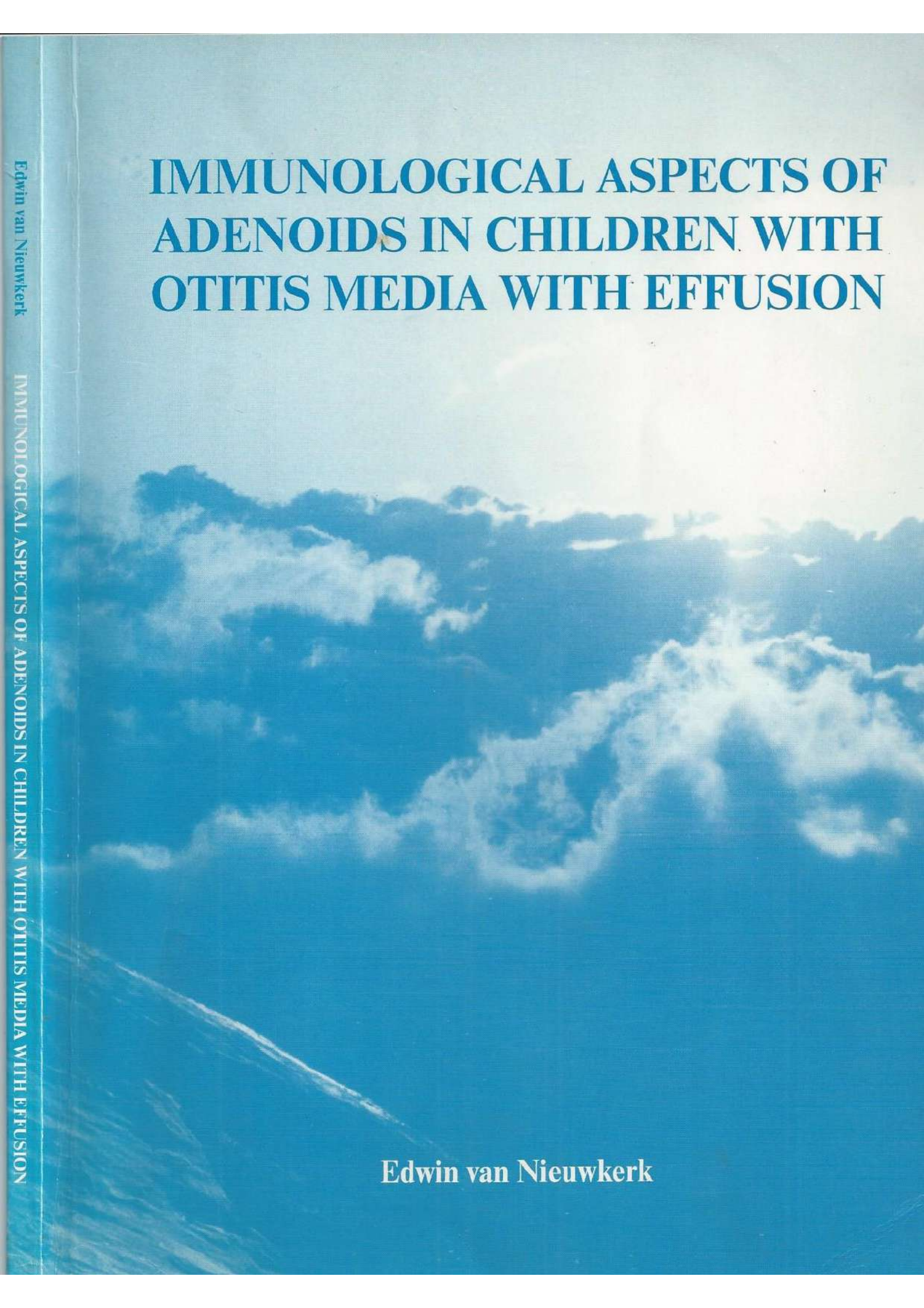
IMMUNOLOGICAL ASPECTS OF ADENOIDS IN CHILDREN WITH OTITIS MEDIA WITH EFFUSION



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I wish to thank all who have contributed to the work described in this thesis; their support was greatly appreciated.

The studies described in this thesis were performed in the Department of Cell Biology, Division of Electron Microscopy (Head: Prof.dr E.C.M. Hoefsmit), Vrije Universiteit.

This doctorate was financially supported by Astra Pharmaceutica BV, Glaxo BV, Roussel BV, Entermed BV, Pfizer BV producent van o.a. Zithromax en Terra-Cortril, UCB Pharma BV, Zambon Nederland BV, Artu Biologicals NV, Abbott BV, Yamanouchi Pharma BV.

Cover: Khumbu, 1983
Drukkerij Elinkwijk BV, Utrecht

VRIJE UNIVERSITEIT

IMMUNOLOGICAL ASPECTS OF THE ADENOID IN CHILDREN WITH OTITIS MEDIA WITH EFFUSION

ACADEMISCH PROEFSCHRIFT

ter verkrijging van de graad van doctor aan
de Vrije Universiteit te Amsterdam,
op gezag van de rector magnificus
prof.dr E.Boeker,
in het openbaar te verdedigen
ten overstaan van de promotiecommissie
van de faculteit der geneeskunde
op vrijdag 16 december 1994 te 13.45 uur
in het hoofdgebouw van de universiteit, De Boelelaan 1105

door

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Aan mijn ouders
Aan Jettie

CONTENTS

ABBREVIATIONS

AOM	acute otitis media
APC	antigen presenting cell
APh	acid phosphatase
ATCC	american type culture collection
BG	Birbeck granulum
BHI	brain heart infusion
BSA	bovine serum albumin
CD	cluster of diferentiation
CFU	colony forming units
con A	concanavalin A
cpm	counts per minute
DAB	diaminobenzidine-tetrahydrochloride
DC	dendritic cell(s)
DNA	deoxyribonucleic acid
ET	Eustachian tube
FACS	fluorescein activated cell sorter
FCS	fetal calf serum
FDC	follicular dendritic cell
Gy	gray
HBSS	hanks balanced salt solution
HD	high density
HLA	human leukocyte antigen
HPS	human pooled serum
ICAM-1	intercellular adhesion molecule-1
IDC	interdigitating cell
Ig	immunoglobulin
IL-1	interleukin-1
IL2R	interleukin-2 receptor
LC	Langerhans cell
LD	low density
LFA	lymphocyte function-associated antigen
LPS	lipopolysaccharide
MALT	mucosa associated lymphoid tissue
MEE	middle ear effusion(s)
MHC	major histocompatibility complex
MLR	mixed leukocyte reaction
MoAb	monoclonal antibody
Mφ	macrophages
NO	nitric oxide
NTHI	non-typeable Haemophilus influenzae
OME	otitis media with effusion
PB	peripheral blood
PBS	phosphate buffered saline
PS	patient serum
PWM	pokeweed mitogen
s.d.	standard deviation
sIgA	secretory immunoglobulin A
TGF-β	transforming growth factor-β
TNF	tumor necrosis factor
URI	upper respiratory tract infection
VC	veiled cells

GENERAL INTRODUCTION.

OTITIS MEDIA WITH EFFUSION.

Otitis media with effusion (OME) is one of the most common diseases in early childhood. In the 1950s many physicians believed that a new disease had been discovered when OME was recognized in abundance. However, it is generally regarded that Politzer at the end of the last century provided the first comprehensive description of this disease. In fact, references to a condition with the same description are present dating back to the Hippocratic school in 400 BC (Black 1984).

Since Politzer's classic work (1878) more than fifty different names have been identified over the years for OME (Black 1984). At the "Second International Symposium on Recent Advances in Otitis Media with Effusion" an uniform terminology was proposed to improve clinical and scientific communication (Senturia et al. 1980). At the Third International Symposium the consensus of the second symposium was questioned. At this symposium the view was held that individual authors might use whatever nomenclature appeared appropriate, provided that it was defined (Paparella et al. 1985). OME, in this thesis, is defined as the accumulation of fluid in the middle ear cavity behind an intact tympanic membrane without the symptoms and signs of an acute infection. The chronology of OME is not involved in this definition. The effusions can be serous, mucoid or mucopurulent (Senturia 1976).

A large-scale epidemiological study provided a good view into the occurrence and natural course of OME in the Netherlands (Zielhuis et al. 1989). It appeared that 80% of the children experienced at least one episode of OME before the age of four. The improvement rate was about 50% every three months and the cumulative rate of recurrence was about 50% in the two year study period. Earlier epidemiological studies from other countries provided also a good view into the occurrence and natural course of OME in children of various ages (Cauwenberge 1988, Tos et al. 1984, Chalmers et al. 1989, Fiellau-Nikolajsen 1983).

The otological sequelae of persistent OME can vary from atrophy, tympanosclerosis and attic retraction of the tympanic membrane (Tos et al. 1987, Sadé & Berco 1976, Schilder 1993) to more serious conditions such as adhesive otitis or cholesteatoma (Sadé et al. 1981). The principal effect of OME is a conductive hearing loss which generally averages around 15-30

dB, associated with the presence of fluid in the middle ear (Fria et al. 1985, Chalmers et al. 1989, Schilder et al. 1993). Recent studies also suggest the possible development of sensorineural hearing loss during the course of OME (Harada et al. 1992, Schilder 1993).

The relationship between OME during early childhood and delayed development of speech, language and cognition has been debated for over 25 years. Longitudinal studies showed such a relationship between OME and a delay in speech and language development and in social maturity in children up to 4 years of age (Jerger et al. 1983, Teele et al. 1984, Wallace et al. 1988, Rach et al. 1988). Recently, it has been shown that the association between early OME and the delay in language development, found at preschool age, was no longer present at school age (Schilder 1993) although in the Dunedin study (Chalmers et al. 1989) problems with learning, language and development were still present at least until the children were aged 7 to 9. The relationship between OME and the development of the child remains controversial.

Diagnosis of OME is made by clinical, audiometric and tympanometric assessment. These assessments form the basis for selection between treatment or no treatment (wait and see policy).

Treatment for OME varies widely and is dependent on the duration and severity of the condition. It is accepted that mild forms of the disease can resolve spontaneously. As yet, no study has demonstrated any long-term benefit from several medical treatments such as vasoconstrictor substances, anti-allergy remedies, antibiotics and systemic steroids (summarized in Bluestone & Klein 1988).

Treatment with ventilation tubes has become the surgical intervention most practiced for OME and is also the most frequently performed operation upon children (Effective Health Care 1992). Tubes placement improves hearing very effectively. However, its effect on the otological and functional sequelae of OME is largely unknown. Since Pollitzer, adenoidectomy is commonly accepted as a treatment for OME and is still widely performed.

In the following section the pathogenesis of OME will be described more extensively, with special emphasis on the possible role of the adenoid in this process.

PATHOGENESIS.

The major factors in the etiology and pathogenesis of otitis media with effusion are dysfunction of the Eustachian tube and inflammation of the mucosa of the middle ear and Eustachian tube (Bluestone 1983; Bernstein 1985; Sprinkle & Veltri 1986).

1. Dysfunction of the Eustachian tube.

The Eustachian tube (ET) or tuba auditiva forms a connection between the middle ear cleft and the nasopharynx, lined by a mucosal layer. The function of the ET is thought to be: 1) ventilation of the middle ear, 2) drainage of secretions from the middle ear and 3) protection of the middle ear against the nasopharyngeal secretions (Bluestone et al. 1972). The tube also protects the middle ear against noise and pharyngeal pressure changes (Holmquist & Olen 1980).

ET dysfunction as a factor in the pathogenesis of middle ear diseases has been discussed for many years (Bluestone et al. 1972; Renvall & Holmquist 1974; Cantekin et al. 1976; Bluestone & Doyle 1988). Dysfunction of the ET has especially been put forward in the development of OME. In children the ET is more horizontal and shorter than in adults (Proctor 1967). Bluestone and Cantekin (1979) describe dysfunction of the ET, due to either functional or mechanical obstruction. Functional obstruction can be caused by a collaps of the ET with increased compliance, as a result of differences in the structure of tubal cartilages (Bluestone & Beery 1976, Falk & Magnusson 1984) or by an inadequate active opening mechanism (especially the musculus tensor veli palatini)(Bylander et al. 1983) caused by immaturity of the neuromuscular system (Bylander & Tjernström 1983, Bylander 1984). It is also shown that the lumen of the isthmus of the ET increases with age (Sadé et al. 1985). A quantitative histological study of the ET shows no obstruction of the lumen or a significant difference between the size of the lumen in specimens with OME, acute otitis media (AOM) and non pathological specimens (Sadé & Luntz 1989). Mechanical obstruction can be intrinsic, due to edema of the tubal mucosa as in inflammation or allergy, or extrinsic as in the case of nasopharyngeal tumors or adenoid hyperplasia (Bluestone 1982). An abnormally patent ET may also cause middle ear disease; repeated sniffing is thought to create a negative pressure in the nasopharynx which,

via an ET with reduced closing ability, may be transferred into the middle ear (Magnusson 1981, Falk & Magnuson 1984).

All the mechanisms mentioned, leading to ET dysfunction correlate with the hydrops ex vacuo theory which suggests that in tubal dysfunction a negative pressure develops in the middle ear cleft due to gas absorption, resulting in a sterile transudate. This is clearly not the sole mechanism, as it is now realized that bacterial or viral infection of the upper airways with an accompanying inflammatory exsudate is a likely co-factor in the pathogenesis of OME (Bluestone 1983, Bernstein 1985). ET dysfunction can also be secondary to inflammatory processes in the upper respiratory tract.

2. Inflammation of the mucosa of the middle ear and of the ET.

Several studies show a correlation between upper respiratory tract infections (URI) and OME. Tos et al. (1979) state that the common cold due to viral and bacterial infections of the upper respiratory tract is the most frequent and dominant etiological factor in OME. Similar studies confirm these observations (Grote & Kuijpers 1980, Cauwenberge 1988, Casselbrant et al. 1985, Otten 1986). A correlation between acute otitis media (AOM) and OME has also been put forward (Shurin et al. 1979, Teele et al. 1980, Mills 1987). However, AOM is not necessarily preceding the development of OME (Stangerup & Tos 1985, Cauwenberge 1988, Schilder et al. 1992).

OME has long been considered as a sterile process, since in several reports, unsuccesfull attempts to culture bacteria from the middle ear effusions are described (Robinson & Nicholas 1951, Harcourt & Brown 1953, Siirala 1957). Positive bacterial cultures are demonstrated in 40% of middle ear fluid specimens by Senturia et al. (1958). Subsequently, in many other studies positive cultures of the effusions are found in 12-52% (Liu et al. 1975, Healy & Teele 1977, Giebink et al. 1979, Lim & DeMaria 1982, Brook et al. 1983, Cabenda et al. 1988). The most common bacteria isolated are *Streptococcus pneumoniae, Haemophilus influenzae, Moraxella (Branhamella) catarrhalis* and *Staphylococcus aureus*. Contradicting data exist over the presence of anaerobes (Brook et al. 1983, Edström et al. 1985).

Products, derived from micro-organisms, are also found in middle ear effusions (MEE) in OME: endotoxins of *H. influenzae*, even in MEE with negative cultures (DeMaria et al. 1984), interferon (Howie et al. 1982) and

neuraminidase (LaMarco et al. 1984)
Viruses and viral antigens, although playing a role in AOM and upper respiratory tract infections (Klein et al. 1982) which may precede OME, are not found in MEE of OME (Sarkinnen et al. 1982).

The MEE, found in OME, contain the major classes of immunoglobulins. Findings of higher concentrations of sIgA, IgA and IgG in MEE than in corresponding sera suggest a local production. Synthesis of other immunoglobulins in the middle ear, especially IgE is less certain (Juhn et al. 1971, Mogi et al. 1973, Liu et al. 1975, Bernstein 1979, Mogi 1984).

Activation of complement has been shown in MEE in OME (Prellner et al. 1980, Meri et al. 1984, Prellner 1987).
The presence of many other substances that take part in immune or inflammatory reactions have been shown in MEE: immune complexes (Veltri & Sprinkle 1976), histamine (Berger et al. 1984), bradykinins (Bernstein et al. 1978), plasminogen-plasmin system (Bernstein et al. 1979a), prostaglandins (Bernstein 1976), leukotrienes, platelet activating factor and lysosomal enzymes (Bernstein et al. 1979b). Also the presence of cytokines has been shown in MEE (Himi et al. 1992).
Moreover, it has been well established that all types of inflammatory cells are present in the MEE in OME including polymorphonuclear leukocytes, various types of lymphocytes, macrophages, eosinophils, basophils, natural killer cells and mast cells (Palva et al. 1987).

The middle ear mucosa itself shows histological changes. The middle ear cavity is normally lined by ciliated columnar and cuboidal cells. In OME it has been shown that there is an increase in goblet cells and mucous glands, leading to an increased secretory activity of the mucosa (Tos 1974). In animal experiments ET obstruction without infection does not result in transformation of the middle ear mucosa into an actively secreting epithelium, while ET obstruction with supervening infection shows a striking resemblance between the changes in the animal middle ear mucosa and in the middle ear mucosa of patients with OME (Kuijpers & van der Beek 1987). Van der Baan et al. (1988) show the development of MALT like organized lymphatic tissue in the middle ear mucosa in OME, but could not find

immune complexes, as suggested by Veltri & Sprinkle (1976).

All these factors: the relation between URI/AOM and OME, positive bacteriologic cultures and the presence of products of these bacteria in MEE in OME, the presence of immunological and inflammatory factors in the MEE, the presence of inflammatory cells in the MEE and the changes in the mucosa of the middle ear cavity in OME, support the role of inflammation in the pathogenesis of OME.

THE ADENOID AND OTITIS MEDIA WITH EFFUSION.

In 1860 the adenoid had been recognized as entity by Meyer (Black 1985). Thereafter, it was accepted that the nasopharynx played a pivotal role in OME. Adenoidectomy became the principal treatment for OME before the widespread use of ventilation tubes. Adenoidectomy is being used again in the treatment of OME, especially after several recent studies have confirmed its effectiveness (Maw 1983, Gates et al. 1987, Paradise et al. 1990). Several reasons are mentioned for removal of the adenoid: obstruction of the ET by an enlarged adenoid, improvement of ET function after adenoidectomy and/or removal of a chronically infected adenoid to eliminate a source of infection.

1. The adenoid as obstructive factor.
The notion that nasopharyngeal pathology represents a potential danger to middle ear ventilation stems from the association of enlarged adenoids with OME and the association of nasopharyngeal carcinoma with OME. Adenoids usually reach their maximum size in children at about the same age as OME is most prevalent, i.e. between the ages of two and four years. However, there are no studies available demonstrating a mechanical obstruction of the ET by adenoids (Sadé 1994). Several studies show that there is no correlation between adenoid size and the presence of OME (Roydhouse 1980, Hibbert & Stell 1982, Gates et al. 1988). Other clinical studies indicate that the effect of adenoidectomy is independent of its size (Gates et al. 1987, Paradise et al. 1990). Maw (1985) found adenoidectomy to be more effective in children with larger adenoids only in the first 3 months postoperatively. A correlation

between the weight of the adenoids and the presence of OME was not found (Gerwat 1975). Honjo (1988) showed no difference in ET function (opening pressure/positive pressure equiliazation) between children with OME in whom the tubal ostium was obstructed and children with OME in whom the ostium was clearly open. Moreover, there are strong indications that the site of ET obstruction is in the distal part of the cartilaginous portion, 5-15 mm from the orifice, rather than at the orifice itself (Takahashi et al. 1987).
Although there is no correlation between an enlarged adenoid and OME, recent work indicates that the nasopharynx itself, and particularly the nasopharyngeal airway, is significantly smaller in those children with proven OME when compared with matched normal controls (Philips et al. 1987). Recently, significant smaller nasopharynx dimensions were found in children with recurrent acute otitis media compared with controls (Niemelä et al.1994). Moreover, the period in which the nasopharyngeal airway is smallest coincides almost exactly with the ages at which the incidence of OME is highest (Jeans et al. 1981).
For nasopharyngeal carcinomas it has been shown that the middle ear effusion is the consequence of damage to the ET muscles and not a consequence of blocking of the ET opening in the nasopharynx (Meyers et al. 1984, Wei et al. 1988).
In conclusion, there is no clear causal relation between adenoid size and mechanical obstruction of the ET, leading to OME.

2. The adenoid as source of infection.
Since inflammation of the middle ear mucosa and of the ET mucosa is considered as a major factor in the pathogenesis of OME, it is suggested that the nasopharynx in these patients, i.e. the adenoid, is the source of infection.
In the nasopharynx non-pathogenic as well as pathogenic organisms like non-typeable *Haemophilus influenzae* (NTHI), *Streptococcus sp.* and *Moraxella catarrhalis* are present. The occurrence of these pathogens diminishes with age (Stenfors & Räisänen 1990). The period during which these bacteria are most often detected in the nasopharynx corresponds to the period of peak incidence of OMA and OME in children, i.e. between 2-5 years of age (Freijd et al. 1984, Stenfors & Räisänen 1991). Several studies show that the same bacterial species grow simultaneously in the middle ear cavity and in the nasopharynx (Sundberg et al. 1981, Palva et al. 1983, Stenfors &

Räisänen 1992). Furthermore, outer membrane protein and DNA fingerprint analysis of paired nasopharyngeal and middle ear fluid strains in otitis media due to NTHI, reveals that the nasopharyngeal and middle ear strains are identical (Murphy et al.1987, Loos et al. 1989).
If one compares the incidence of pathogens in the nasopharynx (adenoid) of children with OME and control children, conflicting data exist. Ruokonen et al. (1979) showed a 50% incidence of NTHI in children with OME compared with 14% in controls. Similarly, virus isolates were positive in 28% of the OME group versus 3% in controls. The same findings were reported by Tomonaga et al. (1989). However, Maw & Speller (1985) found no significant differences in cultures from the adenoid of children with OME compared with age-matched controls.
These studies all took swabs from the nasopharynx for culture, recent studies show a discrepancy between cultures from the surface of adenoid tissue and homogenized adenoid tissue: NTHI seems to be absent in traditional nasopharyngeal cultures while it can be isolated from homogenized tissue (Forsgren et al. 1993), where they are localized in subepithelial mononuclear cells as shown with in situ hybridization techniques (Forsgren et al. 1994).
The few studies that have been done to investigate the bacterial flora before and after adenoidectomy show a marked decrease of potentially pathogenic organisms after adenoidectomy (Talaat et al. 1989, Tomonaga et al. 1989).
Adherence of bacteria in the nasopharynx appears to be significantly greater in patients with OME than in normal subjects (Shimamura et al. 1990). It has also been shown that the normal decrease in adherence of NTHI to nasopharyngeal cells in growing up, healthy children does not occur in children with OME (Stenfors & Räisänen 1992).
In conclusion, there is increasing evidence from bacteriological studies that the adenoid can function as a source of infection in the pathogenesis of OME.

3. Efficacy of adenoidectomy and OME.
Studies of the efficacy of adenoidectomy in OME have given conflicting results. There is a wide variation in experimental design and methods used in these studies, making comparisons difficult. The great variety of treatments and outcomes makes meta-analysis impossible (Bodner et al. 1991). Other important variations include sample size, observer validation, entry criteria,

randomization and follow up procedures.
The principal studies showing no effect of adenoidectomy are those of Roydhouse (1980), Fiellau-Nikolajsen et al. (1984) and Widemar et al (1985). In these studies, outcome was measured and compared at widely spaced observation times e.g. at 1, 2 and 3 years after adenoidectomy (Roydhouse, 1980) or at 2 years post adenoidectomy only (Widemar et al. 1985), or small sample sizes were used like in the study of Fiellau-Nikolajsen et al. (42 patients)(1984).
Recent large scale, well designed, prospective studies showing a significant effect of adenoidectomy on OME, are those of Maw (1983), Gates et al. (1987) and Paradise et al (1990).
Maw (1983) studied 103 children from 2 to 12 years (mean age 5,25 years) with bilateral OME and randomly assigned them to 3 groups: adenotonsillectomy (N=34), adenoidectomy (N=36), or neither (N=33). At operation, one ear was randomly assigned to receive a tympanostomy tube. The rate of clearance of MEE in the unoperated ear (diagnosed by otoscopy) was the criterion of succes. At 3,6,9 and 12 months after surgery the clearance rates were: 56%, 64%, 58% and 72% respectively for adenoidectomy; 50%, 59%, 62% and 62 % respectively for adenotonsillectomy; 22%, 26%, 19% and 26% respectively for the control group. The difference between the two surgical groups and the control group at 12 months was significant, but there was no significant difference between the adenoidectomy and the adenotonsillectomy group. In a more recent study the patients from the previous study were re-evaluated with 42 additional patients: a significant resolution of effusions was demonstrated and maintained for at least 3 years in the adenoidectomy group. Although similar improvements could be achieved by use of a ventilation tube, reinsertion was required twice as frequently with a tube alone than when the middle ear drainage was combined with adenoidectomy or adenotonsillectomy (Maw 1988). Even after a 5 year follow-up, with a total of 222 children, adenoidectomy had a significant effect in relation to resolution of OME (Maw & Bawden 1994).
Gates et al. (1987) randomly assigned 491 4 to 8 year old children with OME to tympanostomy tubes, adenoidectomy or adenoidectomy plus tubes. Bilateral myringotomy and suction aspiration of the middle ears were performed in all cases. The children in the two adenoidectomy groups experienced significantly less time with effusion and fewer repeated surgical

treatments over the 2 year follow-up period than those who did not undergo adenoidectomy. There was no difference in time with abnormal hearing in the three surgical groups, regardless of placement of tubes. The reduction in morbidity (from control levels) in terms of time spent with recurrent effusion was 29% with tubes only, 38% with adenoidectomy and 47% with adenoidectomy and tubes.
Paradise et al. (1990) studied the effect of adenoidectomy in two groups of children with OME, recurring after prior tympanostomy tubes placement. The children in one group (N=99) were randomly assigned to either an adenoidectomy group or a control group; the treatment in the other group (N=114) was determined by parental choice. In both groups the outcomes for the adenoidectomized children were significant better than for the control children during the first 2 years of follow-up. In addition to significant reduction in time with effusion, the authors noted clinically significant reductions in suppurative (acute) episodes in the adenoidectomy group.
In conclusion, well designed prospective randomized studies support the view that adenoidectomy is effective in improving the natural history of OME.

IMMUNOLOGY AND THE ADENOID.

From the preceding paragraphs it is clear that the adenoid plays a distinctive role in the pathogenesis of OME, possibly by functioning as a source of infection.
For tonsils it has been well established that all the cell types, which are involved in immunologic reactivity, are present (Brandtzaeg 1987). The adenoids themselves have received only limited attention. Although tonsils and adenoids may have similar functions, the unique anatomic location of the adenoid in the nasopharynx and the presence of a respiratory epithelium on the adenoid (the tonsil is covered by a squamous epithelium) which contains secretory component and thus can form secretory IgA, makes insight in the immunology of the adenoids important.
This thesis deals with the cells involved in the immunological reactivity of the adenoid in children with OME. Special attention is paid to the dendritic cells and macrophages. In the next paragraphs a short introduction will be given to the immune response in general and dendritic cells and macrophages

in particular since these aspects play a central role in the following chapters. Finally the structure and function of the adenoid will be discussed.

The immune response.

When pathogens pass the epithelial lining, cells of the mononuclear phagocyte system will recognize and eliminate these pathogens. A first line of defence is thus provided by phagocytic cells, like macrophages and monocytes. Chemoattractive cytokines will be produced by these cells, leading to an influx of neutrophils. Neutrophils eliminate micro-organisms by phagocytosis and by intracellular digestion. This first line of defence guarantees a quick response to invading pathogens and can limit the spreading of infections through the body. At the same time, a specific immune response may develop in the lymphoid tissue to deal with remaining pathogens and to build up immunological memory.

The induction of the specific immune responses to pathogens results in the activation of antigen specific helper T-cells (Unanue et al. 1984), and is essential to start a humoral immune response. This activation is not induced by free antigen but only by so-called antigen presenting cells which degrade antigenic proteins and present the immunogenic peptides to the appropriate lymphocytes (Grey & Chestnut 1985). Although several cells such as macrophages, endothelial cells, epithelial cells and B lymphocytes are capable of antigen presentation, dendritic cells are considered as antigen presenting cells par excellence (Young & Steinman 1988). Helper T cells can only be stimulated by antigens presented to them by antigen presenting cells in the context of major histocompatibility complex (MHC) class II molecules (HLA-DR, -DP and/or -DQ molecules)(Thorsby et al. 1982). After antigen exposure to helper T cells, these T cells will induce B cell responses with subsequent development of immunoglobulin secreting plasma cells (Yewdall & Bennink 1990). Part of the activated B cells reach the follicle centers of lymphoid organs, where under influence of follicular dendritic cells, memory B cells are formed, which may rapidly differentiate into plasma cells after repeated antigen exposure.

Dendritic cells.

The term dendritic cell (DC) is used for a group of bone marrow-derived, mononuclear cells, which differ from lymphocytes and macrophages. These

cells were first isolated and described by Steinman and Cohn (1973) from mouse peripheral lymphoid organs. Nowadays it is accepted that the immune system contains a lineage of DC, the members of which are widely distributed throughout the body. In the body they occupy three compartments:

1. The bone marrow and blood from which DC can be isolated by culture (Knight et al. 1986).
2. Non lymphoid tissues such as Langerhans cells (LC) in the epidermis (Romani & Schuler 1989) and the interstitial DC of lung (Holt et al. 1993), heart (Hart & Fabre 1981) and liver (Prickett et al. 1988). From these tissues DC migrate via the afferent lymph as veiled cells (VC) (Hoefsmit et al. 1980, Pugh et al. 1983) to the regional lymph nodes.
3. Lymphoid organs; primarily the interdigitating cells (IDC) of the T cell dependent areas of lymph nodes (Kamperdijk et al. 1978), spleen and the thymic medulla (Landry et al. 1988).

These pools of DC are interconnected: following deposition of antigen DC migrate with this antigen via lymph to the T cell dependent area of lymphoid organs in which activation of recirculating, resting T lymphocytes occurs (Steinman 1991, Fossum 1988, Kupiec-Weglinski et al 1988). Very recently, the presence of two subsets of DC in blood has been suggested: a CD11c negative subset, representing immature bone-marrow derived precursors migrating to non-lymphoid tissues such as skin, and a CD11c positive subset, derived from tissues where they have been activated by antigen, migrating to the spleen or lymph nodes to stimulate T cell responses (O'Dohorty et al. 1994).

While many cell types are capable of generating MHC-peptide complexes and present these to primed T cells in vitro, it is evident that the DC is the most potent accessory cell to induce primary T cell responses (Inaba & Steinman 1984, King & Katz 1990). Moreover, it has been shown that DC initiate T cell dependent immune responses in vivo, such as contact sensitivity (Macatonia et al. 1986), MHC class I and II restricted T cell responses (Inaba et al. 1990), rejection of transplants (Larsen et al. 1990) and formation of T cell dependent antibodies (Sornasse et al. 1992).

Although there are no specific markers for human DC, the expression of some markers on DC (see below), together with their typical dendritic processes (Knight et al. 1986) and potent accessory activity in primary responses (such as antigen presentation to naive T cells and the mixed leuco-

cyte reaction (MLR)) (Young & Steinman 1988), makes them distinguishable from other cell types. In general, DC express high levels of MHC class I and II molecules, this can explain their potency as APC. There is high expression of adhesion molecules such as LFA-1, ICAM-1 and LFA-3. The Fc receptor and complement receptors are weakly expressed or absent on DC (King & Katz 1990). We identify DC in our laboratory on the basis of a dendritic appearance, the presence of a juxtanuclear concentration of lysosomes, shown by acid phosphatase activity in a spot, and by MHC class II molecules on the cell surface. Moreover, the monoclonal antibody (moAb) EBM11 (anti CD68) also stains in a spot near the nucleus in human DC (Betjes et al. 1991).

DC can be isolated from blood, lymphoid and non-lymphoid organs and afferent lymph by selection for low buoyant density, non-adherence to plastic surfaces, and the absence of cell surface markers found on other cell types (Steinman 1991). From human tonsils DC have been isolated and their cell surface molecules extensively studied (Hart & McKenzie 1988).

Macrophages.

Macrophages belong to the mononuclear phagocyte system (van Furth et al. 1972). These cells originate in the bone marrow from stem cells. The monoblast and promonocyte give rise to monocytes. They enter the circulation and then migrate into the tissues and serous cavities as macrophages, where maturation and differentiation occur in response to environmental stimuli (van Furth & Sluiter 1983).

All macrophages stain for acid phosphatase activity throughout the whole cytoplasm, this in contrast to the DC (Kamperdijk et al. 1989). The endogenous peroxidatic activity pattern is an important tool in classifying the activation and developmental stages of mononuclear phagocytes (Beelen & Fluitsma 1982). Different moAbs have been used to group human monocytes and macrophages into differentiation stages. EBM11 (CD68) recognizes an human antigen which occurs in monocytes and macrophages (Kelly et al. 1988). In contrast, other moAbs are more stage specific. UCHM1 (CD14) reacts with monocytes while RFD7 reacts with well differentiated macrophages (Janossy et al. 1986, Hogg 1989).

Macrophages are essential cells in both non-specific and specific immune responses. In the non- specific immune response they phagocytose invading

organisms and other antigens. They have prominent lysosomal granules containing degradative enzymes to destroy phagocytosed material. Killing will be initiated at the moment of phagocytosis by oxygen-dependent and oxygen independent mechanisms.

In the specific immune response they can act as antigen presenting cells, regulator cells and effector cells (Unanue & Allen 1987, Weaver & Unanue 1990).

MHC class II positive macrophages can, like all other MHC class II positive cells, present antigen to primed T lymphocytes. Unanue et al. (1984) claim that the macrophage is an important APC, because the endocytozing capacity of DC is limited to soluble antigens. However, some studies suggest co-operative interactions between macrophages and DC; macrophages ingest antigen particles, process them partially and provide immunogenic peptides for presentation by DC (Kapsenberg et al. 1986, Pancholi et al. 1992). However, Holt et al. (1988) suggest that lung macrophages have a suppressive effect on DC antigen presentation. Macrophages are generally not able to initiate immune responses by priming naive T cells (Inaba et al. 1990).

Macrophages are involved in the regulation of: 1) the inflammatory responses; in response to infection macrophages produce mediators like IL-1 and TNF which have proinflammatory activity (Dinarello & Savage 1989), 2) the haemopoiesis in the bone marrow (Denkers et al. 1992), 3) the antigen specific limb of the inflammatory response (Unanue & Allen 1987). Macrophages can also provide costimulatory factors to stimulate T cells (Dinarello & Savage 1989). Finally, they may also suppress immune responses by inhibiting T or B cell proliferation directly (Stout & Fisher 1983) or by inhibiting the result of dendritic cell antigen presentation (Holt and al. 1988, van Vught et al. 1992, Havenith 1993). Production of prostaglandin, TGF-β and possible nitric oxide are involved in the macrophage-mediated immuno suppression (Shibata & Volkman 1985, Pierce et al. 1989, Kawabe et al 1992).

As effector cells, macrophages can display strong cytotoxic activity towards potentially harmful micro-organisms, virally infected cells or tumor cells (Pryima 1989).

Structure and histology of the adenoid.

The adenoid (nasopharyngeal tonsil) forms a part of Waldeyer's ring. The

surface of the adenoid is largely covered by a respiratory epithelium, which, however, may be partly replaced by patches of squamous or intermediate type of epithelium (Ali 1965, Friedmann et al. 1972). The epithelium extends into crypts or vertical furrows (Ali 1965, Owen & Nemanic 1978). The crypt epithelium contains reticular parts corresponding to the dome regions of the underlying lymphoid follicles. Influx of foreign material (antigens) takes place mainly through these reticular parts of the crypt epithelium, in which antigen transporting membrane cells, M cells, are present (Karchev & Kabakchiev 1984, Winther & Innes 1994). It is unknown how antigen is translocated from the reticular epithelium, after passing the M cells, into the lymphoid follicles. Such a transfer normally takes place; the formation of germinal centers is in fact dependent on antigenic stimulation (Kraal et al. 1982).

The adenoid contains many lymphoid follicles, which follow the epithelial lining. B lymphocytes form the main cell type in lymphoid follicles. Histomorphometric measurements show that lymphoid follicles form the largest compartment in adenoids (Korsrud & Brandtzaeg 1980). The follicles are in reactive state with extensive germinal centers (secondary follicles). IgM is the most prominent surface isotype, followed by IgA, IgG and IgD (reviewed by Brandtzaeg 1987). Differentiation from B lymphocytes to Ig producing plasma cells, mostly IgG and IgA, takes place in the follicles. The framework of the follicles consist of characteristic follicular dendritic cells. These cells are thought to play an important role in generation of memory B cells (Nieuwenhuis & Opstelten 1984).
In between the follicles are the extrafollicular areas. Both T and B lymphocytes enter the lymphoid tissue through high endothelial venules in the extrafollicular areas (Gowans & Knight 1964). The extravasated T lymphocytes accumulate predominantly in this extrafollicular area. In these T cell areas interdigitating cells are present (Ennas et al. 1984). These cells are often closely surrounded by T lymphocytes, which may lead to the induction of an immune response.

Function of the adenoid.
The adenoid is thought to play an important role in non-immunological and immunological defence mechanism of the upper respiratory tract. The

adenoid has non-immunological defence systems with antimicrobial proteins such as lysozyme and lactoferrine, which act against a broad spectrum of antigens, as do non specific phagocytic cells. Also the mucociliary transport system clears particles that are trapped in the mucous blanket (Brandtzaeg 1984).
The immunological defence system of the adenoid is, in part, a secretory system, that contributes directly to the surface protection of the upper respiratory tract. The production of secretory IgA (sIgA) is an important first line defence against invasion by micro-organisms. The classic report of Ogra (1971) on IgA antibodies to poliovirus in nasopharyngeal secretions of children previously immunized perorally with live vaccin, describes that after removal of the adenoid (and tonsils), the pre-existing average titers decreased 3- to 4-fold, and in some children no antibody could be detected. Attempts to vaccinate a seronegative group of children who had been subjected to tonsillectomy and adenoidectomy resulted in a delayed and poor nasopharyngeal secretory IgA response. Earlier epidemiological studies demonstrated increased incidence of poliomyelitis after adenotonsillectomy in non-immunized children (reviewed in Ogra 1971).
The role of the adenoid in the induction of a secretory response is supported by the decrease of secretory IgA in the nasopharynx after adenoidectomy (D'Amelio et al. 1982). The output of sIgA is most likely of local origin (Korsrud & Brandtzaeg 1981).
In general it is assumed that antigen stimulated IgA bearing B cells migrate to the regional lymph nodes and via the regional lymphatics into the blood circulation. These cells finally home to the site of original antigenic challenge and initiate synthesis of specific antibody as plasma cells. Part of these cells will seed in mucosal sites distant from the site of initial challenge (Piedra et al. 1987). For the adenoid, so far, no prove for this concept has been given.
Adenoids are continously involved in immune responses to micro-organisms of the nasopharyngeal flora as shown by expansion of antigen specific B cell clones, but also polyclonal costimulation of B cells (Freyd & Rynnel-Dagöö 1982, Meistrup-Larsen et al. 1979, Platts-Mills & Ishizaka 1975, Rynnel-Dagöö et al. 1977, Rynnel-Dagöö 1978). It has been speculated that this phenomenon could be a phylogenetically old immunological defence mechanism activating many clones at the same time (Rynnel-Dagöö & Freijd 1988).

AIM OF THE STUDY.

Dysfunction of the Eustachian tube (ET) and inflammation of the middle ear and ET mucosa are considered to be important factors in the pathogenesis of OME. Bacteriological studies strongly suggest that the adenoid can function as a source of infection, which is supported by the positive effect of adenoidectomy, i.e. removal of lymphoid tissue, on the course of OME. This could mean that in adenoids of children with OME inadequate handling of bacteria and/or bacterial antigens leads to a continous exposure of the mucosa in the upper airways (i.e. ET and middle ear mucosa) to these antigens. The development of organized lymphatic tissue in the middle ear mucosa is a sign of this continous exposure (van der Baan et al. 1988). The aim of the study, described in this thesis, is to determine whether all different cells, required for the non-specific and the specific immune response, are present in adenoids of children with OME and possess functional potency to fullfill these functions.

Chapter 2

Several studies describe the lymphocyte subsets in adenoids. However, in none of these studies these subsets are related to the presence of OME. A first question which arises, is whether all cells, involved in immunological reactivity, are present in the adenoids of children with OME. In this chapter we characterize the lymphoid and non-lymphoid cell types from adenoids of children with OME. They are compared with those of the adenoid of children with upper respiratory tract infections without OME and with those of the adenoid of "healthy" children and adults. In the second part of this chapter we investigate in adenoids of children with OME whether there is activation of the different T-lymphocyte populations ($CD4^+$ and $CD8^+$ T cells). Moreover, these $CD4^+$ and $CD8^+$ T cells are divided into subpopulations using monoclonal antibodies. These results are compared with the T lymphocytes in peripheral blood of these children.

Chapter 3 and 4

For initiating adequate immune reactions antigen presenting cells (APC) play a pivotal role. The next questions to be answered are what kind of APC are present in the adenoid of children with OME in vivo, where they are located

and do they differ from adenoids of controls. The presence of Langerhans cells in the respiratory epithelium of the adenoid is described in chapter 3. Their possible function is discussed. In chapter 4 the location of macrophages and different types of DC is described and the possible significance of the presence of dendritic cells in OME is studied.

Chapter 5

An inability of the adenoid to handle bacteria and/or bacterial antigens adequately, might be related to diminished antigen presentation by APC. Antigen presentation to T helper cells is essential for promoting a humoral immune response (Yewdell & Bennink 1990). DC are exclusively engaged in activation of naive T lymphocytes (King & Katz 1990). Only in ver low numbers, DC are present in adenoids of children with OME (chapter 2). In this chapter we describe the development of a method to enrich DC from adenoids of children with OME. They are characterized by immuno- and enzyme-cytochemistry and by ultrastructural criteria. Moreover, we test the functional capacities of these cells in a mixed leukocyte reaction.

Chapter 6

Macrophages play an important role in both the specific and non-specific immune response. They have a large impact on the overall regulation of the specific immune response. In the non-specific defence these cells are important for phagocytosis and killing of micro-organisms. In this chapter the macrophages from adenoids of children with OME are tested for their capacity to kill *Staphylococcus aureus*. We also study the ability of these macrophages to produce nitric oxide as a response to the presence of formol-killed bacteria. This nitric oxide is a molecule which plays an important role in the oxygen-dependent killing of micro-organisms.

Chapter 7

In this chapter the results described in chapter 2 to 6 are summarized. It also provides a general discussion of these results.

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LYMPHOID AND NON LYMPHOID CELLS IN THE ADENOID OF CHILDREN WITH OTITIS MEDIA WITH EFFUSION: A COMPARATIVE STUDY.

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Clin Exp Immunol 1990; 79, 233-239.

SUMMARY.

In the present study we characterized, on immuno- and enzymecytochemical level, the lymphoid and non-lymphoid cells in the adenoid of children with upper respiratory tract infections (URI) and otitis media with effusion (OME) and compared these with the adenoid of children with URI without OME and with the adenoid of "healthy" children and "healthy" adults.
Besides macrophages and dendritic cells we also showed the presence of MHC class II positive, ciliated, epithelial cells. These non-lymphoid cells were present in all adenoids. However, their number was less than 1% of all cells. We found no difference in lymphocyte subsets comparing children suffering from URI + OME with children suffering from URI alone. These two groups showed a significant decrease of CD-8 positive (suppressor/cytotoxic) cells and a slight increase in CD-22 positive B-cells in comparision to "healthy" children. No difference was found in percentages of CD-4 positive (helper/inducer) cells. The localization of the lymphoid subsets in adenoids of children with URI and/or OME did not differ from those of "healthy" children and "healthy" adults.

INTRODUCTION.

Otitis media with effusion (OME) is a very common disease during childhood, characterized by the presence of fluid in the middle ear without signs of an acute infection. The disease causes moderate hearing loss and, if persisting, may lead to a postponed development of language and speech (Teele et al. 1984). Dysfunction of the Eustachian tube (ET) and inflammation of the mucosa of the middle ear and ET are considered to be important factors in the aetiology and pathogenesis of OME (Bluestone 1983, Bernstein 1985, Sprinkle & Veltri 1986).
OME often follows an upper respiratory tract infection (URI) (Tos et al. 1979, Casselbrant et al. 1985), giving rise to an effusion being serous (a transudate) or mucoid (an exudate) (Mogi et al. 1973). Many effusions show positive bacteriologic cultures, especially for H. Influenza and S. Pneumoniae, suggesting a direct role of bacteria in the pathogenesis of OME (Giebink et al. 1979, Lim & DeMaria 1982, Cabenda et al. 1988). Since immune complexes, complement factors and lysosomal enzymes have been found in middle ear effusions (MEE), immunological factors in the pathogenesis of OME, especially emphasizing an immune complex mediated (type III) hypersensitivity reaction, has been suggested (Veltri & Sprinkle 1976, Maxim et al. 1977).
Van der Baan et al. (1988) however were unable to show a vasculitis and an infiltration of polymorphonuclear leukocytes in the middle ear mucosa in OME. This more or less excludes an type III hypersensitivity reaction in OME. Instead they found in 86% of the patients highly organized lymphatic tissue in the middle ear mucosa which could be regarded as part of the mucosa associated lymphoid tissue (MALT).
It has been suggested that the adenoid plays an important role in OME: 1) by mechanical and/or functional obstruction of the ET (Bluestone 1983) and 2) by functioning as a source of bacterial antigens due to an inadequate handling of bacteria during URI (Gates et al. 1988). In addition several well designed studies have shown statistically significant effect of adenoidectomy on the resolution of OME (Maw 1983, Maw & Herod 1986, Gates et al. 1987). Since this effect appeared to be independent of the size of the adenoid (Maw 1985, Gates et al. 1988), mechanical obstruction is apparently of less importance. So it is reasonable to assume that in OME the handling of bacteria by

the adenoid may be disturbed. Since lymphoid and non-lymphoid cells play a key role in the defense against bacteria it is necessary to study these cells in adenoids in OME.
In this investigation we characterized the different subsets of lymphoid cells and non-lymphoid cells (especially macrophages and dendritic cells) in the adenoids of children with URI and OME and of children with URI without OME, using immuno- and enzymecytochemical methods. We compared the relative distribution of these cells in their adenoids and we studied the localization of the different cells in the adenoids using cryostat sections. These results were compared with those obtained from adenoids of "healthy" children and "healthy" adults.

MATERIALS AND METHODS.

Patients.
Adenoids were obtained from 36 children with OME (duration longer than three months), diagnosed by otoscopy, tympanometry and/or audiometry. The adenoids were divided in three groups according to the type of effusion found during the paracentesis: adenoids with serous effusion (N=9), adenoids with mucoid effusion (N=19) and adenoids with no effusion, present at the moment the scheduled middle ear ventilation could be performed (N=8). Adenoids from patients with a history of asthma, bronchitis or an IgE-mediated allergy were excluded.

Controls.
Three control groups were investigated: adenoids of children with frequent upper respiratory tract infections without OME (N=19), adenoid biopsies of "healthy" children (N=8) who were operated for a strabismus correction and adenoid biopsies of "healthy" adults (N=10) who underwent orthodontic surgery.
In this study children and adults were considered "healthy" if they had not used antibiotics or have had upper respiratory tract infections for at least one month prior to the operation and if they have had no middle ear infections or middle ear surgery during the previous half year. People with a history of asthma, bronchitis or an IgE-mediated allergy were excluded.

Table 1 summarizes the composition of all groups.

Table 1. Details of the groups studied for subsets lymphoid and non-lymphoid cells in the adenoid.

			age (months)*	
Group	n	M/F	mean	range
URI with OME	36	25/11	44	14-83
mucoid	19	15/4	52	14-68
serous	9	5/4	36	14-53
no effusion	8	5/3	34	14-83
URI without OME	19	12/7	58	18-108
"healthy" children	8	4/4	57	22-98
"healthy" adults	10	4/6	30	17-55

* Except for adults, where age and range are in years.
URI; Upper Respiratory tract Infection.
OME; Otitis Media with Effusion.

Cell suspensions.
Adenoids or adenoid biopsies were cut into small pieces which were gently pressed through a nylon gauze (± 100μm mesh) to collect the cells in Hanks Balanced Salt Solution (HBSS, Flow Laboratories) with Hepes (pH 7,4). The cell suspensions were washed twice in HBSS and cytocentrifuge preparations were made (Shandon cytospin 2 apparatus) in RPMI (Flow Laboratories) containing 10% foetal calf serum (FCS).

Cryostat sections.
To get more information about the localization of the lymphoid and non-lymphoid cells cryostat sections of adenoid biopsies were made. Biopsies were immediately frozen in liquid nitrogen. Cryostat sections 4 μm thick were cut and mounted on Poly-L-Lysine coated slides, dried overnight above

silica gel and stored at -20°C until use.

Immunoperoxidase techniques.
Indirect immunoperoxidase procedures were carried out on the cytocentrifuge preparations and cryostat sections. The mouse monoclonal antibodies used are listed in table 2.

Table 2. antibodies and their specificity.

antibody	cluster differentiation	specificity	source
Leu 3a + 3b	CD4	Helper/inducer T cells	Becton Dickinson, Mountain View, CA
RIV 4	CD8	Suppressor/cytotoxic T cells	Rijksinstituut voor Volksgezondheid en Milieu hygiene, The Netherlands.
Leu 14	CD22	B lymphocytes, precursors of B lymphocytes	Becton Dickinson
H 2.5.10		HLA-DR[1]	Gift from Dr. P.M. van Lansdorp (Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam).
HLA-DP		HLA-DP	Becton Dickinson
SPV-L3		HLA-DQ[2]	Gift from Dr. G.D. Keizer (The Netherlands Cancer Institute, Amsterdam).

[1] Bos et al. 1989.
[2] Spits et al. 1984.

After fixation in 100% acetone for 10 min at 4°C the sections were incubated with the appropriate antibody for 1 h at room temperature followed by washing three times in phosphate buffered saline (PBS, pH 7,4) containing 1% bovine serum albumin (BSA). Hereafter the preparations were covered for 1 h at room temperature with horseradish peroxidase conjugated to rabbit anti mouse IgG in a dilution of 1:250 in PBS with 1% BSA and 1% normal human serum. After washing three times with PBS incubation was performed in 3,3'-diaminobenzidine-tetrahydro- chloride (Merck) at a concentration of 0,5 mg/ml in Tris/HCl buffer (pH 7,6) containing 0,03% H_2O_2 and 0,05 M Imidazole for enhancing the reaction. Subsequently the slides were rinsed

with distilled water, 0,9% NaCl and 0,5% $CuSO_4$ in 0,9% NaCl for 5 min each at room temperature, washed in distilled water and counterstained with haematoxylin. Finally the preparations were embedded in Entellan. Control preparations were incubated in a medium in which the monoclonal antibodies were replaced by PBS containing 1% BSA.

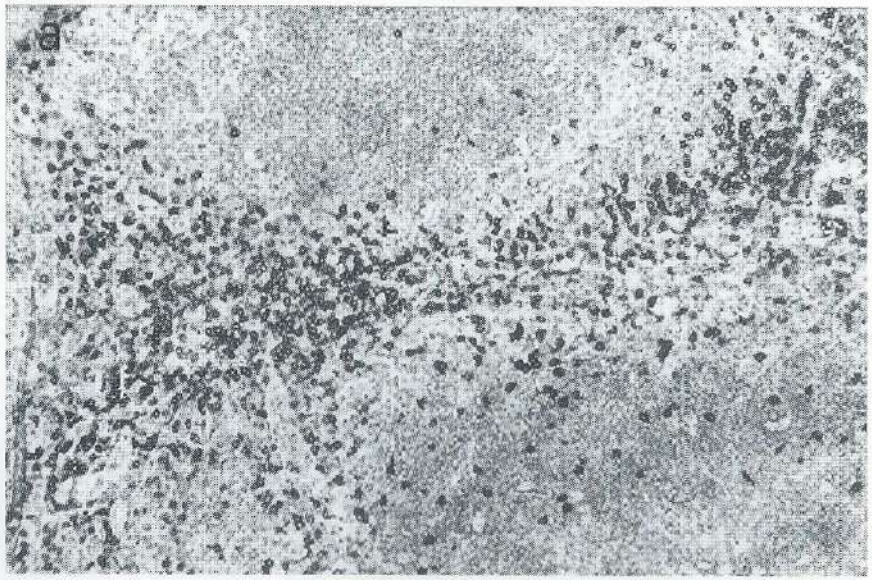
Acid Phosphatase (APh) staining.
The method according to Burnstone (Pearse 1968) was used. Incubation was carried out with naphtol-AS-BI-phosphate (Sigma) as substrate for 60 min at 37°C. APh staining was always preceded by MHC class II immunoperoxidase staining.

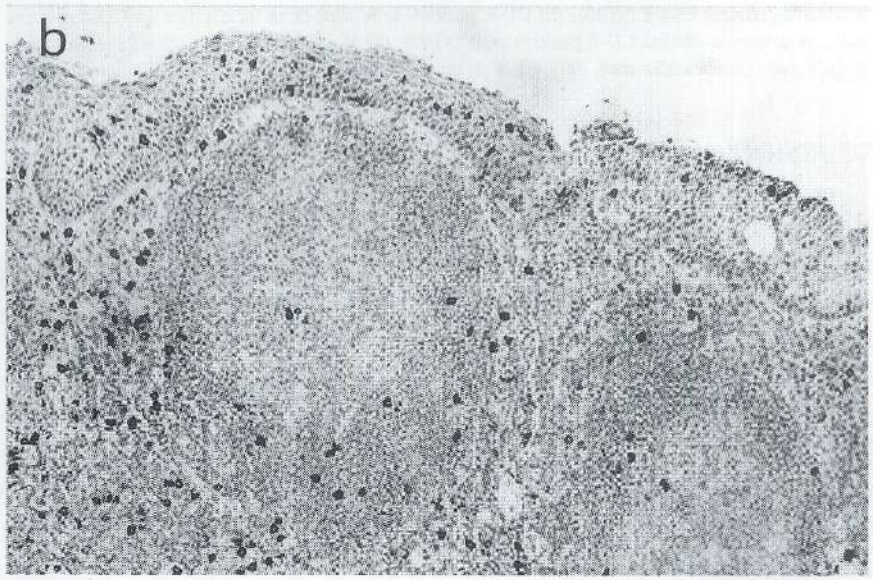
Evaluation.
To identify the different cell populations in cytocentrifuge preparations immuno- and enzymecytochemical stainings were used in combination with morphological criteria. Also the relative number of CD-4, CD-8, CD-22 and HLA-DR positive cells, per 200 cells at least, were counted. The non-lymphoid cells were also screened for the presence of HLA-DP and HLA-DQ antigens.
Biopsies were screened for the localization of the lymphoid subsets and non-lymphoid cells.

Statistical analysis.
Differences were compared using a two sided student's t-test for independent samples. P values of ≤ 0.05 were considered significant.

RESULTS.

Cytocentrifuge preparations.
In all experiments, except for the healthy adults, more than 50% of all cells (lymphocytes and non-lymphoid cells) showed HLA-DR positivity. All cytospin preparations showed few cells with APh activity.





b) cells showing APh activity in a central spot of variable size in the vicinity of the nucleus. They were not only positive for HLA-DR but also for HLA-DP and HLA-DQ. These cells showed an irregular outline. Frequently they also showed characteristic cytoplasmic extensions like veils. They contained an eccentric localized and often lobulated nucleus. The nucleocytoplasmic ratio ranged from 1:1 to 1:3. (fig.1a,b)

c) very large cells with a cuboidal shape and cilia on one side of their cell surface. These cells showed APh activity in a spot always localized between the nucleus and the cell surface (fig.1c). Most of them expressed HLA-DR antigens. Very occasionally these ciliated cells expressed HLA-DP antigens, while HLA-DQ positive ciliated cells could not be detected. The nucleocytoplasmic ratio was ± 1:2 to ± 1:3. These three different cell types comprised less than 1% of all cells present in the adenoid.

Table 3 shows the percentages of the different lymphocyte subsets and HLA-DR positive cells in the adenoids of children with URI and OME, of children with URI alone and of "healthy" adults and "healthy" children.

Table 3. Percentages of lymphocyte subsets and HLA-DR positive cells.

	n	CD4*	CD8*	CD22*	HLA-DR#
URI with OME	36	24±6	7±2	69±7	59±9
mucoid	19	25±6	7±2	68±7	55±9
serous	9	26±6	7±2	67±7	69±4
no effusion	8	20±5	5±1	74±6	58±8
URI without OME	19	23±7	7±2	68±9	59±9
"healthy" adults	10	43±6	15±5	42±8	44±9
"healthy" children	8	29±5	13±4	57±5	55±8

Data are mean ± s.d.
* Percentages of lymphoid cells.
# Percentages of lymphoid and non-lymphoid cells.
URI; Upper Respiratory tract Infection.
OME; Otitis Media with Effusion.

T-helper/inducer (CD-4) / T-suppressor/cytotoxic (CD-8) ratios were calculated and are shown in table 4.

Table 4. T helper/inducer:T suppressor/cytotoxic ratios.

	n	CD4/CD8	P*
URI with OME	36	3.7±0.9	<0.001
mucoid	19	3.6±1.0	<0.01
serous	9	3.7±1.1	<0.02
no effusion	8	4.1±0.6	<0.01
URI without OME	19	3.9±1.0	<0.001
"healthy" adults	10	2.5±0.9	NS
"healthy" children	8	2.4±1.0	-

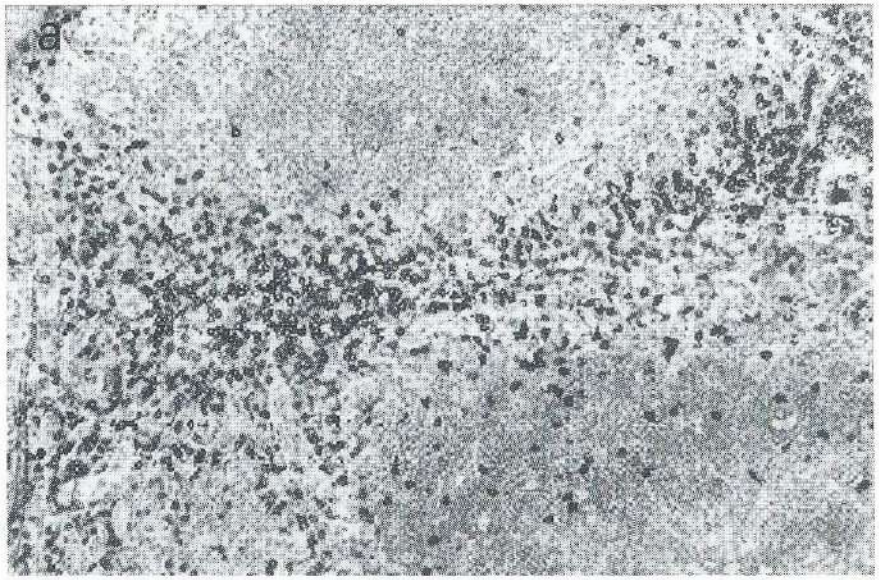
* Compared with "healthy" children; Student's *t*-test.
URI; Upper Respiratory tract Infection.
OME; Otitis Media with Effusion.
NS; not significant.

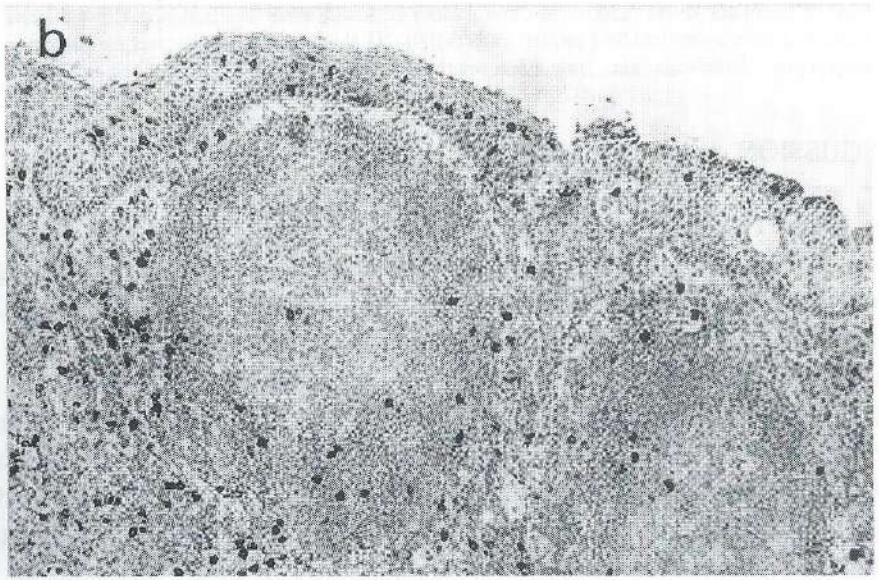
In the control preparations, which were treated with PBS/BSA 1% instead of MAbs, neutrophilic granulocytes were easily recognised by their strong brown cytoplasmic staining due to their endogenous peroxidase activity. Using anti CD-4 MAb some monocytes and macrophages were also weakly positive (Wood et al. 1983), however these cells could be distinguished easily from lymphocytes by their larger cell size, ruffled cell membrane and often eccentric localized nucleus.

Cryostat sections.

The localization of the T helper/inducer, T cytotoxic/supressor cells and B-lymphocytes is shown in fig.2. No gross difference was found between the adenoids of the different groups.

In the epithelium the CD-8 positive cells predominated, whereas CD-4 positive cells were very few. Hardly any CD-22 positive cells were found in the epithelium. Mononuclear cells stained with anti HLA-DR antibody were ocassionally present in the epithelium.





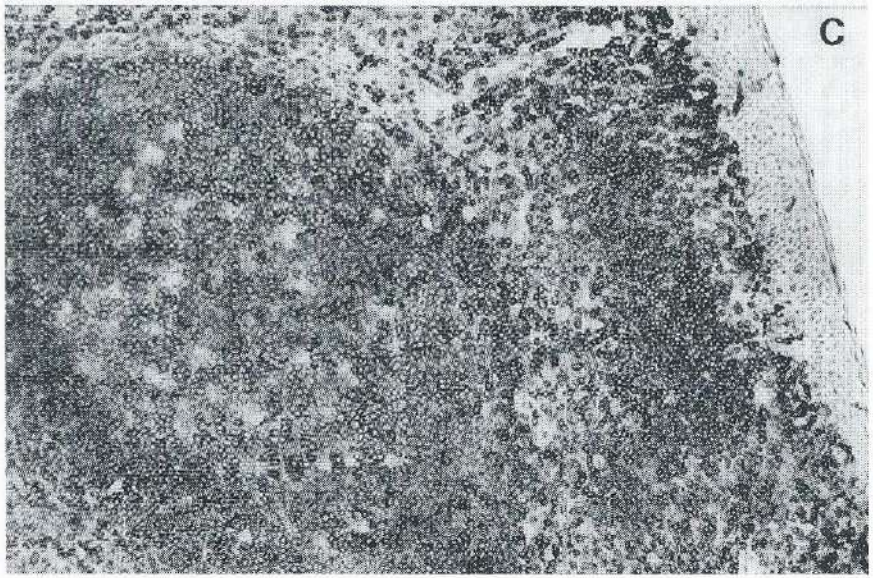


Fig.2 Cryostat sections of the adenoid stained for CD-4, CD-8 and CD-22 expression. (a) CD-4 positive cells clearly present in interfollicular area and to a lesser extent in the follicular mantle zone and germinal centre (x100). (b) CD-8 positive cells with same localization as CD-4 positive cells, note intra-epithelial CD-8 positive cells (x100). (c) Numerous B-lymphocytes are present in follicle and interfollicular area, only a few positive B cells are localized in the epithelium (x100).

DISCUSSION.

Our results clearly show that the population of non-lymphoid cells could be distinguished using MHC class II expression, APh staining and morphology. Based on these criteria we found three different cell types. Cells with APh activity throughout the whole cytoplasm which were either HLA class II positive or negative, these cells represent macrophages (MQ, Beelen et al. 1989). Cells showing cytoplasmatic extensions like veils, which express MHC class II antigens on their cell surface and demonstrate APh in a central spot near the lobulated nucleus: dendritic cells (DC, Kamperdijk et al. 1989). Cells expressing class II antigens with an APh spot between the ciliated surface and the nucleus: epithelial cells.

It has been shown that DC are highly efficient in presenting antigen to immunocompetent T-cells (Van Voorhis et al. 1982). They represent the in vitro equivalent of the interdigitating cells which are characteristic for T-cell

areas in lymphoid organs (Kamperdijk et al. 1985).

In accordance with human blood DC (Gaudernack & Bjercke 1985, Brooks & Moore 1988), synovial DC (Waalen et al. 1987) and tonsillar DC (Hart & McKenzie), DC from adenoids were HLA-DP$^+$, HLA-DQ$^+$ and HLA-DR$^+$.

About 50% of the MQ were HLA-DR$^+$ and only a minor fraction HLA-DP$^+$ or HLA-DQ$^+$. Almost all epithelial cells were HLA-DR$^+$, only very occasionally HLA-DP$^+$ cells were present. The epithelial cells never expressed HLA-DQ.

HLA-DR$^+$ epithelial cells in mucosa associated lymphoid tissue have been shown in tonsils (Brandtzaeg 1987), nasal mucosa (Brandtzaeg 1984) and gut (Scott et al. 1980).

Recently it has been shown that HLA-DR$^+$, -DQ$^+$ and -DP$^-$ epithelial cells in the gut were able to take up, process and present antigen to CD-4 positive cells. Moreover they could directly activate CD-8 positive suppressor cells (Mayer & Shlien 1987). Although adenoid epithelial cells are HLA-DR$^+$, -DP$^+$ and -DQ$^-$ it would be reasonable to assume that the class II positive, ciliated, epithelial cells in the adenoid, apart from macrophages and especially dendritic cells, are involved in antigen uptake and may act as antigen presenting cells.

In all experiments the total numbers of these three types of non-lymphoid cells were less than 1%, independent of the adenoid source. Therefore it is impossible to compare the number of these cells between the different study groups. Further functional characterization (i.e. chemotaxis, phagocytosis, bacterial killing and antigen presentation) of these cells is necessary to get more insight in their possible role in the pathogenesis of OME.

In contrast to the non-lymphoid cells, lymphoid cells were abundantly present. Our investigations revealed that the CD-4/CD-8 ratio in "healthy" children and in "healthy" adults, 2.4 ± 1.0 and 2.5 ± 0.9 respectively, were equal. These values correspond to the percentages of CD-4 and CD-8 positive cells described in other studies using tonsils (Brandtzaeg 1987).

However, our results showed higher percentages of CD-22 positive cells in healthy children compared with healthy adults. This can be explained by the decrease in size and number of the follicles, which mainly consists of B-cells, with increasing age as described earlier (Korsrud & Brandtzaeg 1980).

Remarkably, CD-4/CD-8 ratio's in children suffering from URI with and without OME were similar, the percentages of CD-22 positive cells differed

slightly. It seems likely that in both conditions the immunological properties of the adenoids, as far as quantitative parameters are concerned, are similar.
Children with URI with or without OME, showed a significant higher CD-4/CD-8 ratio than both "healthy" children and "healthy" adults. These results were obtained by comparing the numbers of the different lymphocytes from biopsies from the adenoid in "healthy" children and "healthy" adults with the numbers of the different lymphocytes from the whole adenoid in children with URI with or without OME. This is possible since it has been shown that lymphocyte subsets of biopsies of tonsils are representative for the lymphocyte subsets in whole tonsils, confirming the reliability of using adenoid biopsies from "healthy" children and "healthy" adults as controls (Plum et al. 1986). Cryostat sections of these different adenoid biopsies can be considered as representative for the whole organ since they clearly showed the presence of the different compartments of adenoids (epithelium, numerous follicles and interfollicular areas).
The significant difference in CD-4/CD-8 ratio's between children with URI with or without OME and "healthy" children (and adults) is due to the much higher percentages of CD-8 positive cells found in the adenoid of the "healthy" children (and adults). The same observation was made in the healing phase of chronic sinusitis (Nishimoto et al. 1988). In inflammatory diseases of the tonsils contradicting findings were reported: these studies reported a significant increase not only of CD-4 positive cells but especially of the CD-8 positive cells, resulting in a decrease of CD4/CD8 ratio's in inflammatory tonsillar diseases (Yamanaka et al. 1983, Brodsky et al. 1988).
In our cryostat sections no difference was found in localization of the CD-4, CD-8 and CD-22 positive cells between all the different groups.
The mechanism and significance of imbalance in local T cell subsets are unknown. It is posible that the local immune reactivity to antigens could be harmful to the host. In both "healthy" children and "healthy" adults the higher number of suppressor/cytotoxic (CD-8 positive) cells could be of benefit to them to control an excess immune response. In URI with or without OME the decrease of suppressor/cytotoxic cells could lead to more frequent and intensive immune responses against the different bacterial antigens and so participate in the pathogenesis of URI and/or OME.
In conclusion our results show: 1) there is no difference in the percentages of CD-4, CD-8 and CD-22 positive cells in the adenoid between children with

URI with OME and children with URI without OME. 2) these two groups show a significant lower percentage of CD-8 positive cells compared with the adenoid of "healthy" children. 3) In all groups there is no difference in localization of the different lymphocyte subsets. 4) besides MHC class II positive macrophages and dendritic cells also MHC class II positive epithelial cells with cilia are present.
Functional properties of the lymphoid and especially of the non-lymphoid cells (i.e. phagocytosis, chemotaxis, killing and antigen presentation) in the adenoid will be the subject of future investigations.

Acknowledgements
The authors thank the department of opthalmology (Dr. F.D. Koole) and the department of maxillofacial surgery (Prof. Dr. D.B. Tuinzing) for providing adenoid biopsies for controls, mr S. Paniry for photographical assistance and Prof. Dr. E.C.M. Hoefsmit for critical reading the manuscript.

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IMMUNOPHENOTYPE OF T-LYMPHOCYTE SUBPOPULATIONS IN THE ADENOID AND PERIPHERAL BLOOD OF CHILDREN WITH OTITIS MEDIA WITH EFFUSION.

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Submitted

SUMMARY.

In the adenoid of children with otitis media with effusion (OME) the majority (about 60%) of the $CD4^+$ T cells are of the helper-inducer ($4B4^+$) phenotype, while in peripheral blood the majority (about 60%) of the $CD4^+$ T cells are of the suppressor-inducer ($2H4^+$) phenotype. About 30% of the adenoid T cells are activated (HLA-DR/DQ positive) while significantly more $CD8^+$ T cells ($\pm$ 40%) are activated than $CD4^+$ T cells ($\pm$ 15%). In contrast, in peripheral blood only few T cells ($\pm$ 4%) are activated. The predominance of $CD4^+$ T cells of the helper-inducer phenotype in the adenoid suggests that, in the adenoid, there is a high state of immune reactivity as result of exposure to exogenous antigens locally. The observation that in the adenoid significant more activated $CD8^+$ T cells are present, suggest suppression or down regulation of the immune response in adenoids of children with OME.

INTRODUCTION.

T lymphocytes play a central role either as regulatory or as effector cells in the immune response. Peripheral T lymphocytes are generally thought of as being in one of three states: naive, memory or effector (Bradley et al. 1993). A naive cell is a circulating precursor which has not yet encountered antigen; a memory cell is a resting cell, derived from a naive cell after encountering antigen, which mediates antigen recall responses; an effector cell is a highly activated cell, arosen from either naive or memory cells, and carries out the specialized function of T cells.

T lymphocytes can be divided into $CD4^+$ and $CD8^+$ subsets that are functionally distinct. It is generally believed that activation of naive and/or memory $CD4^+$ T lymphocytes results in secretion of immunoregulatory cytokines (effector function), whereas naive and/or memory $CD8^+$ T lymphocytes develop as suppressor (effector) cells that suppress antigen specific T and B cell responses or develop as cytotoxic (effector) T cells that kill transformed antigen presenting cells (Sprent et al.1988, Yewdell & Bennink 1990). Activation of T cells can result in expression of MHC class II molecules and expression of a receptor for interleukin 2 (IL2R).

Morimoto et al. (1985) developed 2 convenient monoclonal antibodies, 4B4 (CDw29) and 2H4 (CD45R) respectively, that can be used to subdivide the human $CD4^+$ T cell subset into 2 major subpopulations that are phenotypically and functionally distinct. The $CD4^+$, $4B4^+$ subset proliferates poorly upon stimulation with concanavalin A (Con A) and autologous cell antigens in a autologous mixed leukocyte reaction. They proliferate well on exposure to soluble antigens and they provide a good helper signal for pokeweed mitogen (PWM) induced Ig synthesis in B lymphocytes. This is the helper-inducer subset. The $CD4^+$, $2H4^+$ subset proliferates well to Con A stimulation, but poorly to soluble antigen stimulation, and provides poor help to B cells for PWM induced Ig synthesis. They are inducer of $CD8^+$ suppressor cells and called the suppressor-inducer subset.

Comparable to the $CD4^+$ subsets, a functional difference between $4B4^+$ ($CDw29^+$) and $2H4^+$ ($CD45R^+$) $CD8^+$ T lymphocytes has also been reported (de Jong et al.1991).

In a previous study we have shown that in adenoids of children with otitis media with effusion a significant lower percentage of $CD8^+$ T lymphocytes

(7±2%) is present in comparison with adenoids of healthy controls (13±4%)(Nieuwkerk et al. 1990). However, the percentage of $CD4^+$ T lymphocytes is not different in the adenoids of children with OME and healthy controls. In this investigation we characterize the different T cell subsets and state of activation of the T cells from adenoids of children with OME. The results are compared with those from the peripheral blood of the same children.

MATERIALS AND METHODS.

Material.

Adenoids were obtained from 10 children with OME (duration longer than 3 months). During the operative procedure a sample of peripheral blood was taken. This procedure was approved by the Medical Ethical Commision of the Free University hospital and informed consent was obtained from the parents of the children for the use of adenoid and blood.

Adenoid T cell fractions.

Adenoids were cut into small pieces, which were gently pressed through a nylon gauze to collect the cells in Hanks Balanced Salt Solution (HBSS). The cell suspensions were washed in HBSS and applied to a lymphoprep (Nycomed, Oslo, Norway) gradient. After centrifugation the cellular interface was collected and washed. T lymphocytes were isolated from the mononuclear cell suspensions using a rosetting technique with neuraminidase-treated sheep erythrocytes. Part of the mononuclear cells were incubated with CD4 dynabeads (Dynabeads, Dynal AS, Oslo, Norway), part of these cells with CD8 dynabeads for 1 hour on ice. The number of magnetic beads used was 20 dynabeads to 1 positive cell (assuming that approximately 25% of the cells were reactive with CD4 and 10% with CD8). After washing twice in phosphate buffered saline (PBS) supplemented with 2% fetal calf serum (FCS, heat inactivated for 30 min at 56°C), the cells attached to the magnetic beads were removed using a magnet (Dynal SA). Subsequently the obtained cell fractions were incubated with detachabeads (Dynal SA) for 1 hour at room temperature and with the magnet the detachabead-dynabead combinations were removed. The CD4 and CD8 enriched fractions were washed in

PBS and resuspended in αRPMI (RPMI 1640, Flow Lab., Irvine, UK, supplemented with 1mM glutamine, penicillin 100 U/ml, streptomycin 100 μg/ml, gentamycin 50 μg and 10% fetal calf serum (FCS).

Blood T cell fractions.

Blood of the patients was drawn by venapuncture into EDTA syringes. Mononuclear blood cells were isolated on a Lymphoprep gradient (Nycomed). CD4 and CD8 positive lymphocyte fractions were obtained by incubating the mononuclear cells with CD8 dynabeads, followed by removing these cells with the magnet and subsequently incubating the remaining cells with CD4 dynabeads. The magnetic particles were removed from the cell fractions with detachabead as described for the adenoid fractions. Cell viability of all cell populations was always more than 90% as determined by trypan blue exclusion. Cytocentrifuge preparations were made of all fractions.

Immunocytochemistry.

A routine immunoperoxidase staining method was performed on cytocentrifuge preparations of all cell fractions obtained as has been described in detail previously (Nieuwkerk et al. 1990). The monoclonal antibodies used were: anti CD2 (pan T cell marker, Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam), anti CD4 (recognizing helper T cells, Sanbio, Uden, The Netherlands), anti CD8 (RIV4) (recognizing cytotoxic/suppressor T cells, Rijksinstituut voor Volksgezondheid en Milieu Hygiene, Amsterdam), anti IL2 receptor (AM9.27.1) (ATCC, Rockville, NY, USA), anti HLA-DR/DQ (9.3F10) (ATCC), 4B4 (recognizing helper-inducer T cells, Coulter Immunology, Hialeah, Florida) and 2H4 (recognizing suppressor-inducer T cells, Coulter Immunology). A minimum of 200 cells were examined for the presence of the different antigens.

Statistical analysis.

Differences were compared using a two-tailed Student's t-test for independent samples. $P \leq 0.05$ was considered significant.

RESULTS.

Purity of the fractions.

More than 97% of the cells in all obtained T cell fractions were CD2 positive. There was only minor contamination (<4%) of $CD4^+$ and $CD8^+$ positive cells in the $CD8^+$ and $CD4^+$ fraction respectively of both adenoid and peripheral blood T lymphocytes.

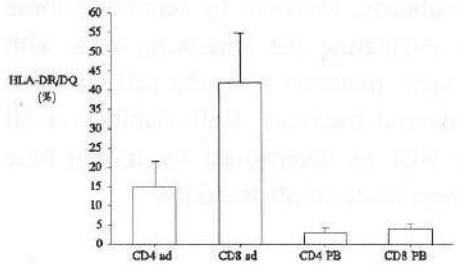

Fig.1a Percentages of HLA-DR/DQ positive T cells in the adenoid (ad) and peripheral blood (PB).

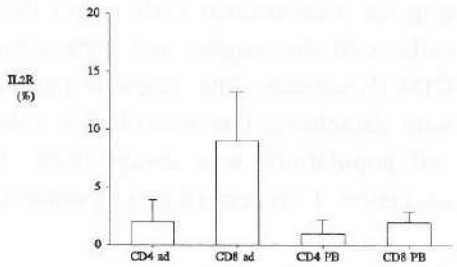

Fig.1b Percentages of IL2R positive T cells in the adenoid (ad) and peripheral blood (PB).

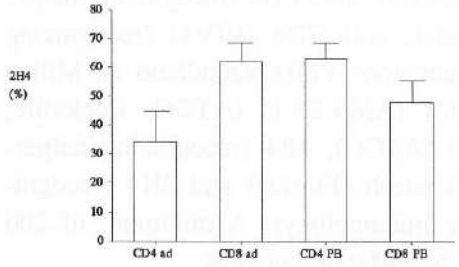

Fig.2a Percentages of 2H4 positive T cells in the adenoid (ad) and peripheral blood (PB).

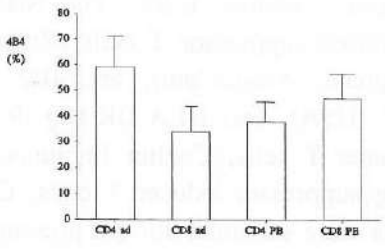

Fig.2b Percentages of 4B4 positive T cells in the adenoid and peripheral blood.

Activated T lymphocytes.

As shown in fig.1a and b, the adenoid contains significantly more activated $CD8^+$ lymphocytes than activated $CD4^+$ lymphocytes. Of the $CD8^+$ lymphocytes $42\pm12\%$ showed HLA-DR/DQ expression, while of the $CD4^+$ lymphocytes $15\pm6\%$ were positive (fig.1a). IL2R expression was observed in $9\pm4\%$ of the $CD8^+$ T cells and in $2\pm2\%$ of the $CD4^+$ T cells (fig.1b) ($p \leq 0.001$). The number of HLA-DR/DQ positive T lymphocytes in the adenoid is significantly higher than in the blood ($28\pm6\%$ vs $4\pm1\%$) ($p \leq 0.001$). IL2R is significantly more expressed on adenoid T cells ($6\pm2\%$) compared with those of peripheral blood ($2\pm1\%$)($p \leq 0.02$).

$2H4^+$ and $4B4^+$ subsets of the $CD4^+$ and $CD8^+$ T lymphocytes.

As shown in fig.2a and b the $CD4^+$ lymphocytes of the adenoid contain significantly less $2H4^+$ cells than the $CD4^+$ lymphocytes of peripheral blood ($34\pm11\%$ vs $63\pm4\%$)($p \leq 0.001$)(fig.2a). In the $CD8^+$ subset there are significantly more $2H4^+$ cells in the adenoid than in the peripheral blood ($62\pm6\%$ vs $48\pm7\%$)($p \leq 0.02$). The $CD4^+$ lymphocytes in the adenoid contain significantly more $4B4^+$ cells than the $CD4^+$ lymphocytes of the peripheral blood ($59\pm11\%$ vs 38 ± 6)($p \leq 0.02$)(fig.2b). $4B4^+$ cells are just significantly less in the $CD8^+$ subset of the adenoid compared with the $CD8^+$ subset of peripheral blood ($34\pm9\%$ vs $47\pm6\%$)($p \leq 0.05$).

DISCUSSION.

The $CD4^+$ T lymphocytes in the adenoid of children with OME are, in contrast to those in the peripheral blood, predominantly of the 4B4 phenotype. This suggests that they might especially be involved in B cell help. This corresponds with the presence of many germinal centers in the adenoid in vivo. In several chronic inflammatory laesions like thyroiditis in Grave's disease (Ishikawa et al. 1987) and atopic dermatitis (Lever et al. 1987) significant higher percentages of this $4B4^+$ (helper-inducer) subset, in comparison with peripheral blood, are also described.

Although it has generally been accepted that $4B4^+$ $CD4^+$ cells represent helper-inducer T cells and that $2H4^+$ $CD4^+$ represent suppressor-inducer T cells, it has also been proposed that the $4B4^+$ $CD4^+$ cells are memory T cells, while the $2H4^+$ $CD4^+$ cells are naive cells, thus representing different maturational stages of T lymphocytes (Sanders et al. 1988). Moreover, it has been shown that a gradual acquisition of memory T cells in children occurs with aging probably because naturally occurring pathogens are sequentially

encountered (Pirucello et al. 1989). In our study the memory cells ($4B4^+$) predominate the naive cells ($2H4^+$). The same observations have been made in gut mucosa (James et al. 1986), skin (Bos et al. 1987) and on lung epithelial surfaces (Saltini et al. 1988). In contrast to the adenoid, it appeared that the blood T lymphocytes in the OME children are predominantly naive $CD4^+$ cells. This may reflect conversion of cells to the memory phenotype at sites of exposure to exogenous antigens and/or the homing of memory cells to areas where they may parcipitate in host defence. However, recently it has been suggested that memory T cells may revert to a resting state in which they may not differ phenotypically from naive cells (Michie et al. 1992). In that case, differentiation between naive, memory and effector cells can only be made by considering phenotype, activation state and analysis of function (Gray 1994).

$CD8^+$ T lymphocytes have also been divided in $2H4^+$ and $4B4^+$ cells representing respectively naive and memory cells (de Jong et al.1991). This would mean that in the adenoid of children with OME most $CD8^+$ cells represent naive cells. However, it is still questionable whether 2H4 can be used as a marker for virgin cells in $CD8^+$ T cells (Okumura et al. 1993).

The expression of HLA-DR/DQ antigens on T cells is a sign of activation. Activated T cells can, in addition, be identified by a receptor for interleukin 2 (IL2R), expressed on their surface. It has been indicated that about 25% of the T cells of human adenoids, removed because of adenoid hypertrophy, express HLA-DR (Ennas et al. 1984). Our results ($28\pm7\%$) are comparable with this. However, only a few percent of the T cells expressed IL2R. The CD8 subset of adenoid T lymphocytes contains significantly more activated cells than the CD4 subset, as is indicated by HLA-DR/DQ and IL2R expression. It may be that there exists some kind of suppression or down regulation of the immune response in the adenoid of children with OME. The observations that adenoid lymphocytes in vitro can only be stimulated succesfully by bacteria when these cells were obtained from children who where apparently free from immediately preceding nasopharyngeal exposure to the same bacterium (Meistrup-Larsen et al. 1979, Rynnel-Dagöö 1978), also indicate that immunosuppression develops once stimulation has occurred. However, it was shown recently that adenoid T cells were unable to turn on B cells to mature in immunoglobulin secreting plasma cells (Bernstein et al. 1993). This was not due to suppressor activity of the adenoid T cells, but related to

a defective IL2 production. This may explain the discrepancy between the high number of HLA-DR/DQ positive T cells ($28\pm6\%$) and the low number of IL2R positive T cells ($6\pm2\%$).

In conclusion, in adenoids of children with OME the majority of the $CD4^+$ T cells are helper-inducer T cells, while in the peripheral blood the majority of the $CD4^+$ T cells are suppressor-inducer T cells, indicating a higher state of immune reactivity in the adenoid than in the blood. The $CD8^+$ T cells are significantly more activated in the adenoid than the $CD4^+$ T cells. In the blood only few T cells are activated in comparison with the adenoid.

Acknowledgements.

The authors wish to thank Ms. M. van Pelt and Mr. C.W. Tuk for their excellent technical assistance.

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LANGERHANS CELLS IN THE RESPIRATORY EPITHELIUM OF THE HUMAN ADENOID.

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Eur J Cell Biology 1991; 54, 182-186.

SUMMARY.

Langerhans cells are known to be distributed in stratified, squamous epithelia in man. They resemble dendritic cells, express CD-1 and are characterized by the presence of Birbeck granules. In this study characteristic Langerhans cells are described on light microscopical and ultrastructural level in the respiratory epithelium of human adenoid.

INTRODUCTION.

Langerhans cells (LC) are epidermal, bone-marrow derived cells, which belong to the family of dendritic cells (DC) (Hanau et al. 1987, Stingl et al. 1980). The most important and best documented function of the LC is antigen presentation to T lymphocytes (Braathen & Thorsby 1980, Silberberg-Sinakin et al. 1976). LC have Fc and complement receptors, surface ATP-ase activity and immunoreactivity for T6 (CD-1a), M241 (CD-1c), CD-4, MHC class II, and S-100 antigens (Breathnach 1988, Macatonia et al. 1987). However LC are determined by the presence of Birbeck granules (BGs) and this organelle can only be visualized in the electron microscope Birbeck et al. 1961).

The function of the BG is not known, and its origin is still disputed, either from the Golgi complex, or from endocytosis of T6 from the cell surface (Fokkens et al. 1989, Nieuwkerk et al. 1990). Cells with the same characteristics as LC but lacking BGs are called 'indeterminate cells' and may be intermediate forms between DC and LC.

LC are especially described in stratified squamous epithelium, which is present in the epidermis, oro- and nasopharynx, esophagus, conjunctiva and genito-urinary tract.

Recently the presence of LC in respiratory epithelium has been shown (Ray et al. 1989). In contrast to Sertl et al. (1986), who were unable to show LC in bronchial epithelium, Richard et al. (1987) described dendritic shaped, BGs containing cells, i.e. LC, in this epithelium.

Both tonsils and adenoids form part of the lympho-epithelial structures of Waldeyer's ring. This ring is thought to play a major role in the mucosal immunity of the upper respiratory tract. In tonsils, which are covered with squamous epithelium, LC have been described (Weinberg et al. 1987). However LC have never been demonstrated in adenoids, an organ which is largely covered with a typical respiratory epithelium, i.e. a columnar, ciliated epithelium with goblet cells. The presence or absence of LC in the epithelium of the adenoid could have important implications for the immune function of the adenoid. The present study was performed to find out whether LC are present in the epithelial and sub-epithelial layers of human adenoids.

In this paper we describe for the first time characteristic LC in the respirato-

ry epithelium of the human adenoid by immunocytochemistry and by electronmicroscopy.

MATERIALS AND METHODS.

Material.
Adenoids were obtained from children who underwent adenoidectomy because of recurrent upper respiratory tract infections.

Cryostat sections.
Adenoid tissue pieces were frozen in liquid nitrogen. Cryostat sections 6μm thick were cut and mounted on gelatin-chromaluin coated slides. These slides were dried overnight above silica gel and stored at -20 °C until use.

OKT-6 labelling.
Indirect immunoperoxidase procedures were carried out on the cryostat sections. After fixation in 100% acetone for 10 min. at 4 °C and blocking of endogenous peroxidatic activity with 0,3% H_2O_2 the sections were overlayered with a monoclonal mouse anti human OKT-6 (Dakopatts) antibody in a 10x dilution in phosphate buffered saline (PBS, 0,15 M, pH 7,4) containing 1% bovine serum albumine (BSA) for 45 min. at room temperature. Thereafter the preparations were rinsed 3 times in PBS, followed by incubation for 30 min. with horseradish peroxidase conjugated to rabbit anti mouse IgG 2a in a dilution of 1:250 in PBS with 1% BSA and 1% normal human serum. After washing three times with PBS, incubation was performed in 3,3'-diaminobenzidine-tetrahydrochloride (Merck) at a concentration of 0,5 mg/ml in 0,05 M Tris/HCl buffer (pH 7,6) containing 0,03% H_2O_2 and 0,05 M Imidazole for enhancement of the reaction. Subsequently the sections were rinsed with distilled water, 0,9% Na Cl and 0,5% $CuSO_4$ in 0,9% NaCl for 5 min. each , washed in distilled water and counterstained with haematoxylin. In controls the monoclonal antibody was replaced by PBS containing 1% BSA. No background label was visible.

Electron microscopy.
Tissue specimens were fixed in 1,5% glutaraldehyde in phosphate buffer

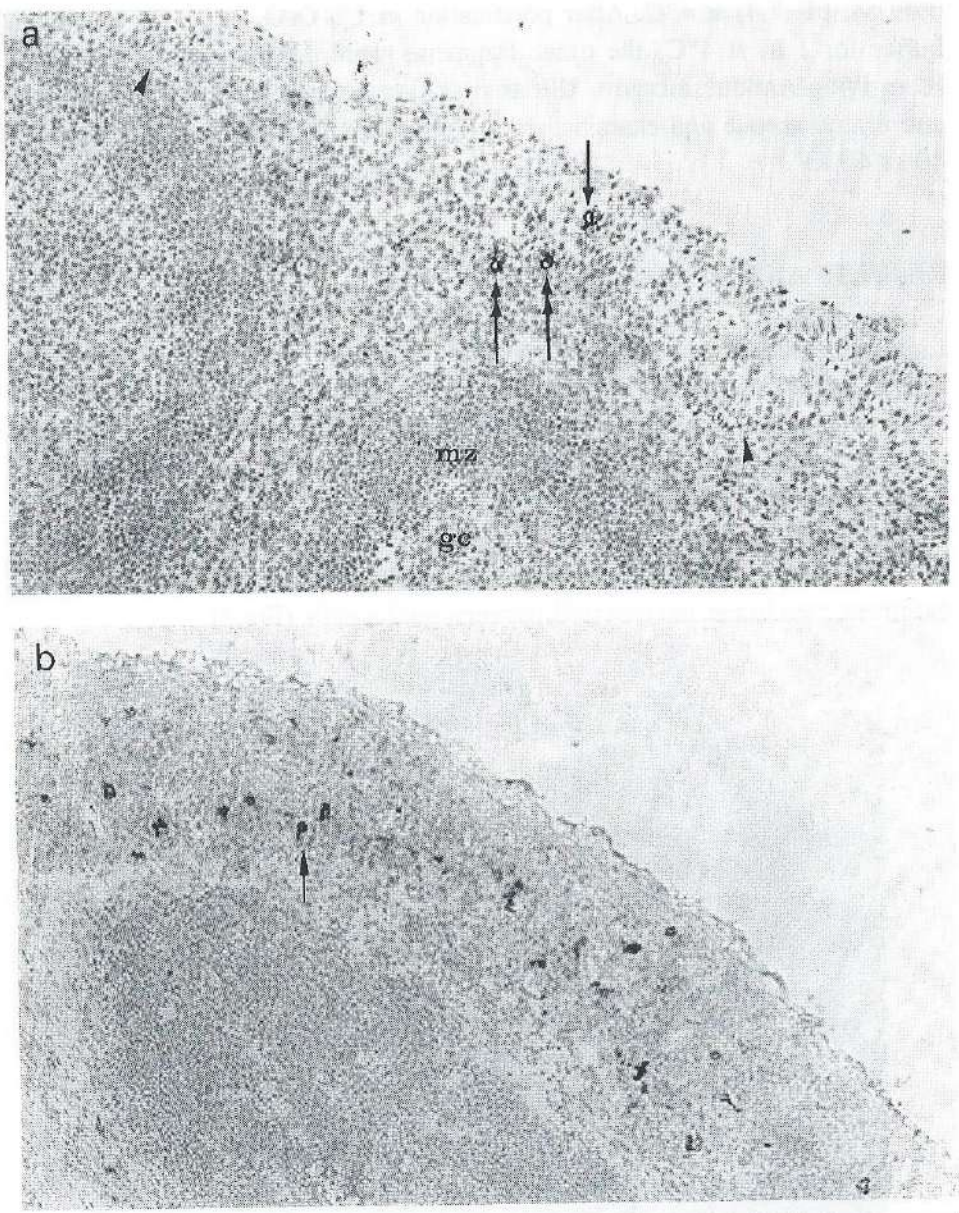

Fig 1 Cryostat section of the adenoid showing intra-epithelial (arrow) and sub-epithelial (double arrows) CD-1 positive cells. Arrow head indicates basement membrane. Note germinal centre (gc) and mantle zone (mz) of lymph follicle. x 250 (a). Cryostat section of the tonsil showing numerous CD-1 positive cells (arrow). x 200 (b).

0,09 M, pH 7,4) at 4°C. After postfixation in 1% OsO_4 in 0,1 M cacodylate buffer for 1 h. at 4°C, the tissue fragments were dehydrated and embedded in an Epon/Araldite mixture. Ultrathin sections were stained with lead citrate and uranyl acetate and examined in a Philips EM 301 electron microscope at 40 or 60 kV.

RESULTS AND DISCUSSION.

Light microscopical observation revealed only a few T6 (CD-1) positive cells in the epithelium of the adenoid. In the subepithelial layer the number of these cells was quite equal. The CD-1 positive cells were scattered throughout both regions (Fig.1) and showed slender cytoplasmic processes. Relatively great numbers of dendritic like cells could easily be recognized by their electron lucent cytoplasm at the electron microscopical level. In the epithelium they were localized in the pseudostratified epithelium just above the basement membrane interspersed between goblet cells (Fig.2).

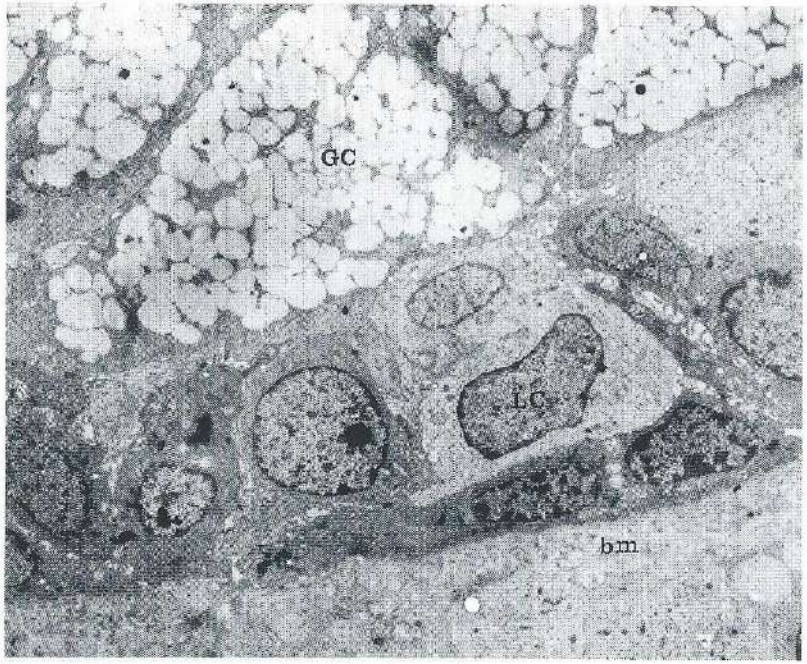

Fig 2. Electron microscopical section of the epithelium of the adenoid. GC: goblett cell; LC: Langerhans cell; bm: basement membrane. x 4500

Only occasionally BGs could be observed in the intraepithelial cells and, if present, were localized near the plasmamembrane (Fig.3). The nucleus was usually reniform or slightly indented. The nucleus/cytoplasmatic ratio varied from 1:1 to 1:3. They had a nucleus, which was euchromatic and sometimes contained a
nucleolus. All cells contained small numbers of polyribosomes and some mitochondria. There were only short strands of rough endoplasmatic reticulum. A distinct Golgi apparatus was often present. Occasionally we found a dense body resembling a monocyte granule (Fig.3). If endogenous peroxidatic activity in this granule could be demonstrated, than a relation between monocytes and LC could be suggested.
Subepithelial cells, with the same ultrastructural characteristics, did not contain BGs, and resemble small dendritic cells.

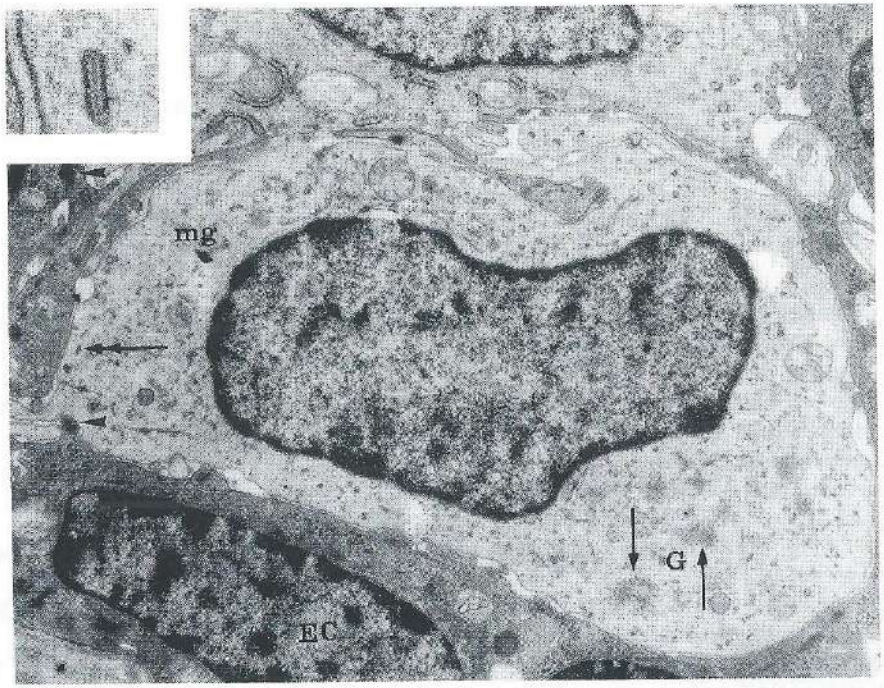

Fig 3. Electron micrograph showing higher magnification of LC. The electron lucent cytoplasm contains a well developed Golgi apparatus (G) composed of several Golgi stacks (arrows), a Birbeck granule (double arrow, see inset) and a dense body resembling a monocyte granule (mg). Surrounding cells are epithelial cells (EC), as demonstrated by the presence of desmosomes (arrow heads). x 21.000 Inset: Birbeck granule. x 145.000.

Although adenoids and tonsils both belong to the Waldeyer ring which forms an immune barrier in the upper respiratory tract, they differ with reference to the epithelium. In contrast to adenoid, tonsils are covered with a squamous epithelium. Weinberg et al (1987) showed the presence of characteristics of LC in this epithelium, but were unable to show BGs in these cells. However Yamamoto et al (1988) described BGs containing cells in the tonsil.
Our results clearly show that in the epithelium of the adenoid LC are present. It could be that these cells present antigen to immunocompetent T-cells in the epithelium itself. These T-cells are mostly of the CD-8 (suppressor/cytotoxic) phenotype (Murphy 1985). Another possibility could be that they migrate and transport antigens to the inner part of the adenoid or regional lymph node and stimulate there immunocompetent T-cells (Katz et al. 1979, Sertl et al. 1986). The immunological significance of the presence of LC in normal or infected adenoids is in study.

Acknowledgement.
The authors wish to thank S. Paniry for photographical assistance.

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LOCALIZATION AND MORPHOLOGY OF ANTIGEN PRESENTING CELLS IN THE ADENOID OF CHILDREN WITH OTITIS MEDIA WITH EFFUSION.

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Clin Immunol Immunopathol: in press.

SUMMARY.

This study describes the localization of antigen presenting cells (APC) in the different compartments in adenoids of children with otitis media with effusion (OME) and "healthy" children and adults. It is shown that the adenoid of children with OME contains a relatively high number of OKT6 and RFD1 positive cells. Moreover, accumulations of these cells are present in the extrafollicular areas of these adenoids. Very occasionally dendritic cells in the epithelial microenvironment contain Birbeck granules, indicating characteristic Langerhans cells. These OKT6 positive cells are not seen in the adenoids of the control group. Our results clearly show a relation between the presence of dendritic cells and the occurrence of OME. No differences are seen in localization and morphology of the APC in the studied adenoids.

INTRODUCTION.

Otitis media with effusion (OME) is one of the commonest diseases of childhood, characterized by fluid in the middle ear without signs of an acute infection. The resulting hearing impairment may lead to postponed development of language and speech (Friel-Patti & Finitzo 1990, Teele et al. 1984, Wallace et al. 1988).

The adenoid has long been recognized as an important factor in the pathogenesis of OME. It is thought that upper respiratory tract infections and/or acute otitis media are necessary antecedents of OME and that the adenoid plays an important role in these processes as a source of bacteria and as a factor contributing to dysfunction of the Eustachian tube (Bernstein 1985, Bluestone 1983, Gates et al. 1988, Stangerup & Tos 1985, Tos et al. 1979).

The adenoid is a lymphoepithelial organ adjoining the nasopharyngeal orifice of the eustachian tube, the only entrance to the middle ear. An imbalance between the microbial colonization of the adenoid and the immune mechanisms of the adenoid could induce OME or its antecedents (van Nieuwkerk et al. 1990).

Antigen presenting cells (APC) play a central role in initiating immunological reactions by antigen presentation in both the extrafollicular zones as well as in the germinal centers in lymphoid tissues such as adenoids (Tew et al. 1982). All cells that express MHC class II activity, such as dendritic cells, macrophages, epithelial cells and B lymphocytes can function as APC. The family of dendritic cells (DC) have been shown to be very potent APC (Grey & Chestnut 1985) and they play a key role in primary immune responses, i.e. stimulation of naive T cells (Inaba et al. 1990). Besides these migrating DC, there are follicular dendritic cells (FDC) in germinal centers which play a role in immunoglobulin isotype switch and generating memory B cells. There are strong indications that this type of cell belongs to the stroma of this area (Heusermann et al. 1980, Dijkstra et al. 1984).

DC are localized in different tissues such as the epithelial environment (Langerhans cells, LC), afferent lymph (veiled cells, VC), thymic medulla and T cell areas of lymphoid organs (interdigitating cells, IDC). They are also present in non lymphoid tissues such as blood (van Voorhis et al. 1982, Knight et al. 1986), synovial fluid (Zvaifler et al. 1985) and peritoneal cavity (Betjes et al. 1991).

Since APC are responsible for signalling T cells and B cells it is important to know the number and distribution of the APC in the various compartments of adenoids of children with OME in comparison with those of healthy persons. In this paper the localization of APC in adenoids of children with OME is studied and compared with the distribution of APC in adenoids of "healthy" children and adults, using immunohistochemistry and enzymehistochemistry. Special attention was paid to the ultrastructure of these cells in the adenoids of children with OME.

MATERIALS AND METHODS.

Patients.

Adenoids were obtained from 15 children with OME (duration longer than three months), diagnosed by otoscopy, tympanometry and/or audiometry. Adenoids from patients with a history of asthma, bronchitis or an IgE-mediated allergy were excluded.

Controls.

Two control groups were investigated: adenoid biopsies of "healthy" children (N=9) who were operated for a strabismus correction and adenoid biopsies of "healthy" adults (N=10) who underwent orthodontic surgery.

Children and adults were considered "healthy" if they had not used antibiotics or suffered from upper respiratory tract infections for at least one month prior to the operation and if they have had no middle ear infections or middle ear surgery during the previous half year. People with a history of asthma, bronchitis or an IgE-mediated allergy were excluded. Table 1 summarizes the composition of all groups.

The use of the adenoids from patients and controls was approved by the Medical Ethical Commission of the hospital and informed consent from the parents was obtained.

Cryostat sections.

Adenoids of patients and adenoid biopsies of controls were immediately frozen in liquid nitrogen. Four μm thick cryostat sections were cut and mounted on Poly-L-Lysine coated slides, dried overnight above silica gel and

stored at -20°C until use.

Table 1. Demographics of patients and controls.

	n	M/F	Age; mean/range (months)*
OME	15	9/6	43/ 14-83
"healthy" children	9	5/4	54/ 22-98
"healthy" adults	10	4/6	30/ 17-55

M/F: male/female

* Except for adults where age; mean/range is in years

Monoclonal antibodies.

The monoclonal antibodies used were: RFD1 which reacts with activated dendritic cells and activated B cells (gift from L.W. Poulter, Poulter et al. 1986); RFD7 which reacts with macrophages (gift from L.W. Poulter, Poulter et al. 1986); OKT6 (CD1a, Dakopatts) which is specific for Langerhans cells and cortical thymocytes; EBM11 (CD68, Dakopatts) which is specific for macrophages; DRC1 (Dakopatts) which stains follicular dendritic cells and S100 (Dakopatts) which reacts with Langerhans cells and interdigitating cells.

Staining techniques.

Indirect enzymtechniques using horseradish peroxidase conjugated immunoglobulin or alkaline phosphatase - anti alkaline phosphatase (APAAP) complexes as immunolabels were carried out on the cryostat sections. The immunoperoxidase technique was used as described elsewhere (van Nieuwkerk et al. 1990). Endogenous peroxidase activity was blocked by periodate. For S100 horseradish peroxidase conjugated to Cow anti Mouse IgG was used.

The APAAP method was used after Cordell et al.(1982). After fixation the preparations were incubated subsequently with the antibody, with Rabbit anti Mouse Ig (Dakopatts, for S100; Cow anti Mouse IgG) and with APAAP immune complexes (consisting of alkaline phosphatase and monoclonal anti-alkaline phosphatase). After each incubation the slides were washed in Tris-HCl buffer. As a substrate naphtol-AS-BI-phosphate was used for 15 min..

All slides were counterstained with haematoxylin. Finally the preparations were embedded in Entellan.
Control preparations were incubated in a medium in which the monoclonal antibodies were replaced by PBS or Tris-HCl buffer containing 1% BSA. In these control preparations no reaction product was visible.

Acid Phosphatase (APh) staining.
The method according to Burnstone (Pearse 1968) was used. This method visualizes APh activity which is normally present in (phago)lysosomes; in macrophages throughout the whole cytoplasm , in DC localized in a spot near an eccentrically localized nucleus (van Nieuwkerk et al. 1990). Incubation was carried out with naphtol-AS-BI-phosphate (Sigma) as substrate for 60 min at 37°C.

Evaluation.
The cryostat sections were coded and evaluated independently by three persons with conventional light microscopy at different magnifications. The number of positive stained cells detected in 6 representative sections of each adenoid were scored respectively: negative (-): when no positive cells were visible; occassionally present (±): when less than 10 positive cells were seen; present (+): when more than 10 but less than 100 positive cells were visible; abundantly present (++); more than 100 positive stained cells were present.

Electron microscopy.
From all studied groups tissue specimens were fixed in 1,5% glutaraldehyde in phosphate buffer (0,09 M, pH 7,4) at 4°C for at least 1 h.. After post-fixation in 1% OsO_4 in 0,1 M cacodylate buffer for 1 h. at 4°C, the tissue fragments were dehydrated and embedded in an Epon/Araldite mixture. To obtain an overview of the adenoid tissue, semithin (1 μm thick) sections were cut on a Reichert OmU3 microtome, stained with toluidine blue and studied lightmicroscopically. Ultrathin sections of selected areas were stained with lead citrate and uranyl acetate and examined in a Philips EM 301 electron microscope at 60 kV.

RESULTS.

Lightmicroscopy.
Patients:
As shown in table 2 only very occasionally OKT6 positive cells were present in the epithelium. However, accumulations of this cell type were observed in the extrafollicular zones especially in the deeper parts of the adenoids (fig 1a). All adenoids of the patients with OME showed these accumulations however, there was a variation in the frequency of these accumulations. In 2 of the 15 studied patients OKT6 positive cells were exclusively present in the extrafollicular area.
S100 positive cells were mainly seen in the extrafollicular zones and occasionally present in the crypt epithelium. The germinal centers and mantle zone never contained these cells.
RFD1 positive cells, indicating activated dendritic cells, were found in extrafollicular zones occasionally. However, they were never observed in germinal centers, mantle zones or crypt epithelium (fig. 1b). Accumulations of these cells showed the same extrafollicular localization as the OKT6 positive cell accumulations.
EBM11 positive cells were only rarely seen in the crypt epithelium. They were seen in the extrafollicular zone and germinal centers. Especially the latter structures showed strong reactivity with EBM11 .
RFD7 positive cells were seen in the extrafollicular areas and sometimes in the epithelium. They were never observed in the mantle zone or germinal center.
DRC1 positive cells were exclusively present in germinal centers (fig. 1c).
Cells showing APh activity were visible in all areas. However, the number of these cells was higher in the epithelium and the follicles than in the extrafollicular areas and mantle zones (fig. 1d). In the epithelium, areas of APh positive ciliated epithelial cells were alternated with areas of APh negative squamous epithelium.

Controls:
In healthy adults the pseudostratified respiratory epithelium was largely replaced by a squamous epithelium. In the epithelium of the adenoid of adults APh activity was rarely seen.

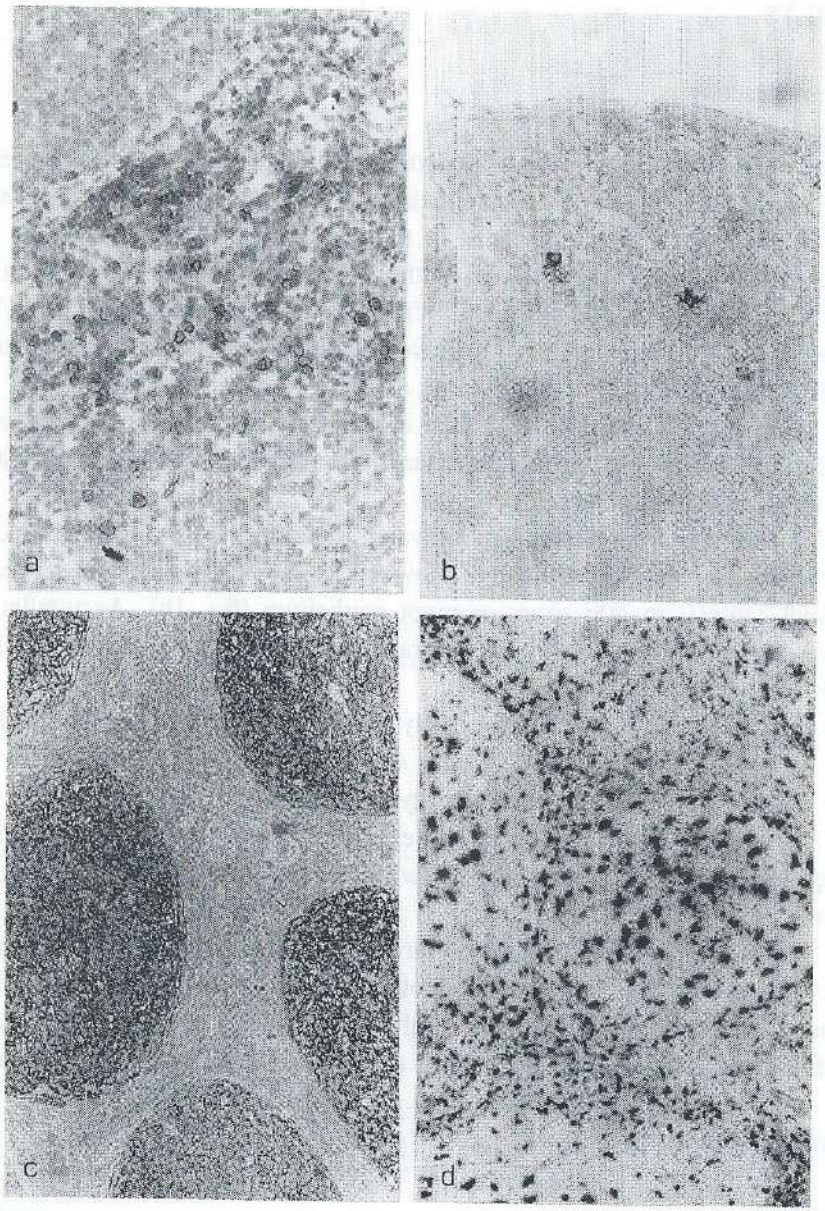


Fig.1 Cryostat sections of the adenoid of patients. (a) accumulation of OKT6 positive cells in extrafollicular zone. 125x (b) two RFD1 positive cells extrafollicularly localized. 110x (c) DRC1 reactivity of follicles, note the small extrafollicular areas. 62x (d) especially in the follicles large APh positive cells are seen, reflecting tingible body macrophages. 80x

OKT6 positive cells were neither observed in healthy children nor in adults.

Table 2: The presence of cells stained with the different monoclonal antibodies and for APh activity in the different compartments of the adenoid in OME and in "healthy" control children.

	OKT6	S100	RFD1	EBM11	RFD7	DRC1	APh	
epithelium	±	±	-	±	±	-	+	patients
	-	-	-	±	±	-	+	controls
germinal center	-	-	-	++	-	++	++	patients
	-	-	-	++	-	++	++	controls
mantle zone	-	-	-	+	-	-	-	patients
	-	-	-	+	-	-	-	controls
extrafollicular	+	+	+	+	+	-	+	patients
zone	-	±	±	+	+	-	+	controls

-: absent
±: very occasionally present
+: present
++: abundantly present

Moreover accumulations of these cells were absent. S100 and RFD1 positive cells were occasionally observed in healthy children and adults in the extrafollicular areas. Accumulations of RFD1 positive cells were never seen. EBM11 and RFD7 positive cells were seen at the same localizations and frequency as in the patient group. DRC1 positive cells were seen in the follicles of all adenoid biopsies. However, the size of these follicles the adults was clearly smaller than in both the patients and healthy children.

Electronmicroscopy.

Patients:

Electron microscopical observations revealed that in the epithelium DC like cells were scattered between the epithelial cells. They lacked tonofilaments and desmosomes. In general they had an electronlucent cytoplasm and an eccentrically localized nucleus. In many cases well developed Golgi complexes were seen in the cytoplasm; in this area relatively small (phago)lysosomes were present (fig. 3a). Only very occasionally Birbeck granules (BG), the characteristic cellorganelle of the epidermal LC (Birbeck et al. 1961), were present in the cytoplasm of these cells (Fig. 2).

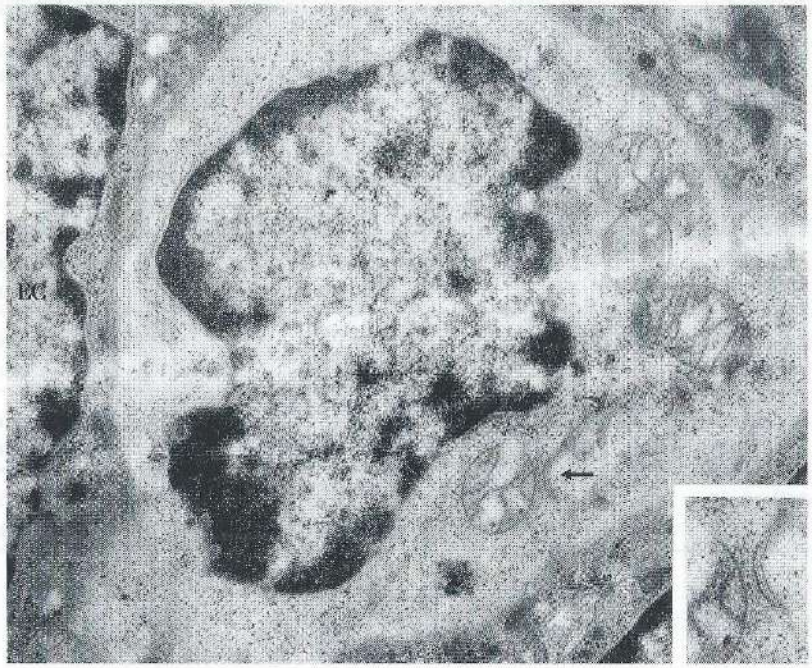


Fig.2 Langerhans cell in epithelium of the adenoid of an OME patient. The cytoplasm is electronlucent with a Birbeck granule (arrow, inset). EC:epithelial cell. 42000x, inset 80000x

These organelles never showed any relationship with other cellorganelles or with the cell membrane. The cells regularly showed dense bodies resembling phagocytic activity. Remnants of bacteria were never observed. Sometimes these cells were very small with a relatively large nucleus and they lacked the cytoplasmatic extensions. DC like cells were also seen penetrating the basal membrane (fig. 3b).

The extrafollicular areas, characterized by the presence of high endothelial venules (HEV), showed well developed dendritic cells i.e. interdigitating cells. They had a strikingly lucent cytoplasm and processes which interdigitated with extensions of adjacent lymphocytes, most probably T-cells. Sometimes these cells were in close apposition with lymphoblasts (Fig. 3c). They never contained Birbeck granules. Sometimes transitional forms between DC like cells in epithelial areas and well developed DC, were present in this area indicating a relationship between these different cell populations. Cells with the morphological characteristics of macrophages

were present in all areas of the adenoid. Remnants of bacteria were only sporadically seen. The macrophages of the germinal centers showed the characteristic morphology of tingible body macrophages; a quadrangular, mainly centrically situated nucleus, containing many pycnotic lymphocytes in various stages of digestion and other recognizable cellular detritus.

In the same areas FDC were present which had a characteristic morphological feature. They had peculiar slender cell processes which interdigitated with cell processes of adjacent FDC and/or cells of the lymphoid lineage. In some cases these cytoplasmic extensions were interconnected by adherent junctions. Especially in well developed germinal centers, these processes showed many invaginations covered with electron dense material, probably immune complexes. The cells commonly had a quadrangular to multilobulated nucleus with finely dispersed heterochromatin, surrounded by a small rim of cytoplasm. The presence of a well developed Golgi system is striking.

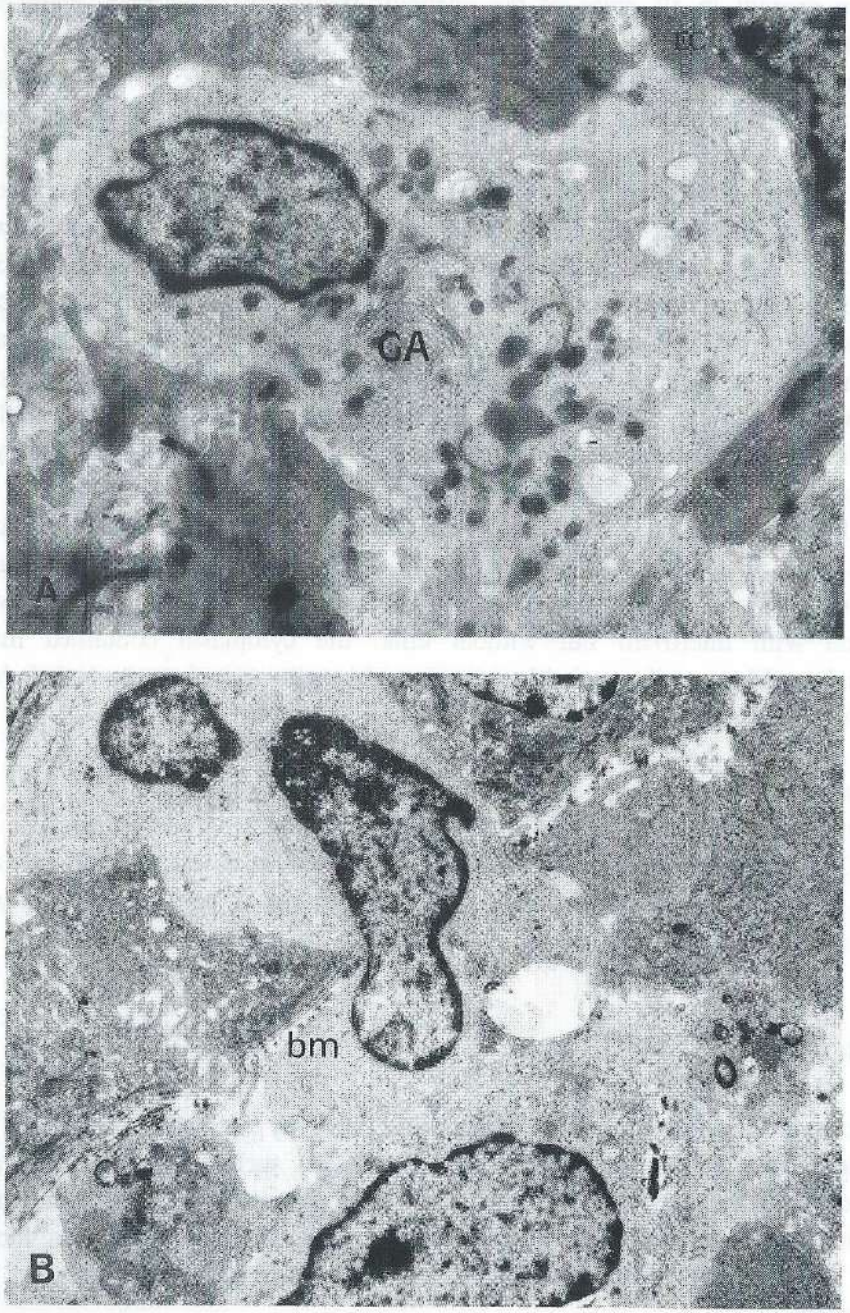
In the epithelium, epithelial cells were stretched with at their surface microvilli and cilia. They had a basal localized nucleus with frequently (phago)-lysosomes in a central area (fig. 4). Between these cells cubic cells were found with microvilli but without cilia, the cytoplasm contained many vesicles. These cells probably represent M cells (Karchev & Kabakchiev 1984).

Controls:

At the ultrastructural level no distinct differences were observed between the APC of the OME patients and the controls. The total absence of BG containing DC like cells in the epithelial microenvironment of controls was the only different observation.

DISCUSSION.

The induction of an immune response occurs by activation of helper T-lymphocytes by antigen presenting cells (APC) such as macrophages and dendritic cells. These cells present the immunogenic fragments of antigenic proteins to immunocompetent lymphocytes (Unanue et al. 1984). As shown for all lymphoid organs these APC are distributed in specific areas of the adenoid.



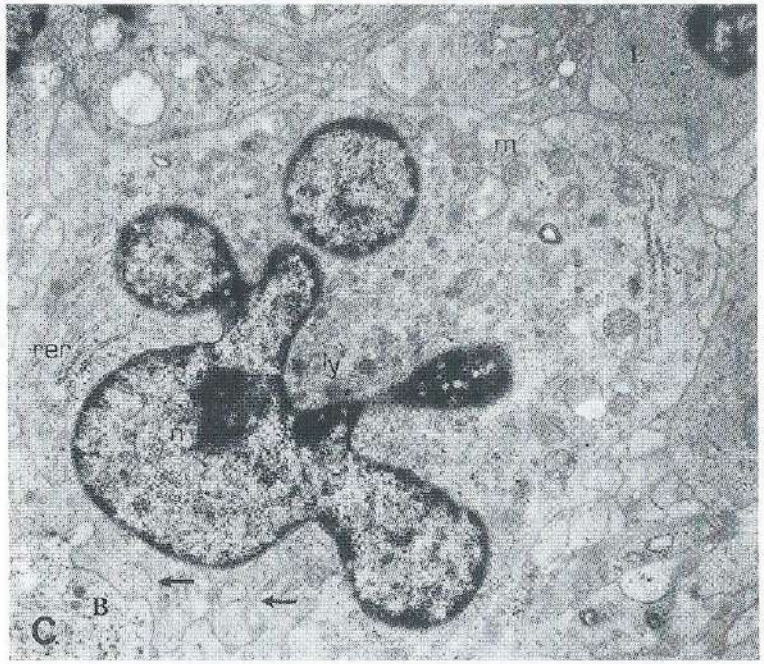


Fig.3 Dendritic like cells in the adenoid of OME patients; (A) indeterminate cell in the epithelium. The nucleus is eccentrically localized, in the centre many dense bodies representing phagolysosomes are present. GA:Golgi apparatus, EC:epithelial cell. 20000x (B) dendritic like cell penetrating the basal membrane (bm). 8100x (C) characteristic IDC in the extrafollicular or T cell area of the adenoid. The cell shows a bizarre shaped nucleus with a nucleolus (n). The electronlucent cytoplasm shows lysosomes (ly) in the vicinity of the nucleus, mitochondria (m) and rough endoplasmatic reticulum (rer). The cell membrane possesses tubular invaginations (arrows), L:lymphocyte, B:lymphoblast. 18700x

Only patients with OME showed OKT6 positive dendritic cells in the epithelium and subepithelial layers. In the deeper parts of these adenoids relative high numbers of these cells were present. Our ultrastructural observations of the epithelium revealed that only a very small part of the OKT6 positive cells contained Birbeck granules (BG). This means that there exists a discrepancy between the number of OKT6 positive cells and the number of BG containing cells. Most of the OKT6 positive cells represent indeterminate cells, i.e. cells which are phenotypically and functional identical to LC but lack the characteristic BG (Breathnach 1988). These granules were never

observed in DC like cells localized in other parts of the adenoid. These results confirm the suggestion that the epithelial microenvironment is responsible for the formation of BG intracellularly in this type of cell (Kamperdijk et al. 1990). However the number of BG containing DC of all DC in the pseudostratified respiratory epithelium of the adenoid is much lower than in the epidermis of human skin. This could mean that the type of epithelium influences the formation of BG. High numbers of indeterminate cells were described by Weinberg et al. (1987) in the epithelium of palatine tonsils. In other reports the presence of characteristic LC in the epithelium of both palatine tonsils and adenoids was described, however on ultrastructural level distinct BG were not shown (Gallo et al. 1991, Yamamoto et al. 1988).

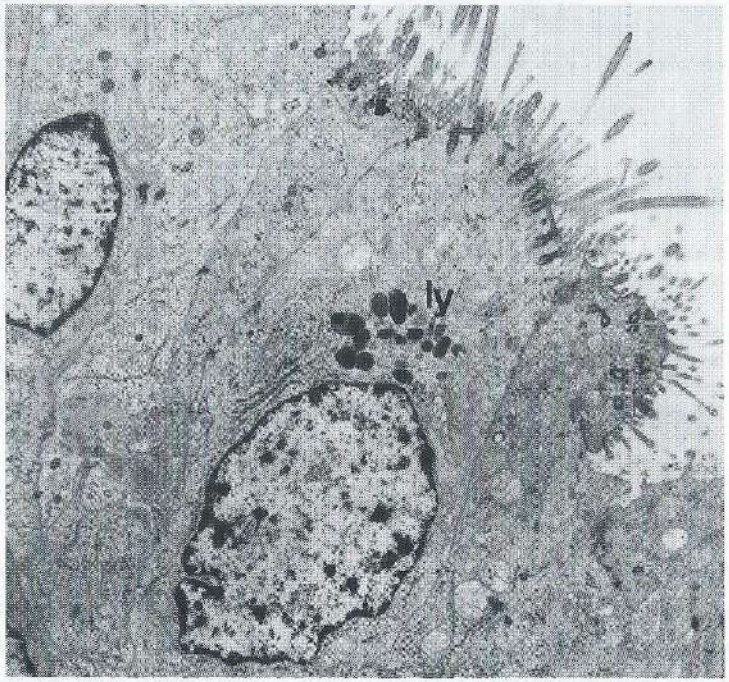


Fig.4 Ciliated epithelial cell showing (phago)lysosomes (ly) centrically localized. 12000x

In both control groups no OKT6 positive cells were present. This indicates that the presence of these cells in adenoids of children is related to the occurrence of OME (van Nieuwkerk et al. 1992). The presence of transitional forms of DC like cells between the epithelium and the extrafollicular

areas, penetrating the basal membrane, and the accumulations of OKT6 and RFD1 positive dendritic cells in the extrafollicular areas strongly suggest a migration of these cells from the periphery towards the inner parts of the adenoids. It is well known that, after antigenic stimulation, LC migrate from outside into the T cell areas of lymph nodes. In both the animal and human system an increase of these cells (IDC) is observed (Kamperdijk et al. 1985, Macatonia et al. 1987). Moreover it appeared that the number of dendritic accessory cells in tonsils of adults is largely reduced (Siegel et al. 1982). This is in accordance with the low frequency of upper respiratory tract infections and thus the immunoreactivity of these tonsils.

In the extrafollicular areas dendritic cells were found with the characteristics of interdigitating cells (RFD1 and/or S100 positive). They were found in close apposition of lymphocytes. Moreover, in contrast to the controls, clusters of RFD1 positive cells were seen in the extrafollicular areas of the adenoids of the OME patients. RFD1 positive cells are described in several chronic inflammatory diseases. It has been postulated that there exists a relationship between the number of activated DC (RFD1 positive cells) and some diseases such as sarcoidosis, colitis and asthma (Poulter & Janossy 1993). This suggests that OME is related to an inflammatory reaction in the adenoid (van Nieuwkerk et al. 1992).

Macrophages were present in the epithelium, as indicated by both RFD7 and EBM11 reactivity. RFD7 is a marker for mature macrophages (Poulter et al. 1986). Our results showed that RFD7 never stained tingible body macrophages, cells which are specific for germinal centers. The macrophages in all areas, including tingible body macrophages, appeared to be EBM11 positive. It has been described that there exists a difference in localization of EBM11 or APh between macrophages and DC; macrophages in cell suspensions showed EBM11 and APh activity throughout the cytoplasm while isolated DC showed this activity in a central spot (van Nieuwkerk et al. 1990). However in our frozen adenoid sections it was impossible to discriminate between these macrophages and DC using these criteria.

In our experiments we seldom observed bacteria or bacterial remnants. However it has been shown that bacteria, absent in traditional adenoid cultures of OME patients, can be isolated from homogenized adenoid tissue of the same individual (Forsgren et al. 1993). Recently Forsgren et al (Forsgren et al. 1994) showed the presence of *Haemophilus influenzae* in subepithelial

mononuclear cells of adenoids of children with OME using in situ hybridization techniques. This implies that the bacteria are harboured within the lymphatic tissue.

On electronmicroscopical level we observed ciliated epithelial cells with distinct, centrically localized phagolysosomes. Previous obserations have shown that these cells are MHC class II positive (van Nieuwkerk et al. 1990). Moreover, CD8 positive T lymphocytes are present in the adenoid epithelium. So it cannot be excluded that these epithelial cells are involved in activation of suppressor T cells as has already been shown for gut epithelial cells (Bland & Warren 1986, Mayer & Shlien 1987). This indicates that these cells might play an immunoregulatory role.

In conclusion; adenoids of OME patients showed, in contrast to controls, OKT6 positive cells. At the ultrastructural level occasionally BG were observed in DC in the epithelium of the adenoid of the OME group, indicating characteristic LC. Accumulations of RFD1 and OKT6 positive cells were present in the extrafollicular areas of these adenoids. Our results show a relation between OME and the presence of DC in adenoids of these children.

Acknowledgements.

We thank the department of ophthalmology (Dr. F.D. Koole) and the department of maxillofacial surgery (Prof. Dr. D.B. Tuinzing) for providing adenoid biopsies for controls, Mrs. M. Verdaasdonk for technical and Mr. S. Paniry for photographical assistance.

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ISOLATION AND CHARACTERIZATION OF DENDRITIC CELLS FROM ADENOIDS OF CHILDREN WITH OTITIS MEDIA WITH EFFUSION.

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Clin Exp Immunol 1992; 88, 345-349.

SUMMARY.

Dendritic cells (DC) were enriched from adenoids of children suffering from otitis media with effusion (OME) by density gradient centrifugation and culture techniques.
An enrichment of 40-140 fold was obtained for DC. These cells were identified using morphology, enzymecytochemistry, immunocytochemistry and functional criteria. DC could be easily distinguished from macrophages (MQ). It appeared that the monoclonal antibody (MoAb) EBM11 (CD68) discriminated between DC and MQ; in DC this activity was localized in a spot, whereas in MQ it was found throughout the whole cytoplasm. The DC enriched fractions showed a strong stimulatory effect on allogeneic T cells. These responses were MHC class II dependent since they could be blocked by anti HLA-DR/DQ monoclonal antibodies. The data clearly show that DC from adenoids of children with OME still have functional capacities.

INTRODUCTION.

Otitis media with effusion (OME) is characterized by the presence of fluid in the middle ear without signs of an acute infection. OME is the most frequent cause of hearing loss in children.
It has been suggested that the adenoid plays an important role in the pathogenesis by functioning as a source of bacterial antigens (Gates et al. 1988, Sprinkle & Veltri 1986). In this organ the (immunological) defence is probably not sufficient to conquer these bacteria.
Dendritic cells (DC) play a key role in the induction of an immune response to antigen as antigen presenting cells (Tew et al. 1982, Unanue et al. 1984). This function of DC in OME may be inadequate.
In a previous study we described that DC were occassionally present in the adenoids of children with OME (Nieuwkerk et al. 1990). This means that, to get more information about the functional activity of these cells in OME, enrichment is necessary.
In the present study a method is described to obtain purified DC fractions from adenoids of children with OME. To identify DC and to discriminate them from macrophages (MQ) immune-, enzyme-cytochemistry, ultrastructural- and functional- criteria were used.

MATERIALS AND METHODS.

Preparation of adenoid cell suspensions.
Adenoids were obtained from children undergoing adenoidectomy for OME. The use of the adenoids was approved by the Medical Ethical Commission of the hospital and consent from the parents was obtained. The adenoids were cut into small pieces which were gently pressed through a nylon gauze (± 100 μm mesh) and collected in Hanks Balanced Salt Solution (HBBS) with Hepes (pH 7,4). The cell suspensions were washed in HBSS and applied to a Lymphoprep gradient (Nycomed, Oslo, Norway). After centrifugation the cellular interface was collected and washed.

Preparation of the different fractions.
Removal of T lymphocytes was performed using a rosetting technique with

neuraminidase treated sheep erythrocytes. Erythrocyte-rosette-positive (Er^+) and negative (Er-) fractions were separated over a Lymphoprep gradient. The Er^- fraction was cultured for 24-48 hours in RPMI 1640 (Flow Lab., Irvine, Scotland), supplemented with 1 mM glutamine, penicillin (100 units/ml)-streptomycin (100 μg/ml), gentamycin (50 μg/ml) and 10% fetal calf serum (which was heat inactivated for 30 min. at 56°C). Culture was performed in Teflon beakers (Savillex, Minnetonka, MN)(2 x 10^6 cells/ml in 10 ml culture medium) to prevent monocyte/macrophage adherence. After culture the fraction (Er^-) was washed in HBBS, resuspended and brought to a concentration of 4 x 10^7 cells/ml in RPMI 1640 containing 10% FCS. The cells were layered over a continuous Percoll gradient (Pharmacia, Uppsala, Sweden) and centrifuged at 3360 rpm for 25 minutes at 4°C, to obtain a low density (LD) and an high density (HD) fraction.
Removal of B lymphocytes was performed by a panning technique using a CD22 monoclonal antibody. The non adherent cells, obtained after this panning, were indicated as LD B^- fraction.
The number and viability of the cells were determined for all fractions.

Immunocytochemistry.
Cytocentrifuge preparations of the cell fractions were fixed in buffered formaldehyde/acetone for 10 min. at 4°C, incubated for 1 hour with one of the monoclonal antibodies (MoAb) as mentioned in table III, washed, incubated with rabbit anti-mouse Ig horseradish peroxidase diluted in PBS with 1% bovine serum albumin (BSA) and 1% normal human serum for 60 min., and stained for peroxidase in diaminobenzidine-tetrahydrochloride (DAB) and 0,03% H_2O_2. Staining reactions were graded as definitely positive (+, ++, or +++, according to the intensity of staining) or negative (-) compared with the control, and uncertain weak staining was recorded as ± (table III). In control preparations, where incubation with the primary antibody was omitted, no reaction product was visible.

Enzymecytochemistry.
On cytocentrifuge preparations acid phosphatase (APh) staining was performed according to Burnstone (Pearse 1968). Incubation was carried out with naphtol-AS-BI-phosphate as substrate for 60 min. at 37°C. APh staining was always preceded by MHC class II immunoperoxidase staining. At least 200

cells were examined for the detection of DC and MQ.

Identification of dendritic cells.
DC were identified using criteria already described. Briefly, cells were scored as DC if fulfilling three criteria: 1) APh activity in a spot, 2) persistent MHC class II positivity and 3) dendritic cell morphology. In contrast, MQ had APh activity throughout the cytoplasm and only half of them expressed MHC class II antigens (Nieuwkerk et al. 1990).

Electronmicroscopy.
Cells from the LD fractions were fixed in 1,5 % glutaraldehyde in phosphate buffer (0,09 M, pH 7,4) at 4°C. After fixation in 1% OsO_4 in 0,1 M cacodylate buffer for 1 hour at 4°C, the cell suspensions were dehydrated and embedded in an Epon/Araldite mixture. Ultrathin sections were stained with lead citrate and uranyl acetate.

Table 1. Viability and number of cells in the different fractions.

	Cell yield (x10^6)	Viability (%)
Unfractionated	200-850	97-100
E^+	85-210	86-100
E^-	90-360	90-99
E^{+*}	24-110	77-97
High density	4-10	93-100
Low density	3-7	66-98
Low density B^-	0.1-0.4	41-78

T-cell isolation from peripheral blood.
For the functional analysis (mixed leukocyte reactions) of the different adenoid fractions, T cells were used from blood of healthy individuals. Human blood was drawn by venipuncture into EDTA syringes and mononuclear peripheral blood cells were obtained by Ficoll-Paque centrifugation. These cells were adjusted to 5 x 10^6 cells/ml in culture medium and incubated at 37°C for 90 min. in petridishes. The nonadherent cells were removed

and subsequently panned to remove B lymphocytes using a CD22 monoclonal antibody. The cell suspension thus obtained, contained more than 90% T lymphocytes.

Allogeneic mixed leukocyte reaction.
Allogeneic purified peripheral blood T lymphocytes (5 x 10^4) were cocultured with the different fractions (which were irradiated with 3000 rad) in 200 μl RPMI 1640 supplemented with 10% inactivated FCS, 1 mM glutamin, 100 U/ml. penicillin, 100 μg/ml. streptomycin and 5 x 10^{-5} M 2-mercaptoethanol. The irradiated cells and the responder cells, allogeneic T lymphocytes, were cultured for 6 days in 96 well microtiter plates at 37°C in a 5% CO_2 atmosphere. Sixteen hours before cell harvesting tritiated thymidin (0,5 μCi/well) was added. Incorporation of the isotope was measured by means of a liquid scintillation counter and expressed as mean counts per minute of triplicate cultures ± standard deviation.

RESULTS.

Viability and numbers of cells in the different fractions.
Unfractionated adenoid cell suspensions contained 2 - 8,5 x10^8 cells depending on the size of the removed adenoid. Total cell recovery was ± 1% of the unfractionated cells for the LD and ± 0,1% for the LD B$^-$ (table 1).

Table 2. Percentages MQ and DC as determined by APh pattern, MHC class II positivity and morphology.

	Macrophages (%)	Dendritic cells (%)	No. experiments
Unfractionated	< 1%	<0.1%	54
High density	< 0.5%	0.5-1%	5
Low density	1-4%	4-14%	5

Enzyme- and immunocytochemistry of cytocentrifuge preparations.
Using the criteria described earlier, both DC and MQ were identified.
Table 2 shows the distribution of DC and MQ in the different fractions. The

percentage of DC in the LD fractions of the different adenoid varied from 4% to 14% (an enrichment of 40-140 fold) (Fig.1)
Table 3 shows the staining results of DC and MQ with the MHC class II MoAb and several non-lymphoid cell markers.

Table 3. Characterization of DC and MQ using different monoclonal antibodies.

CD/specificity	MoAb	Reactivity with dendritic cells	Reactivity with macrophages	Source
HLA-DP	HLA-DP	+++	+++	Becton Dickinson[1]
HLA-DQ	SPV-L3	+++	+++	Gift from G.D. Keizer[2*]
	RFD1	++	±	L.W. Poulter[3]
HLA-DR	H 2.5.10	+++	+++	Gift from P.M. van Lansdorp[4]
CD1	OKT6	-	-	Dakopatts[5]
CD68	EBM11	+++	+++	Dakopatts
Macrophage subset	RFD7	-	+	L.W. Poulter
Follicular DC	DRC1	-	-	Dakopatts

[1] Becton Dickinson, Mountain View, CA
[2] Gift from G.D. Keizer, The Netherlands Cancer Institute, Amsterdam
[3] Poulter (Poulter et al, 1986)
[4] Gift from P.M. van Lansdorp, Central laboratory of the Netherlands Red Cross Blood Transfusion service, Amsterdam
[5] Dakopatts, Glostrup, Danmark

The monoclonal antibody EBM11 stained both DC and MQ, however in DC the EBM11 activity was localized in a spot (Fig.2), while in MQ it was localized throughout the whole cytoplasm.

Functional properties of the different fractions.
As shown in table 4, cells with accessory capacity were present in the LD fraction. In all cases the responses could be blocked by anti HLA-DR/DQ MoAbs.

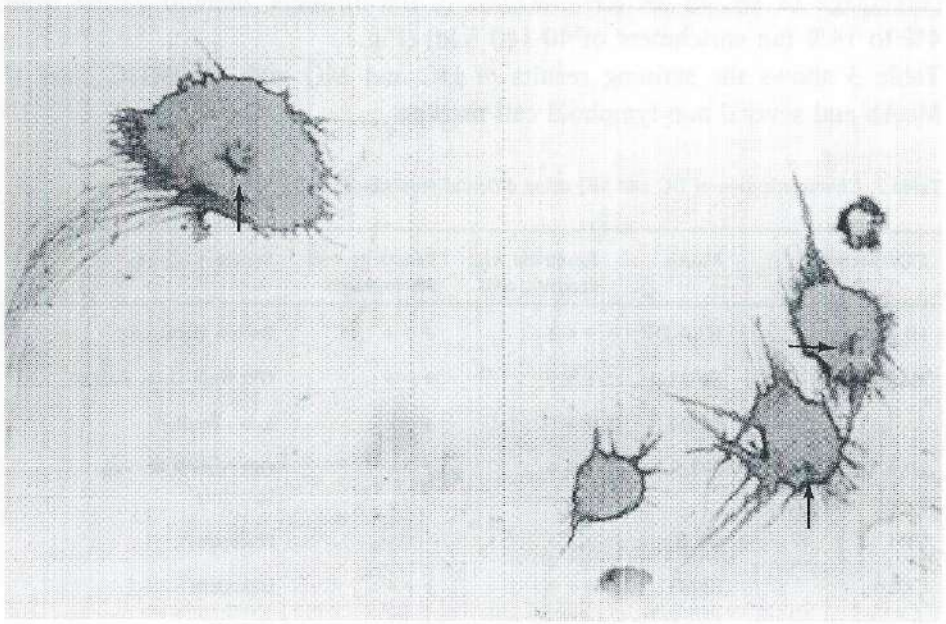

Fig.1 cytocentrifuge preparation of LD fraction showing three DC. They are MHC class II positive with cytoplasmic extensions. The cells show APh activity in a spot (arrows) (x630).

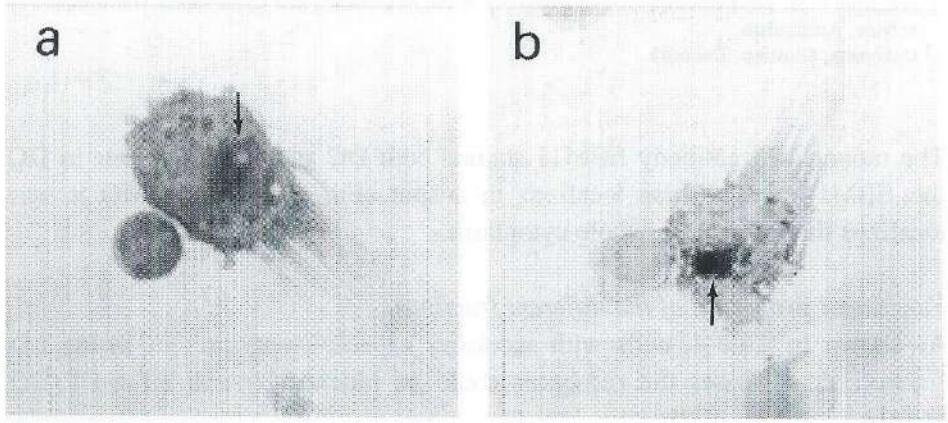

Fig.2 (a) cytocentrifuge preparation showing a characteristic DC. The cell is MHC class II positive (brown reaction product, not visible) and shows cytoplasmic extensions. APh activity is localized in a central spot (arrow) (x630). (b) cytocentrifuge preparation showing a DC stained with monoclonal antibody EBM11. Activity is localized in a spot (arrow), present at the same localization as APh (x630).

Table 4. Allogeneic T-cell responses*, as measured by ^{3}H thymidin incorporation (cpm ± sd), induced by (un)fractionated adenoid cells in absence or presence of an αHLA-DR/DQ monoclonal antibody**.

	Number of stimulator cells/well		
	10	25	50 (x 10^3)
Unfractionated	407±339	605±359	932±215
Anti-HLA-DR/DQ	380±110	334±262	442±155
High density	840±349	2055±559	2578±408
Anti-HLA-DR/DQ	711±182	480±78	408±131
Low density	6944±1419	9985±361	23164±2752
Anti-HLA-DR/DQ	815±239	2636±412	4262±922

* Different numbers of fractionated adenoid cells were co-cultured with 50.000 allogeneic T-cells.
** Mean values (of representative experiment) of triplicate cultures are expressed as cpm ± sd..

Electronmicroscopy.

The LD fraction contained relatively higher numbers of cells with ultrastructural characteristics of DC (Fig.3). DC never contained Birbeck granules, the characteristic cell organelle of the epidermal Langerhans cells (Birbeck et al. 1961).

DISCUSSION.

In this study "the antigen presenting" DC (Tew et al. 1982) were enriched from adenoids of children with otitis media with effusion (OME). In man dendritic cells have also been isolated and characterized from peripheral blood (Van Voorhis et al. 1982), synovial fluid (Tyndall et al. 1983), peritoneal effluents of patients with renal insufficiency undergoing continuous ambulatory peritoneal dialysis (Bos 1989) and from solid tissues such as tonsils (Hart & McKenzie 1988), thymus (Landry et al. 1988) and synovial membranes (Waalen et al. 1986). However, none of the isolation procedures described are suitable to enrich DC from adenoids.

Identification of DC is mainly based on their typical dendritic morphology and strong MHC class II expression (Austyn 1987). However, discrimination of these cells from MHC class II positive MQ and MHC class II positive B lymphocytes (Corradi et al. 1987) is very difficult. Moreover, there are no suitable MoAb specific for human DC. Poulter et al (1986) developed a MoAb (RFD1) staining DC and not MQ, but it appeared that a subset of B lymphocytes is also positive for this MoAb. For that reason we characterized DC not

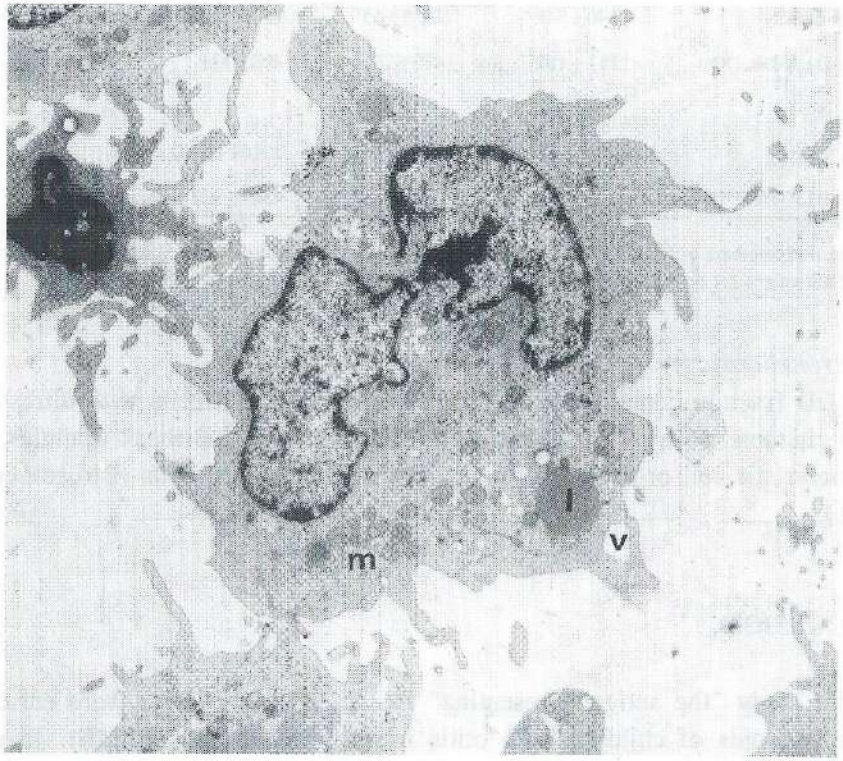

Fig. 3 electronmicroscopical picture of a DC. The cell has an irregular outline and the nucleus is eccentrically localized with identations (x6100).(m:mitochondria; l:lipid droplet; v:vacuole).

only by morphology, immunecytochemistry and expression of MHC class II molecules, but also by enzymecytochemistry (APh activity) and functional activity (MLR). Our results show that DC from adenoids, just as DC isolated from other tissues or fluids, were persistent MHC class II positive for HLA-DP, HLA-DQ and HLA-DR.

In DC APh activity was present in a spot. Staining of DC, from the LD fraction, with MoAb EBM11 shows a reaction product in a spot in the vicinity of the nucleus. This spot has exactly the same localization as the APh spot. In contrast MQ express EBM11 throughout the whole cytoplasm (Kelly et al. 1988). This would mean that EBM11 can be used to discriminate between DC and MQ, as described for these cells of peritoneal effluents of patients undergoing continuous ambulatory peritoneal dialysis (Betjes et al. 1991).

Our functional studies showed that the LD fraction had a strong stimulatory effect on allogeneic T cells. This confirmed the presence of DC in this fraction, as indicated by phenotypical analysis. This phenomenon is class II dependent, since the responses could be blocked by anti HLA-DR/DQ MoAb. The responses of the enriched fractions show that the DC from these adenoids still have functional capacities.

During the isolation procedure a dramatic decrease of the number and viability of cells after B cell panning was observed. For that reason we mainly used the LD fractions. It was shown that this LD fraction contained 4-14% characteristic DC, which is comparable with the results described after isolation of DC from human tonsils using density gradients and FACS separation (Hart & McKenzie 1988). However, several other studies claim a much higher enrichment of human tonsil DC (King & Katz 1989, Le et al. 1986). These fractions were probably contaminated with B lymphocytes, since in culture these cells can assume a dendritic morphology and express MHC class II molecules (Corradi et al. 1987). The variation in enrichment we observed might be due to the state of immune reactivity of the studied adenoid, since it has been shown that the immune reactivity of lymph nodes in the animal system determines the number of these cells in this organ (Kamperdijk et al. 1985).

In the respiratory epithelium of human adenoids characteristic Langerhans cells are present as indicated by the MoAb CD1 and the presence of Birbeck granules ultrastructurally (Nieuwkerk et al. 1991). These cells belong to the family of DC (Tew et al. 1982). However, the cells in the enriched fractions were negative for CD1 and did not contain Birbeck granules. It may be that during culture, which is necessary for DC enrichment, these characteristic parameters disappeared, a phenomenon which is also described for epidermal Langerhans cells (Schuler & Steinman 1985, Teunissen et al. 1990).

In conclusion this study descibes a method to purify DC from adenoids of children with OME. These DC still have stimulatory capacaties in a MLR. Further functional studies of these cells from adenoids of children with OME are now in progress.

Acknowledgements.
We thank Mr S. Paniry for photographical and Mrs. M. Verdaasdonk for technical assistance. The authors also wish to thank R.A. Scheeren, M.D. and P. Patel, M.D. for critical reading the manuscript.

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ANTIBACTERIAL PROPERTIES OF MACROPHAGES FROM ADENOIDS OF CHILDREN WITH OTITIS MEDIA WITH EFFUSION.

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Submitted

SUMMARY.

The adenoid as source of infections in children with OME might be explained by an impaired antibacterial capacity of adenoid macrophages in these patients. Therefore, in the present study some antibacterial properties of adenoid macrophages, obtained from children with OME, are investigated. It was demonstrated in vitro that adenoid macrophages kill *Staphyloccocus aureus* very efficiently. This killing was higher in presence of serum of patients with OME than in pooled serum of "healthy" donors. Furthermore, human adenoid macrophages were able to generate nitric oxide as measured by nitrite production. In the presence of the formol-killed bacteria *Streptococcus pneumoniae*, non-typeable *Haemophilus influenzae* and *Moraxella catarrhalis*, the production of nitric oxide increases, indicating activation of these adenoid macrophages. These results suggest that the antibacterial properties of adenoid macrophages from children with OME are not hampered.

INTRODUCTION.

Otitis media with effusion (OME) is among the most common illnesses that affect young children. Approximately 80% of children experiences at least one episode of OME during the first 4 years of life and about 20% suffers from persisting middle ear effusions (Zielhuis et al. 1989). Dysfunction of the Eustachian tube and inflammation of the middle ear are considered to be important factors in the etiology and pathogenesis of OME (Bluestone 1983, Bernstein 1985). Traditionally, it has been felt that OME was a sterile process since several reports described unsuccesful attempts to culture bacteria from the middle ear effusions (Harcourt & Brown 1953, Robinson & Nicholas 1951). However, in recent bacteriological studies 22-52% of the effusions were found to contain micro-organisms (Giebink et al. 1979, Healy & Teele 1977, Mills et al. 1985). The micro-organisms most commonly isolated in these and other studies were: *Streptococcus pneumoniae, Haemophilus influenzae, Moraxella (Branhamella) catarrhalis* and *Staphylococcus aureus* (Riding et al. 1978). The adenoid, localized at the entrance of the Eustachian tube, is thought to be the focus of bacteria and/or bacterial antigens or toxins. After viral infection of the upper respiratory tract commensal and pathogenic bacteria can invade these adenoids. Subsequently, the bacteria or bacterial antigens may ascend the Eustachian tube and contribute to dysfunction of this Eustachian tube. The adenoid is presumed to be a source of immunocompetent cells that can prevent or control infections: it has been established that adenoids have all the cell types required to produce immunological reactivity (Nieuwkerk et al. 1990). On the other hand, several well designed studies have shown that removal of the adenoid can have a significantly beneficial effect on the resolution of OME (Gates et al. 1987, Paradise et al. 1990).

Macrophages play an important role in both specific and non-specific immunity. They have a large impact on the overall regulation of the specific immune response: they are mobile cells, concentrate antigens, transport them and finally present them to immunocompetent T cells (Unanue et al. 1984). In the non-specific immune response macrophages are important for phagocytosis and killing of micro-organisms. Phagocytosis is enhanced by opsonization of the micro-organism with complement and/or antibodies (Roitt 1988). One of the mechanisms involved in the killing of micro-organisms by

macrophages is the oxygen-dependent pathway, in which nitric oxide plays a pivotal role (Liew & Cox 1991).
In adenoids of children with OME less than 1% of all cells are macrophages (Nieuwkerk et al. 1990). So far, no attention has been paid to the function of these macrophages in adenoids. In the present study the impact of these macrophages on the antibacterial host defence is addressed by studying their capacity to kill bacteria. Furthermore, the ability of these macrophages to produce nitric oxide as a response to the presence of formol-killed bacteria is evaluated. If the antibacterial defence of the adenoid macrophages is hampered, this may explain why the adenoid can function as a potential source of infection and thereby can contribute to the development of OME.

MATERIALS AND METHODS.

Patients and controls.
Adenoids were obtained from children with OME (duration longer than 3 months). During the operative procedure a blood sample was taken. The sera obtained were stored at -70°C before use. Control sera were obtained from healthy children who underwent surgery for a strabismus correction and pooled serum was formed of sera from 4 healthy adults. Approval for this procedure was obtained from the Medical Ethical Commision of the hospital and informed consent was obtained from the parents of the children for the use of adenoid and sera.

Adenoid cell suspensions.
Adenoids were cut into small pieces which were gently pressed through a nylon gauze (about 100μm mesh) to collect the cells in Hanks Balanced Salt Solution (HBBS, Flow Laboratories, Irvine, UK) with HEPES (pH 7.4) at 4°C. The cell suspensions were washed twice in HBBS at 4°C and applied to a Lymphoprep gradient (Nycomed, Oslo, Norway). After centrifugation for 20 min. at 520 g at 4°C the cellular interface was collected and washed twice in HBBS and finally suspended in RPMI (Flow Laboratories) /0,1% gelatin (Merck, Darmstadt, Germany) to a concentration of 5 x 10^7 per ml. mononuclear cells.

Micro-organism.
Staphylococcus aureus ATCC (American Type Culture Collection, Maryland, USA) 25923 was used throughout the study. Before each experiment a fresh 18 hr. culture in brain heart infusion broth (BHI, Oxoid, Basingstoke, UK) was made in a shaking waterbath at 37°C. Next, the bacteria were washed twice with phosphate buffered saline (PBS, pH 7.5). At that time the number of bacteria ranged between 6 to 8 x 10^8 Colony Forming Units (CFU) per ml.

Killing assay.
To study the killing of bacteria by adenoid macrophages, the adenoid cell suspension was brought to a concentration of 10^6 macrophages per ml. suspended in RPMI/0,1% gelatin. 100 μl of this cell suspension was added to 60 μl RPMI/0,1% gelatin, 20 μl of a suspension of 10^7 CFU of *Staphylococcus aureus* ATCC 25923 in RPMI/0,1% gelatin and either 20 μl patient serum or 20 μl pooled serum. As a control, instead of macrophages, 100 μl RPMI/0,1% gelatin was used.
Immediately thereafter, a sample of 100 μl was taken and appropriate dilutions in ice cold destilled water, to lyse the macrophages, were plated on Bouillon (Oxoid) agar plates. The test tubes, containing the remaining 100 μl, were shaken in a rotator at 37°C for 30 minutes. Afterwards 900 μl of ice cold destilled water was added to the tubes and vortexed for 60 seconds. Appropriate dilutions were plated on Bouillon agar plates. After overnight incubation at 37°C the number of viable bacteria was determined as CFU.

Short term growth experiments.
For short term growth a 1:40 dilution in BHI of an 18 hr culture of *Staphylococcus aureus* was incubated for 60 min in a shaking waterbath at 37°C to obtain log-phase bacteria. Next, aliqouts of 20 μl of these bacteria were washed twice with PBS and dissolved in 600 μl patient serum or 600 μl pool serum for a reincubation for 300 minutes at 37°C in the same waterbath. Samples were taken at 1 hr intervals and plated in appropriate dilutions on Bouillon agar. After overnight incubation of the plates at 37°C, the bacteria were counted as colony forming units. The outcome is presented as the percentage of increase of the total number of bacteria over 5 hours. Three separate experiments were done.

Production of nitrite by adenoid cells.

To determine the activation status of adenoid macrophages the amount of nitrite (NO_2^-), a product of nitric oxide (NO), was determined using the coloric assay as described by Green et al. (1982). Briefly, a total number of 10^5 adenoid macrophages per ml. α-RPMI (RPMI 1640 medium with 10% heat-inactivated fetal calf serum; 2mM L-glutamine, 50 U/ml penicillin, 50 μg/ml streptomycin) was introduced in separate wells either in absence or presence of 10^7 formol-killed bacteria per ml. α-RPMI and cultured in α-RPMI. The formol-killed bacteria used were *Moraxella catarrhalis*, non-typeable *Haemophilus influenzae* and *Streptococcus pneumoniae* type 3 (kindly donated by dr. H. Snippe, University Hospital, Dept. of Medical Microbiology, Utrecht, Holland).

Every 24 hr during a culture period of 7 days a sample of 100 μl of the supernatant was taken and stored at -70°C before measurement. To measure the concentration of nitrite, this supernatant was incubated with an equal volume of Griess reagent for 10 min. at room temperature. The absorbance was determined by means of a microplate reader (EL 312, Biotek Instruments, Winooski, USA). Absorbance was set at 540 nm; sodium nitrite ($NaNO_2$, Merck, Darmstadt, Germany) dissolved in α-RPMI was used for the standard curve. These assays were performed in triplicate.

Immunochemistry.

Total IgG, IgA and complement factor C3 in serum were measured by rate nephelometry, using the automated Beckman Array System and reagents supplied by the manufacturer, (Beckman Instruments, Brea, CA, USA). IgG subclasses were quantitated by radial immunodiffusion (RID) on commercial plates, containing IgG-subclass specific monoclonal antibodies. Kits were obtained from Serotec, Oxford, England, UK. These determinations were performed in serum of children with OME (n=10) and serum obtained from 4 "healthy" adults. The level of detection for IGg1, IgG2, IgG3 and IgG4 is 100 mg/L, 80mg/L, 80 mg/L and 50 mg/L respectively. Kits were used according to the manufacturer's instructions.

Statistical Methods.

The time-dependent outgrowth of *Staphylococcus aureus* in the in vitro growth curves was evaluated by means of the Kendall rank correlation

(Siegel 1956). Differences in the killing percentages of bacteria by adenoid macrophages between the different sera used were tested with Mann-Whitney U-test. Student's *t*-test was applied for the detection of differences in the concentration of immunoglobulin and C3 in the serum between patient and control sera. For comparing NO production values between macrophages alone and the addition of formol-killed bacteria, the Wilcoxon matched-pairs, signed-rank test was used. The level of significance was set at 0,05.

RESULTS.

*Outgrowth of **Staphylococcus aureus** in sera.*

To detect any effect of serum of children with OME on bacterial outgrowth, in vitro growth curves of *Staphylococcus aureus* in either 10% serum of patients or in 10% pooled serum were performed (Table 1). In both sera there was a time-dependent increase in the bacterial numbers ($p<0.01$). The increase in patient serum was significantly ($p<0.05$) higher than in pooled serum.

Table 1: Fold increase in the number of *Staphyloccoccus aureus* after 5 hours of culture in 10% human pooled serum (HPS) and patient serum (PS).

inoculum	HPS	PS
1.6 x 10^5 CFU	75	188
7.8 x 10^5 CFU	129	308
2.5 x 10^5 CFU	72	132

Immunoglobulins and complement.

The concentration of both immunoglobulins and complement 3 in serum patients and in pooled serum was evaluated as a possible explanation for the difference in outgrowth of *Staphylococcus aureus* in the in vitro growth curves. No differences were obtained, except for the concentrations of IgG4 and IgA. As is shown in table 2 there is a significant lower level of IgG4 and IgA in the serum of patients compared with those of the pooled serum (both

$p < 0.05$). For the other immunoglobulins and complement 3 tested, no significant differences were found between patient serum and pooled serum.

*Killing of **Staphylococcus aureus** by adenoid macrophages.*
As shown in fig 1 in pooled serum and in patient serum there was outgrowth of *Staphylococcus aureus*, being significantly ($p<0.02$) higher in patient serum than in pooled serum. Addition of adenoid macrophages to pooled serum showed a significant ($p<0.01$) decrease in the number of bacteria in the pooled serum. In the patient serum this effect was also highly significant ($p<0.0001$). The killing of *Staphylococcus aureus* by adenoid macrophages was significantly ($p<0.03$) higher in patient serum than in pooled serum.

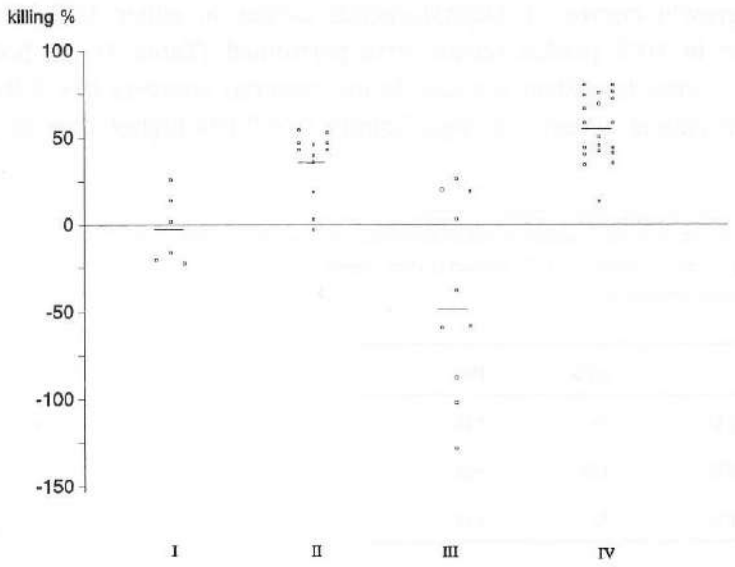

Fig.1 Percentages of killing of *Staphylococcus aureus* determined after 30 minutes of incubation: I; as control in pooled serum without macrophages (-3±20%, mean ± s.d.). II; in pooled serum with macrophages (36±19%). III; as control in patient serum without macrophages (-40±102%). IV; in patient serum with macrophages (55±18%).

Table 2: Immunoglobulin and complement values expressed in mean values ± s.d. (mg/ml)

	IgG1	IgG2	IgG3	IgG4	IgA	C3	age
patients n=10	6.35±1.75	0.98±0.50	0.54±0.22	0.16±0.16	1.02±0.37	1.28±0.20	3,3 yrs.(2,1-4,8)
pooled serum n=4	5.14±1.65	1.97±0.63	0.53±0.27	0.63±0.21	3.40±0.71	1.31±0.18	24 yrs.(19-27)

Nitrite production by adenoid macrophages.
To investigate whether human adenoid macrophages could be activated by formol-killed bacteria the amount of nitrite produced by these cells was determined. Although the concentration was relatively low, in all the experiments conducted, a peak value was established after 5 days of culturing. In the presence of killed bacteria this production was significantly increased; for *Streptococcus pneumoniae* type 3 $p<0.05$ (fig 2b), for non-typeable *Haemophilus influenzae* $p< 0.01$ (fig 2c) and for *Moraxella catarrhalis* $p<0.001$ (fig 2d).

DISCUSSION.

The adenoid functions in the local immune response to exogenous micro-organisms and other antigenic substances. In the pathogenesis of OME the adenoid is thought to be the focus of bacteria and/or bacterial antigens. If the epithelial barrier of the adenoid is altered in response to viral or bacterial infections or changes in the mucociliary clearance, bacteria can intrude the adenoid tissue (Forsgren et al. 1994). After penetration of micro-organisms the humoral and cellular defence systems become active. In the nonspecific cellular defence phagocytic cells (granulocytes, monocytes and macrophages) play an important role in phagocytozing and killing of the micro-organisms (Bainton 1988, Lasser 1983).
The aim of the present study was to investigate whether some antibacterial properties of adenoid macrophages in children with OME are hampered, which could explain the role of the adenoid as a source of infection in the pathogenesis of OME.

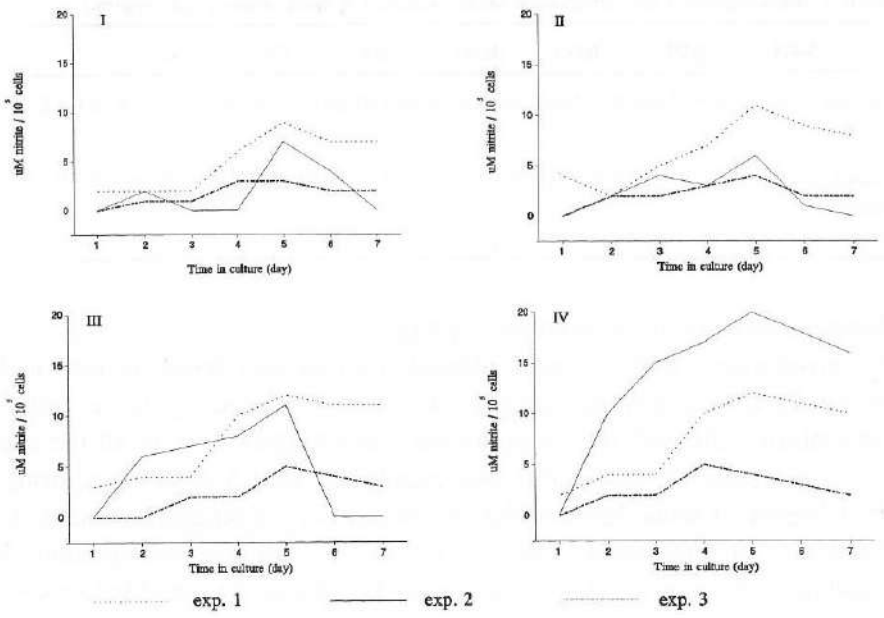

Fig.2 Nitrite production by adenoid macrophages (10^5/ml) alone (I) and in presence of formol-killed bacteria measured at various time intervals: II; in coculture with *Streptococcus pneumoniae* type 3; III; with non-typeable *Haemophilus influenzae*; IV; with *Moraxella catarrhalis*. The results of 3 experiments are shown

The main conclusions to be drawn from this study are: human adenoid macrophages are able to kill *Staphylococcus aureus*, this killing is even better in patient serum than in pooled serum of "healthy" adults. Furthermore, it was found that human adenoid macrophages secrete nitrite. Moreover, the macrophages can be activated in the presence of formol-killed bacteria as is shown by the increase in nitrite production.

In the present study a significantly higher outgrowth of *Staphylococcus aureus* in patient serum than in pooled serum was found. This was not only observed in the bacterial growth curves but also in the killing assays. IgG4 and IgA concentrations were the only serum proteins studied which showed a significant difference between patient and pooled serum which could explain this finding. IgG4 and IgA deficiencies are associated with recurrent respiratory tract infections (Moss et al. 1992, Umetsu et al. 1985). It has been shown that IgA can activate complement by the alternative pathway (Suzuki

et al. 1981) and thereby affect the lytic activity by complement on bacteria. A lower concentration of IgA might be accompanied by a lower eradication of bacteria and thereby an increased outgrowth.

Serum protein measurements, including IgG4 and IgA, in "healthy" children gave no significant differences compared to the values we obtained for the children with OME (results not shown). Therefore it is unlikely that, under physiological conditions, there will be any difference in bacterial outgrowth between patients and "healthy" children. Unfortunately, we could not obtain enough serum of "healthy" children to perform growth curves with this serum.

Regarding functional capacities of adenoid macrophages as possible explanation for a hampered non-specific immune response, we found that adenoid macrophages were able to kill *Staphylococcus aureus* in respectively patient serum and pooled serum. The bacterial killing of adenoid macrophages is comparable with that of other macrophages such as peritoneal macrophages (Verbrugh et al. 1983), lung macrophages (Hoidal et al. 1981) and peripheral blood monocytes (Verbrugh et al. 1978). It is well known that for killing of *Staphylococcus aureus* opsonization with IgG and complement is important (Verbrugh et al. 1984). The significantly higher killing in patient serum vs pooled serum can not be totally explained by the lower level of IgG4 and IgA in patient serum, especially since IgG4 is considered to be a weak opsonin (Fattom et al. 1988) and IgA activates phagocytic cells (Gorter et al. 1987). The most likely explanation for the difference in killing is that serum of patients contain more antigen specific Ig. Therefore it is unlikely that the killing capacity of adenoid macrophages in OME is impaired. Combining these results, a higher outgrowth of *Staphyloccocus aureus* was observed in patient's serum than in pooled serum, whereas the killing by adenoid macrophages was also higher in patient's serum than in pooled serum.

There is little evidence that human macrophages produce nitric oxide. To our knowledge nitric oxide production by human macrophages has only been shown in some parasitic infections (Liew & Cox 1991) and in the killing of *Mycobacterium avium* (Denis 1991). In our study adenoid macrophages were able to produce nitrite, a metabolic product of nitric oxide, a molecule which contributes to the antimicrobial activity of mononuclear phagocytes (Green et al. 1991). Nitric oxide is produced also by neutrophils (Kaplan et al. 1989). However, throughout our study we extremely seldom observed neutrophils in

adenoid material, neither on cytocentrifuge preparations and cryostat sections nor in ultrastructural observations. This is also found by Forsgren et al. (1994). So the most likely source of the measured nitrite production is indeed the adenoid macrophages. The nitrite production could be increased by the addition of formol-killed bacteria, especially *Moraxella catarrhalis* and non-typeable *Haemophilus influenzae*. These bacteria are most found in OME. Nitrite production can be induced in macrophages by exposing them to gamma-interferon and bacterial lipopolysaccharides (LPS) (Ding et al. 1988, Stuehr & Marletta 1987). The presence of LPS in *Moraxella catarrhalis* and non-typeable *Haemophilus influenzae* in contrast to *Streptococcus pneumoniae* might explain the observed increase in nitrite production in the presence of these bacteria. The results of the killing assays and the nitrite production measurements show that adenoid macrophages from children with OME are functionally active.

In conclusion, macrophages of adenoids of children with OME have intact antibacterial properties with respect to bacterial killing. Moreover, the macrophages are able to generate nitrite and addition of formol-killed bacteria can even activate the macrophages in this production. We conclude that there are no indications that these antibacterial functions of adenoid macrophages from children with OME are hampered.

Acknowledgements.

The authors wish to thank Mrs. J.J.E. Brouwer-Steenbergen and Mr. R. Hendrickx for excellent technical assistance.

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SUMMARY AND GENERAL DISCUSSION

SUMMARY.

The adenoid is supposed to function in the local immune response to exogenous micro-organisms and other inhaled or ingested antigenic substances in the presence of a normal nasopharyngeal flora that colonizes the adenoids. In healthy children, a balance must be maintained between the clearance of antigenic material and the processing of antigens. If the epithelial barrier of the adenoids is altered in responses to viral or bacterial infections or changes in mucociliary clearance, clinical significant infection may occur. Changes in the local immunity may then occur due to excessive antigenic load or changes in immune modulation, particularly by antigen presenting cells (APC) and T cell subsets. In children with OME adenoidectomy improves the natural course of OME. This is in general not due to removal of an enlarged adenoid but most likely as a result of removal of the source of infection.
The principal question in this thesis is whether there are indications that in adenoids of children with OME the non-specific and/or specific immune responses are hampered, so that the adenoid can become a source of infection and can contribute to Eustachian tube (ET) dysfunction.

Chapter 1.
In this chapter a general introduction of OME is given with special emphasis on the role of ET dysfunction and inflammatory reactions of both the middle ear mucosa and the ET mucosa in the pathogenesis of OME. We also summarize the arguments dealing with the possible role of the adenoid in OME. This is followed by some relevant basic immunological questions related to the structure and function of the adenoid. Finally, the aim of the study is given together with an introduction to the chapters 2-6.

Chapter 2.
Several studies classify the lymphocyte subsets in adenoids. However, in none of these studies the subsets are related to the existence of OME. To answer the question which different types of cells, generally involved in the defence against pathogens, are present in the adenoids of children with OME, we characterized these lymphoid and non-lymphoid cells from adenoids of children with upper respiratory tract infections (URI) and OME and from adenoids of children with URI without OME. The controls consisted of adenoids from children and adults

who were, for at least one month, free of URI.
It appears that besides DC and macrophages, MHC class II positive, ciliated epithelial cells are present. These different non-lymphoid cells were observed in all adenoids. However, they form only a very small part of the total cell population of the adenoid. We find no difference in lymphocyte subsets in children suffering from upper respiratory tract infections (URI) and OME in comparison with children suffering from URI alone. However, both groups show a significant decrease of CD-8 positive (suppressor/cytotoxic) cells and a slight increase in B-cells, as detected by the monoclonal antibody CD22, in comparison with "healthy" children. The percentages of CD-4 positive (helper/inducer) cells are similar in all groups. The localization of the lymphoid subsets in adenoids of children with URI and/or OME does not differ from those of "healthy" children and "healthy" adults. From these data it is concluded that all cells, neccessary for immunological reactivity against pathogens, are present in adenoids of children with OME.

In the second part of this chapter we show that the majority (about 60%) of the adenoid $CD4^+$ T cells are of the $4B4^+$, helper-inducer phenotype, while in peripheral blood the majority of the $CD4^+$ T cells are of the $2H4^+$, suppressor-inducer phenotype. This could mean that the adenoid shows a relatively high state of immune reactivity as a result of local exposure to exogenous antigens. About 30% of the total adenoid T cell population appears to be activated as shown by the HLA-DR/DQ positivity. Significantly more $CD8^+$ T cells ($\pm$ 40%) are activated in comparison to $CD4^+$ T cells ($\pm$ 15%), suggesting that the immune response in adenoids of children with OME is actively suppressed or down regulated. In contrast, peripheral blood contains only few activated T cells ($\pm$ 4%).

For initiating adequate immune reactions APC play an important role. Cells of the DC lineage are APC par excellence. In chapter 3 and 4 we describe the location of different cell types of the DC lineage and the macrophages ($M\varphi$) present in the adenoids of children with OME in comparison with adenoids of "healthy" children and adults.

Chapter 3.
Langerhans cells (LC), which are supposed to belong to the family of DC, are

known to be distributed in stratified, squamous epithelia in man. They express CD1 and are characterized by the presence of Birbeck granules in their cytoplasm. In this chapter we show, by lightmicroscopical and electron-microscopical studies, that this type of cell is present in the respiratory epithelium of adenoids obtained from children with recurrent upper airway infection. CD1 positive cells are not found in adenoid biopsies of "healthy" children and adults. The function of LC in the adenoids is unknown: LC are capable to present antigen to immunocompetent T-cells. Whether they stimulate T cells, in the epithelium itself, is not known. These T-cells are mostly of the CD-8 (suppressor/cytotoxic) phenotype (chapter 2). However, LC could also migrate and transport antigens to the inner part (extrafollicular area) of the adenoid or regional lymph node, where they stimulate immunocompetent T-cells (chapter 4).

Chapter 4.
In this chapter we describe the location of APC in the different compartments in adenoids of children with OME and controls in vivo. It is shown that, in contrast to "healthy" children, a relatively high number of OKT6 (CD1)(specific for LC) and RFD1 (specific for activated DC) positive cells populates the adenoids of children with OME. Moreover, accumulations of these cells are present in the extrafollicular areas of these adenoids. Very seldom, DC like cells in the epithelial microenvironment contain Birbeck granules, so representing characteristic LC. These types of cells are not seen in the adenoids of the control groups. The results of this study clearly show a relation between the presence of DC and the occurrence of OME.

Chapter 5.
The possible inability of the adenoid to handle bacterial antigens adequately might be related to a diminished potency of APC to function as stimulator cells in the immune response. Antigen presentation to T helper cells is essential to start a humoral immune response (Yewdell & Bennink 1990). In this chapter a cell separation procedure is designed, resulting in a considerable enrichment of DC from adenoids of children suffering from OME. These cells have a dendritic morphology, showed acid phosphatase- and EBM11 activity in a spot and were persistent MHC class II positive, by which they easily can be distinguished from Mφ. Moreover, the DC enriched fractions show a strong stimulatory effect on

allogeneic T cells. These responses are MHC class II dependent since they can be blocked by anti HLA-DR/DQ monoclonal antibodies. The results of this study show that DC from adenoids of children with OME are potent accessory cells and are probably not responsible for inadequate immune effector functions.

Chapter 6.
In the non-specific immune response Mφ are important for phagocytosis and killing of micro-organisms. It might be that the antibacterial properties of Mφ in adenoids of children with OME are hampered. If so, this can explain why these adenoids function as a source of infection. In this chapter we show that adenoid Mφ in vitro have the capacity to kill *Staphylococcus aureus*. This killing is better in the presence of serum of patients with OME than in pooled serum of "healthy" donors. Most likely this is caused by the presence of more antigen specific immunoglobulins in the serum of the children with OME. In addition, Mφ from the adenoids of children with OME have the capacity to produce nitrite. Moreover, in the presence of the formol-killed bacteria *Streptococcus pneumoniae*, non-typeable *Haemophilus influenzae* and *Moraxella cattarhalis*, the production of nitrite is even higher. This indicates that these bacteria activate these Mφ. These results suggest that it is unlikely that the antibacterial properties of adenoid Mφ from children with OME are hampered.

GENERAL DISCUSSION.

The non-specific immune response is a defence mechanism which is activated after penetration of micro-organisms through an epithelial barrier. When this system is not sufficient, the antigen specific immune response takes place. In the non-specific immune response macrophages and neutrophils play an important role. The specific immune response is induced by APC which present antigen to T cells, followed by T cell-B cell interactions and the synthesis of antigen specific immunoglobulins. In this thesis, the location and some functional aspects of these different lymphoid and non-lymphoid cells from adenoids of children with OME are studied. The results may explain the role of the adenoid in the pathogenesis of OME.

The non-specific immune response.

It has been shown that phagocytic cells, including neutrophilic granulocytes and monocytes/macrophages play an important role in the non-specific immune response. In chapter 6 we have shown that adenoid Mφ from children with OME are able to kill *Staphylococcus aureus*. This is comparable with the killing capacity of human Mφ obtained from other localizations like peripheral blood (van Furth et al. 1979) and from peritoneal effluents (Verbrugh et al. 1983).
We have also shown that adenoid Mφ from children with OME produce NO, as measured by the nitrite production. This means that they have the potency to perform antimicrobial activity. Moreover, the state of activation of these Mφ could be upregulated by the presence of the formol-killed bacteria non-typeable *Haemophilus influenzae, Moraxella catarrhalis* and *Streptococcus pneumoniae,* which are the most common isolates in the middle ear effusions found in children with OME. In contrast to their killing potency and nitrite production as sign of activation, only about 50% of these adenoid Mφ are MHC class II positive. After bacterial infection both in the human system (Betjes et al. 1993, 1994) as well as in the animal system (van Vugt et al. 1992) the expression of MHC class II by Mφ is much higher. This suggests that activation by bacterial stimuli in the adenoid is limited. This is also supported by our in vitro and in vivo observations that bacteria or bacterial remnants are seldom present in the adenoid. However, it has been shown that bacteria, absent in adenoid cultures taken by swab from children with OME, could be isolated from homogenized adenoid tissue of the same individual (Forsgren et al. 1993). They showed non-typeable *Haemophilus influenzae* by in situ hybridization techniques in the subepithelial mononuclear cells of adenoids obtained from children with OME and from children with adenoid hypertrophy (Forsgren et al. 1994). This would imply bacterial penetration of the epithelial barrier. An explanation for this discrepancy could be the detection level of the different techniques used.
Neutrophilic granulocytes are seldom observed in adenoids, neither in "healthy" children nor in children with OME. The absence of these cells is also observed by others (Forsgren et al. 1994).
From our results no arguments are obtained that in OME the non-specific immune response is disturbed. The absence of neutrophils in the adenoid and the fact that an increased number of DC is seen in adenoids of children with OME (chapter 4) strongly indicate a chronic inflammatory state in these adenoids, in which bacterial penetration is limited.

The specific immune response.

It has been shown that in a specific immune response an interaction takes place between antigens, lymphocytes and APC. In several studies using diseased adenoids, T- and B-cell distribution has been described (reviewed Brandtzaeg 1987). However, in none of these studies adenoids of "healthy" controls were included. Our experiments show that in adenoids of children with OME a significant higher CD4/CD8 ratio is present compared to "healthy" children. This is due to the significant lower percentage of T-suppressor cells. Similar observations are made in chronic adenoid infection (Brodsky & Koch 1993).
It may be that in "healthy" children the higher number of suppressor cells can be of benefit to prevent an excess immune response,as has also been suggested for the gastrointestinal tract in which epithelial cells selectively activate suppressor T cells, leading to a general suppression of immune responses to antigens (Mayer & Shlien 1987, Bland & Warren 1986). However, the relative decrease in suppressor cells in adenoids of children with OME may also be caused by an excessive antigenic load, which might primarily induce a stimulation of $CD4^+$ T cells. This is in accordance with the predominance of $4B4^+$ $CD4^+$ T cells (chapter 2.2) in the CD4 subset, since these T cells are thought to be involved especially in B cell help. The high number of MHC class II positive, $CD8^+$ T cells can mean that, although there is a decrease in $CD8^+$ T cells in children with OME, these cells are highly activated to prevent an excess immune response.

Stimulation of (naive) T cells in a primary immune response is primarily induced by DC (Inaba & Steinman 1984). Our results clearly show that the different cell types which belong to the DC lineage, including LC, are present in the adenoids of children with OME. The presence of characteristic LC in the epithelial microenvironment, transitional forms of DC like cells penetrating the basal membrane and the accumulations of characteristic interdigitating cells (IDC) in the extrafollicular areas of the adenoid suggest migration from the epithelium towards the T cell areas of the adenoid in children with OME. A comparable migration pattern is described for antigen loaded DC from the skin, via the afferent lymph, to the T cell areas of the lymph node (Kamperdijk et al. 1978, Macatonia et al. 1986, Silberberg-Sinakin 1976).
Our results clearly show that in adenoids of children with OME an increased number of DC is present, indicating a relation between these cells and the

occurrence of OME. The individual variations in number of DC, described in vivo (chapter 4) and in vitro (chapter 5), may be related to differences in the state of immune reactivity of the adenoids studied. Such a relationship has also been found in other lymphoid organs such as lymph nodes (Kamperdijk et al. 1985) and in the peritoneal cavity (van Vugt et al. 1992). An increase in the number of DC has also been described for chronic inflammatory diseases such as sarcoidosis, rheumatoid arthritis and atopic dermatitis (Poulter & Janossy 1993). Together with the absence of neutrophils in the adenoid, this suggests a chronic inflammatory state of the adenoid in children with OME.

In the human system the accessory activity of DC is often determined by the primary mixed leukocyte reaction (Steinman & Inaba 1985). Our studies of DC, enriched from adenoids of children with OME, show that these cells have the potency to induce a MLR. Moreover, preliminary experiments, using the enriched DC fractions, indicate that these cells are also able to present antigen both in a primary and in a secondary T cell response using Purified Protein Derivate and Tetanus Toxoid respectively (data not shown). However, adenoids of "healthy" children are not available, so it is not possible to correlate the magnitude of this accessory potency of DC with the occurrence of OME. Although biopsies can be taken from "healthy" children and adults, which are representative for the whole organ (Plum et al. 1986) and therefore can be used for histological and morphological studies, the total number of DC and Mφ from these biopsies is too low to perform functional studies.

Conclusions.

In this thesis indications are found that in children with OME there is a chronic inflammation of the adenoid. No data are obtained supporting the hypothesis that in the adenoid of children with OME the non-specific and/or specific immune responses are hampered and that as a consequence the adenoid functions as a source of infection.

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SAMENVATTING

Otitis media met effusie (OME; vochtophoping in het middenoor) is een van de meest frequent voorkomende aandoeningen bij kinderen en gaat gepaard met een gering tot matig gehoorverlies. De otologische gevolgen van OME kunnen variëren van geringe trommelvliesafwijkingen tot ernstigere afwijkingen zoals cholesteatoom. Het gehoorsverlies kan leiden, indien langer bestaand, tot een vertraagde spraak- en taalontwikkeling. Het plaatsen van trommelvliesbuisjes is de meest toegepaste behandeling bij OME. Over de oorzaak van OME bestaat nog onduidelijkheid. Algemeen wordt aangenomen dat een niet goed functionerende buis van Eustachius hierbij een rol speelt. Daarnaast wordt een ontsteking van het slijmvlies van deze buis en het middenoor als oorzakelijke factor genoemd. Bacteriologische studies wijzen er sterk op dat het adenoid (de neusamandel) fungeert als bron van infectie. Bovendien heeft verwijdering van de neusamandel een gunstig effect op het beloop van OME. Het adenoid wordt verondersteld een rol te spelen in de lokale immunologische afweer tegen micro-organismen en andere lichaamsvreemde stoffen (antigenen). Als het epitheel van het adenoid is beschadigd als gevolg van virale of bacteriële infecties of als veranderingen in mucociliar transport plaats vinden, kunnen micro-organismen het adenoid binnendringen. Een eerste verdedigingslinie tegen binnendringende micro-organismen wordt gevormd door zgn. fagocyterende cellen; cellen die deze binnendringende organismen onschadelijk kunnen maken. De belangrijkste vertegenwoordigers uit deze groep zijn de neutrofiele granulocyten en macrofagen. Naast deze niet specifieke afweerreactie kan een specifieke afweer reactie optreden. Deze specifieke afweer reactie wordt ingang gezet doordat zgn. antigeen presenterende cellen het antigeen aanbieden aan lymfocyten. Tot de antigeen presenterende cellen worden gerekend o.a. macrofagen en dendritische cellen. Deze laatsten worden algemeen beschouwd als de belangrijkste antigeen presenterende cellen. Het antigeen wordt gepresenteerd aan antigeen specifieke T lymfocyten (helper T cellen) die vervolgens geactiveerd raken en immunoregulerende stoffen uitscheiden waardoor B lymfocyten gaan proliferen en differentiëren tot antilichaam producerende plasmacellen. Deze antilichamen (antistoffen) zijn instaat te hechten aan antigenen en spelen daarmee een belangrijke rol in het onschadelijk maken van deze antigenen.
In dit proefschrift wordt onderzocht of de afweer in de neusamandel bij kinderen met OME gestoord is waardoor het adenoid als infectiebron bij het ontstaan van OME optreedt.

In hoofdstuk 1 wordt een algemeen literatuur overzicht gegeven over OME waarbij enerzijds de nadruk wordt gelegd op de rol van de buis van Eustachius en anderzijds op ontstekingsreacties van het slijmvlies van het middenoor en de buis van Eustachius. Vervolgens wordt de mogelijke rol van de neusamandel in het ontstaan van OME besproken. Hierna wordt de bouw en functie van de neusamandel gerelateerd aan de immunologische afweer waarbij speciaal aandacht wordt besteed aan dendritische cellen en macrofagen.

In hoofdstuk 2.1 worden de verschillende celtypen, die een rol spelen bij de niet specifieke en specifieke afweer, beschreven zoals aanwezig in de neusamandel van kinderen met OME. Deze cellen worden vergeleken met die aanwezig in neusamandelen van gezonde kinderen en volwassenen. De niet lymfoide cellen omvatten dendritische cellen, macrofagen en trilhaardragende epitheelcellen. Deze laatste populatie blijkt deels MHC klasse II positief te zijn waardoor ze mogelijk in staat zijn tot antigeen presentatie. De niet lyfoide cellen bleken aanwezig te zijn in alle bestudeerde adenoiden maar vormden slechts een klein deel ($<1\%$) van de totale celpopulatie van het adenoid. Het grootste deel van de celpopulatie wordt gevormd door B- en T-lymfocyten. Hieruit kan worden geconcludeerd dat alle niet lymfoide en lymfoide cellen, nodig voor niet specifieke en specifieke afweer reacties tegen pathogenen, aanwezig zijn in de neusamandel van kinderen met OME. Kinderen met bovenste luchtweg infecties (BLI) met of zonder OME toonden een significant lager percentage CD8 positieve T lymfocyten (suppressor cellen) in vergelijking met gezonde kinderen. Geen verschil werd aangetoond in aantal noch in lokalisatie van CD4 positieve T lymfocyten (helper cellen) en B lymfocyten.
In hoofdstuk 2.2 worden de T lymfocyten uit de neusamandel verder gekarakteriseerd en vergeleken met geisoleerde T lymfocyten verkregen uit bloed van hetzelfde kind. De meerderheid van de T lymfocyten blijken helper-inducer cellen te zijn, terwijl in bloed de meerderheid suppressor-inducer cellen zijn. Dit suggereert dat in de neusamandel een hoge immunologische activiteit mogelijk is als gevolg van lokale blootstelling aan antigenen. Verder zijn $\pm$ 30% van de T cellen in het adenoid geactiveerd waarbij aanzienlijk meer CD8 cellen dan CD4 cellen geactiveerd zijn hetgeen wijst op actieve suppressie van de immunologische reacties in het adenoid van kinderen met

OME. In bloed blijken slechts een gering aantal geactiveerde T cellen (± 4%) aanwezig te zijn.

Om immunologische reacties in gang te zetten spelen de antigeen presenterende cellen een belangrijke rol. Cellen behorende tot de familie van dendritische cellen, zijn de belangrijkste antigeen presenterende cellen. In de volgende 2 hoofdstukken wordt zowel op lichtmicroscopisch als ultrastructureel niveau de lokalisatie in vivo van de verschillende typen dendritische cellen en macrofagen zoals aanwezig in de neusamandel van kinderen met OME beschreven en vergeleken met die aanwezig in adenoiden van gezonde kinderen en volwassenen.

In hoofdstuk 3 wordt de aanwezigheid van karakterisieke Langerhans cellen aangetoond middels CD-1 expressie op lichtmicroscopisch niveau en aanwezigheid van Birbeck granula op ultrastructureel niveau. Deze populatie cellen behoren tot de familie van dendritische cellen en zijn aanwezig in het respiratoire epitheel van adenoiden van kinderen met recidiverende bovenste luchtweginfecties. Deze cellen blijken afwezig in adenoiden van gezonde kinderen en volwassenen.

In hoofdstuk 4 wordt de lokalisatie bepaald van de verschillende typen antigeen presenterende cellen in het adenoid van kinderen met OME. Hierbij blijkt dat in adenoiden van kinderen met OME ophopingen van CD-1 positieve dendritische cellen aanwezig zijn m.n. in de zgn extrafolliculaire gebieden. In adenoiden van gezonde kinderen blijken deze cellen niet aanwezig. Deze resultaten tonen een duidelijke relatie tussen de aanwezigheid van dendritische cellen en het optreden van OME. Bovendien werden er aanwijzingen verkregen dat de dendritische cellen migreren vanuit het epitheel naar de extrafolliculaire gebieden waar vnl. T lymfocyten aanwezig zijn. De aanwezigheid van ophopingen van dendritische cellen in adenoiden van kinderen met OME én het vrijwel volledig ontbreken van neutrofiele granulocyten in de adenoiden wijzen op een chronische ontstekingsreactie in de neusamandelen van kinderen met OME.

In hoofdstuk 5 wordt een techniek beschreven om dendritische cellen, die slechts in geringe mate (<0,1%) deel uitmaken van alle aanwezige cellen,

uit adenoiden te verrijken. Dit is noodzakelijk om het functionele vermogen van deze cellen in OME te kunnen vaststellen. Met deze methode wordt uiteindelijk een verrijking van deze cellen van ± 100x verkregen. Deze cellen worden herkend door een dendritische uiterlijk, de aanwezigheid van zure fosfatase activiteit in een spot en EBM11 activiteit op deze zelfde lokatie en MHC klasse II expressie. De dendritische cellen blijken in staat tot functionele activiteit (als goede stimulatoren in een "mixed leukocyte reaction" (MLR)).

In hoofdstuk 6 wordt de mogelijkheid van bacteriële killing door adenoid macrofagen getoetst. Hierbij blijkt dat macrofagen uit de adenoiden van kinderen met OME in staat zijn om *Staphylococcus aureus* te doden. De aanwezigheid van eigen serum geeft een verhoogde activiteit in tegenstelling tot toevoeging van serum van gezonde volwassenen. Verder zijn er aanwijzingen dat de macrofagen in staat zijn tot produktie van nitriet, een molekuul dat een rol speelt bij het doden van bacterieën. Deze produktie neemt toe in aanwezigheid van de bacterieën die een rol spelen bij OME zoals *Streptococus pneumoniae*, non-typeable *Haemophilus influenzae* en *Moraxella cattarhalis*.
Op grond van de experimenten beschreven in hoofdstuk 5 en 6 kunnen geen aanwijzingen verkregen worden dat de afweer in de neusamandel bij kinderen met OME gestoord is wegens dysfunctie van dendritische cellen en /of macrofagen. Over de hoogte van de functionele activiteit van deze cellen kan geen uitspraak gedaan worden gezien het ontbreken van adenoiden van gezonde kinderen.

In conclusie: er zijn in ons onderzoek geen aanwijzingen gevonden dat in het adenoid van kinderen met OME de specifieke of niet specifieke afweer verstoord is. Wel lijkt er sprake te zijn van een chronische ontstekingsreactie in het adenoid van kinderen met OME.

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STELLINGEN

behorende bij het proefschrift:

Immunological aspects of adenoids in children with otitis media with effusion.

1. De aanwezigheid van dendritische cellen in adenoiden vertoont een duidelijke relatie met het optreden van OME.

2. Karakteristieke Langerhans cellen zijn aanwezig in respiratoir epitheel van adenoiden bij kinderen met chronisch recidiverende bovenste luchtweg infecties.

3. De beste manier om snel een afspraak voor specialistische hulp te krijgen is de EHBO van een academisch ziekenhuis binnen te lopen.

4. Debridement en/of amputatie bij ernstige bevriezingen moet ten minste 3 maanden uitgesteld worden tot volledige mummificatie verkregen is.

5. Bij kortdurend verblijf op grotere hoogtes is 1x daags 500mg Diamox SR een goede prophylaxe tegen hoogteziekte.

6. De enige manier om gezond te blijven is te eten wat je niet wil, te drinken wat je niet lekker vindt en te doen waar je geen zin in hebt (Mark Twain).

7. Een promovendus is iemand die veel weet van wat anderen niet weten, maar niets weet van wat anderen weten.

8. Orkestratie van beurskoersen is nog geen garantie voor succes.

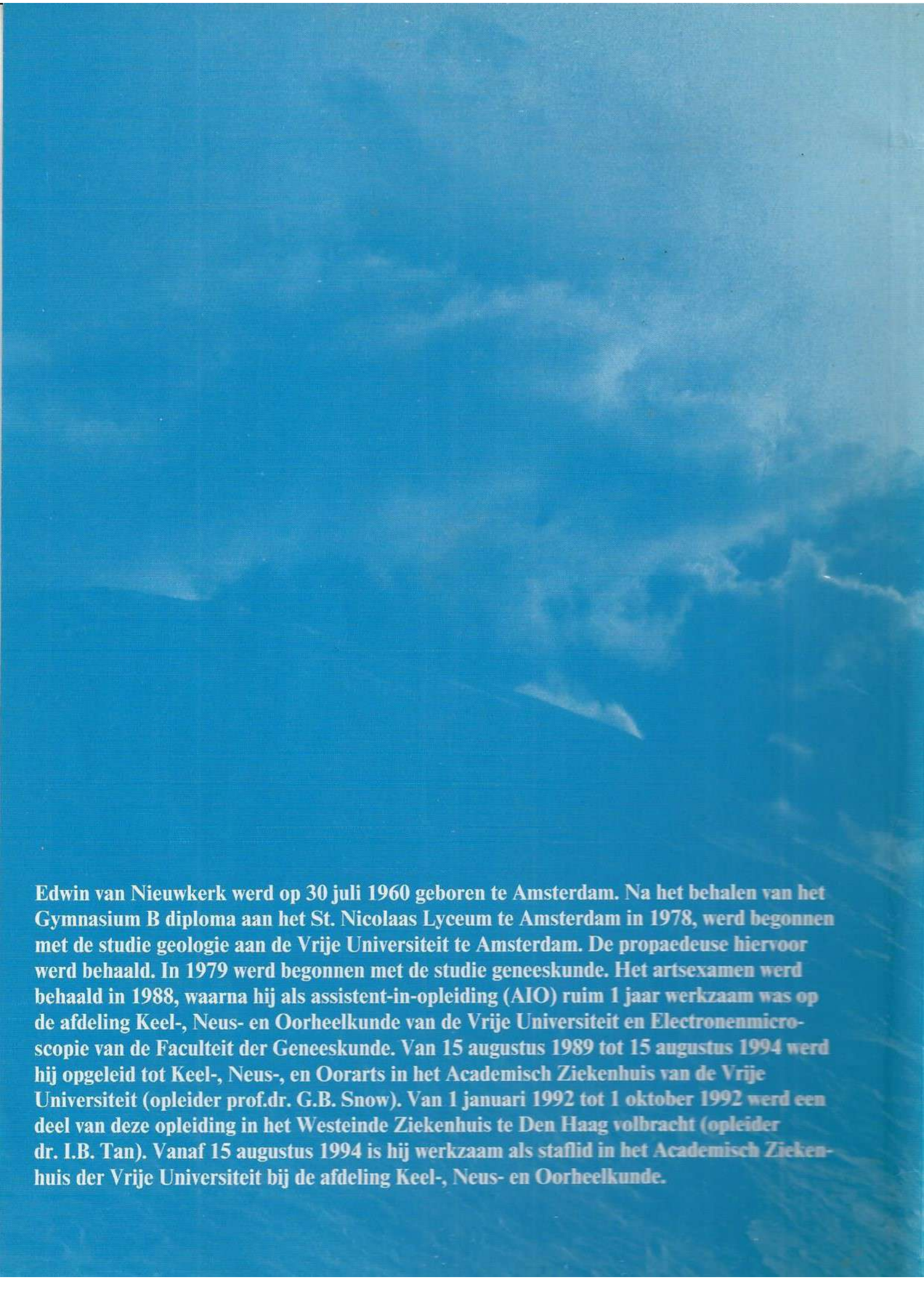
9. De maandelijkse beursverwachting van institutionele beleggers, uitgedrukt in de beurssentimentsfactor, is in de regel tegengesteld aan de werkelijke koersbeweging.

10. De waarde van gestempelde postzegels is 7 cent per kilogram, de rest is fantasie.

11. Indien Scott zijn Zuidpool expeditie had overleefd was hij niet een veel geprezen en beroemde ontdekkingsreiziger geworden maar zou hij waarschijnlijk uit de marine gezet zijn en vervolgd zijn geworden voor roekeloos gedrag.

12. De toename van het aantal parkeerplaatsen voor invaliden in Amsterdam suggereert een sterke stijging van het aantal invaliden zelf.

E.B.J. van Nieuwkerk
Amsterdam, 16 december 1994.



Edwin van Nieuwkerk werd op 30 juli 1960 geboren te Amsterdam. Na het behalen van het Gymnasium B diploma aan het St. Nicolaas Lyceum te Amsterdam in 1978, werd begonnen met de studie geologie aan de Vrije Universiteit te Amsterdam. De propaedeuse hiervoor werd behaald. In 1979 werd begonnen met de studie geneeskunde. Het artsexamen werd behaald in 1988, waarna hij als assistent-in-opleiding (AIO) ruim 1 jaar werkzaam was op de afdeling Keel-, Neus- en Oorheelkunde van de Vrije Universiteit en Electronenmicroscopie van de Faculteit der Geneeskunde. Van 15 augustus 1989 tot 15 augustus 1994 werd hij opgeleid tot Keel-, Neus-, en Oorarts in het Academisch Ziekenhuis van de Vrije Universiteit (opleider prof.dr. G.B. Snow). Van 1 januari 1992 tot 1 oktober 1992 werd een deel van deze opleiding in het Westeinde Ziekenhuis te Den Haag volbracht (opleider dr. I.B. Tan). Vanaf 15 augustus 1994 is hij werkzaam als staflid in het Academisch Ziekenhuis der Vrije Universiteit bij de afdeling Keel-, Neus- en Oorheelkunde.