threshold significance for the groups were respectively: CD3 epithelium (EP) 1.57, CD3 lamina propria (LP) 1.47, CD4 EP 1.78, CD4 LP 1.5, CD8 EP 1.88, CD8 LP 1.73, CD25 EP 2.63, CD25 LP 2,38.

Langerhans cells

This large dendritic cell was found mostly in the epithelium. Only a few were present in the lamina propria. The numbers of CD1-positive cells are shown in table 2. No significant

differences were found. The calculated ratios indicating threshold significance for the groups were: CD1 EP 1.86, CD1 LP 2.20

Cell type	Controls Median(25 %-75%)	Patients Median(25%-75%)	p-value
Epithelium			
CD1	48(15-130)	54(15-110)	0.82
CD3	512(299-867)	630(347-1079)	0.27
CD4	545(341-755)	424(223-584)	0.18
CD8	305(173-431)	446(163-762)	0.11
CD14	310(130-497)	215(179-316)	0.68
CD25	8(0-30)	0(0-25)	0.43
BMK13	0(0-0)	0(0-0)	0.60
Tryptase	0(0-4)	0(0-4)	0.88
Chymase	0(0-0)	0(0-8)	0.06
IgE	0(0-0)	0(0-28)	0.30
CD68	165(89-293)	214(136-378)	0.06
Lamina propria			
CD1	3(1-8)	5(1-13)	0.54
CD3	678(486-832)	552(300-872)	0.31
CD4	464(181-885)	426(259-611)	0.65
CD8	269(160-345)	295(147-476)	0.51
CD14	232(143-367)	196(161-271)	0.58
CD25	7(2-58)	3(0-13)	0.30
BMK13	0(0-0)	0(0-3)	0.18
Tryptase	65(41-71)	69(38-97)	0.30
Chymase	54(47-71)	63(46-100)	0.35
IgE	8(2-62)	22(4-64)	0.67
CD68	145(74-195)	152(101-250)	0.30

Table 2. Median (25th and 75th percentile) of positive cells/mm^2 in epithelium and lamina propria of the nasal mucosa.

Macrophages and monocytes

The CD68 positive cells were large cells with a bright staining cytoplasm. These cells were found to be equally distributed in both layers, as was CD14. The number of CD68 and CD14 cells are shown in table 2. No significant differences were found. The calculated differences indicating threshold significance for the groups were: CD14 EP 1.68, CD14 LP 1.55, CD68 EP 1.44, CD 68 LP 1.52.

Mast cells and other IgE-positive cells

The chymase and tryptase and IgE positive cells (figure 6, page 106: C, D) were found mainly in the lamina propria. The numbers are shown in table 2. No significant differences were found. The calculated differences indicating threshold significance for the groups were: anti-IgE EP 4.22, anti-IgE LP 3.17, tryptase EP 2.62, tryptase LP 1.57, chymase EP 2.47, chymase LP 1.61.

Eosinophils

The number of BMK13 positive cells found in the nasal mucosa of both patients and controls were negligible (figure 6: page 106: A, B). No significant differences were found. The numbers are shown in table 2. The calculated differences indicating threshold significance for the groups were: BMK13 EP 1.96, BMK13 LP 2.21.

DISCUSSION

NANIPER is an intriguing disorder. By strict selection and by using a complaint threshold value, we succeeded in achieving a homogenous group of patients. As NANIPER is reported to account for between 30% and 70% of cases of chronic perennial rhinitis (19), we were surprised to find that only sixty-five out of the 300 selected patients satisfied our inclusion criterions such as nasal complaints for more than 1h/day. This, once again, underlines the importance of the use of nasal symptom scores to characterize the patients objectively (18, 20). The 2 patients, of the total of 65 with negative allergy tests, with a substantial typical cellular allergic infiltrate in the nasal mucosa were classified as possible sufferers of an occult local allergy. This would mean a maximum prevalence of three percent of occult allergy in this group that can be discerned by nasal biopsies. In this NANIPER group no signs of inflammation were found. This contrasts with the findings of Lacroix (4). However, his patients, underwent either functional sinus surgery or were suffering from a drug-induced rhinitis and so probably cannot be characterized as typical NANIPER patients. Moreover, the reported increase of inflammatory cells in his biopsies could well be the result of infection. The presented data is in accordance with recent data by Sanico who was unable to find an

increased responsiveness to capsaicin in a group of 8 non-allergic rhinitis patients. He therefore discarded a central role for capsaicin sensitive nerves (pivotal in the concept of neurogenic inflammation) in the pathophysiology of NANIPER (22). The question then arises as to whether this immunohistochemical evaluation method is sensitive enough to detect significant differences between the groups. The calculated ratios between the geometric means of both groups indicating threshold significance at the 5% level are within the range found in patients with chronic allergic rhinitis (13-15). In these studies, which compare symptomatic allergic patients with asymptomatic controls, cellular differences between patients and controls were indeed found, while the distribution of the number of immunocompetent cells/mm^2 was in the same order of magnitude as in this NANIPER study. We therefore think it is justified to assume that if significant mucosal inflammation would be present, we would have detected it. The lack of differences in cell numbers does not exclude a functional cellular involvement. However, in two recent studies we failed to ascertain a relation between the number of immunocompetent cells and nasal complaints in NANIPER patients (20,21). A significant reduction of immunocompetent cells in the nasal mucosa of NANIPER patients treated with nasal steroids (fluticasone aqueous nasal spray) was not accompanied by a reduction in nasal complaints (20) and ,vice versa, a significant reduction in nasal complaints in a group of NANIPER patients treated with topical capsaicin aqueous nasal spray was not accompanied by a change in inflammatory mediators (21) or a reduction in the numbers of inflammatory cells.

Considering the aforementioned we conclude that inflammatory cells are not involved in NANIPER.

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Chapter 4

The Effect of Nasal Steroid Aqueous Spray on Nasal Complaint Scores and Cellular Infiltrates in the Nasal Mucosa of Patients with a Non-Allergic Non-Infectious Perennial Rhinitis.

H. M. Blom, T. Godthelp, W. J. Fokkens, A. KleinJan, P. G. H. Mulder, E. Rijntjes.

SUMMARY

The efficacy of topical steroids on nasal complaints and the effect on mucosal cell densities was studied in a group of 65 NANIPER patients. Topical corticosteroids are the therapy of choice in non-allergic non-infectious perennial rhinitis (NANIPER). However, the efficacy of the steroid therapy in NANIPER is controversial, as is its mode of action. To our surprise, out of 300 patients initially diagnosed as suffering from NANIPER, only 65 patients with NANIPER reached threshold nasal symptom scores. Patients were randomized into four different treatment regimes. Placebo bi daily (BD) for 8 weeks, fluticasone 200 mg once daily (OD) and placebo OD for 8 weeks, fluticasone 200 mg OD and placebo OD for 4 weeks followed by fluticasone 200 mg BD for 4 weeks, and fluticasone 200 mg (BD) for 8 weeks. A small decrease in nasal symptomatology was found which only reached significance for sneezing. A significant dose dependent decrease in immunocompetent cells was found in nasal biopsies obtained after 4 weeks, and after 8 weeks of treatment. We conclude that topical corticosteroids do not significantly improve nasal symptoms in this group of selected NANIPER patients, even though a significant effect was seen on cells in the nasal mucosa.

INTRODUCTION

Topical corticosteriods became firmly established as the therapy of choice in the treatment of allergic rhinitis in the last decades. Patients suffering from this disorder do greatly benefit by this treatment (1,2). The effects of local steroids in the nasal mucosa in allergic rhinitis has been well documented (3-5). The first studies showing efficacy of topical steroids in NANIPER were performed in the late seventies and in the beginning of the eighties (6-8). Recent studies using fluticasone propionate aqueous nasal spray for the treatment of NANIPER have shown an efficacy comparable to the efficacy of topical steroids in allergic rhinitis (9). Philips in 1995 stated, however, that although some clinical efficacy has been demonstrated in non-allergic non-infectious perennial rhinitis (NANIPER), these agents often do not provide the same relief as they do in allergic rhinitis (10). The etiology of NANIPER has been attributed to, basically, 2 theories (11). The first theory assumes an imbalance between adrenerge and cholinerge innervation of the nasal mucosa (12). In this scenario, underactivity of the sympathetic nervous system leads to nasal obstruction; whereas overactivity of the parasympathetic nervous system leads to rhinorrhoea (13). Support for this theory was found by Wilde in 1996 who showed an abnormal response to isometric exercise in NANIPER, possible due to relative nasal sympathetic hyposensitivity (14).

According to the second theory, NANIPER, could be the result of an "over-active" non-adrenergic non-cholinergic (NANC) system, resulting in a neurogenic inflammation (15, 16). Stimulation of sensory neurons results in sensory nasal changes, rhinorrhoea (17), nasal blockage and sneezing. Sensory neural stimulation may produce these effects either through a central neural reflex, associated with efferent parasympathetic neurotransmission, or via anti-dromic release of neuropeptides from sensory neurons (18). To support this hypothesis, Lacroix reported an increased concentration of neuropeptides in a group of chronic rhinitis patients (19). A theoretical basis, in line with the second theory, for the efficacy of steroid therapy was found when steroids were reported to upregulate neutral endo peptidase, which degrades neuropeptides (20) and inhibit neurogenic plasma extravasation (21).

By its nature as a diagnosis made by exclusion, NANIPER probably represents a heterogeneous group of pathophysiological conditions. To study this disorder (in a second echelon setting) we applied strict selection criteria. We excluded patients with systemic, medical and anatomical disorders that could explain complaints of rhinorrhoea, sneezing, and nasal obstruction. This remaining group was further homogenized on the basis of a daily record chart on which patients had to reach a minimum symptom score.

Using modern immunohistochemical staining methods, no data are available on the effect of local corticosteroid therapy on cellular infiltrates in the nasal mucosa in NANIPER patients.

We studied the effect of different treatment regimes in NANIPER on nasal complaints and cellular infiltrates.

MATERIALS AND METHODS

Patients were studied from 1988 to 1993 in the outpatient ENT departments of the Leyenburg Hospital in the Hague and the Dijkzigt University Hospital in Rotterdam, The Netherlands. Patients were admitted to the study if they had a history of nasal complaints such as nasal obstruction, sneezing, and rhinorrhea for a period of over 1 year which could not be attributed to allergic rhinitis, nasal or paranasal sinus infection, anatomical disorders affecting nasal function, pregnancy or lactation, systemic disorders and/or the use of medication affecting nasal function (table 1). Patients with nasal polyps were excluded, since they may belong to a different pathophysiological group and their polyps may contribute to a higher symptom score for nasal blockage and/or rhinorrhea. Three-hundred patients, with the diagnosis of NANIPER, scored the duration of their nasal complaints, twice daily, for a period of 2 weeks using a daily record chart (DRC) (fig 1).

Possible scores on the daily record chart	
Nasal blockage:	0 = absent
(not being able to breathe freely through the nose)	1 = between 0-1h per half day
Clear nasal discharge: (runny nose)	2 = between 1-2 h per half day
	3 = more than 2 h per half day
Sneezing	0 = absent
Coughing	1 = less than 5 periods per half day
	2 = between 5-10 periods per half day
	3 = more than 10 periods per half day
Mucus production:	0 = absent
(yellow, green or brown)	1 = present

Figure 1. Scheme of the daily record card for defining nasal symptoms in patients with NANIPER (VMR).

In affected patients periods of nasal discharge, sneezing and congestion had to persist for an average of at least 1h per day for at least 5 days during a period of 14 days. The duration of complaints during the day was used as the prime criterion for further study. At every visit the subjects also rated the intensity of their nasal symptoms during the last three days on a visual analogue scale (VAS) (0-10 cm, 0 represented absence of symptoms and 10 represented severe intensity of symptoms). Sixty-five of the 300 patients were found eligible for our study and participated under conditions of informed consent (male/female: 32/33); mean age was 34 years (17-62y). Twenty patients had never smoked, 16 were ex-smokers (had not

smoked for more than 1 year), 29 were current smokers. Ethnic origin of the patients: Oriental 1, Caucasian 56, Negroid 2, Asian 6.

Inclusion criteria
- Age between 16 and 64 years.
- Negative skin prick test: house dust mite, tree pollen mix, grass pollen mix, bijvoet, alternaria, aspergillus, cladosporium, penicillum, dog, cat, parakeet, rabbit, hamster, horse, guinea pig. (ALK-Diephuis, Holland)
- Negative Phadiatop (Pharmacia, Uppsala, Sweden)
- Symptoms for more than 1 year.
- Periods of nasal discharge, sneezing and congestion for an average of at least 1 h per day for at least 5 days during a period of 14 days.

Exclusion criteria
- The use of systemic or inhaled corticosteroids within the previous month.
- Use of inhaled sodium cromoglycate or nedocromil sodium within the previous month.
- Use of astemizole within the previous month.
- Inability of the patient to stop taking medication affecting nasal function.
- A serious and/or unstable disease.
- Nasal surgery within the previous 6 weeks.
- Nasal polyps or a history of nasal polyps.
- Significant anatomical abnormalities affecting nasal function.
- Nasal or paranasal sinus infection (abnormal sinus X-ray).
- Pregnancy or lactation
- Abnormal findings at physical examination.
- Abnormal laboratory results for:
blood: Na, K, Ca, total protein, albumin, urea, creatinine, bilirubin, alkaline phosphatase, aspartate aminotransferase, alanine aminotransferase, gammaglutamyl transpeptidase, hemoglobin, red blood cell count, plasma cell volume, mean corpuscular volume, platelets, total white blood cellcount, neutrophils, lymphocytes, monocytes, eosinophils, basophils.
urine: blood, protein, glucose.

Table 1. Selection criteria for non-allergic non-infectious rhinitis.

Study design

A single investigator, multi-center double blind, placebo controlled study. All patients started with a run-in period of 2 weeks in which they received placebo aqueous spray and recorded their nasal complaints. Eligible patients were randomized into one of the four different treatment regimes; placebo bi daily (BD) for 8 weeks, fluticasone 200 mg once daily (OD) and placebo OD for 8 weeks, fluticasone 200 mg OD and placebo OD for 4 weeks followed by fluticasone 200 mg BD for 4 weeks, and fluticasone 200 mg (BD) for 8 weeks (Fig 2). The treatment period was divided in 2 periods of 4 weeks. Terfenadine tablets (60mg) were used as rescue medication. The study protocol was approved by the ethical review committees, and conducted in accordance with the declaration of Helsinki.
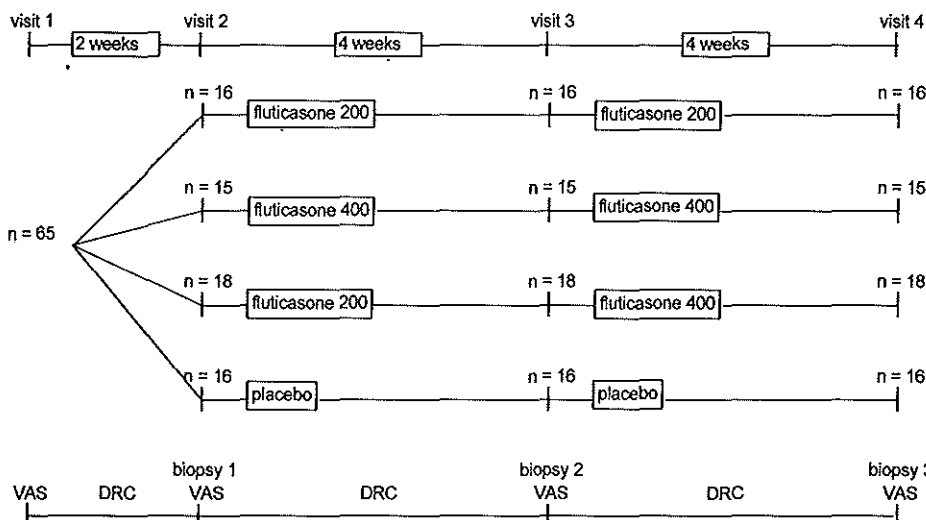
Figure 2. Study design. 200 = FPANS 200 µg once daily, 400 = FPANS 200 µg bi-daily, VAS = visual analogue scale, DRC = daily record chart

Symptomscores

For each of the symptoms of nasal blockage on waking, and nasal blockage during the rest of the day, sneezing on waking and during the rest of the day, and rhinorrhoea on waking and during the rest of the day, the scores were summarized separately by the percentage of symptom-free days as was done by Scadding (9).

The mean sumscore for blockage, sneezing, and congestion was calculated during the week previous to visit 2, the week previous to visit 3, and the week previous to visit 4, since the effect of fluticasone on nasal symptoms in our clinical experience reaches a steady state 1 to 2 weeks after the start of treatment. In order to moderate the fluctuations in DRC scores per patient the mean sumscore of one week was used.

The mean scores for coughing; mucus production; eye irritation; and the number of terfenadine tablets used were recorded.

The VAS was scored each visit, during which the patient was asked to rate the intensity of nasal symptoms during the last 3 days. This was pragmatically considered to be the golden mean between moderating the extremes in nasal symptoms per patient by using several days, and having a reasonable reliable recollection period for the patients' nasal symptoms by taking only 3 days. At each clinic visit the investigator scored the patients' symptoms of nasal blockage, sneezing, rhinorrhoea and post-nasal drip on a severity scale of 0-3 (0= no symptoms; 1 = mild; 2 = moderate; 3 = severe). The nose was assessed by rhinoscopy.

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Turbinate swelling; crusting; bleeding; colour of the mucosa; and secretions were noted as normal or abnormal.

Safety was assessed by monitoring adverse events at each clinic visit. Biochemistry, hematology and urinalysis were evaluated at baseline and at the end of treatment.

Nasal biopsies.

Nasal biopsies were performed after the run-in period, after 4 weeks of treatment and after 8 weeks of treatment in each patient (fig.2). After randomization of the biopsy side, specimen of nasal mucosa were taken from the lower edge of the inferior turbinate, about 2 cm posterior to the front edge, using a Gerritsma forceps with a cup diameter of 2.5 mm (22). Local anaesthesia was obtained by placing a cotton-wool carrier with 50 mg of cocaine and one drop of adrenaline (1:1000) under the inferior turbinate without touching the biopsy site. The specimen were embedded in Tissue-Tek II O.C.T. compound and frozen immediately.

Nasal brush cytology

Contralateral to the biopsy side a nasal brush sample was taken, after the run-in period, from the middle nasal fossa using the Gynobrush (Medeco, Eindhoven, The Netherlands). The brush was immediately placed in RPMI. Within 3 days cytospin preparations were made and cells were stained with Giemsa and toluidine blue (23).

Staining procedures

Monoclonal antibodies (mAb) directed against CD1, CD3, CD4, CD8, CD25, IgE, MBP, Chymase, and Tryptase (table 2) were used together with the super sensitive immuno-alkaline phosphatase (ss-AP) method. Sections of nasal mucosa were cut in 6

Frigocut 2800E/20/40), transferred to poly-L-lysine-coated microscope slides, dried, and fixed in acetone for 10 min at room temperature (RT). They were next rinsed in phosphate-buffered saline (PBS, pH 7.6), placed in a half-automatic stainer (Sequenza, Shandon), incubated with 2 % bovine serum albumin in PBS for 10 min and incubated with normal goatserum (CLB, Amsterdam, The Netherlands) for 10 min. Following this the slides were incubated with the mAb for 60 min at RT. The sections were then rinsed again in PBS for 5 min and incubated for 30 min with a biotinylated goat anti-mouse (1:50) immunoglobulin antiserum, rinsed successively in PBS, incubated with strept Avidin AP (1:50) (Biogenics, Klinipath, Duiven, The Netherlands) for 30 min at RT, rinsed in PBS and TRIS buffer (pH 8.5), and incubated for 30 min with a new fuchsin substrate (Chroma, Kongen, Germany). Finally, sections were rinsed with distilled water, counterstained with Gills hematoxylin and mounted in glycerin-gelatin. Control staining was performed by substitution with PBS and incubation with an irrelevant mAb of the same subclass. Toluidine blue, an analine dye,

stains mast cells metachromatically. The cytospin preparations were stained with toluidine blue at pH 0.5 for 5 min and counts were performed immediately (24). Separate cytospin preparations were stained with May Grunwald-Giemsa (MGG) to study eosinophils.

Antibody	Specificity	Titer	Source
OKT6	CD1	1:100	Dept. Immunology, Erasmus University, Rotterdam
leu4	CD3	1:25	BD, Dorset, UK
leu3	CD4	1:50	BD, Dorset, UK
leu2	CD8	1:100	BD, Dorset, UK
	IgE	1:250	Central laboratory of the Netherlands Red Cross Blood Transfusion service (CLB), Amsterdam, NL
IL-2r	CD25	1:150	BD, Dorset, UK
BMK13	MBP	1:200	Sanbio, Uden, The Netherlands
B7	Chymase	1:100	Chemicon, Temecula, Calif, USA
G3	Tryptase	1:250	Chemicon, Temecula, Calif, USA

Table 2. Monoclonal antibodies used to study nasal mucosal biopsies of patients and controls.

Light-microscopic evaluation

Stained cells were counted in two sections of each biopsy specimen. The epithelium and lamina propria were evaluated separately. The total surface area of a section and its main parts (i.e. the epithelium and the lamina propria) were estimated with the use of the Kontron Image Analysis System Videoplan. The number of cells/mm^2 was calculated for the epithelium and the lamina propria.

Statistics

Statistical analysis of symptomatology was carried out using differences from baseline (Kruskal-Wallis 1-way anova).

Assessment by the investigator was analyzed using Mantel-Haenszel test for linear association.

The biopsy data of the fluticasone 200 OD and the fluticasone 200 OBD was pooled for biopsy number 2 since these groups received the same treatment up to that moment. The Mann-Whitney U-test was used to compare the differences in cell counts between the groups. A P value < 0.05 was considered to indicate a significant difference.

We calculated the Speerman rank correlations between changes in cell numbers and the changes in the VAS scores per randomization group.

RESULTS

Symptom scores

Figure 3 shows the changes in the percentage of symptom free days during the treatment compared with baseline. A small decrease in symptomatology was found, which only reached significance for sneezing. The mean increase in the percentage of symptom free days for sneezing in the FP BD was significantly better than in the placebo group comparing baseline and 8 weeks of treatment. (28 increase in percent points for FP BD vs 5 percent decrease in percent points for placebo). No significant difference between the four treatment groups was seen for coughing; mucus production; eye irritation; and the number of terfenadine tablets used. No significant changes were seen for the mean sumscores (fig. 4) (1 week before each visit) and the VAS score (fig. 5) between the four treatment groups. There were no statistically significant differences between the four treatment regimes in the investigators's assessment of symptoms and rhinoscopy at Clinic visits. We found no correlation larger than 0.7 absolutely which approximately coincides with testing at alpha = 0.01 between cell counts and nasal symptoms, given the size of the randomization groups.

No major adverse events occurred and there were no relevant changes in the routine biochemical, and hematological tests and urinalysis.

Nasal brushes

A total of 500 cells were counted per cytospin. Toluidin blue-positive cells were found in just one cytospin (50 toluidine-blue positive cells per 500). Eosinophils were not found in cytospins stained with MGG.

Biopsy specimens

The sections of the nasal mucosa had an average surface area of 1.6 mm^2 and usually showed a lining of ciliated columnar epithelium with or without goblet cells and/ or partially stratified cuboidal epithelium. The lamina propria consisted usually of a looser subepithelial cell-rich layer with most of the mucous glands and a deeper collagenous cell-poor layer. All sections were sufficiently deep to assess both layers. The sections were generally of good quality. Two biopsy specimen could not be evaluated, one got defrosted, and one was misplaced. The mAb-ss AP staining showed red cells against a blue counterstained background. T-lymfocytes, small round cells, were abundantly present in the epithelium as well as the lamina propria. Sometimes clusters of T-cells were found in epithelium or lamina propria. The occurrence of these clusters did not differ between the groups. Langerhans cells, large dendritic cells, were found mostly in the epithelium. Only a few were present in the lamina propria. Mast cells were found mostly in the lamina propria and hardly in the epithelium.
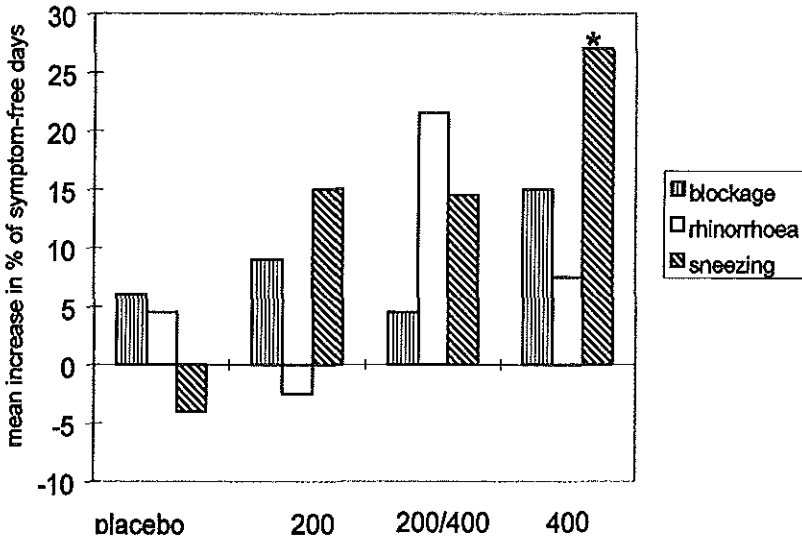
Figure 3
The mean increase in the percentage of symptom-free days recorded on the DRC during days 1-70. * P<0.05: fluticasone treated patients versus placebo.
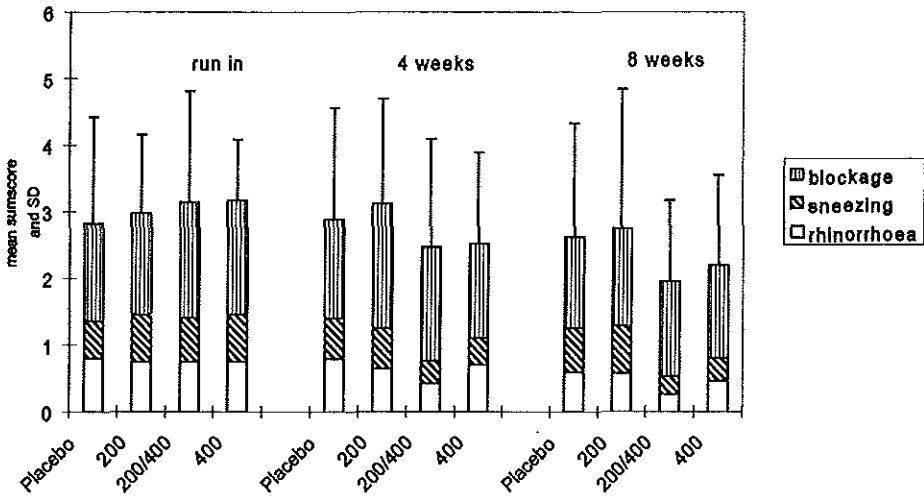Compared were the first 14 days (between visit 1 and 2), and the last 14 days (between visit 3 and 4).

Figure 4
The mean sumscore and standard deviation (SD) are shown for the different treatment regimes in the week preceding each visit.
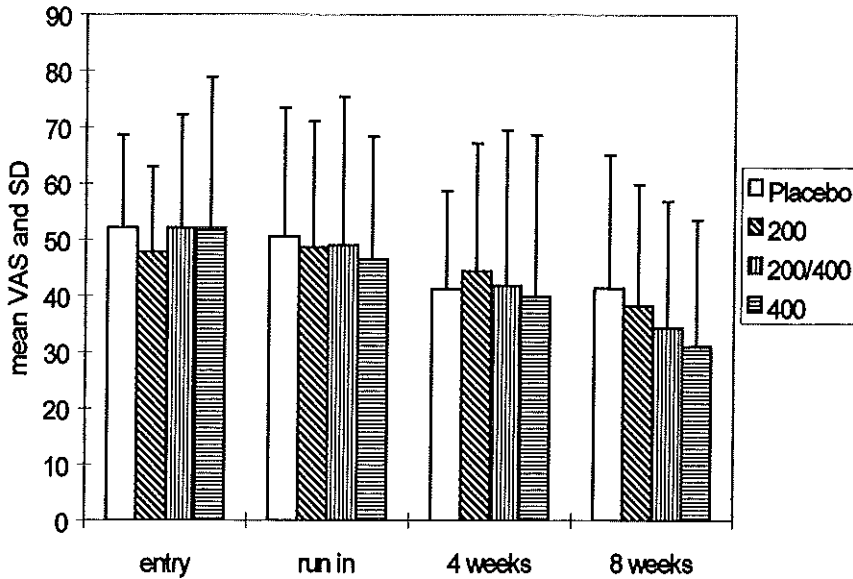
50

Figure 5
The mean VAS and SD are shown as recorded during each visit.

Eosinophils were hardly present in our material. Sometimes moderate infiltrates were found in the mucosa. The occurrence did not differ between the groups in the first biopsies.

Median cell numbers and 25 percentile and 75 percentile are shown for CD1, CD3, CD4, CD8, CD25, IgE, MBP, Tryptase, and chymase positive cells in the epithelium and lamina propria in tabel 3 a and b. Table 4 (after 4 weeks of treatment) and 5 (after 8 weeks of treatment) show the results after statistical evaluation for the different biopsy moments and treatment regimes.

No significant difference was found between the different groups before treatment (biopsy number 1). A marked effect was seen on the number of Langerhans cells and T-cells. The effect of the double steroid dose was more marked than that of the single dose. No additional effect of 4 consecutive weeks of steroid treatment was found after the first 4 weeks of treatment in the epithelium. In the lamina propria 4 extra weeks of treatment seems to effect the mast cells and eosinophils if present.

EPITHELIUM			run in median	25%- 75%	4 weeks median	25%- 75%	8 weeks median	25%- 75%
CD1	placebo		55	22-134	23	0-122	50	27-126
	200		52	4-150	0	0-3	3	0-17
	200/400		83	35-110	0	0-16	0	0-3
	400		50	8-129	0	0-0	0	0-0
CD3	placebo		936	423-1143	488	250-848	347	215-656
	200		623	471-800	115	60-477	145	50-463
	200/400		652	331-1208	192	71-383	65	14-114
	400		403	257-789	151	38-348	210	10-317
CD4	placebo		386	190-544	255	104-514	187	86-331
	200		486	327-1192	63	14-162	78	10-167
	200/400		453	277-674	195	43-343	30	11--76
	400		336	184-716	20	6--93	33	13-98
CD8	placebo		518	288-1018	224	129-629	92	37-241
	200		531	156-997	135	102-335	128	57-145
	200/400		448	127-663	141	73-228	24	9--80
	400		310	133-680	69	14-206	24	7-103
CD25	placebo		20	2—39	15	6--38	20	5--34
	200		0	0-20	4	0-36	4	0-13
	200/400		8,5	0-24	0	0-59	7	0-32
	400		0	0-18	0	0-20	4	0-27
BMK 13	placebo		0	0-7	0	0-5	0	0-10
	200		0	0-8	0	0-0	0	0-0
	200/400		0	0-0	0	0-0	0	0-0
	400		0	0-1	0	0-0	0	0-0
Tryptase	placebo		0	0-20	0	0-0	0	0-2
	200		0	0-0	0	0-0	0	0-0
	200/400		0	0-12	0	0-0	0	0-0
	400		0	0-3	0	0-0	0	0-0
Chymase	placebo		0	0-10	0	0-0	0	0-0
	200		3	0-22	0	0-0	0	0-0
	200/400		2	0-8	0	0-0	0	0-0
	400		0	0-5	0	0-0	0	0-0
IgE	placebo		7,5	0-45	4	0-38	19	0-75
	200		0	0-6	0	0-0	0	0-14
	200/400		0	0-100	4	0-33	10	0-53
	400		0	0-80	0	0-16	0	0-0

Table 3a.
Median cell numbers and 25th percentile and 75th percentile for the various treatment regimes at the end of the run in period, after 4 weeks, and after 8 weeks of treatment in the epithelium.

LAMINA PROPRIA		run in median	25%-75%	4 weeks median	25%-75%	8 weeks median	25%-75%
CD1	placebo	4	1—33	5	0-7	7	2.5-30
	200	3	0-9	0	0-1	1	0-4
	200/400	7	3—14	0	0-6	0	0-2
	400	4	1—18	0	0-0	0	0-0
CD3	placebo	586	446-1104	352	270-613	543	366-688
	200	446	274-804	397	147-537	386	237-814
	200/400	769	495-921	440	253-686	278	199-476
	400	405	226-741	347	271-576	355	125-1137
CD4	placebo	416	289-644	337	211-451	372	267-749
	200	309	206-536	187	139-518	393	161-748
	200/400	479	319-766	335	161-392	300	187-506
	400	325	162-586	152	73-517	284	204-529
CD8	placebo	376	200-655	279	188-329	186	111-399
	200	324	140-415	142	92-340	195	123-401
	200/400	292	174-603	296	216-479	155	108-229
	400	329	151-476	136	87-348	208	93-412
CD25	placebo	10	1—35	12	8--46	30	12--49
	200	0,5	0-5	3	1--3	17	3--38
	200/400	6	0-13	7	0-43	14	6--22
	400	2	0-75	15	1--33	10	5--34
BMK13	placebo	6	0-18	3	1--19	8	1--25
	200	2,5	0-8	0	0-3	0	0-9
	200/400	0	0-3	0	0-13	0	0-2
	400	1,5	0-4	1	0-11	0	0-3
Tryptase	placebo	94	54-131	39	28-75	50	19-78
	200	86	43-117	18	1--58	17	8--68
	200/400	59	33-94	31	16-39	24	14-37
	400	66	34—79	36	13-55	19	10--35
Chymase	placebo	86	47-125	73	46-109	53	25-106
	200	69	39-139	59	27-97	46	18-86
	200/400	61	51-105	53	35-63	49	27-66
	400	62	53-75	43	27-67	25	12--37
IgE	Placebo	36	5—98	24	5--79	32	8--121
	200	6	1—43	19	4--37	11	4--27
	200/400	31	17-99	21	3--59	26	6--69
	400	9	1—52	21	14-47	9	0--27

Table 3b.
Median cell numbers and 25th percentile and 75th percentile for the various treatment regimes at the end of the run in period, after 4 weeks, and after 8 weeks of treatment in the lamina propria.

Epithelium	400 vs 200 + 200/400	200 +200/400 vs placebo	400 vs placebo
CD 1		↓↓	↓↓
CD 3		↓	↓↓
CD 4	↓		↓↓↓
CD 8		↓	↓↓
CD 25			
BMK 13		↓	
Tryptase		↓	
Chymase			
IgE			

Lam Prop	400 vs 200 + 200/400	200 +200/400 vs placebo	400 vs placebo
CD 1		↓	↓↓
CD 3			
CD 4			
CD 8			
CD 25			
BMK 13			
Tryptase		↓	
Chymase			
IgE			

Table 4.
Statistical evaluation after 4 weeks of treatment. The biopsy data of the fluticasone 200 once daily group and the data of the fluticasone 200 once daily for 4 weeks followed by fluticasone 200 bi-daily for 4 weeks is pooled since these groups received the same treatment untill that moment. Arrows indicate significant decrease. One arrow: p<0.05. Two arrows p<0.01. Three arrows p<0.001.

Epithelium	200 vs placebo	400 vs placebo	200/400 vs placebo	400 vs 200	400 vs 200/400
CD 1	↓↓	↓	↓↓↓	↓	
CD 3	↓	↓	↓↓↓		
CD 4		↓↓	↓↓↓		
CD 8		↓	↓↓	↓	
CD 25					
BMK 13	↓				
Tryptase					
Chymase					↓
IgE		↓			↓

Lam Prop	200 vs placebo	400 vs placebo	200/400 vs placebo	400 vs 200	400 vs 200/400
CD 1	↓	↓↓↓	↓↓↓	↓	
CD 3			↓↓		
CD 4					
CD 8					
CD 25					
BMK 13	↓	↓↓	↓↓		
Tryptase		↓			
Chymase		↓↓	↓		↓
IgE		↓			↓

Table 5.
Statistical evaluation after 8 weeks of treatment.
Arrows indicate significant decrease. One arrow: p<0.05. Two arrows p<0.01. Three arrows p<0.001.

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DISCUSSION

Patients.

We were surprised that out of the 300 selected patients, only sixty-five satisfied our conditions for inclusion of nasal complaints for more than 1h a day. This underscores the importance of the use of nasal symptom scores to characterize the patients objectively.

Symptoms.

The efficacy of normal or double dosed fluticasone propionate in treating nasal symptoms in this, second echelon, strictly selected group proved to be no greater than that of placebo. This contrasts with previous reports. However, of these early studies, only Malm's study (n=22) was placebo controlled, while half of his patients showed a nasal eosinophilia (NARES patients)(6), which is known to be associated with a good responds to steroids (25). In our study no eosinophilia was shown. Furthermore, in Malm's study, the reduction of baseline complaints by placebo was larger than the additional effect of steroid therapy. The efficacy of placebo has to be attributed to wetting the nose twice a day with the spray (26). Scadding reported about the clinical efficacy of topical steroids in a combined group of 371 patiens with allergic and non-allergic rhinitis. She concluded that topical steroids are efficacious in the treatment of allergic and non-allergic rhinitis. Unfortunately no distinction was made between allergic and non-allergic patients as they were pooled together in the seperate treatment groups (9). The efficacy of treatment versus placebo was not seperately tested for the non-allergic patients. The reported overall efficacy might perhaps be attributed to the known clinical efficacy in allergic rhinitis patients. Furthermore a significant reduction in nasal eosinophilia was seen in the non-allergic group similar to that of the allergic group, again suggesting a substantial number of NARES patients.

The only significant decrease in the percentage of symptom free days we found was for sneezing, not the most important complaint of our patients. For none of the other assessment methods (VAS, DRC mean sumscores, assessment of nasal symptoms by investigator, rhinoscopy) a significant effect was found. A small dose dependent effect on symptoms that was not significant can be seen in the different graphs. Considering the fore mentioned, we feel that the effect of fluticasone on nasal symptoms in NANIPER patients as we selected them is not clinically relevant. We feel that the NANIPER patients seen by the specialists these days are not suffering from a cellulary mediated disease (27). Nowadays, nasal steroids are often used as the first line of treatment by the general practitioner before referral to a specialist. It is thus possible that the referred NANIPER patients are mostly the non-steroid responders, which agrees with our clinical experience.

Cells.

The concept of NANIPER being a neurogenic inflammation, as presented by Wolff and others, is not supported by our findings. For one, we found no differences in the numbers of inflammatory cells between patients and controls (27). Two, the lack of effect of nasal steroids in this group while these steroids have been reported to be efficacious in induced neurogenic inflammation (21).

The absence of correlation between the marked reduction in cell numbers of the immunocompetent cells and nasal complaints could be the result of two different phenomena. The groups could be too small to measure an effect on nasal complaints. However our group is larger than those of Malm (n=22) (6), and Pipkorn (n=12) (8) who did find a significant reduction in nasal complaints. Or the reduction in cell numbers by the steroid therapy in NANIPER is not clinically relevant. The reported reductions in cell numbers in allergic rhinitis, in responds to the steroid, are preeceded by an increase of immunocompetent cells in response to the allergic stimulus. In NANIPER however, no significant differences were found, in immunocompetent cell numbers, between patients and healthy controls (27) Therefore it is more likely that the absence of correlation in this study is relevant.

The steroid effect in the nose seems to be cell specific and not disease specific. The Langerhans cell seems to be most sensitive to steroid therapy, as it is in allergic rhinitis (4). The T-cells in the epithelium are also sensitive, but to a lesser extend. Although our NANIPER data suggests only a moderate effect on eosinophils and mast cells, which is not in line with the allergic data, this is probably due to the relative absence of these cells in NANIPER if compared to allergic rhinitis. If, even in small numbers, eosinophils and mast cells are present in NANIPER patients they are also reduced. The effect of doubling the dose of fluticasone on the cells is more marked than the single dose effect. This is in agreement with data from Godthelp (4) in patients with perennial allergic rhinitis.

The additional effect of the higher dosage of local steroid in NANIPER on the reduction in cell numbers has not been described before. If this has implications in the treatment of (steroid responsive) patients is questionable.

The marked effect of a wetting agent (i.e. placebo) in NANIPER as seen by Malm and Spector is not seen in this study. Our findings as far as the placebo effect is concerned are more in line with those of Scadding. This might reflect the change in NANIPER population as seen by the specialist nowadays.

To conclude, the "rhinitis" specialist is increasingly confronted with a non-steroid-responsive NANIPER group. Doubling the treatment dose does not have a significant effect on nasal symptomatology. Although there is a significant dose dependent steroid effect on nasal immunocompetent cells, this does not seem to be of clinical relevance.

One has to bear in mind that this is a referred and therefore selected group. In a "virgin"(no previous local steroid) NANIPER patient, local steroids are still first-line treatment. Topical capsaicin therapy might be a new therapy for the non-steroid sensitive group.

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Chapter 5

Intranasal Capsaicin is efficacious in Non-Allergic Non-Infectious Perennial Rhinitis. A Placebo-Controlled Study.

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SUMMARY

The efficacy of topical aqueous capsaicin spray in the treatment of NANIPER patients was studied. Several authors described capsaicin, the pungent substance in red pepper, as an efficacious therapy for non-allergic non-infectious perennial rhinitis (NANIPER). Repeated capsaicin application induces peptide depletion and specific degeneration of the unmyelinated sensory C-fibers in the nasal mucosa. We performed a placebo (NaCl 0.9%) controlled study with 25 NANIPER patients. Daily record charts and visual analogue scales (VAS) were used for clinical evaluation. Nasal lavages were obtained before, during, and after treatment. There was a significant and long-term reduction in the VAS scores in the capsaicin group. No significant difference was found between the placebo and capsaicin treated groups for the mean group concentrations of leukotriene (LT) $C_4/D_4/E_4$, prostaglandin D_2 (PGD_2), and tryptase. The levels of mast cell mediators, tryptase and PGD_2, and leukotrienes, mediators derived from a variety of inflammatory cells, were low at baseline and comparable with levels observed in nasal lavages obtained from normals. As involvement of inflammation could not be demonstrated, it is not surprising that capsaicin has no effect on inflammatory mediators. This suggests that inflammatory cells do not play a major part in the pathogenesis of NANIPER.

INTRODUCTION

The knowledge of non-allergic non-infectious perennial rhinitis (NANIPER) or vasomotor rhinitis is limited. This condition is unrelated to allergy, infection, structural lesions and/or other systemic disease (1). The diagnosis is made by exclusion. Patients within this classification may complain of symptoms such as sneezing, watery rhinorrhea and/or nasal obstruction. Treatment of this condition is more difficult than that of allergic rhinitis, a disease that can be relieved by use of antihistamines and nasal steroids.

The pathophysiology of non-allergic rhinitis is largely unknown (1). Several hypotheses have been put forward. A subgroup of patients may react to cold dry air with release of inflammatory mediators from mast cells involving a non-IgE-dependent mechanism (2). Inflammatory cells appear to play a minor part in the vast majority of patient's (3). However, Knani reported a significant increase in tryptase levels, and increased levels of LTC4 and PGD2 in nasal lavage in symptomatic NANIPER patients versus control subjects. (4). Neurogenic mechanisms may be important since some patients, who react with watery discharge to spices and change of temperature, may benefit from use of anticholinergics (5).

Lacroix has shown that repetitive administration of capsaicin - the pungent agent in hot pepper - reduces nasal symptoms of patients with a rhinosinusitis, for which they underwent sinus surgery, or patients suffering from a drug-induced rhinitis (6). This reduction is accompanied by a decrease in positive immunoreactivity to calcitonin gene-related peptide (CGRP) in nasal biopsies. This observation is consistent with the observation that capsaicin induces peptide depletion and specific degeneration of the sensory C-fibers in the nasal mucosa of rodents (7). Several studies have been published showing that capsaicin desensitization might be an important therapeutic modality in NANIPER (6,8-10). However, no placebo-controlled studies have been performed. Moreover, the reported studies lack well-defined criteria for having NANIPER, with the risk of heterogeneity of the patients used.

The purpose of this study was to evaluate capsaicin treatment in a placebo-controlled fashion using a homogeneous group of well-characterized patients suffering from NANIPER. Second, by measuring mediators of inflammation in nasal lavage fluid, we investigated the involvement of inflammation in NANIPER and the possible modulation by capsaicin.

MATERIALS AND METHODS

Subjects

Patients were admitted to the study if they had a history of nasal complaints such as nasal obstruction, sneezing, and rhinorrhea for a period of over 1 year which could not be attributed to allergic rhinitis, nasal or paranasal sinus infection, anatomical disorders affecting nasal function, pregnancy or lactation and/or systemic disorders (tabel 1).

Inclusion criteria
- Age between 16 and 64 years.
- Negative skin prick test: house dust mite, tree pollen mix, grass pollen mix, bijvoet, alternaria, aspergillus, cladosporium, penicillum, dog, cat, parakeet, rabbit, hamster, horse, guinea pig. (ALK-Diephuis, Holland)
- Negative Phadiatop (Pharmacia, Uppsala, Sweden)
- Symptoms for more than 1 year.
- Periods of nasal discharge, sneezing and congestion for an average of at least 1 h per day for at least 5 days during a period of 14 days.

Exclusion criteria
- The use of systemic or inhaled corticosteroids within the previous month.
- Use of inhaled sodium cromoglycate or nedocromil sodium within the previous month.
- Use of astemizole within the previous month.
- Inability of the patient to stop taking medication affecting nasal function.
- A serious and/or unstable disease.
- Nasal surgery within the previous 6 weeks.
- Nasal polyps or a history of nasal polyps.
- Significant anatomical abnormalities affecting nasal function.
- Nasal or paranasal sinus infection (abnormal sinus X-ray).
- Pregnancy or lactation
- Abnormal findings at physical examination
- Abnormal laboratory results for:
blood: Na, K, Ca, total protein, albumin, urea, creatinine, bilirubin, alkaline phosphatase, aspartate aminotransferase, alanine aminotransferase, gammaglutamyl transpeptidase, hemoglobin, red blood cell count, plasma cell volume, mean corpuscular volume, platelets, total white blood cellcount, neutrophils, lymphocytes, monocytes, eosinophils, basophils.
urine: blood, protein, glucose.

Table 1. Selection criteria for patients with NANIPER.

They were non-smokers not using medication affecting nasal function. Patients with nasal polyps were excluded, since they may belong to a different pathophysiological group and their polyps may contribute to a higher symptom score for nasal blockage and/or rhinorrhea. Thirty-five patients, with the diagnosis of NANIPER, scored their nasal complaints for a period of 2 weeks using a daily record card (DRC) (figure 1) (11). In affected patients periods of either nasal discharge, and/or sneezing and/or congestion had to persist for an average of at least 1h per day for at least 5 days during a period of 14 days. Coughing and coloured mucus production were used as indicators of upper airway infection and thus used as exclusion criterion.

Possible scores on the daily record chart	
Nasal blockage:	0 = absent
(not being able to breathe freely through the nose)	1 = between 0-1h per half day
Clear nasal discharge: (runny nose)	2 = between 1-2 h per half day
	3 = more than 2 h per half day
Sneezing	0 = absent
Coughing	1 = less than 5 periods per half day
	2 = between 5-10 periods per half day
	3 = more than 10 periods per half day
Mucus production:	0 = absent
(yellow, green or brown)	1 = present

Figure 1. Scheme of the daily record card for defining nasal symptoms in patients with NANIPER (VMR).

The duration of complaints during the day was used as the prime criterion for further study. Twenty-five of the 35 patients were found eligible for our study and participated under conditions of informed consent (male/female: 16/9); mean age was 36 years (18-60). Procedures were approved by the local Medical Ethics Committee.

Study design

Patients were randomised and treated with placebo (11 persons) or capsaicin (14 persons) as depicted in figure 2. This study was performed in a single-blind placebo controlled fashion. Three applications of xylometazolinehydrochloride 0,1% (Otrivin[R] (1 mg/ml Zyma, Breda, Holland), nebulisator) were given for decongestion in each nostril. The nasal airway was anesthetized by 3 applications (10mg/puff) of lidocainebase (100mg/ml) (Xylocaine[R] 10 % spray (Astra, Rijswijk, Holland)) in each nostril. To ensure good anesthesia a pause of 15 minutes was introduced. Lips, columella, and philtrum were covered with petrolatum/lanolin/glycerin salve. Capsaicin test puff was done in an exhaust hood to avoid eye irritation. Patients were instructed to inhale deeply before, hold their breath during and to exhale after substance application. The capsaicin solution (0.1 mmol/l) consisted of pelargonic acid vanillylamide (Fluka, Buchs, Germany) dissolved in 3 ml alcohol (96%) and diluted in 1 L NaCl solution (0.9%) (Wolf, personal communication). For 'placebo therapy' we used NaCl solution (0.9%). During provocation 0.5 ml solution was sprayed in each nostril (0.15 mg capsaicin). Blood and urine samples were taken during visits 1 and 9 (table 1) to monitor changes during therapy. At every visit the subjects rated overall nasal symptoms since the last visit on a visual analogue scale (VAS) (0-10 cm, 0 represented absence of symptoms and 10 represented high intensity of symptoms). DRC scoring was continued untill two weeks after treatment.
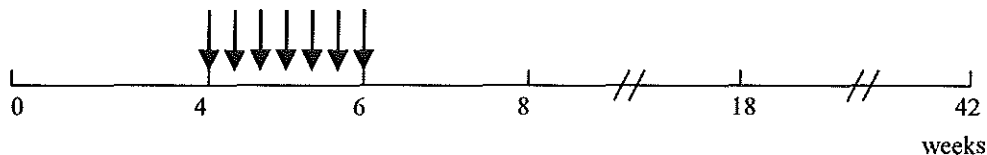
Time schedule of hospital visits (in weeks). Every visit a VAS-score was obtained.
0-4   Selection period, 4 weeks, during which patients recorded nasal syptoms for 2 weeks
4–6   Capsaicin treatment ($\downarrow$), every 2 or 3 days. Totally 7 treatments.
6–8   Evaluation period, 2 weeks after therapy
8–18   Evaluation period, 3 months after therapy
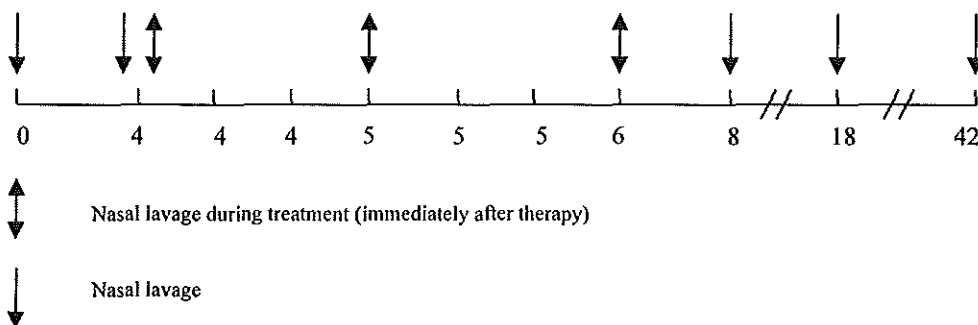18–20   Evaluation period, 9 months after therapy

Nasal lavage during treatment (immediately after therapy)

Nasal lavage

Figure 1. Study design.

Nasal lavage was performed according to the method of Greiff (12), using a modified nasal pool device. Experience with nasal lavage has been obtained in several studies (13). Nasal lavage was performed (figure 2) with 14 ml saline, preheated to 37 °C. Seven ml saline was instilled into each nostril. After 10 seconds, the lavage fluid was expelled and collected in tubes, stored on ice and centrifuged for 10 minutes at 400 x g. The supernatant was stored at 20 °C until analysis.

Mediator assays
The levels of leukotriene (LT) $C_4/D_4/E_4$ and prostaglandin (PG) D_2 were measured by Biotrak[R] and Radioimmunoassay (RIA), respectively (Amersham, UK). The limits of sensitivity of the assays were approximately 10 pg/ml for both assays. Cross reactivity of $LTC_4/D_4/E_4$ assay: LTC_4 (100%), LTD_4 (100%), LTE_4 (70%), LTB_4 (0.4%) and prostaglandins (<0.006%); PGD_2 assay: PGD_2
(100%), PGJ_2 (7%), TxB_2 (0.3%), PGF_2 (0.04%) and other prostaglandins (<0.02%).

Tryptase was determined by RIA according to the manufacturer's instructions (Pharmacia, Uppsala, Sweden). The detection limit was 0.5 mU/ml. Cross reactivity for heparin (<0.01%). (14)

Statistical analysis
VAS data during treatment were analysed using a repeated measures analysis of variance. In the model, time was included as a quantitative variable; the interaction between time and treatment group was also included. Hence, a difference in time trend between the two treatment groups can be estimated and tested. The within-subject (co)variance matrix of the residuals is supposed to be unstructured. Leukotrienes, prostaglandin D_2, and tryptase are analysed after log transformation.

Measurements after treatment (visits 9, 10, and 11) were analysed separately as changes from baseline using t-tests, between groups (unpaired) as well as within groups (paired). DRC data are summarized as within patient averages over two-week periods: a first period before randomization/treatment, a second period after randomization (during therapy) and a third period after cessation of treatment. Between groups differences are tested using the Mann-Whitney test. P values < 0.05 were considered significant.

RESULTS

The application of Xylocaine[R] spray in the nasal airway was immediately followed by a painful sensation that was described by all subjects as most unpleasant. Patients did not complain of irritation of nose and lips during or after capsaicin/placebo application.

One of the 14 capsaicin patients could not continue after three capsaicin applications because of influenza with fever.

SYMPTOM SCORES

Daily record card
The mean score (± standard error of the mean) on the DRC of the included patients was 2.0 (± 0.049) for blockage, 1.4 (± 0.044) for clear nasal discharge, and 1.5 (± 0.033) for sneezing before therapy. No significant difference was found for the individual symptoms as well as the mean sumscore before, during or after therapy.
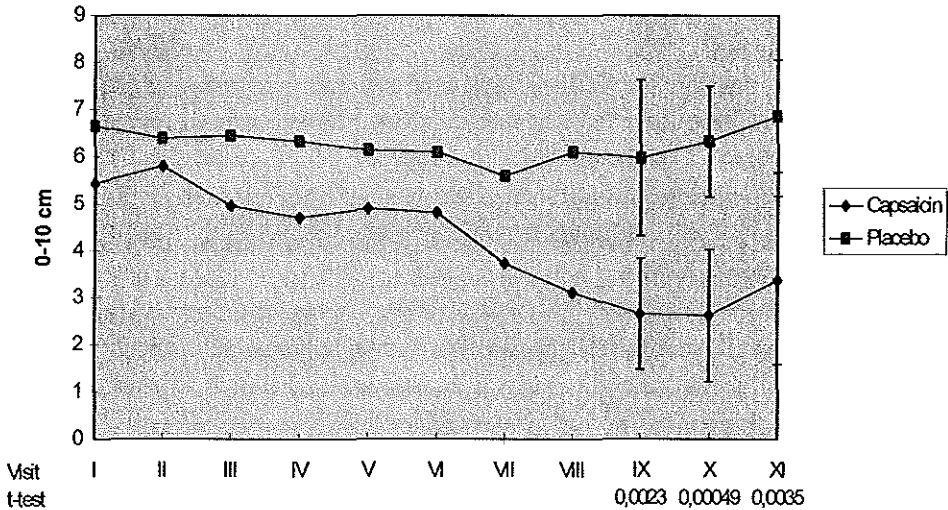
Figure 2. The mean of the symptom score measured on a VAS (0-10 cm, 0 represented absence of symptoms and 10 severe intensity of symptoms) for nasal complaints. Error bars indicate 2.07SE.

Visual analogue scale

The mean of VAS is shown in figure 3. There was no significant difference between the groups in the VAS score before treatment. During treatment a smaller trend with time (-0.40 per two days) was seen in the capsaicin group than in the placebo group (+0.019 per two days), the difference being significant (p=0.0007). At visits 9, 10 and 11 the difference between the groups remained significant. Also, the difference from baseline remained significant within the capsaicin group from visit 9 on. This was not the case in the placebo group (figure 2).

Nasal lavage

The median return, of the 14 ml NaCl instilled, was 10 ml.

The mean baseline levels (± standard error of the mean) of tryptase, $LTC_4/D_4/E_4$, and PGD_2 were 1.98 (± 0.422) mU/ml, 7.70 (± 3.10) pg/ml, and 16.2 (± 2.00) pg/ml, respectively, in the nasal lavage fluid of the treated group.

The mean baseline levels ($\pm$ standard deviation) of tryptase, $LTC_4/D_4/E_4$, and PGD_2 were 2.09 ($\pm$ 0.611) mU/ml, 2.62 ($\pm$ 1.12) pg/ml, and 16.4 ($\pm$ 2.85) pg/ml, respectively, in the nasal lavage fluid of the placebo group. The mean baseline levels ($\pm$ standard deviation) of tryptase, $LTC_4/D_4/E_4$, and PGD_2 were 2.09 ($\pm$ 0.611) mU/ml, 2.62 ($\pm$ 1.12) pg/ml, and 16.4 ($\pm$ 2.85) pg/ml, respectively, in the nasal lavage fluid of the placebo group.

During treatment no significant difference in time trend between the two groups was found for the concentrations of tryptase, $LTC_4/D_4/E_4$, and prostaglandin D_2 in the nasal lavage fluid. At visits 9, 10 and 11 no significant changes from baseline or significant differences between the groups were found either.

Safety data

None of the patients had a relevant change of blood and/or urine chemistry outside the normal range.

DISCUSSION

There is a dearth of information regarding the pathophysiology of NANIPER. The limited understanding of this condition hampers the development of therapeutic modalities. An imbalance in the nonadrenergic, noncholinergic peptidergic neuronal system has been proposed as the underlying mechanism of NANIPER (15). Treatment with capsaicin may fit in with this hypothesis (16). This study showed that seven treatments in a 14-day period ameliorated symptoms during a follow-up of 9 months. It is possible that reduction of symptoms will last longer; however, we feel that it is unethical to maintain a placebo-treatment for many months, so we ended the trial after nine months of follow-up. This longterm placebo-controlled study confirmed the efficacy and safety observations made during open uncontrolled studies (6,8-10).

The study has several limitations. The study was designed in a placebo-controlled double blind fashion. However, we did not expect that we could blind the treatment for the patients, as this was considered impossible by several authors. In contrast to our expectations patients complained severely about the Xylocaine® spray. Therefore they were not able to discriminate between the active and the placebo substance. Furthermore the immediate respons to treatment did not permit us to discriminate between patients receiving capsaicin or placebo.

Second, since we used saline as placebo treatment rather than the solution used for dissolving capsaicin (which contained saline with 0.3 % alcohol 96%), we cannot exclude the possibility that an effect of alcohol biased the therapeutic efficacy of capsaicin. It is, however, unlikely that instillation of these minute quantities of alcohol will induce a significant reduction in

nasal symptoms during 9 months. Moreover, saline containing a fivefold dose of 1.5% alcohol has no effect on nasal conductance (17).

Finally we encountered a discrepancy between the reduction in VAS score and the absence of effect on DRC, which might be explained by the difference in nature between the scoring methods. The VAS scores the severity of the complaints whilst the DRC scores the duration of the complaints.

Furthermore, as the study proceeded patients compliance (in filling in the DRC) seemed to grow less, since scoring the DRC is a time consuming and daily returning task. At the end of the trial some patients even reported that they had filled in their DRCs all at once just prior to their hospital visit.

In contrast, the VAS score is a quick and easy method for the patient. Also the fact that the placebo group showed no evidence of improvement in the VAS combined with the finding that the duration of the treatment's effect is quite impressive and consistent with what all the previous uncontrolled studies have suggested (6,8-10), we feel the VAS is more reliable than the DRC.

In animals capsaicin stimulates sensory C-fibers with the resultant release of substance P (SP) (7,18) and calcitonin gene related peptide (CGRP) (19). However, after several stimulations this is followed by depletion of these fibers and results in desensitisation to capsaicin and other stimuli (16). As tachykinins (20) and capsaicin (21) induce the recruitment of inflammatory cells in the nose in allergic rhinitis and SP releases histamine and TNF-alpha from peritoneal mast cells in animals (22), capsaicin may modulate inflammation of the nasal mucosa. The levels of tryptase, PGD_2, and leukotrienes, mediators derived from several inflammatory cells such as eosinophils, basophils, and mast cells (23,24), were low at baseline and comparable with levels observed in nasal lavages obtained from normals (C. de Graaf-in 't Veld, submitted of personal communication). This contrasts with the results presented by Knani (4) However, 6 out of 14 patients in Knani's study showed a prominent eosinophilia in nasal secretions and may have been NARES patients. Our data concords with the findings of Roche, however this paper describes the results in asymptomatic patients. As involvement of inflammation could not be demonstrated, it is not surprising that capsaicin has no effect on inflammatory mediators. Perhaps the absence of inflammation, also demonstrated in a recent study (3), is an explanation of the moderate efficacy of nasal steroids in non-allergic rhinitis.

To conclude, capsaicin is an efficacious substance in the treatment of NANIPER. In our placebo-controlled study a therapeutical effect lasted more than 9 months. No effect was found on inflammatory mediators. No adverse side effects were noted.

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Chapter 6

The Long Term Effects of Capsaicin Aqueous Spray on the Nasal Mucosa.

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R. Gerth van Wijk, W. J. Fokkens.

SUMMARY

The long term effects of capsaicin spray on the nasal mucosa are studied. Capsaicin has been shown previously to reduce nasal complaints in patients with a non-allergic non-infectious perennial rhinitis. Proposed pathophysiologic mechanisms for non-allergic non-infectious perennial rhinitis include a chronic inflammatory disorder of antigenic or neurogenic nature as well as the possibility of a functional neuronal disorder. We hypothesized that the benificial effect of capsaicin might be the result of a down regulation of inflammation (by a reduction of inflammatory cells) or through a modulation of neural tissue density. Patients were treated with either a placebo or capsaicin spray solution delivering 0.15 milligrams of capsaicin per nostril once every second or third day for a total of seven treatments. Both sides were treated each visit. Biopsies were taken before, 2 weeks after, 3 months after, and 9 months after the treatment period. Immunohistochemical staining of the biopsy specimen was performed to ascertain the effect of treatment on immunocompetent cell densities (quantitative) and neural tissue densities (semi-quantitative) in the nasal mucosa. Nasal complaints were significantly reduced in the capsaicin treated group. The number of CD1+, CD25+, CD3+, CD68+, BMK13+, IgE+, Tryptase+, and Chymase+ cells did not significantly differ between capsaicin and placebo group. No significant differences between both groups were found in pan-neurogenic staining of nasal mucosa using neurofilament and synaptophysine. Capsaicin aqueous nasal spray has been shown previously to reduce nasal complaints without affecting cellular homeostasis or overall neurogenic staining upto 9 months after treatment, furthermore immunocompetent cells are not involved in non-allergic non-infectious perennial rhinitis.

INTRODUCTION

In a double blind placebo controlled study we recently demonstrated that capsaicin is highly effective in controlling non-allergic non-infectious rhinitis (1). A long-lasting relief in symptoms was obtained for at least 9 months.

Capsaicin is the pungent agent in red peppers. Its mode of action is well documented in rodents, where it affects mainly the thin unmyelinated sensory nerve fibers. It causes initial stimulation (with release of endogenous neuropeptides), followed by desensitisation to capsaicin and other sensory stimuli (2) With higher doses long term functional or even morphological ablation of the thin sensory neurons occur (3). In humans the effect of capsaicin has not been fully documented (4). Moreover the pathophysiologic mechanism for non-allergic non-infectious perennial rhinitis is not understood. Proposed mechanisms include a chronic inflammatory disorder of antigenic or neurogenic nature, or a functional neuronal disorder (5,6).

To study wether capsaicin reduces inflammation or modulates nasal neuronal tissue densities we performed a nasal biopsy study in 24 patients with non-allergic non-infectious perennial rhinitis. Cells were quantified per square millimeter and the sections stained with neuronal markers were scored semi-quantitatively for morphometric changes.

MATERIALS AND METHODS

Subjects

Patients were admitted to the study if they had a history of nasal complaints such as nasal obstruction, sneezing, and rhinorrhea for a period of over 1 year which could not be attributed to allergic rhinitis, nasal or paranasal sinus infection, anatomical disorders affecting nasal function, pregnancy or lactation and/or systemic disorders (7,8) They were non-smokers not using medication affecting nasal function. Patients with nasal polyps were excluded, since they may belong to a different pathophysiological group and their polyps may contribute to a higher symptom score for nasal blockage and/or rhinorrhea.
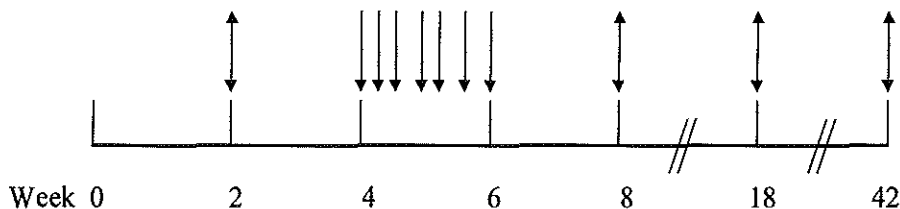
Study design

Thirty-five patients, with the diagnosis of non-allergic non-infectious perennial rhinitis, scored nasal blockage, clear discharge, sneezing and coughing on a 4 point scale and mucus production (absent or present) for a period of 2 weeks using a daily record card (1,7,8). Mucus production or coughing were used as indicators of upper airway infection. If present they led to exclusion of the patient. Patients were included in this study if periods of clear nasal discharge, sneezing and nasal blockage persisted for an average of at least 1h per day for at least 5 days during a period of 14 days. The duration of complaints during the day was used as

the prime criterion for further study. Twenty-five of the 35 patients were found eligible for our study and participated under conditions of informed consent (male/female: 16/9); mean age was 36 years (18-60). One of the 14 capsaicin patients could not continue after three capsaicin applications because of influenza with fever.

Procedures were approved by the local Medical Ethics Committee.

Patients were randomised in a double-blind placebo controlled fashion and treated with placebo (11 persons) or capsaicin (14 persons). A total of seven treatments over a period of two weeks were given.

Time schedule of hospital visits, Every visit a visual analogue scale score was obtained
↓ : indicates either capsaicin treatment (n=14) or placebo (n=11), every 2 or 3 days.

↑ : indicates biopsy moment

Figure 1. Study design. During the entire study period patients scored their nasal complaints such as nasal blockage, clear discharge, sneezing, coughing and mucus production on a daily record card. After the first 2 weeks (0-2, run in) patients with sufficient nasal complaints (> 1 hour/day for at least 5 days during the 14 days period) were selected. Mucus production and coughing were used as indicators of upper airway infection. If present they led to exclusion of the patient. A biopsy was taken in included patients. Weeks 2-4 were used to allow healing of the nasal mucosa before treatment. During weeks 4-6 a total of 7 treatments was given. During the evaluation period three nasal biopsies were taken.

Treatment procedure

The nose was decongested with Xylometazolinehydrochloride 0,1% and anaesthetised with lidocaine base-spray (100mg/ml). Capsaicin aqueous nasal spray (0.15 mg) or placebo was instilled in each nostril (1). The application of Xylocaine[R] spray in the nasal airway was immediately followed by a painful sensation that was described by all subjects as most unpleasant. Patients did not complain of irritation of nose and lips during or after capsaicin/placebo application. At every visit the subjects rated nasal symptoms on a visual analogue scale (0-10 cm, 0 represented absence of symptoms and 10 represented high intensity of symptoms). Daily record card scoring was continued for up to two weeks after treatment (1). Nasal biopsies were taken four times. At the run-in period, and after 2 weeks, 3 months, and 9 months after the treatment period (figure 1).

After randomisation of the biopsy side, specimens of nasal mucosa were taken from the lower edge of the inferior turbinate, about 2 cm posterior to the front edge, using a Gerritsma forceps with a cup diameter of 2.5 mm. (9). Local anaesthesia was obtained by placing a cotton-wool carrier with 50 mg of cocaine and one drop of adrenaline (1:1000) under the inferior turbinate without touching the biopsy site. The specimens were embedded in Tissue-Tek II O.C.T. compound and frozen immediately.

Blood: (Na, K, Ca, total protein, albumin, urea, creatinine, bilirubin, alkaline phosphatase, aspartate aminotransferase, alanine aminotransferase, gamma-glutamyl transpeptidase, haemoglobin, red blood cell count, plasma cell volume, mean corpuscular volume, platelets, total white blood cell count, neutrophils, lymphocytes, monocytes, eosinophils, basophils) and urine: (blood, protein, glucose) samples were taken during visits 1 and 9 to monitor changes during therapy.

Staining procedures

Monoclonal antibodies (mAb) directed against synaptophysine, neurofilament, CD1, CD3, CD25, CD68, IgE, MBP, chymase, and tryptase (table 1) were used together with the super sensitive immuno-alkaline phoshatase (ss-APAAP) method. Sections of nasal mucosa were

Antibody	Specificity	Titre	Source
OKT6	CD1	1:100	Dept. Immunology, Erasmus University, Rotterdam
leu4	CD3	1:25	BDH, Dorset, UK
KIM-6	CD68	1:100	Behring, Rijswijk, NL
2F11	Neurofilament	1:50	Sanbio, Uden, NL
Sy38	Synaptophysine	1:20	Dakopatts, ITK, Uithoorn, NL
	IgE	1:250	Central laboratory of the Netherlands Red Cross Blood Transfusion service (CLB), Amsterdam, NL
IL-2r	CD25	1:150	BDH, Dorset, UK
BMK13	MBP	1:200	Sanbio, Uden, The Netherlands
B7	Chymase	1:100	Chemicon, Temecula, Calif, USA
G3	Tryptase	1:250	Chemicon, Temecula, Calif, USA

Table 1. Monoclonal antibodies used to stain biopsy specimen.

transferred to poly-L-lysine-coated microscope slides, dried, and fixed in acetone for 10 min at room temperature . They were next rinsed in phosphate-buffered saline (PBS, pH 7.2), placed in a half-automatic stainer (Sequenza, Shandon), and incubated with normal goat serum (CLB, Amsterdam, The Netherlands) for 10 min. Following this the slides were incubated with the mAb for 60 min at room temperature. The sections were then rinsed again

in PBS for 10 min and incubated for 30 min with a goat anti-mouse (1:50) biotin, rinsed successively in PBS, incubated with streptavidin alkaline phophatase supersensitive (1:50) (Biogenex, Klinipath, Duiven, The Netherlands) for 30 min at room temperature, rinsed in PBS and TRIS buffer (pH 8.5), and incubated for 30 min with a new fuchsin substrate (Chroma, Kongen, Germany). Finally, sections were rinsed with distilled water, counterstained with Gill's haematoxylin and mounted in glycerin-gelatin. Control staining was performed by substitution with PBS and incubation with an irrelevant mAb of the same subclass.

Light-microscopic evaluation

Stained cells were quantified ("blinded") in two sections of each biopsy specimen. The epithelium and lamina propria were evaluated separately. The total surface area of a section and its main parts (i.e. the epithelium and the lamina propria) were estimated with the use of the Kontron Image Analysis System Videoplan. The number of cells/mm^2 was calculated for the epithelium and the lamina propria. The intensity, number and dimensions (width and length) of neuronal staining was semi-quantified by 3 separate observers. Biopsies were ranked 1 to 24 by continuously comparing the biopsies amongst another until all were ranked, by each seperate observer. In practice: a section would be taken (at random), evaluated and put down. The next section would be taken (at random) and be graded for stronger or weaker staining compared with the previous section. The next section would be stronger, weaker, or in between the two previous sections. At the end all sections would be "on the table" and the weakest stained section would receive rank 1 and the strongest stained section would receive rank 24.

Statistical analysis

The non-parametric Mann-Whitney U-test was used to compare the differences in cell counts between the groups. A p-value < 0.05 was considered to indicate a significant difference.

The Spearman rank correlation's between changes in cell numbers and the changes in the visual analogue scale scores per randomisation group were calculated.

For the inter-observer variation the rank correlation between the rankings of any two observers was calculated per visit for synaptophysine and neurofilament. Also differences in ranking between any two observers were calculated. The mean rank averaged over the three observers was used to compare the two treatment groups per visit, using the Mann-Whitney U test.

RESULTS

Biopsies

The sections of the nasal mucosa had an average surface area of 2.0 mm^2 and usually showed a lining of ciliated columnar epithelium with or without goblet cells and/ or partially stratified cuboidal epithelium. The lamina propria consisted usually of a looser subepithelial cell-rich layer with mucous glands and a deeper collagenous cell-poor layer. All sections were sufficiently deep to assess both layers. The sections were generally of good quality. No structural damage to the mucosa was seen after capsaicin treatment (thickness of the epithelium, thickness of basal membrane, number and size of glands)

Inflammatory cells

The results are shown in tables 2a and 2b. The mAb-ss APAAP staining showed red cells against a blue counterstained background. T-lymphocytes, small round cells, were abundantly present in the epithelium as well as the lamina propria (figure 2, page 103). Sometimes clusters of T-cells were found in the epithelium or lamina propria (500-1000). The occurrence of these clusters did not differ between the groups. Langerhans cells, large dendritic cells, were found mostly in the epithelium. Only a few were present in the lamina propria . Mast cells were found mostly in the lamina propria and hardly ever in the epithelium (figure 3, page 103). Eosinophils were hardly ever present in our material. Sometimes moderate infiltrates were found in the mucosa. The occurrence did not differ between the groups. The CD68 positive cells were large cells with a bright staining cytoplasm. This cell type was found to be equally distributed in both layers. No significant changes were found between treatment and placebo for any of the cells.

Neuronal staining

Synaptophysine and neurofilament staining showed red fibers cut at different angles (figure 4 and 5, page 103). The rank correlation between any 2 observers varied from r = 0.8 to r = 0.96. The differences in ranking between any 2 observers varied between -8 and 11, with a mean and a median (almost) equal to zero, as expected. No significant differences between the two treatment groups were found for either synaptophysine or neurofilament staining.

Blood and urine

No significant changes were found for any of the blood or urine parameters.

Epithelium	Run in		2 weeks		18 weeks		42 weeks	
Treatment	plac	caps	Plac	Caps	plac	caps	plac	Caps
CD1	379	341	451	447	275	355	230	350
25%	346	159	221	269	211	261	93	100
75%	846	425	970	791	591	529	278	621
CD3	1457	624	943	821	732	1340	1244	1339
25%	561	438	378	677	572	737	762	301
75%	2105	1432	1049	1557	1013	2992	1955	2667
CD25	84	34	59	86	52	50	44	36
25%	53	15	29	32	16	30	18	19
75%	128	92	67	176	182	195	64	129
CD68	455	578	512	587	480	1037	595	487
25%	228	340	334	379	305	472	421	377
75%	1934	800	709	856	1440	2071	875	1842
BMK13	8	3	25	9	9	7	13	3
25%	1	2	0	2	0	0	0	0
75%	65	22	29	110	67	152	56	37
Tryptase	11	6	11	25	18	27	15	13
25%	0	4	0	0	0	11	0	0
75%	26	17	50	115	109	100	51	45
Chymase	0	3	16	12	7	15	14	36
25%	0	0	0	0	0	2	3	0
75%	8	56	26	61	18	42	19	105
IgE	74	123	57	36	153	56	29	20
25%	9	6	0	3	7	2	0	0
75%	311	206	420	160	182	269	344	185

Lamina propria	Run in		2 weeks		18 weeks		42 weeks	
Treatment	plac	caps	Plac	Caps	plac	caps	plac	Caps
CD1	58	31	27	52	48	35	9	18
25%	51	15	18	16	21	17	9	18
75%	67	63	54	79	70	95	46	90
CD3	1372	591	616	1151	964	1262	856	1386
25%	1055	407	366	576	759	632	433	639
75%	3191	1237	1125	1457	1451	2172	2139	2552
CD25	50	36	47	82	24	43	28	45
25%	30	20	23	41	17	8	13	11
75%	93	75	79	141	51	97	54	83
CD68	384	321	267	477	320	573	380	544
25%	200	257	161	395	278	274	244	300
75%	911	675	752	571	831	984	524	965
BMK13	23	11	16	51	18	15	16	39
25%	6	8	5	21	2	6	13	8
75%	45	43	53	89	52	77	38	93
Tryptase	199	192	194	302	212	205	171	248
25%	98	60	94	177	94	113	121	169
75%	281	402	494	445	387	395	213	479
Chymase	158	164	146	282	178	285	148	285
25%	100	93	62	112	123	119	98	189
75%	270	377	334	479	410	343	273	427
IgE	102	47	112	140	131	117	85	150
25%	55	30	45	75	34	36	35	55
75%	281	265	234	160	390	253	180	316

Table 2a and b. Median cell numbers (in the epithelium and the lamina propria) and 25th percentile and 75th percentile for capsaicin (caps) and placebo (plac) treatment at the end of the run in period, after 2 weeks, 18 weeks and 42 weeks after treatment.

DISCUSSION

The effect of capsaicin on nasal complaints and cellular mediators has already been described (1). To summarise, a nine months amelioration of nasal complaints was seen without an effect on cellular mediators.

The mode of action of capsaicin is not clear, neither is the aetiology of non-allergic non-infectious perennial rhinitis. Proposed mechanisms for non-allergic non-infectious perennial rhinitis include the possibility of a chronic inflammatory disorder. We hypothesised that the beneficial effect of capsaicin-treatment could be the result of down-regulation of the inflammation, resulting in a reduction in the number of inflammatory cells. Knowledge on the effect of capsaicin *provocation* on nasal cellular homeostasis is limited to lavage studies following a single capsaicin dose. Philip (10) described biphasic inflammatory-cell influx with neutrophils, eosinophils and mononuclear cells (in nasal lavage) upto 4 hours following capsaicin provocation. Roche (4) described an increase in neutrophils but not in other cells 10 min. after capsaicin challenge. Whether or not this reflects a 'wash out' by increased nasal secretion, or an increase in mucociliary activity which could sweep cells out of the sinuses, or a transmigration of immunocompetent cells from the vessels through the nasal mucosa in the nasal lumen remains open for discussion (10). Our nasal biopsy study circumvents the "wash out problem" since it allows study of all mucosal layers. In a pilot study with 3 patients (unpublished data) we learned that 2 weeks after the last capsaicin treatment a new steady state in nasal symptomatology was reached. We hypothesised that if a correlation was to be found between the number of immunocompetent cells and nasal symptomatology this would be the moment to ascertain it. We realised that we would miss an opportunity to study the direct effect of capsaicin on nasal immunocompetent cells ("provocation effect") since two weeks after cessation of the neurogenic stimulus the supposed capsaicin induced neurogenic inflammation may have withered and will therefore not be detected. A biopsy taken directly after the first treatment was not considered opportunistic, because six more treatments would follow.

No correlation was found between nasal symptomatology and any of the immunocompetent cells, for any of the biopsy moments. Nor did we find any significant cellular differences between the placebo and the treatment group for any of the biopsy time points. This could mean that, a): cells are not an intregral part of the neurogenic response, or b): as Greiff stated " The animal concept of neurogenic inflammation is not valid for the nasal airway, not even in inflamed airways when a neural hyperresponsiveness has developed". (11)

The absence of correlation between cells and symptoms which is congruent with our previous study in which a reduction in nasal immunocompetent cell numbers was found in a group of non-allergic non-infectious perennial rhinitis patients treated with fluticasone aqueous nasal spray without a reduction in nasal symptomatology (8), the absence of significant differences

in the number of immunocompetent cells between capsaicin and placebo group, and the absence of an increase in cellular mediators (as a sign of cellular activation) following capsaicin challenge (1,12) raises the question of the relevance of the increase of immunocompetent cells in nasal lavage after capsaicin challenge.

We, again conclude that immunocompetent cells are not involved in non-allergic non-infectious perennial rhinitis (7,8).

Reports on the effect of capsaicin *treatment* on neuronal tissue are not consistent. Lacroix (13) showed a 50% decrease in CGRP-like immunoreactivity after capsaicin treatment of 16 patients with a drug induced rhinitis suggesting depletion or atrophy of the unmyelinated sensory nerve fibers. In contrast, Wolf (14) in a study of 123 patients failed to show any reduction of peptidergic neurones within the nasal mucosa of 16 selected (uncharacterised) patients. He suggested a blockage of receptors as the mode of action. To quantify neuronal staining is difficult and often open to discussion, as nerve fibers may have a different diameter and can be cut at different angles, resulting in an abundant variation in staining morphology (figures 4 and 5). We used a continuous ranking system. We found a very high Kappa for interobserver variability suggesting a reliable quantification method. No significant differences were found between placebo and treatment group for neurofilament or synaptophysine staining. These antibodies are pan-neurogenic markers. They do not allow discrimination between the adrenergic, the cholinergic and or the peptidergic system. The data thus, allows us to conclude that capsaicin does not induce gross changes in nervous tissue in the nasal mucosa in non-allergic non-infectious perennial rhinitis patients. Other signs of capsaicin induced mucosal damage were not seen, and inflammatory cell densities were not affected. Possible changes in the peptidergic system (the supposed site of action of capsaicin) might not have been detected with these pan-neurogenic markers.

To conclude capsaicin aqueous nasal spray does significantly improve nasal symptomatology in non-allergic non-infectious perennial rhinitis patients, without affecting cellular homeostasis or overall neurogenic staining upto 9 months after treatment.

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Chapter 7

General Discussion

GENERAL DISCUSSION

In the introduction the goals of this thesis were outlined. Stepwise I will discuss the answers and elaborate on future research.

Did we accurately define, select, and study a group of NANIPER patients?

Unfortunately, there is no litmus test for NANIPER. The diagnosis is and was made above all through exclusion of the known causes. This means that the studied patient group is probably a melting pot of patients suffering from nasal complaints, with presumably variable pathogenesis. To study, select and define a group of patients, and more, measure the effects of interventions, positive selection criteria needed to be formulated. Several methods have been used to quantify nasal complaints. Basically these methods can be subdivided into two scoring methods. Either the intensity (1) or the duration of the nasal complaints is graded. The grading system can either be continuous such as the visual analogue scale (VAS) or it can be semi-quantitative. From previous studies and pilot studies it was learned that a semi-quantitative daily record card (DRC), scoring duration of complaints together with the VAS suited our purposes most. It allowed the use of stringent selection criteria as suggested by Mygind (2), patients did not experience the daily returning task of filling in the record card as too great a burden, and the use of the VAS allowed for a sensitive statistical analyses of the interventional effects. During the presented studies it was noticed that the longer the period between the visits the weaker the compliance of the patients in filling in the DRC (3, 4). We have now set a maximum of two weeks between patient and researcher contact in our studies to monitor patient compliance. For the evaluation of interventional effects the VAS proved to be most sensitive (3, 4). This has resulted in the use of the DRC as a tool to include patients; the VAS is used to monitor interventional effects in present studies.

It seems that the above stated goal was reached. A key question however is; "was a representative group of NANIPER patients gathered?" We feel they were representative for the NANIPER patients seen nowadays in the second echelon (3, 4). Doubts remain wether this group is also representative of the first echelon NANIPER patient. The increased prescription of local steroids in the first echelon, during the last decade, to which a subgroup of NANIPER patients were reported to respond well, may have biased the studied group as these patients were probably not referred to the second echelon.

Are inflammatory cells involved in the pathogenesis of NANIPER?

The involvement of inflammatory cells is well accepted in allergic rhinitis (5-11). In NANIPER the involvement of inflammatory cells is under discussion. Although some pathophysiological concepts, such as local allergy (12) and NANIPER induced by the non-adrenergic non-cholinergic system (NANC) (13-20), may agree with an involvement of inflammatory cells others such as autogenic imbalance (21-25) and dys- or hyperaesthesia (Togias, personal communication) do not.

Knani reported increased mediator levels in NANIPER suggesting an involvement of inflammatory cells (26). Mast cells were implicated by Abe and Terrahe (27, 28). However, Braunstein and Hua failed to find any evidence for neurogenically induced mast cell degranulation (29, 30).

Several methods are available to study nasal immunological cells. All three were used in the presented studies. To ascertain the percentage of NARES patients in the studied patients group a nasal brush was taken allowing quantification of eosinophils in the harvested cells. By measuring mediators of inflammation in nasal lavage fluid, inflammatory mediators in NANIPER were investigated. Biopsy specimens were taken to allow study of the deeper layers of the nasal mucosa (31). In chapter 2 and 3 a comparison was made of the nasal mucosa cell densities in NANIPER patients versus asymptomatic controls. No significant differences were found. Moreover we failed to find a single NARES patient in our group. The question then arises as to whether this immunohistochemical evaluation method is sensitive enough to detect significant differences between the groups. The calculated ratios between the geometric means of both groups indicating threshold significance at the 5% level are within the range found in patients with chronic allergic rhinitis (9, 10, 32). In these studies, which compare symptomatic allergic patients with asymptomatic controls, cellular differences between patients and controls were indeed found, while the distribution of the number of immunocompetent cells/mm^2 was in the same order of magnitude as in this NANIPER study. It seems justified to assume that if significant mucosal inflammation would be present, it would have been detected. The lack of differences in cell numbers does not exclude a functional cellular involvement. However, a relation between the number of immunocompetent cells and nasal complaints in NANIPER patients could not be ascertained (3, 4). A significant reduction of immunocompetent cells in the nasal mucosa of NANIPER patients treated with nasal steroids (fluticasone aqueous nasal spray) was not accompanied by a reduction in nasal complaints (3) and, vice versa, a significant reduction in nasal complaints in a group of NANIPER patients treated with topical capsaicin aqueous nasal spray was not accompanied by a change in inflammatory mediators (4) or a reduction in the numbers of inflammatory cells. Considering the aforementioned we conclude that inflammatory cells are not involved in NANIPER.

Were local steroids effective, and if so, how are they effective in NANIPER, and in which subgroup?

The efficacy of normal or double dosed fluticasone propionate in treating nasal symptoms in this, second echelon, strictly selected patient group proved to be no greater than that of placebo. This contrasts with previous reports. However, of these early studies, only Malm's study (n=22) was placebo controlled, while half of his patients showed a nasal eosinophilia (NARES patients) (33), which is known to be associated with a good responds to steroids (34). In our study no eosinophilia was shown. Furthermore, in Malm's study, the reduction of baseline complaints by placebo was larger than the additional effect of steroid therapy. The efficacy of placebo could be attributed to wetting the nose twice a day with the spray (35). Scadding reported about the clinical efficacy of topical steroids in a combined group of 371 patients with allergic and non-allergic rhinitis. She concluded that topical steroids are efficacious in the treatment of allergic and non-allergic rhinitis. Unfortunately no distinction was made between allergic and non-allergic patients as they were pooled together in the separate treatment groups (36). The efficacy of treatment versus placebo was not separately tested for the non-allergic patients. The reported overall efficacy might perhaps be attributed to the known clinical efficacy in allergic rhinitis patients. Furthermore a significant reduction in nasal eosinophilia was seen in the non-allergic group similar to that of the allergic group, again suggesting a substantial number of NARES patients.

We have tried to correlate cells and nasal complaints. A combined average score for running, blockage and sneezing was calculated for the first week (run-in) and for the last study week (prior to the last visit). No correlation was found between the calculated scores and changes in the mucosal densities of lymphocytes (CD3, CD4, CD8) and activated lymphocytes (CD25), mast cells (tryptase and chymase), IgE positive cells, and eosinophils (BMK13 and EG2), and CD1 positive cells for any of the randomization groups. However when the partial correlation (the correlation between cells and complaints corrected for the possible treatment effects for the entire patient group) was calculated, a weak (0.38 and 0.38) but significant (0.007 and 0.008) partial correlation was found for CD1 in the lamina propria and for BMK13 (eosinophils) in the epithelium consecutively. Considering the weak correlation, the clinical relevance of these findings is questionable.

To conclude: steroids are not effective in ameliorating nasal complaints in this group of NANIPER patients. Care has to be taken to extrapolate this data to the patients seen in the first echelon of which a subgroup (e.g. NARES) is reported to respond to steroid treatment.

Is local capsaicin therapy effective in NANIPER? What could be the working mechanism?

Capsaicin is the pungent agent in sharp food such as red peppers. This chemical has the ability to stimulate sensory nerve fibers, especially nonmyelinated c-fibers that act predominantly as nociceptors. On acute exposure to capsaicin, these fibers generate action potentials; at the same time, they are stimulated to release neuropeptides that are stored in granules, at nerve endings. These include tachykinins, CGRP, GRP, and possibly others. When capsaicin is applied chronically and/or at high doses at nerve endings, defunctionalization occurs. Proposed mode of actions of capsaicin include; vanilloid receptor modulation (capsaicin receptor) (37), destruction of the cells of the c-afferent system (38, 39) and/or depletion of neuropeptides (19, 40).

Several studies have been published showing that capsaicin desensitization might be an important therapeutic modality in NANIPER (13, 16, 37, 40-42). However, no placebo-controlled studies have been performed. Moreover, the reported studies lack well-defined criteria for having NANIPER, with the risk of heterogeneity of the patients used.

In chapter 5 and 6 the effects of local repeated capsaicin versus placebo applications in a well-defined group of 24 NANIPER patients is described. We were able to show in a double-blind placebo controlled fashion that capsaicin treatment ameliorated nasal symptoms for at least nine months without affecting nasal mucosal cell and/or general nerve densities and/or inflammatory mediators.

Can it now be concluded that the therapy for NANIPER is found? The treatment period lasts 2 weeks during which 7 treatment visits of an average of 1 hour are required. Surgery aimed at a reduction of the inferior turbinate takes just one treatment visit and is reported to relieve nasal symptoms for a period of 1-year (43). The vidian neurectomy propagated by Golding and Wood in the early sixties has more recently been reported to reduce nasal complaints in 92% of the patients. This study extended over a 5-year period (44). The procedure also requires just one treatment visit. Unfortunately the vidian neurectomy is not very effective in relieving congestion whilst the surgery of the inferior turbinate does not relieve the runny nose and/or sneezer and not all reports present such good results (45, 46). Moreover both techniques seem crude and could be considered as "overdoing it" and serious complications were reported (blindness, adhesions and atrophic rhinitis). Hence the interest in capsaicin. It must, however, be obvious that a lot of work needs to be done to optimize the capsaicin treatment and dosage scheme. One could consider a "rush treatment" during the first treatment visit (e.g. 5 treatments in one morning) with monthly or bi-monthly maintenance visits. Another interesting scheme is the one suggested by Eberle (47) in which the patients apply a low-dose capsaicin solution 3 times a day during a period of 4 weeks at home. One can also conceive a combination of the previous schemes.

What is NANIPER?

The question of the mode of action of capsaicin in NANIPER cannot be answered without the answer to the question: "what is NANIPER?". As mentioned before NANIPER is probably not one disease but a collection of diseases inducing the same nasal symptoms.

NARES and occult allergy

One cannot elaborate on the effect of capsaicin in NARES patients since there is no data on these patients. Furthermore due to the good response of these patients to local steroids there is simply no need for capsaicin therapy in NARES. The same argumentation applies for the subgroup of patients possibly suffering from an occult allergy.

Chronic inflammation.

Chronic inflammation can be the result of the underlying disorder in NANIPER. It is known that allergic rhinitis patients with complaints where an allergic infiltrate can be observed in the nasal mucosa exhibit a nasal hyperreactivity (48). Therefore it is reasonable to raise the possibility of "a disorder" inducing an inflammatory infiltrate in the nasal mucosa of NANIPER patients resulting in their nasal complaints. Vice versa it can also be argued that the underlying disorder such as the postulated over-active NANC system (13) is directly responsible for the nasal complaints and that an eventually noted increase in inflammatory cell densities in the nasal mucosa is seen as a consequence of the underlying disorder without having any relation with the nasal complaints. Fortunately we can short-circuit this discussion since we have showed that inflammatory cells are not involved in NANIPER. Capsaicin therefore does not exert its effect in NANIPER through modulation of inflammatory cells.

Neurogenic imbalance

The theory of a neurogenic dysfunction is very attractive and elegant. It appeals to the modern descartian medical professional since cause and consequence are neatly ordered. Unfortunately hard evidence is scarce. One of the firsts to suggest the involvement of the NANC-system in NANIPER was Lundblad in 1983 (49). However he extrapolated from the animal model. Lacroix in 1992 was able to show an increased concentration of neuropeptides in a group of non-allergic rhinitis patients (19), improvement of symptoms by local treatment of capsaicin giving a 50% reduction in CGRP-Li content in nasal biopsies (40), and a correlation between symptoms intensity and CGRP-Li concentration in nasal mucosa (50). Regrettably these patients were either abusing sympathico-mimetic nose-drops or underwent surgery for paranasal sinus pathology. Graf described an increase in hyperreactivity following the use topical decongestants (51). And so, it is very imaginable that the increase of CGRP-Li levels is the result of an overactive peptidergic system induced by a suppressed sympathetic

system, or the result of chronic infection. In contrast, Wolff was recently unable to show a reduction of NANC-fibers in the nasal mucosa in NANIPER patients after successful capsaicin treatment (37). However his method of quantifying the NANC-fibers is not made clear. Fang reported on neuropeptide tissue concentrations and neuroendocrine cell densities in normals and NANIPER patients. No significant differences were found. Unfortunately, in spite of elegant neuropeptide quantification methods, patients are simple characterized as suffering from chronic hypertrofic rhinitis (53). Hyperresponsiveness of mucosal elements to neuronal stimulation has been putatively cited as a pivotal mechanism in NANIPER. Sanico was unable to show an increased (secretory) responsiveness to capsaicin provocation in a group of NANIPER patients and concludes that neurovascular involvement in NANIPER is unlikely (52). Unfortunately obstruction was not evaluated in this study. In our patients obstruction was the dominant complaint. Eberle was able to show a rhinomanometric increase in nasal flow after modulation of the neurovascular components of the nasal mucosa by capsaicin treatment, suggesting involvement of the neurovascular system in NANIPER. A recent study by Wilde showed an abnormal response to axillary pressure and isometric exercise in intrinsic rhinitis (NANIPER), perhaps due to relative nasal sympathetic hyposensitivity (24, 25). Since sensory efferent, cholinergic and adrenergic neural pathways are anatomically linked and interact a compensatory change (increase) in the sensory neural pathway (NANC-fibers) due to hyposensitivity of another neural (sympathetic) pathway is a plausible option. However in our own studies with capsaicin we were unable to show a change in overall neurogenic staining between placebo and capsaicin treatment in NANIPER patients. Nevertheless we feel that functional results prevail over morphological data. To conclude: it seems likely that capsaicin exerts its effect through a depletion of sensory neuropeptides either through atrophy of the NANC-system via neurotoxity (39) or through receptor modulation (37). Regrettably decisive data is missing.

Dys- or hyper- esthesia at the CNS level
If NANIPER patients are suffering from no more than a misguided perception due to a faulty interpretation of afferent signals originating in the nasal mucosa, than capsaicin is an ideal chemical that can either through atrophy or receptor modulation of the afferent neural system in the nasal mucosa trick the central nervous system into a "feel good" perception. The bandwidth of successful neural modulation is small. A total deafferentation of the c-afferent system, e.g. by applying a local anesthetic such as Xylocaine to the nasal mucosa, results in an increased perception of nasal blockage. Furthermore for a correction of a faulty perception suggesting a reduced nasal passage at CNS level an increase in afferent signals is required. This can be compared with the perception of an increased nasal flow after the consumption of menthol. Obviously, this train of thought is false if all the afferent signals responsible for the perception of nasal flow are generated by the myelinated C-afferent system.

CONCLUSIONS

- A well-defined group of NANIPER patients was selected.
- NARES patients were not included in our group. This is probably the result of a good response to therapy in the first echelon.
- Patients with a typical allergic infiltrate in the nasal mucosa (despite negative allergy tests) constituted a maximum of 3% of the study population
- Fluticasone propionate aqueous nasal spray (FPANS) does not reduce nasal complaints in NANIPER.
- Fluticasone propionate aqueous nasal spray (FPANS) does significantly reduce the normal inflammatory cell densities in NANIPER. This has no clinical bearing.
- Inflammatory cells are not involved in NANIPER
- Capsaicin aqueous nasal spray significantly reduces nasal complaints in NANIPER patients.
- Capsaicin aqueous nasal spray does not affect inflammatory cell densities or inflammatory mediator concentrations in the nasal mucosa.
- The etiology of NANIPER is still unclear.

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Chapter 8

Summary
Samenvatting

SUMMARY

Chapter 1 contains a general introduction to the thesis. NANIPER is defined as a chronic nasal disorder of unknown pathogenesis. The epidemiology, pathogenetical models and treatment modalities of NANIPER are described. The key-questions of this thesis are presented.

In **chapter 2** forty patients suffering of NANIPER were carefully selected on the basis of inclusion and exclusion criteria proposed by Mygind and Weeke. Nasal biopsy specimens were taken in the patient group as well as a group of ten controls. Brush cytology was also taken in the NANIPER group. Inflammatory cells were identified and counted in the nasal mucosa with the use of immunohistochemical techniques and a panel of monoclonal antibodies. Eosinophils were studied with the use of BMKI3, RG2, and Giemsa. Mast cells were studied with antichymase (B7), anti-tryptase (G3) and toluidine blue. Sections were stained with IgE as well. There was no significant difference in the number of eosinophils, mast cells and IgE-positive cells between the two groups. Additionally in contrast with other reports, in sections that were double-stained with anti-chymase and anti-tryptase, single chymase positive cells were found.

In **chapter 3** mucosal inflammatory cell densities are correlated with nasal complaints in NANIPER. Some authors suggest inflammation of neurogenic or immunogenic nature as underlying disorder for NANIPER. We examined whether inflammatory cells are involved in the pathogenesis of NANIPER. Nasal biopsies were taken of sixty-five patients with significant nasal complaints and twenty controls without nasal complaints. Inflammatory cells were quantified, using monoclonal antibodies directed against lymphocytes, antigen presenting cells, eosinophils, mast cells, macrophages and monocytes. No significant differences were found, for any cell, between patients and controls. We conclude that inflammatory cells are not involved in NANIPER

In **chapter 4** the efficacy of topical steroids on nasal complaints and the effect on mucosal cell densities are studied in a group of 65 NANIPER patients. Topical corticosteroids are the therapy of choice in non-allergic non-infectious perennial rhinitis (NANIPER). However, the efficacy of the steroid therapy in NANIPER is controversial, as is its mode of action. To our surprise, out of 300 patients initially diagnosed as suffering from NANIPER, only 65 patients with NANIPER reached threshold nasal symptom scores. Patients were randomized into four different treatment regimes. Placebo bi daily (BD) for 8 weeks, fluticasone 200 mg once daily (OD) and placebo OD for 8 weeks, fluticasone 200 mg OD and placebo OD for 4 weeks followed by fluticasone 200 mg BD for 4 weeks, and fluticasone 200 mg (BD) for 8 weeks. A small decrease in nasal symptomatology was found which only reached significance for sneezing. A significant dose dependent decrease in immunocompetent cells was found in nasal biopsies obtained after 4 weeks, and after 8 weeks of treatment. We conclude that

topical corticosteroids do not significantly improve nasal symptoms in this group of selected NANIPER patients, even though a significant effect was seen on cells in the nasal mucosa.

In **Chapter 5** the efficacy of topical aqueous capsaicin spray in the treatment of NANIPER patients was studied. Several authors described capsaicin, the pungent substance in red pepper, as an efficacious therapy for non-allergic non-infectious perennial rhinitis (NANIPER). Repeated capsaicin application induces peptide depletion and specific degeneration of the unmyelinated sensory C-fibers in the nasal mucosa. We performed a placebo (NaCl 0.9%) controlled study with 25 NANIPER patients. Daily record charts and visual analogue scales (VAS) were used for clinical evaluation. Nasal lavages were obtained before, during, and after treatment. There was a significant and long-term reduction in the VAS scores in the capsaicin group. No significant difference was found between the placebo and capsaicin treated groups for the mean group concentrations of leukotriene (LT) $C_4/D_4/E_4$, prostaglandin D_2 (PGD_2), and tryptase. The levels of mast cell mediators, tryptase and PGD_2, and leukotrienes, mediators derived from a variety of inflammatory cells, were low at baseline and comparable with levels observed in nasal lavages obtained from normals. As involvement of inflammation could not be demonstrated, it is not surprising that capsaicin has no effect on inflammatory mediators. This suggests that inflammatory cells do not play a major part in the pathogenesis of NANIPER.

In **Chapter 6** the long term effects of capsaicin spray on the nasal mucosa are studied. Capsaicin has been shown previously to reduce nasal complaints in patients with a non-allergic non-infectious perennial rhinitis. Proposed pathophysiologic mechanisms for non-allergic non-infectious perennial rhinitis include a chronic inflammatory disorder of antigenic or neurogenic nature as well as the possibility of a functional neuronal disorder. We hypothesized that the benificial effect of capsaicin might be the result of a down regulation of inflammation (by a reduction of inflammatory cells) or through a modulation of neural tissue density. Patients were treated with either a placebo or capsaicin spray solution delivering 0.15 milligrams of capsaicin per nostril once every second or third day for a total of seven treatments. Both sides were treated each visit. Biopsies were taken before, 2 weeks after, 3 months after, and 9 months after the treatment period. Immunohistochemical staining of the biopsy specimen was performed to ascertain the effect of treatment on immunocompetent cell densities (quantitative) and neural tissue densities (semi-quantitative) in the nasal mucosa. Nasal complaints were significantly reduced in the capsaicin treated group. The number of CD1+, CD25+, CD3+, CD68+, BMK13+, IgE+, Tryptase+, and Chymase+ cells did not significantly differ between capsaicin and placebo group. No significant differences between both groups were found in pan-neurogenic staining of nasal mucosa using neurofilament and synaptophysine. Capsaicin aqueous nasal spray has been shown previously to reduce nasal complaints without affecting cellular homeostasis or overall neurogenic staining upto 9 months after treatment, furthermore immunocompetent cells are not involved in non-allergic non-infectious perennial rhinitis.

SAMENVATTING

Hoofdstuk 1 bevat de algemene introductie van dit proefschrift. NANIPER wordt gedefinieerd als een chronische rhinopathie met een onduidelijke pathogenese. De epidemiologie, bestaande pathogenetische modellen en behandelings-mogelijkheden van deze aandoening worden besproken. De doelstellingen van dit proefschrift worden beschreven.

In **hoofdstuk 2** konden, gebruik makend van de in en exclusie criteria van Mygind en Weeke 40 patiënten met NANIPER geselecteerd worden. Zowel in deze patiëntengroep als bij 10 controles zonder neusklachten werden neusslijmvliesbiopten genomen. In de NANIPER groep werd eveneens een neusbrush afgenomen. Met behulp van monoclonale antilichamen werden inflammatoire cellen geïdentificeerd en gekwantificeerd in de biopten. Eosinofielen werden bestudeerd met behulp van BMK13, EG2 en Giemsa. Mestcellen werden bestudeerd met behulp van anti-chymase, anti-tryptase en toluidine blauw. De coupes werden eveneens gekleurd met anti- IgE. Er werd voor geen van de verschillende kleuringen significante verschillen gevonden. Verder werden in tegenstelling tot de bekende literatuur enkel chymase-positieve cellen gevonden in coupes dubbelgekleurd met anti-chymase en anti-tryptase.

In **hoofdstuk 3** wordt onderzocht in hoeverre inflammatoire cellen betrokken zijn bij de neusklachten van NANIPER patienten. Inflammatie van neurogene dan wel immunogene aard wordt door sommige auteurs als het onderliggend lijden van NANIPER gezien. Wij onderzochten of inflammatoire cellen betrokken zijn bij het ontstaan van NANIPER. Bij 65 patiënten, met voldoende neusklachten, en 20 controles zonder neusklachten werden biopten van het neusslijmvlies genomen. De aantallen lymfocyten, antigeenpresenterende cellen, eosinofielen, macrofagen, monocyten en mestcellen werden bepaald met behulp van monoclonale antilichamen. Er werden geen significante verschillen gevonden tussen patiënten en controles. Wij concluderen dat inflammatoire cellen niet betrokken zijn bij NANIPER.

In **hoofdstuk 4** wordt de effectiviteit van locale steroiden bij de behandeling van neusklachten en het effect op inflammatoire in het neusslijmvlies bestudeerd. Lokale steroïden zijn de therapie van keuze in NANIPER. De effectiviteit en het werkingsmechanisme van de steroïden bij NANIPER zijn echter stof voor discussie. Tot onze verrassing bleken slechts 65 van de 300 patiënten, die aanvankelijk als NANIPER geboekt waren, voldoende neusklachten te hebben om aan onze inclusie criteria te kunnen voldoen. Deze 65 patiënten werden gerandomiseerd in 4 verschillende behandelingsschema. Placebo twee maal daags (BD) gedurende 8 weken, FPANS 200 mcg een maal daags (OD) en placebo (OD) gedurende 8 weken, FPANS 200 mcg OD en placebo OD gedurende 4 weken gevolgd door FPANS 200 mcg BD gedurende 4 weken, en FPANS 200 mcg gedurende 8 weken. Er werd een kleine afname van neusklachten gevonden die alleen significant bleek voor niezen. Een significante dosis afhankelijke afname van het aantal immuuncompetente cellen werd gevonden na 4 en 8 weken behandeling. Wij concluderen dat locale steroïden de neusklachten van deze groep

streng geselecteerde patiënten niet significant verbeterd ondanks een significante afname van het aantal immuuncompetente cellen in het neusslijmvlies.

In **hoofdstuk 5** wordt het effect van capsaicine nevel op de neusklachten van NANIPER patienten en op ontstekingsmediatoren bestudeerd. Capsaicine wordt door verschillende auteurs beschreven als een effectief middel bij NANIPER. Herhaalde capsaicine applicaties induceren neuropeptide depletie en specifieke degeneratie van de niet gemyeliniseerde C-afferente vezels in het neusslijmvlies. Bij 25 patiënten werd een placebo gecontroleerde studie verricht. Dagkaarten en visual analogue scales (VAS) werden gebruikt voor de klinische evaluatie. Voor, na en gedurende de behandeling werden neuslavages verricht. Er werd een lang aanhoudende, significante vermindering van neusklachten gevonden in de met capsaicine behandelde groep. Er werd geen verschil gevonden tussen de gemiddelde groep concentraties van leukotriënen C4/D4/E4, prostaglandine D2, en tryptase voor de verschillende behandelingen. De concentraties van mest cel mediatoren, tryptase en prostaglandine D2, en leukotriënen, mediatoren afkomstig van verscheidene inflammatoire cellen waren laag en vergelijkbaar met concentraties gevonden bij normale controles. Capsaicine had dan ook geen effect heeft op de ontstekingsmediatoren. Dit suggereert dat inflammatoire cellen geen grote rol spelen in de pathogenese van NANIPER.

In **hoofdstuk 6** worden de lange termijn effecten van capsaicine nevel op het neusslijmvlies van NANIPER patienten onderzocht. De effectiviteit van capsaicine behandeling bij NANIPER werd in hoofdstuk 5 beschreven. Veronderstelde pathogenetische mechanismen omvatten een chronisch inflammatoire aandoening van antigene dan wel neurogeen origine alsmede een mogelijke functionele neurogene aandoening. Wij veronderstelden dat het gunstige effect van capsaicine bij NANIPER ontstaat ten gevolge van een vermindering van inflammatie (via een reductie van inflammatoire cellen) of dankzij een vermindering van de dichtheid van zenuwweefsel in het neusslijmvlies. Patiënten werden behandeld met placebo of met een waterige capsaicine oplossing waarbij 0.15 mg capsaicine per neusgat werd verneveld om de een of twee dagen met in totaal 7 behandelingen. Neusslijmvlies biopten werden genomen voor, 2 weken na, 3 maanden na en 9 maanden na behandeling. Coupes van deze biopten werden immunohistochemisch bewerkt zodat het effect van de behandeling op inflammatoire celdichtheid (kwantitatief) en zenuwweefsel dichtheid (semi-kwantitatief) bepaald konden worden. Het gunstige effect van capsaicine is reeds in hoofdstuk 5 beschreven. Er werd geen verschil gevonden in de mediane aantallen van CD1+, CD25+, CD3+, CD68+, BMK13+, IgE+, Tryptase+ en Chymase+ cellen tussen de placebo en de met capsaicine behandelde groep. Ook met behulp van de neuromarkers "neurofilament" en "synaptofysine" konden geen verschillen worden aangetoond. Capsaicine heeft een gunstig effect op de neusklachten bij patiënten met NANIPER. Dit resulteert niet in een meetbaar effect op inflammatoire cellen of zenuwweefsel dichtheid in het neusslijmvlies.
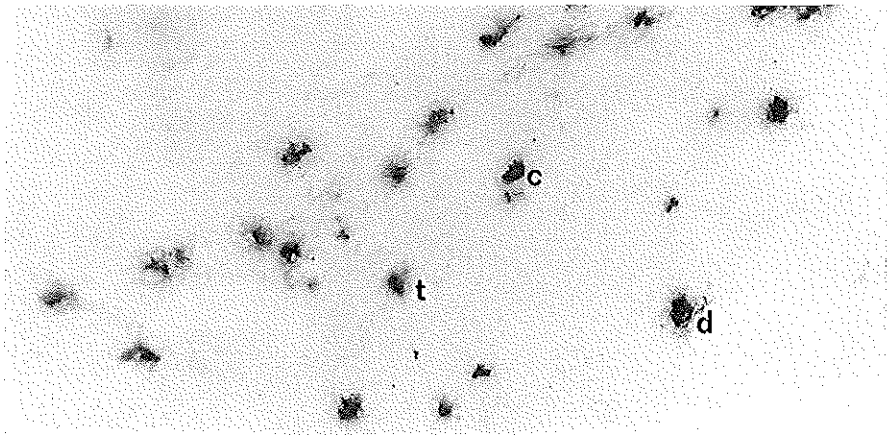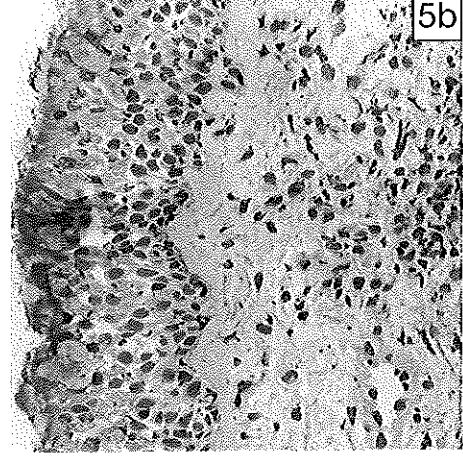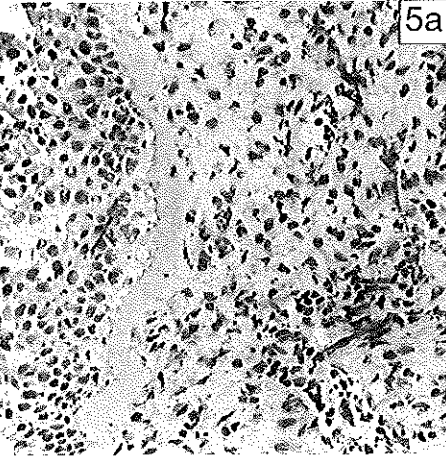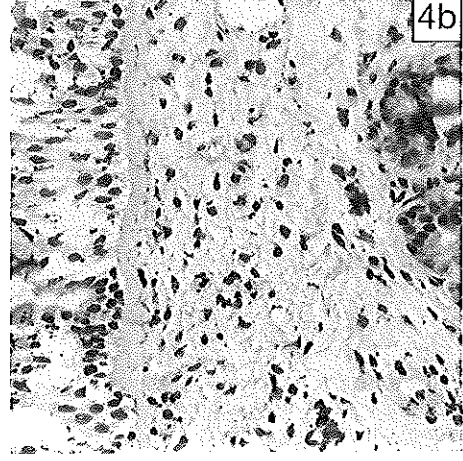
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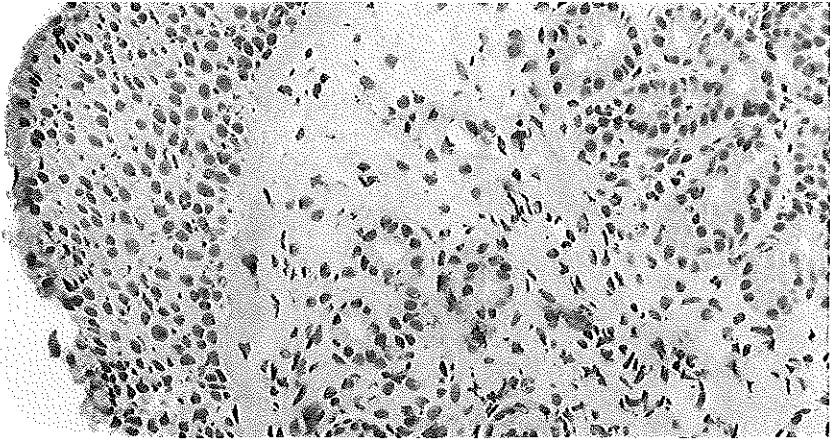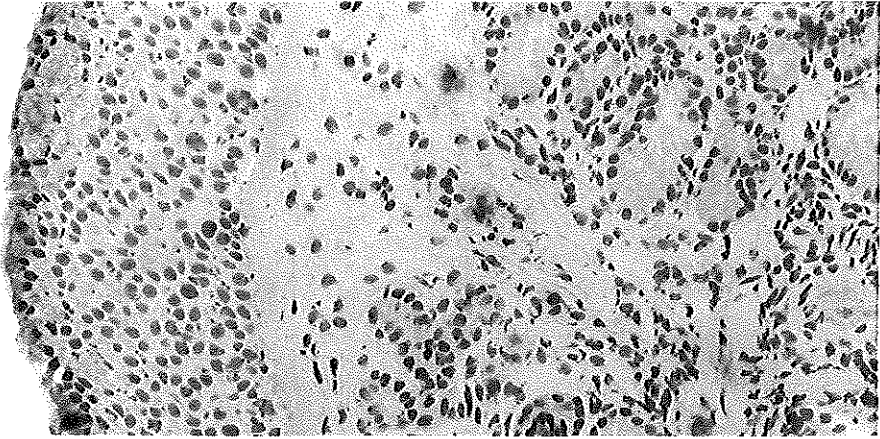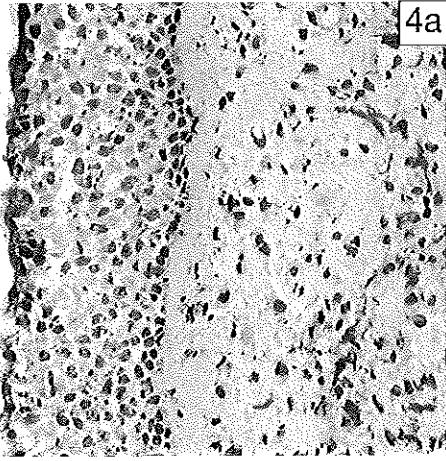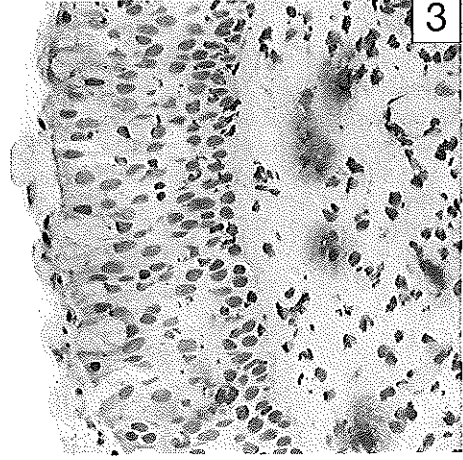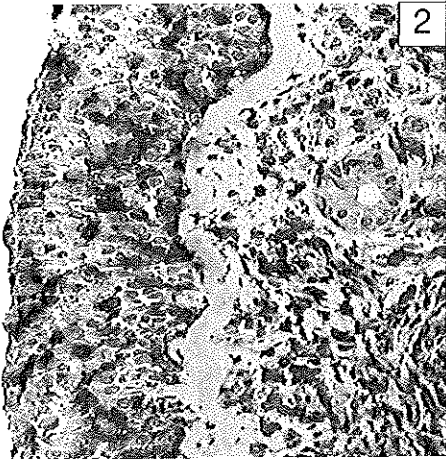
Figure 1 a-c. Serial sections of nasal mucosal biopsies. 1a Nasal mucosa stained with anti-chymase. 1b Nasal mucosa stained with anti-tryptase. 1c Nasal mucosa stained with anti-chymase (blue) and anti-tryptase (red). The double stained cells can be clearly discerned. Single-chymase positive cell marked (c), single-tryptase positive cells marked (t), double positive cells marked (d).The sections are slightly counterstained with hematoxylin and eosin (magnification 160 x).

Figure 2. Lymphocytes in the nasal mucosa (CD3 +). The epithelium, basal membrane, and lamina propria can be distinguished. Positive cells stain red. Lymphocytes are abundantly present in both layers (magnification 160 X).

Figure 3. Mast cells in the nasal mucosa (tryptase+). The different mucosal layers can be distinguished. Mast cells are not present in the epithelium, but are frequently present in the lamina propria (magnification 160 X).

Figure 4a and b.   Synaptophysine in the nasal mucosa (magnification 160 X).
                    4a. Strong staining. Axial, transversal and longitidinal cut fibers can be
                    distinguished. No signal is seen in the epithelium
                    4b. Weak staining. Mostly axial cut fibers are seen.
Figure 5a and b.   Neurofilament in the nasal mucosa (magnification 160 X).
                    5a. Strong staining. Mostly longitudinal cut fibers are seen in both
                    the epithelium and lamina propria.
                    5b. Weak staining. Some longitudinal cut fibers can be
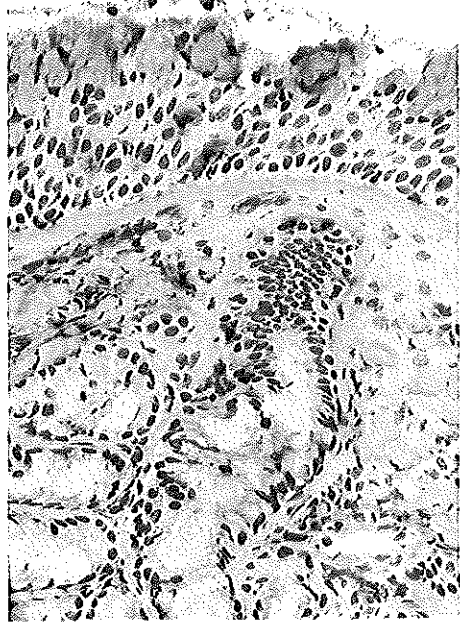   distinguished.

Figure 6. Sections of nasal mucosa of patients and controls. Eosinophils (A, B), IgE positive (C, D) and CD4 (G, H) and CD8 (E, F) positive cells are shown. No significant differences were found between both groups for the various cells. (A, C, E, G: NANIPER patients; B, D, F, H: CONTROLS; magnification 160 X)
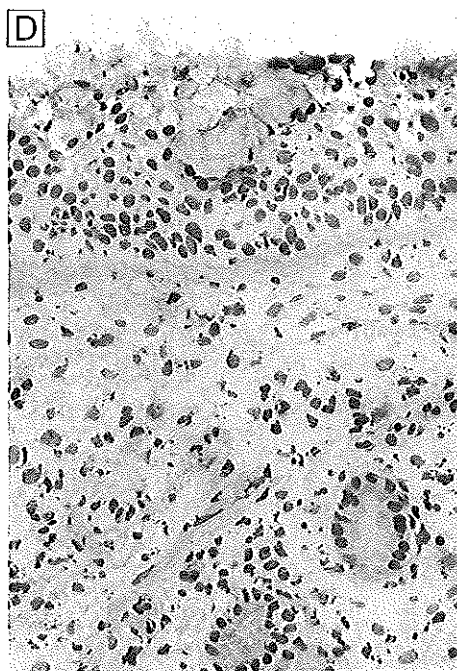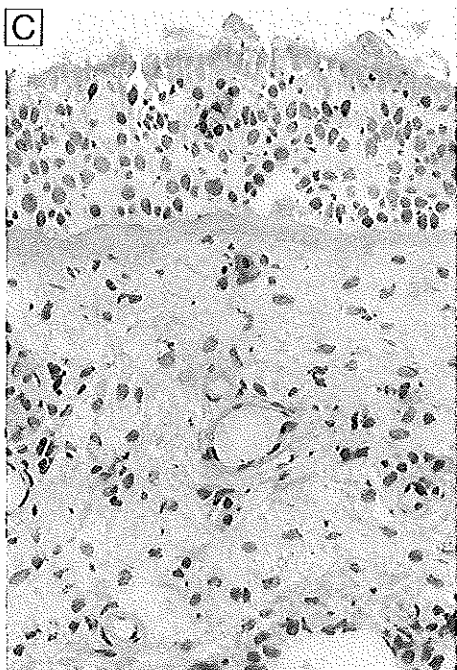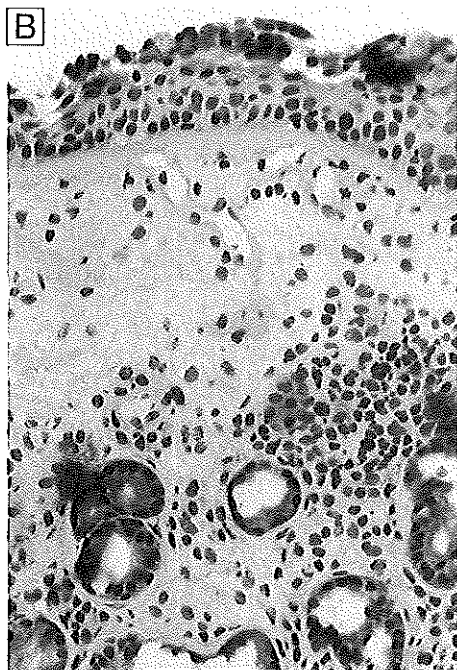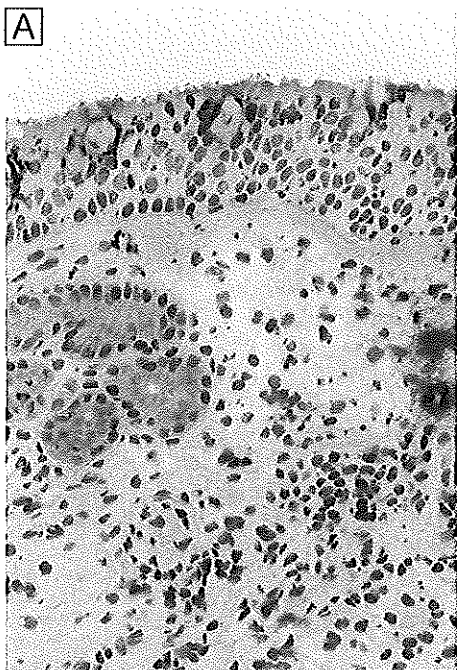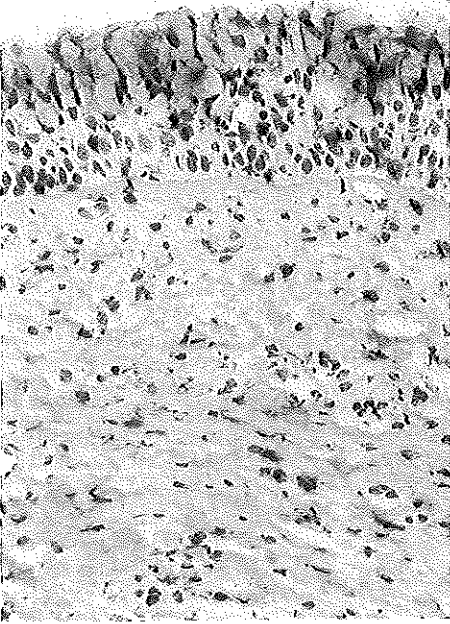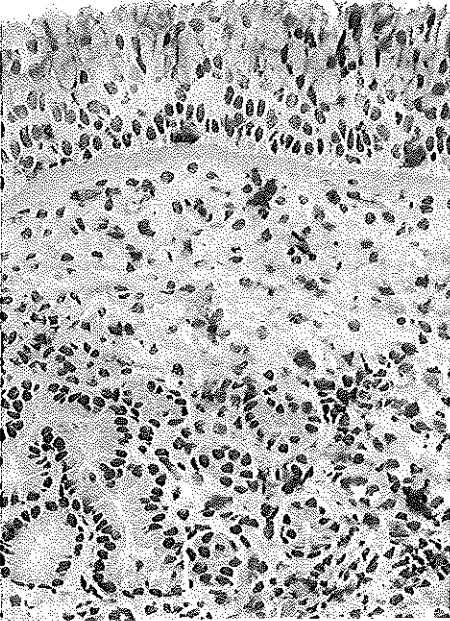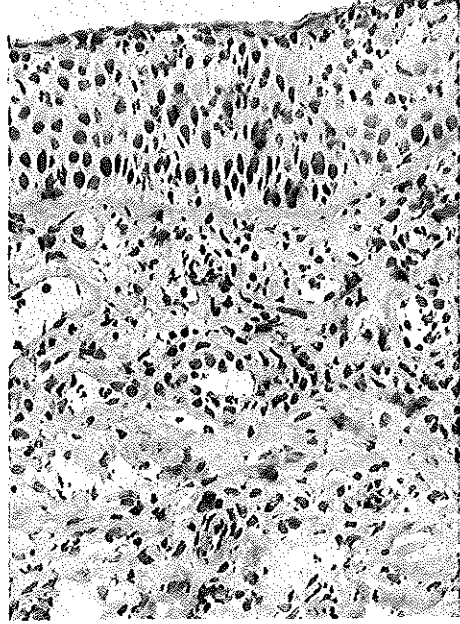
DANKWOORD

De in dit proefschrift beschreven onderzoeken werden verricht binnen de afdelingen Keel-neus-oorheelkunde van het Leyenburg Ziekenhuis, Den Haag en het Dijkzigt Ziekenhuis, Rotterdam en de afdelingen Immunologie en het Keel-neus-oorheelkundig research laboratorium van de Erasmus Universiteit Rotterdam.
Ik wil graag eenieder bedanken die me geholpen heeft bij de totstandkoming van dit proefschrift. Een aantal van hen wil ik hier met name noemen.

Prof. Dr. C.D.A. Verwoerd. Hartelijk dank voor het gestelde vertrouwen. Gelukkig heeft U het dreigement dat mijn opleiding minstens 10 jaar zou gaan duren niet uitgevoerd.

Dr. W.J. Fokkens. Beste Wytske, ik ben je zeer erkentelijk voor je begeleiding en je aandeel in mijn vorming tot KNO-onderzoeker. Je hebt de gave om dingen scherp te bekijken.

Dr. E. Rijntjes. Beste Evert, allereerst dank, dat je me uit de klauwen van de minister gered hebt. Verder heb ik in het Leyenburg de KNO "ontdekt". Jouw omgang met patiënten is bijzonder stimulerend. Ik heb een perfecte wetenschappelijke en geneeskundige start bij je kunnen maken.

Prof. Dr. J.H. de Jongste. Beste Johan, de snelheid en het enthousiasme waarmee je het manuscript hebt beoordeeld, doet vermoeden dat we veel en plezierig zullen samenwerken.

Prof Dr. H.C. Hoogsteden. Beste Henk, zeer veel dank voor je razendsnelle hulp.

Prof. Dr. A. Änggård. Dear professor Änggård, I would like to thank you very much for your stimulating words during various international meetings. I'm honored that you are prepared to come to Holland to "join the opposition". I look forward to the next meetings.

Dr. R. Gerth van Wijk. Beste Roy, niet alleen heb je een belangrijk aandeel gehad in de capsaicine onderzoeken, ook heb je me geholpen met de puntjes op de "i" van de discussie. Ik waardeer het enorm dat je jezelf op 23 oktober hebt willen vrijmaken.

Elly van Schaik. Beste Elly, een dankwoord zou niet compleet zijn als jij niet vermeld zou zijn. Je hebt je het vuur uit de sloffen gelopen om patiënten te verzamelen, motiveren en behandelen.

Jeroen van Rijswijk. Beste Jeroen, ik vind het geweldig hoe jij je voor eenieder, in voor jou toch zeer moeilijke tijden, inzet. Ik hoop dat ik voor jou hetzelfde terug kan doen. "Nema problema"

Alex KleinJan en Lisanne Severijnen. Jullie is nooit wat teveel; de duizenden coupes, kleuringen en een groot gedeelte van de tellingen. En natuurlijk de goede ideeën. Petje af.

Ewout Baarsma en Cock Hoogerwerf. Hartelijk dank voor het stimuleren van jullie patiënten om aan de onderzoeken mee te doen.

Paul Mulder. Beste Paul, met jou (soms onnavolgbare) kijk op de data, heb je mij menig maal de relevantie van significantie doen inzien.

Tom Godthelp. Beste Tom, jij was mijn laboratorium vraagbaak in de eerste jaren. Je veelomvattende gezellige persoonlijkheid zal zeker tot zijn recht komen in het "Brabantse".

Adriaan Holm. Beste Adriaan, ik begrijp niet waarom ze ons voortdurend verwisselen. Jij wel?

Hans Hoeve. Capo di tuti capi van het Sophia, ik werk erg graag met je samen.

De patiënten. Zonder jullie was dit boekje er niet geweest. Biopten, brushes, bezoeken; placebo, peper, patiëntenboekjes; het was allemaal geen enkel probleem. Heel veel dank.

De pàh, de màh en fratel. Bedankt dat jullie BS, op gezette momenten, de spiegel voorhouden.

Maja, moja prva i poslednja ljubav.

Curriculum Vitae

Hendrikus Maria Blom werd op 3 mei 1964 te Rijnsburg geboren. Het diploma Atheneum B werd in 1982 behaald aan het Bonaventura College te Leiden. Van 1982 tot 1990 studeerde hij Geneeskunde aan de Rijks Universiteit te Leiden. In 1986 deed hij een clinical clerkship plastische chirurgie in St. Vincent's Hospital, Sydney, Australie. Van 1 januari 1990 tot 1 januari 1991 was hij werkzaam als basisarts op de vliegbasis Soesterberg. Van 1 januari 1991 tot juli 1993 was hij werkzaam als AGNIO KNO in het Ziekenhuis Leyenburg te Den Haag. Hier werd onder supervisie van dr. E. Rijntjes begonnen met het onderzoek dat resulteerde in dit proefschrift. Van 1 november 1993 tot 1 mei 1998 werd de opleiding tot KNO-arts gevolgd in het Academisch Ziekenhuis te Rotterdam onder leiding van Professor Verwoerd. Sinds 1 mei 1998 is hij als staflid verbonden aan de afdeling KNO-heelkunde van het AZR, met als aandachtsgebied de pediatrische KNO-heelkunde. Hij is getrouwd met Maja Stefanovic. Zij hebben een zoon, Nikola.