Immunology of the upper respiratory tract: studies on rat nasal-associated lymphoid tissue (NALT) and human nasal mucosa

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VRIJE UNIVERSITEIT TE AMSTERDAM

IMMUNOLOGY OF THE UPPER RESPIRATORY TRACT: STUDIES ON RAT NASAL-ASSOCIATED LYMPHOID TISSUE (NALT) AND HUMAN NASAL MUCOSA

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De neus is het uiteinde van een slang die uit het lichaam steekt om zuurstof in de longen te brengen

Uit: *De ondraaglijke lichtheid van het bestaan,* van Milan Kundera

In herinnering aan mijn vader
Aan mijn moeder

IMMUNOLOGY OF THE UPPER RESPIRATORY TRACT: STUDIES ON RAT NASAL-ASSOCIATED LYMPHOID TISSUE (NALT) AND HUMAN NASAL MUCOSA

CONTENTS

Chapter 1

GENERAL INTRODUCTION

1.1. The respiratory tract

The respiratory system in air-breathing animals includes the nasal cavity, the pharynx, the larynx and continues through the trachea into the bronchi, bronchioli and ultimately the alveoli. The most evident function of the respiratory tract is oxygen intake. Oxygen from the inspired air is exchanged for carbon dioxide and is transported by the blood and body fluids to the tissues and organs. The oxygen-carbon dioxide exchange takes place in the lungs which form the respiratory portion of the system. Apart from oxygen the inspired air also brings many 'unwanted' substances in contact with the respiratory tract mucosa. The site of inhalation contact between the external and internal environment extends from the tip of the nasal cavity, the vestibule, as far as the smallest alveoli in the lung. The nasal passages, pharynx, larynx, trachea, bronchi and bronchioli together form the conducting part of the respiratory tract. In the pharynx the respiratory tract converges with the alimentory tract but they diverge again in the larynx. Anatomically the respiratory system is divided into upper and lower respiratory tract. The larynx, situated between pharynx and trachea, is usually considered a part of the upper respiratory tract (Mygind et al. 1982). Sometimes even the upper part of the trachea is included (McCaffrey 1989). From an ontogenic point of view the larynx could be considered a part of the lower respiratory tract, as both originate from the embryologic pharynx (entoderm) while the nasal cavity is from ectodermal origin (Langman 1976, Romer 1970). The Eustachian tube, although part of the upper respiratory tract, is entodermal from origin (Langman 1976). To avoid any confusion the larynx should be considered on its own, interposed between upper and lower respiratory tract (Proctor 1982). Although they may be distinct anatomically, upper respiratory tract pathology may have great clinical relevance to lower respiratory tract symptoms and *vice versa* (Cole and Stanley 1983).

1.2. Mucosal defence mechanisms with special emphasis on the respiratory tract

In comparison to the skin, mucosal membranes form a weak mechanical barrier. Therefore, it is essential that the mucosae are provided with an adequate mucosal defence apparatus. The integrity of mucosal membranes is maintained by non-immunological as well as immunological defence systems. The former comprise

Table 1. Functions and properties of IgA

Resists proteolysis
Blocks bacterial or viral adherence
Inhibits complement-dependent lysis via IgG
Neutralization of toxin activity
Limits penetration of antigen
Helps to clear absorbed antigen from the tissue
Suppresses inflammatory effect of antibody-dependent cell-mediated cytotoxicity and allergic reactions

(Mestecky et al. 1986, Ernst et al. 1987, Russell and Mestecky 1988)

antimicrobial proteins such as lysozyme and lactoferrine, which act against a broad spectrum of antigens, as do non-specific phagocytic cells. In addition, the mucociliary transport system clears particles that are trapped in the mucous blanket overlying the mucosal surfaces. The immunological defence system includes lymphocytes of B- and T-cell subpopulations and antigen presenting cells, essential to generate an immune response, as well as specific immunoglobulins.

1.2.1. Secretions

In the secretions specific and non-specific mechanisms cooperate to combat invading antigens. The mucosae together comprise farmost the largest area of contact with the external environment and this is reflected in a vast production of secretory immunoglobulin A (IgA) which exceeds the production of the other immunoglobulin isotypes (For a review on IgA and its functions see Mestecky and McGhee 1987). Some functions and properties of IgA are summarized in Table 1.

IgA is produced by plasma cells residing in the lamina propria. Underneath the epithelium IgA but also IgG producing cells are found (Nakashima and Hamashima 1980, Brandtzaeg 1985, Brandtzaeg and Bjerke 1989). The produced IgA is of the dimeric or polymeric type, containing J-chain (Kutteh et al. 1982). After binding to the secretory component on luminal and glandular epithelial cells it is transported across the epithelium to the exterior as secretory IgA (Fig. 1; For a review see Underdown and Schiff 1986, Mestecky and McGhee 1987, Brandtzaeg 1987a). Specific anti-bacterial secretory IgA may cause aggregation of microorganisms (Arnold et al. 1976), thereby increasing the possibility that these are caught in the mucous layer. Thus specific antibody sustains the non-specific mucociliary system. When comparing the IgA subclass distribution in the nasal mucosa and the intestines a shift is seen from IgA1 to IgA2 predominance (Kett et al. 1986). This is an important finding with respect to bacteria colonizing mucosal surfaces, as several bacteria are known to produce IgA1 proteases (Kilian et al. 1983). Among them are frequent

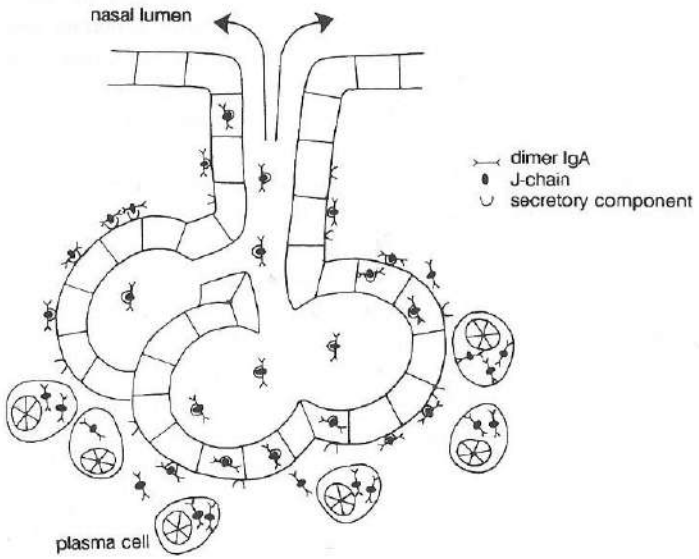

Fig. 1. Glandular translocation of secretory IgA

colonizers of the respiratory tract such as *Haemophilus influenzae*, and *Streptococcus pneumoniae*. The IgA1 protease-mediated breakdown of secretory IgA1 may be important in the pathogenesis of infection, particularly so in the upper respiratory tract where IgA1 is the predominant subclass. Naturally occurring antibodies to a given antigen are predominantly associated with either IgA1 or IgA2 (Brown and Mestecky 1985). The implications of bacterial enzymes for respiratory tract infections have been reviewed by Kilian and Reinholdt (1986). Not only IgA is detected in secretions, the other immunoglobulin classes, IgG, IgM and IgE are found as well (Widdicombe and Wells 1982, Cumella and Ogra 1987, Kurono and Mogi 1987, Brandtzaeg 1984). However, agglutinating antibodies specific for *Streptococcus mutans* were found to belong exclusively to the secretory IgA class (Arnold et al. 1976).

1.2.2. Epithelium

Epithelial cells

If an antigen passes the defensive apparatus of the secretion and the mucociliary system, it encounters the epithelium of the mucous membrane. The intact epithelium

forms a new line of defence. Adjacent epithelial cells cohere very tightly, thus providing a mechanical barrier. The junctional complex around the apex of the cells often consists of three distinct components: zonula occludens, zonula adherens and macula adherens. In the zonula occludens the outer leaflets of the adjoining unit membranes actually fuse, hence the intercellular space is closed.

Nowadays it is recognized that the epithelial cells may act as antigen presenting cells. Under certain circumstances epithelial cells are induced to express the Ia antigen (or HLA-DR antigen in man) antigen, both under pathological conditions such as inflammatory bowel disease (Hirata et al. 1986, Pallone et al. 1988) and Sjögren's syndrome (Lindahl et al. 1985, Savage et al. 1987), and in healthy nasal epithelium (Brandtzaeg 1985, Winther et al. 1987) and gut epithelium (Cerf-Bensussan et al. 1984, Spencer et al. 1986). Intratracheal challenge with horseradish peroxidase induced Ia expression by BALT (bronchus-associated lymphoid tissue) epithelial cells in rat (Van der Brugge-Gamelkoorn et al. 1986a). Ia expression by enterocytes is restricted to the upper two-thirds of the villus (Bland 1988). Recently Mayer and Shlien (1987) showed that human Ia+ gut epithelial cells can pick up tetanus toxoid and present it to T lymphocytes of the suppressor subtype. Similar results had been obtained for rat Ia+ enterocytes presenting ovalbumin (Bland and Warren 1986a,b). These are *in vitro* observations but they strongly suggest an immunological role for the Ia expressing epithelial cells *in vivo*. A correlation between the expression of Ia antigen on epithelial cells and the number of infiltrated intra-epithelial lymphocytes (IEL; see below) has been suggested (Barclay and Mason 1982, Cerf-Bensussan et al. 1984, Bland 1988).

In epithelia covering lymphoid follicles a specialized type of epithelial cell, the so called M-cell occurs. Originally the M-cell was described in the epithelium covering lymphoid accumulations in the gut (Peyer's patches, PP); M meant 'microfold' as it was thought that the M-cell possessed luminal microfolds instead of microvilli (Owen and Jones 1974). Now that microvilli are detected on M-cells, M stands for membraneous epithelial cell (Brandtzaeg 1984, Pankow and Von Wichert 1988). M-cells and M-like cells have been described later in the epithelium covering BALT (Fournier et al. 1977, Van der Brugge-Gamelkoorn et al. 1986a), appendix (Rosner and Keren 1984), tonsils (Pappo and Owen 1988) and also in the epithelium overlying lymphoid tissue in the rat nasal cavity (NALT; Spit et al. 1989). The function of M-cells is to take up antigen from the lumen and to discharge it in intact form into the extracellular space where it can be picked up by infiltrating macrophages/dendritic cells and presented to lymphocytes locally or in the draining lymph nodes. Whether or not M-cells constitutionally carry the Ia (HLA-DR) marker and thus can present antigen, is still a matter of controversy. Some authors state they do (Ernst et al. 1987), although more recently it was denied (Russell and Mestecky 1988) or questioned (Bjerke and Brandtzaeg 1988, Brandtzaeg and Bjerke 1989).

Non-epithelial cells

The epithelia of the mucosae are highly infiltrated by lymphocytes (IEL) and also by

dendritic or Langerhans cells. IEL form a current subject of investigation. They occur in the mucosal linings throughout the body. In 1975 Jeffery studied the distribution of various cell types, including lymphocytes, in the rat airway epithelium. However, most of the data published on IEL apply to the (small) intestine. It is now generally accepted that these lymphocytes are mainly T cells, that among IEL the CD8 (suppressor/cytotoxic) phenotype usually predominates (Selby et al. 1981, 1983, Cerf-Bensussan et al. 1983, Van der Heijden 1986, Trejdosiewicz et al. 1987) and that a considerable proportion of IEL lack general T-cell markers (Petit et al. 1985). Colonic mucosa contains CD4+ (T helper cells) and CD8+ IEL in virtually equal numbers (Trejdosiewicz et al. 1987, Smart et al. 1988). In 1987, Winther and coworkers reported a predominance of CD4+ over CD8+ cells in the epithelium of healthy human nasal mucosa.

In mice IEL express T-cell receptors (TCR) composed of γ and δ chains whereas mature circulating T cells generally have receptors composed of α and β chains (Goodman and Lefrançois 1988). During thymocyte development $\gamma\delta$ TCR precedes $\alpha\beta$ TCR (Pardoll et al. 1987). Nevertheless, IEL are supposed to be mature T lymphocytes (Lefrançois 1987). The finding that IEL are also present in nude mice, albeit in lower numbers (Rell et al. 1987), is further evidence that IEL are an extraordinary population of T cells. Most likely they enter the epithelium through the lamina propria because they have been detected while passing through the basement membrane (Loo and Chin 1974). Precursors of IEL have entered the mucosa through high endothelial venules (see section 'MALT'). Isolated gut IEL exhibit a homing ability which is highly specific for mucosal sites (Schmitz et al. 1988). With respect to their function in mucosal immunity, the subject is still open for discussion. The predominance of CD8+ IEL suggests a role as cytotoxic or suppressor T cells. Cytotoxicity may be performed against virus infected epithelial cells or foreign antigens, as suggested by Cerf-Bensussan and coworkers (1983). Cytotoxic activity of IEL was demonstrated by Flexman and coworkers (1983). However, the finding that only few CD8+ jejunal IEL express a marker of cytotoxic cells (Trejdosiewicz et al. 1987) favours the presumed suppressor function, as does the observation that inoculation of tumour cells results in little cytotoxicity by IEL compared to lamina propria T cells (Davies and Parrott 1981). Recently Monk and coworkers (1988) reported that activation of lamina propria T cells can cause an increase in IEL numbers.

It is well documented that Langerhans cells in skin play a major role in antigen uptake and antigen presentation. These dendritic cells of bone marrow origin (Frelinger et al. 1979, Katz et al. 1979) pick up antigen and transport it towards the draining lymph nodes (Silberberg-Sinakin et al. 1976). Langerhans cells have been described in the bronchial mucosa (Richard et al. 1987) and at several other sites in the mucosae (Schmitt et al. 1989). In the oral and nasal mucosa dendritic HLA-DR+ cells have been classified as Langerhans cells because they simultaneously expressed CD1. The presence of the characteristic Birbeck granules, however, was not reported

(Cruchley et al. 1987, Fokkens et al. 1989).

1.2.3. Lamina propria

After being stimulated by antigen IEL may reenter the lamina propria and activate B cells directly or indirectly through regulatory lamina propria T cells (Brandtzaeg 1984). Moreover, antigen that has penetrated into the lamina propria may be picked up by macrophages and dendritic cells, processed and presented to B cells or regulatory T cells, locally or in the draining lymphoid organs. The proportion of CD4+ lamina propria T cells outnumbers the proportion of CD4+ IEL (Selby et al. 1983, Van der Heijden 1986, Trejdosiewicz et al. 1987). CD4+ T cells play an important role in B cell activation. Activated B cells migrate towards the draining lymph nodes where they proliferate and differentiate into specific memory cells and plasmablasts. The latter preferentially return to the mucosal tissues and further develop into mature plasma cells which produce immunoglobulins.

IgA producing plasma cells have developed from virgin B cells bearing surface IgM and IgD. They switched to surface IgA and IgA production after induction by α-specific switch T cells (Kawanishi et al. 1983a,b). As mentioned, locally produced J chain containing IgA dimers and polymers are secreted as secretory IgA. In the lamina propria of the rat small intestine 75% of the plasma cells contain IgA, 20% IgG and 5% IgM (Sminia and Plesch 1982). The percentages of IgA producing cells in the human nasal mucosa differs between glandular and subepithelial areas and is 70% and 35%, respectively (Brandtzaeg 1985). IgG producing cells are predominantly found underneath the epithelium, the glandular area contains only few IgG producing cells (Brandtzaeg 1985). The predominant role of IgA in mucosal defence mechanisms has led to extensive mucosal studies on cases of selective IgA deficiencies. Some of these patients show an increase in numbers of IgG and IgM producing cells in the nasal mucosa, which may have developed from precursors out of gut-associated lymphoid tissue (GALT; Brandtzaeg et al. 1986). Other patients compensated with IgG and IgD producing cells presumably originating from precursors out of tonsils and BALT (Brandtzaeg 1985, Brandtzaeg et al. 1986). Also in healthy nasal mucosa IgD producing cells are frequently found (9-10% of the immunoglobulin producing cells; Brandtzaeg et al. 1979, Brandtzaeg and Korsrud 1984, Brandtzaeg 1985), in contrast to the gut lamina propria where IgD producing cells are rarely encountered (Brandtzaeg and Korsrud 1984). In tonsillar tissue the percentages of IgD producing cells vary from 0.2% of the immunoglobulin producing cells in the oral (lingual) tonsils (Matthews and Basu 1982) to 3.3% in the nasopharyngeal tonsils (Korsrud and Brandtzaeg 1980). The palatine tonsils had 1.7% IgD producing cells (Korsrud and Brandtzaeg 1980). IgE producing cells are virtually absent from healthy mucosal tissues, as shown in gut (Sminia and Plesch 1982) and in nasal mucosa (Ganzer and Bachert 1988). However, IgE antibodies play an important role in parasitic infections and in allergic reactions.

Apart from immunoglobulin producing cells, other lymphoid and non-lymphoid cells, such as macrophages, dendritic cells, mast cells, neutrophilic, basophilic and eosinophilic granulocytes are present in the lamina propria.

1.2.4. Mucosa-associated lymphoid tissue (MALT)

At several sites in the mucosal lining organized accumulations of lymphoid and non-lymphoid cells are encountered directly underneath the epithelium. Together with solitary lymphoid nodules these form the mucosa-associated lymphoid tissue (MALT). Comprehensive studies have been undertaken on GALT which is composed of PP, solitary follicles and appendix. In the lungs BALT was described more than 15 years ago (Bienenstock et al. 1973a, 1973b). Recently Sminia and coworkers (1989) reviewed the data on BALT. Also the upper respiratory tract is provided with components of MALT. Well-known in man is Waldeyer's ring (Waldeyer 1884; cited by Oláh 1978), consisting of palatine, lingual and nasopharyngeal tonsils (adenoid). Sometimes the term tubular tonsil is used to indicate the part of the adenoid adjacent to the auditory tube (Wyburn 1978). Also in other mammalian species as horse (Mair et al. 1987, 1988) and rat (Spit et al. 1989) nasal lymphoid tissue has been described. A more elaborate description on these tissues is given in section 1.3.4. In chickens, the local immune function of the orbital, nasal and upper respiratory tract areas is served by the Harderian glands, situated behind the eyes (Eerola et al. 1987, Baba et al. 1988). Species specific differences exist, as the source of IgA in tears of rats was shown not to be the Harderian gland but the extraorbital gland (Sullivan and Allansmith 1984).

PP are considered to play a central role in the mucosal immune system. As much as 70% of the cells in PP germinal centres bear surface IgA (Butcher et al. 1982). IgA precursor cells from PP can migrate and repopulate other mucosal sites in the gut (Craig and Cebra 1971) and even in the lung (BALT) as was shown in cell transfer experiments (Rudzik et al. 1975). Other *in vivo* studies confirmed these observations (Weisz-Carrington et al. 1987, Dahlgren 1987, Gregory and Filler 1987). Specific antibody in nasal secretions and saliva of monkeys was elicited by intestinal stimulation with killed influenza virus (Bergmann et al. 1986). Montgomery and coworkers (1983) elegantly showed that the IgA molecules seeding the mucosae after gastro-intestinal priming probably arise from a common clone of precursor cells. Isoelectric focussing of the produced IgA showed complete homology in tears, saliva and bronchial fluid after gastro-intestinal immunization and not after subcutaneous or ocular/topical immunization. In addition, Brandtzaeg and coworkers proposed that the secretory sites of the upper respiratory tract are mainly seeded by B cells from BALT and tonsils (Brandtzaeg et al. 1986, Brandtzaeg 1988).

Migration of lymphocytes is a continuous phenomenon. Lymphocytes enter the blood via the efferent lymph vessels through the thoracic duct and extravasate from the blood vessels at the high endothelial venules. Receptor molecules on both lymphocytes and high endothelial cells (homing receptors) guide a selective entry of recirculating lymphocytes into the tissues (Kraal et al. 1987). In this way mucosal lymphocytes preferentially repopulate mucosal tissues. Lamina propria lymphoblasts selectively bind to mucosal high endothelium *in vitro* (Jalkanen et al. 1989). The

performance of the high endothelial venule is influenced by antigenic load. (For a review on the mechanisms and regulation of lymphocyte recirculation see Duijvestijn and Hamann (1989). Some non-immunological factors are also known to influence the regulation of lymphoblast traffic to, from and among mucosal tissues, e.g. hormones and iron supply (Bienenstock et al. 1983). Recirculation of lymphocytes between mucosal tissues has led to the proposal of a common mucosal immune system (Bienenstock 1974). In this view specific lymphoblasts induced somewhere in MALT can repopulate other mucosal tissues. A common feature of the mucosal lymphoid tissues is the lack of afferent lymphatics. Mucosal repopulation, thus, can solely occur through specialized venules. Sometimes the migratory pathways appear complicated. Animals primed intra-intestinally get memory cells in BALT, but if they are primed intratracheally no specific memory is formed in the gut (Sminia et al. 1987). Furthermore, lymphocytes adhere in different B:T ratios to high endothelial venules in BALT (Van der Brugge-Gamelkoorn and Kraal 1985) and tonsils (Pals et al. 1986) than to PP high endothelial venules. To the latter relatively more B cells adhere.

1.3. The nasal mucosa

1.3.1. Morphology of the nasal cavity

The nose is a hollow organ with a complicated internal structure that narrows from floor to roof. It is externally covered by skin and internally by a mucous membrane. Various epithelial cell types can be found in the nasal cavity, thus, reflecting the multiple nasal functions of air conditioning and smell. Lengthwise the nasal cavity is divided into two cavities. Anteriorly these cavities are in open contact with the surrounding air and posteriorly they combine into the pharynx, which continues into the larynx and further into the trachea.

The most anterior part of the nasal cavity is called vestibule and is reaching from the external to the internal nares. Hairs growing in the vestibule act as a first coarse filter to retain larger particles from the inspired air. Posteriorly, the nasal cavity widens into the respiratory region. From the lateral wall in the human nose three laminae protrude into the lumen. These are the superior, middle and inferior turbinates, also called conchae. The passages underneath the turbinates are the correspondingly named meati: superior, middle and inferior meatus. Of the three human turbinates the superior is the smallest and the inferior the largest. The latter is composed of a separate bone, while the other two arise from the ethmoid bones. Their mucosal lining also shows a connection with the ethmoid region; nasal polyps which are the most common nasal tumors in man, usually originate from the ethmoid area. The inferior turbinate is rarely involved in polypous formations. The turbinates substantially enlarge the surface area of the nasal cavity, thereby facilitating an efficient warming and moistening of the inhaled air. Furthermore, the laminae cause turbulence in the air stream passing through the nasal cavity. In this way particles which move slower than the air, are caught against the nasal walls and are thus excluded from the inspired air.

Some differences occur between the nasal cavities in man and rat. While the

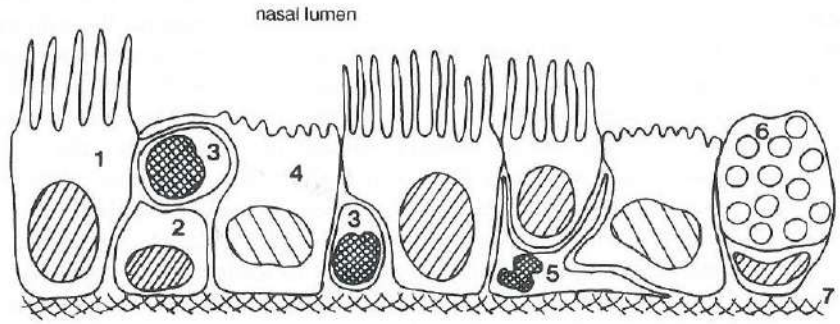

Fig. 2. Respiratory epithelium. 1: ciliated epithelial cell; 2: replacement (basal) cell; 3: intra-epithelial lymphocyte; 4: non-ciliated epithelial cell; 5: HLA-DR+ cell with dendritic processes extending towards the lumen; 6: goblet cell; 7: basement membrane.

former has a vertical orientation, the conchae one above the other, the rat nose is built rather horizontally. The turbinate structures are even more complex with an inner row of four entoturbinates and an outer row of three ectoturbinates (Liebich 1975). None of the ethmoturbinates is continuous with the nasoturbinates (Hebel and Stromberg 1986). In rats the vomeronasal organ (of Jacobson) is situated at the base of the nasal septum. Its exact function is uncertain but it may serve to smell foods (Romer 1970). In man the vomeronasal organ appears at the embryonic age of five or six weeks, but atrophies by about six months of embryonic age and is vestigial in the adult (Wyburn 1978).

1.3.2. Air-conditioning properties of the nasal cavity

The epithelial linings in rat (Liebich 1975, Montiero-Riviere and Popp 1984) and man (Mygind et al. 1982) bear great similarities. As mentioned, the various functions of the nasal cavity are reflected in the variety of epithelia present. With respect to air-conditioning three functions may be distinguished: filtering, moistening and warming of the inspired air. The filtering process starts in the vestibule where the coarse hairs form a first filter for larger particles. Here a stratified squamous mildly keratinized epithelium is observed. Keratinization completely disappears caudad. In the adjacent part of the nasal cavity a vast area of respiratory epithelium is seen (Fig. 2). This is pseudostratified columnar ciliary epithelium. Basically four cell types occur which are typical of respiratory epithelium, basal (and intermediate) cells,

which are the so called replacement cells, goblet cells and ciliated as well as non-ciliated columnar cells. The columnar cells are provided with microvilli which are bifunctional. They increase the surface area of the epithelial cells and thus enhance the exchange and transport processes in the epithelium. Furthermore, the microvilli prevent drying of the surface. Mygind has used the analogy of the garden lawn which keeps the morning dew much better than a surfaced road (cited by Brain 1989). A moist surface is a prerequisite for adequate ciliary function. The cilia penetrate somewhat into the overlying mucous blanket. Around the base of the cilia a more serous fluid is found, called periciliary fluid, which allows free movement of the cilia (Sadé 1970, Eccles 1983). The cilia and the mucous blanket together form the mucociliary system of which the main function is clearance of the conductive airways. Its importance is emphasized by the fact that in Kartagener's syndrome or in primary ciliary dyskinesia the abolished mucociliary transport is linked to chronic rhinosinusitis (Pedersen and Mygind 1982, Van der Baan et al. 1983). As expected, the absence of nasal cilia has a similar effect (Babin and Kavanagh 1985). By the ciliary beat the mucous blanket is moved towards the oral cavity where it can be swallowed. Foreign material trapped in the mucous layer is thus cleared from the nose. Particles larger than 1-3 µm are to a large extent trapped in the nasal mucous blanket (Brandtzaeg 1984, Stuart 1984, Dahl et al. 1988). The mucus is produced by goblet cells and submucosal nasal glands. Antigen specific secretory IgA molecules enhance trapping of the material by aggregating smaller particles. The immunoglobulin concentrations in nasal secretions vary during the day. These variations may be caused by variations in secretory activity of the glands which is greatest during the morning (Mygind and Thomsen 1976). Other studies reported an increased production of IgA during the night (Hughes and Johnson 1973, Passàli and Bellusi 1988).

Despite the nasal trapping, large sized allergens (3-5 µm) may cause an asthmatic reaction, thus involving the lower respiratory tract (Brain 1989). This may be an indication that larger particles can pass the nasal filter but probably they mostly reach the lungs by mouth-breathing without nasal involvement. The speed at which trapped particles are cleared is not influenced by weight nor by density or surface properties of the particles, indicating that foreign bodies travel on top of the mucous blanket as passive passengers (Sadé 1970). The transport rapidity varies among individuals from less than 1 mm to over 20 mm per minute (Eccles 1983).

Besides trapping foreign particles, the mucous layer is important in holding the fluid which moistens the inspired air. According to Cauna and coworkers (1972, 1982) this fluid is not produced by glands or goblet cells but extravasated from fenestrated subepithelial capillaries. The blanket of the nasal secretion thus conducts heat and water vapour from the mucosa to the inspired air (Eccles 1983).

Apart from air-conditioning the nose is a sensory organ, namely the organ of smell. This purpose is served by olphactory epithelium which is found on the cranial parts caudad in the nasal cavity. Normally only 5-10 % of the inhaled air reaches the olphactory epithelium. This rate may be increased to 20 % when sniffing (Moore-Gillon 1989).

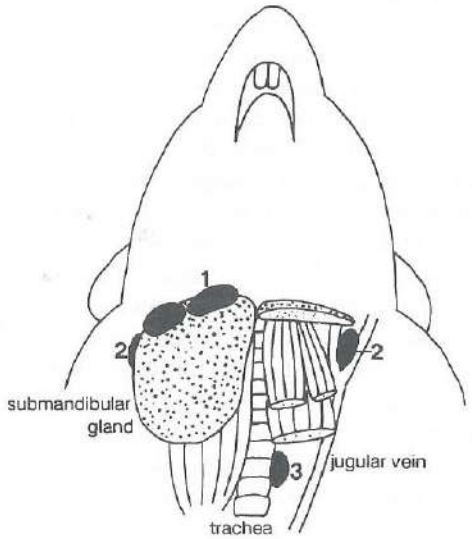

Fig. 3. Localization of cervival lymph nodes in the rat. 1: superficial cervical lymph node; 2: facial lymph node; 3: posterior cervical lymph node

1.3.3. Vascularization

The nasal mucosa is a well vascularized tissue. The arterial blood is supplied by periosteal and perichondrial arteries, giving rise to a subepithelial plexus. This plexus opens up into a wide venous 'blood-space'. Such an architecture allows rapid swelling of the nasal mucosa. As mentioned before, an extensive network of fenestrated subepithelial capillaries provides fluid to moisten the inspired air (Cauna 1970, Cauna et al. 1972). Around the submucosal glands also a large venous cavernous plexus can be found. Direct anastomoses between arteries and venous sinuses frequently occur (Wyburn 1978, Cauna 1982). In the cat more than 50% of the normal nasal blood flow was shown to be shunted through arteriovenous anastomoses (Änggård 1974).

Lymph vessels form an irregular network located superficially in the mucosa. The main lymph vessels are directed backwards and in man are ultimately collected into two trunks, one passing to a retropharyngeal lymph node, the other passing to one or two deep cervical lymph nodes (Wyburn 1978). Also in the rat the head and neck regions are drained by cervical lymph nodes (Fig. 3). The deep cervical lymph nodes, called the posterior cervical lymph nodes, drain all peripheral nodes in the neck as well as deep cervical structures (Tilney 1971).

1.4. Mucosa-associated lymphoid tissue in the respiratory tract

1.4.1. Lower respiratory tract

As mentioned before, the lower respiratory tract contains distinct units of mucosa-associated lymphoid tissue, denoted BALT (Bienenstock 1973a,b, Sminia et al. 1989), which occurs at well defined sites between an artery and bronchus epithelium. It makes contact with the bronchus epithelium through the locally interrupted muscularis mucosae (Plesch 1982). BALT is detected in the rat from day 4 after birth. Histochemical studies on its ontogeny have been published previously (Gregson et al. 1979, Plesch et al. 1983). It was observed that B cells precede T cells in the development of BALT. As the number of lymphocytes increases, high endothelial venules become visible. *In vitro* adherence studies revealed that T and B cells adhere in about equal numbers to BALT high endothelial venules (Van der Brugge-Gamelkoorn and Kraal 1985). This is similar to the adherence ratio of T and B cells found in mesenteric lymph nodes, but it is different in other lymph nodes where preferentially T cells adhere and in PP where preferentially B cells adhere to the high endothelial venules. Distinct areas of T and B cells are seen from 4 weeks post-partum (Plesch et al. 1983). This compartmentalization may be due to antigenic stimulation. T- and B-cell areas in PP occur around day 10 after birth (Sminia et al. 1983), thus indicating that BALT develops later than GALT.

Immunoglobulin bearing lymphocytes in BALT are mainly of the IgM or IgG isotype, each comprising about 40%. IgA bearing lymphocytes comprise only about 15% of the immunoglobulin bearing cells, similar as in the corona of PP (Sminia and Plesch 1982). Plasma cells are rare and occur mainly in the periphery of BALT or outside BALT in the surrounding connective tissue. Lymphocytes and macrophages infiltrating the epithelium are seen inconsistently (Plesch 1982).

Immunization studies revealed some interesting features of BALT. Firstly, intratracheal priming with the T independent antigen lipopolysaccharide (LPS) and the partly T independent antigen paratyphoid vaccin (PTV) caused an increase in plasmablasts and peripherally located plasma cells along with an infiltration with lymphocytes of the epithelium and the area under the epithelium. Also an enlargement of the subepithelial area occurred. Germinal centres, which are major sites of proliferation and differentiation of antigen-specific B cells *in vivo* (Kraal et al. 1982), appeared 6 to 8 weeks after immunization. Upon intratracheal administration of T dependent antigens such as horseradish peroxidase (HRP), bovine serum albumin (BSA) and *Baccillus Calmette Guérin* (BCG), lymphocytes infiltrated the epithelium while also the area under the epithelium was enlarged and more filled with lymphocytes. However, no increase in plasmablasts, plasma cells or germinal centres could be observed (Van der Brugge-Gamelkoorn et al. 1985a). Similarly, intratracheal challenge with 2,4,6-trinitrophenylated keyhole limpet haemocyanin (TNP-KLH) hardly resulted in specific anti-TNP forming cells. Intratracheal boosting after the priming enhanced the immune response and mainly so in the draining paratracheal lymph nodes (Van der Brugge-Gamelkoorn et al. 1986b). Several other studies revealed that intratracheal immunization induced specific antibody forming cells in the draining lymph nodes (Kaltreider et al. 1987, Sminia et al. 1987, Thepen et al. 1989). Specific antibody forming cells in the lung parenchyma were seen after a second intratracheal boost with TNP-KLH (Van der Brugge-Gamelkoorn et al. 1986b). They were also elicited after subcutaneous priming and intratracheal boosting with HRP (Van der Brugge-Gamelkoorn et al. 1985b). Intra-intestinal priming with TNP-KLH followed by intratracheal boosting with the same antigen showed specific anti-TNP forming cells in both BALT and lung, whereas immunizing in the reverse order did not elicit specific antibody forming cells in Peyer's patches or gut lamina propria. These results underline that the lower respiratory tract is a rather closed system (Sminia et al. 1987). In this respect it is worth mentioning that the height of an immune response in the lungs following intratracheal antigen administration is to a large extent determined by a suppressive action of alveolar macrophages (Holt 1986, Thepen et al. 1989).

In summary, the characteristics of the immunological reactivity in the lower respiratory tract known thus far are:

-Intratracheal antigen administration induces an enlargement of the area under the epithelium and induces lymphocytes to infiltrate the epithelium.

-Primary induction of blast cells and plasma cells occurs mainly after administration of T independent antigens.

-The majority of specific antibody forming cells are seen in the draining lymph nodes and not in the lung.

-The mucosal immune response in the respiratory tract is a rather local phenomenon.

-If any, antibody forming cells in BALT are located in the periphery of this lymphoid tissue or in the surrounding connective tissue.

These data concern the lower respiratory tract. With respect to the upper respiratory tract little information on the effects of local immunization was available at the start of the present study.

1.4.2. Upper respiratory tract

Mucosa-associated lymphoid tissue in the respiratory tract is not restricted to the lungs. In the aeroalimentory tract several entities of lymphoid tissue may be observed. As mentioned before, in man Waldeyer's ring is found. Most of its components encounter respiratory as well as dietary antigens. The epithelium covering the tonsils is of a squamous type, the adenoid is covered by respiratory epithelium. Tonsils are provided with epithelial crypts composed of reticular epithelium wherein lymphocytes and non-lymphoid cells gather. Like other components of MALT the tonsils show structural similarities with lymph nodes with the exception that afferent lymphatics are lacking. Antigens are transported either actively or passively from the exterior via the epithelium to subepithelial lymphoid cells. M-cells are present in the tonsillar epithelium to take up antigen from the lumen (Brandtzaeg 1984, Pappo and Owen 1988). Apart from M-cells the specialized antigen-presenting Langerhans cells were

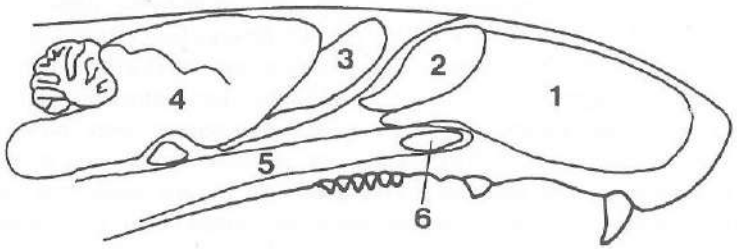

Fig. 4. Longitudinal section through the head of a rat. The lower jaw has been removed. 1: respiratory epithelium; 2: olfactory epithelium; 3: bulbus olfactorius; 4: brain; 5: nasopharyngeal duct; 6: nasal-associated lymphoid tissue (NALT). By courtesy of Dr. B.J. Spit.

described in the deep layers of the epithelium of the palatine tonsil (Brandtzaeg 1984). Langerhans cells migrate towards the draining lymph nodes. Therefore, their presence is an indication that not all antigens are dealt with in the subepithelial tissue.

In several other mammalian species lymphoid tissue has been described in the upper respiratory tract. As early as in 1947 Kelemen observed massive lymphoid accumulations in the floor of the nasal cavity of rats. In other species similar structures have been found. In monkeys and slow lorises large as well as smaller aggregations of lymphoid cells were described (Loo and Chin 1974). In monkeys mucosa-associated lymphoid tissue is also described around the ducts of the minor salivary glands (duct-associated lymphoid tissue: DALT; Nair and Schroeder 1986), which is not found as such in murine oral mucosa (Lacasse et al. 1987). In man lymphoid accumulations in salivary glands have been observed under pathological conditions (Lindahl et al. 1985). Carter and coworkers (1975) reported that mice would not have nasal lymphoid tissue as was described in the rat by Kelemen (1947), but two years later murine nasal lymphoid tissue was observed (Belal et al. 1977). Mair and coworkers (1987) used the term nasal-associated lymphoid tissue (NALT) to denote collective nodular lymphoid aggregates in the equine upper respiratory tract, as did Harkema and coworkers (1987) to denote lymphoid tissue in the nasopharynx of bonnet monkeys. Independent hereof Spit and coworkers (1987) were the first to introduce the term NALT for lymphoid accumulations in the rat nasal cavity (Fig. 4). Data were provided on localization and morphology, including an electron microscopic survey of the lymphoid tissue and its epithelium (Spit et al. 1989). This nasal lymphoid tissue was described as a rat equivalent of the human Waldeyer's ring (WRE; De Jong et al. 1985, Koornstra et al. 1987, 1989). An age related decrease in B-cell numbers accompanied by an increase in T-cell numbers was observed. Moreover, they found that the homing of WRE lymphoid cells to WRE, PP, mesenteric and deep cervical lymph nodes had decreased in older animals. Based on these results it was suggested that WRE is most active at a younger age (Koornstra et al. 1987). In this respect the nasal lymphoid tissue would indeed resemble human tonsils. In ageing the tonsillar B-cell compartment decreases from 33% to 26% while the T-cell compartment rises from 51% to 70% of the tissue as determined by histomorphometric analyses (Brandtzaeg 1987b). A further similarity is the predominance of T helper cells (CD4+) in both WRE (Koornstra et al. 1989) and in tonsils, where they comprise 70% of the lymphoid cells in the T-cell areas (Brandtzaeg 1987).

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Chapter 2

INTRODUCTION TO THE EXPERIMENTAL WORK

The aim of this thesis was to get a better insight into the immunological role of the upper respiratory tract mucosa. Prior to performing immunization studies we decided to study the normal structure and ontogeny of the nasal mucosa, as the morphology and development of a tissue can provide valuable information as to its functional role.

Comparing the ontogeny of mucosa-associated lymphoid tissue in the rat gut (GALT; Sminia et al. 1983) and lower respiratory tract (BALT; Gregson et al. 1979, Plesch et al. 1983), it appears that the latter develops more gradually. Before inspired antigens reach the lungs they pass the nasal cavity, as rats are nose-breathers. The nasal cavity thus plays a protective role with respect to inhaled antigens. Presumably this protective function is reflected in an early development of lymphoid tissue. In **chapter 3.1** an immuno- and enzyme-histochemical study on the postnatal development of NALT is described.

With respect to the adult NALT, a light and electron-microscopic survey was performed by Spit et al. (1989). Further morphological information was obtained by immuno- and enzyme-histochemical staining techniques so as to detect various lymphoid and non-lymphoid cell populations. The results of this study are reported in **chapter 3.2.**

Although morphology and function are closely linked, the question remained: 'what happens when antigen is administered in the nasal cavity?' The effects in rats and mice of several intranasally applied antigens have been determined through the years, in serum (Morag et al. 1974, Ivanoff et al. 1982), skin (DTH; Tamura et al. 1988) or lungs (Morag et al. 1974, Ivanoff et al. 1982, Kumagai et al. 1985, Khavkin and Tabibzadeh 1988), but not in the nose. Only few studies concerned the rat nasal mucosa (Ostrow et al. 1979) or murine nasal wash (El Guink et al. 1989). In the latter study specific IgA antibodies in nasal wash were elicited by intranasal immunization with vaccines of influenza or Sendai virus. Intranasally administered antigens that reach NALT may induce a cellular and a humoral immune response. In **chapters 4.1** and **4.2** the humoral response to several antigens is reported. The antigens chosen are 2,4,6-trinitrophenyl (TNP) conjugated carriers, which provide an elegant model to study anti-TNP antibody forming cells *in situ* (Claassen and Van Rooijen 1984, Van Rooijen and Kors 1985). Apart from NALT also serum, spleen and lungs were examined, as were the cervical lymph nodes, which drain the nasal cavity. From the study by Tilney (1971) it was known that the posterior cervical lymph nodes drain the nasal cavity, which was confirmed by own observations. Moreover, injection of

^{125}I-labelled human IgG-aggregates into NALT pointed to the posterior cervical as the draining lymph nodes of NALT (Koornstra et al., in preparation). In addition, the superficial cervical lymph nodes were assigned an important role in IgE suppression in the respiratory tract (Sedgwick and Holt 1985).

Direct extrapolation from the animal experiments to the human situation is impossible as man does not have NALT as such. As suggested NALT may be an equivalent of Waldeyer's ring (De Jong et al. 1985, Koornstra et al. 1987, 1989). Especially the adenoid or nasopharyngeal tonsil is a candidate for this counterpart, since both NALT and adenoid exclusively encounter airborne antigens. The nasal mucosa itself also contains numerous lymphoid and non-lymphoid cells. Although well studied in the gut, few studies have been undertaken on the distribution of leukocytes in the mucosa of the upper respiratory tract. Nishimoto et al. (1988) studied the lymphocyte distribution in the inflamed maxillary mucosa and Winther and coworkers (1987) did so in normal human nasal mucosa. Recently Stoop and coworkers (1990) studied the distribution of leukocytes in the nasal mucosa of polyposis patients. In this thesis the human nasal mucosa was subject of an immuno- and enzyme-histochemical study. Tissue specimens from ear, nose and throat patients were compared to control samples to describe the normal situation and to allow the detection of pathologically induced deviations. As mentioned in the general introduction it is known that the superficial epithelium plays an important role in immunological defence of the mucosa. Therefore, the intra-epithelial lymphocytes and non-lymphoid cells in the human nasal mucosa are first described in **chapter 5.1.** The survey was then extended to the deeper layers of the nasal mucosa, viz. the subepithelial connective tissue and the glandular area. The results of the latter study are given in **chapter 5.2.**

Chapter 6 provides a general discussion of the findings described in the preceding chapters. The data on the rat nasal cavity are compared to data on other parts of MALT. Special attention is paid to the immunological role of NALT and its position in the mucosal immune system.

The observations on human samples are also compared to results obtained in other studies. Moreover, clinical implications are considered.

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Chapter 3

STRUCTURE AND DEVELOPMENT OF RAT NASAL-ASSOCIATED LYMPHOID TISSUE (NALT)

3.1.
An immunohistochemical study on the postnatal development of rat nasal-associated lymphoid tissue (NALT)

3.2.
Lymphoid and non-lymphoid cells in nasal-associated lymphoid tissue (NALT) in the rat. An immuno- and enzyme-histochemical study

AN IMMUNOHISTOCHEMICAL STUDY ON THE POSTNATAL DEVELOPMENT OF RAT NASAL-ASSOCIATED LYMPHOID TISSUE (NALT)

Dona M.H. Hameleers, Marja van der Ende,

Jeike Biewenga, Taede Sminia

SUMMARY. This study concerns the development of nasal-associated lymphoid tissue in the rat, using immuno- and enzyme-histochemical staining techniques on cryostat sections. Nasal-associated lymphoid tissue is present at birth as a small accumulation of mainly T lymphocytes and non-lymphoid cells; B cells are rare. Distinct areas of T and B cells appear at 10 days after birth; by that time high endothelial venules are also observed. Intra-epithelial lymphocytes are present, most of them being T helper cells. ED1+ macrophages are seen throughout the tissue. The proportion of ED1+ cells does not change during ontogeny. ED2+ cells (tissue macrophages) are present predominantly at the border between the lymphoid tissue and the surrounding connective tissue, in all age-groups. ED3+ macrophages are scattered throughout the nasal-associated lymphoid tissue of young animals. Later on, the ED3+ cells migrate into the border-area between lymphoid and connective tissue. Ia+ non-lymphoid cells in the nasal lymphoid tissue increase in number during ontogeny. Only a few of them show acid phosphatase activity, indicating that the proportion of classical scavenger macrophages is low. Some of them may be antigen presenting (dendritic) cells. Ia+ dendritic cells also occur between the epithelial cells. Moreover, some epithelial cells express the Ia marker.

INTRODUCTION

The fetus greatly relies on maternal immunity. Once born, the young animal must be able to mount an immune response by itself. Its capacity to do so increases gradually, as is shown experimentally, *in vivo* and *in vitro* (Nadler et al. 1980; Ohara et al. 1985; Van Rees et al. 1987). The immunological maturation parallels an increase in the number of lymphoid cells and the development of non-lymphoid cell types; it is accompanied by a gradual compartmentalization of lymphoid tissues. In Peyer's patches (PP) in rat, T and B cells are present at birth, but separated areas of T and B cells are observed from day 12 onwards (Sminia et al. 1983). Similarly, rat bronchus-associated lymphoid tissue (BALT) is detectable four days after birth (Plesch et al. 1983); it is easily found around the second week of life (Gregson et al. 1979), although discrete areas of T and B cells appear later, viz. after 4 weeks (Plesch et al. 1983). Concentrating on the upper respiratory tract, Kelemen described, as early as 1947, massive lymphoid cell-accumulations in the mucosa of the two opposite lateral walls near the anterior orifice of the nasopharyngeal duct. Instead of elucidating the role and development of this nasal lymphoid tissue, later studies were merely confined to descriptions of epithelial cell types and conchae structures (Kelemen 1947, 1962; Liebich 1975; Monteiro-Riviere and Popp 1984; Popp and Martin 1984).

The present paper deals with the ontogenic development of the aforementioned lymphoid cell-accumulation, which is called nasal-associated lymphoid tissue (NALT; Spit et al. 1989). The lymphoid and non-lymphoid cell populations have been determined by using monoclonal antibodies. With respect to the latter, a panel of monoclonal antibodies (ED1, ED2, ED3; Dijkstra et al. 1985), specific for three macrophage subsets, has been used. In short, ED1+ cells include almost all types of macrophages, ED2+ cells include tissue macrophages and ED3+ cells form a small subset of tissue macrophages present in lymphoid organs.

MATERIALS AND METHODS

Animals

Pregnant white Wistar rats obtained from Harlan/CPB (Zeist, The Netherlands) were kept under routine laboratory conditions. Two or more animals were sacrificed at the following ages: day of birth (0 days) and 2, 4, 7, 10, 14 and 21 days after birth. The youngest animals were killed by decapitation, the others died following CO_2 inhalation. The heads of animals up to 14 days of age were quickly frozen in liquid nitrogen; they were either intact (up to 4 days of age) or in two halves, after the skin had been partly stripped off. The mandibulae were removed from heads of animals aged 7 days or more. The extent of bone calcification in animals aged 10 and 14 days

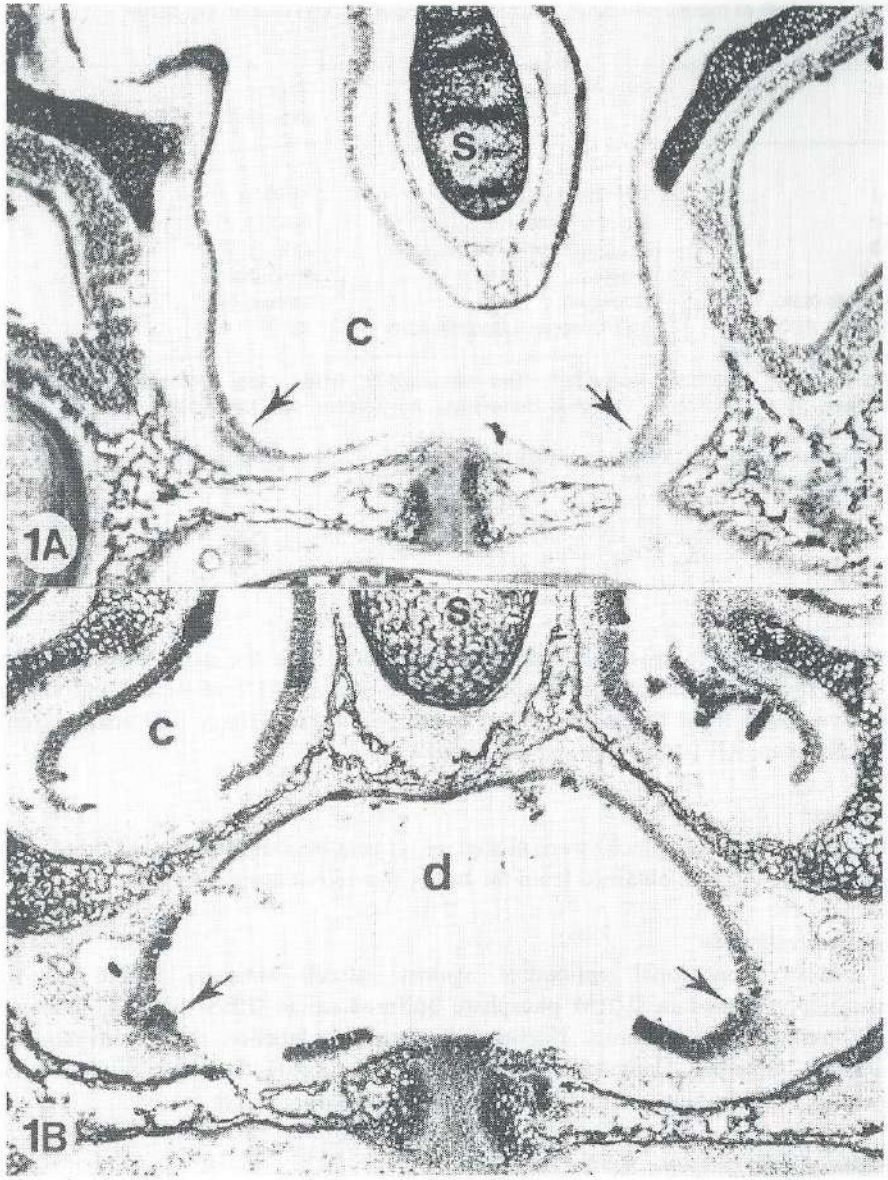


Fig. 1A,B. Frontal sections through the head of a rat aged 4 days. NALT is indicated by *arrows*. (**A**) NALT is located in the nasal floor and (**B**) extends caudally into the nasopharyngeal duct. *c* Nasal cavity; *d* nasopharyngeal duct; *s* nasal septum. Toluidine blue staining. A x30, B x50.

Table 1. Details of mouse anti-rat monoclonal antibodies (mabs) used in this study

Mab	Directed against	Source (reference)
ER-1	T lymphocytes	EUR (1)
ER-2	T helper cells	EUR (1, 2)
ER-3	T suppressor/cytotoxic cells	EUR (1, 2)
His 14	B cells	RUG (3)
OX4; Mas 029c	Ia antigen	Seralab (4)
ED1, ED2, ED3	macrophage subpopulations	VU (5)

EUR: Erasmus University, Rotterdam, The Netherlands; RUG: State University of Groningen, Groningen, The Netherlands; VU: Vrije Universiteit, Amsterdam, The Netherlands; Seralab: Seralab, Crawley Down, UK.
(1) Joling et al. 1983; (2) Joling et al. 1985; (3) Kroese et al. 1985; (4) McMaster and Williams, 1979; (5) Dijkstra et al. 1985.

made it necessary to split the head sagittally, parallel to the nasal septum, thereby reducing the proportion of bone to be sliced. To study NALT of 21-day-old animals, it was removed from the nasal cavity, embedded in liver tissue and snap-frozen in liquid nitrogen. All tissues were stored at -20°C.

Sections

Cryostat sections (8µm thick) were picked up on gelatin-coated slides and dried above silica gel. All sections obtained from the heads were frontal sections (Fig. 1).

Immune reagents

The mouse monoclonal antibodies against rat-cell antigens (Table 1) were appropriately diluted in 0.01M phosphate buffered saline (PBS) pH 7.4, containing 0.5% bovine serum albumin. Horseradish-peroxidase-labelled rabbit anti-mouse Ig (Dakopatts, Glostrup, Denmark) was used as the conjugate. This was diluted in PBS containing 0.5% bovine serum albumin and 1% normal rat serum.

Immuno- and enzyme-histochemistry

The sections were fixed in pure acetone for 10 min, then incubated horizontally with the appropriate monoclonal antibody for 1h, thoroughly rinsed in PBS, incubated with the conjugate for 30 min and washed again in PBS. The sections were stained for peroxidase activity with 3,3'-diaminobenzidine-tetrahydrochloride (DAB, Sigma, St. Louis, Mo, USA) at a concentration of 0.5 mg/ml in TRIS-HCl buffer pH 7.6, containing 0.02% H_2O_2, for 10 min. To enhance the staining of the brown reaction product, slides were rinsed in distilled water and 0.9% NaCl successively, and incubated in 0.5% $CuSO_4$ in 0.9% NaCl for 10-15 min. After being washed in distilled water, the sections were counterstained with haematoxylin (3-10 s), rinsed, dehydrated, cleared and mounted in Entellan (Merck, Darmstadt, FRG). The entire staining procedure was carried out at room temperature.

Control slides were treated similarly, omitting the first-step antibody. These slides were examined for non-specific staining. Cryostat sections of both spleen and peripheral lymph node were used as positive controls. Acid phosphatase (AcPh) activity was demonstrated according to Burstone (Pearse 1968), using naphthol-AS-BI phosphate (Sigma) as the substrate and hexazotized pararosaniline as the diazonium salt. For double staining of Ia antigens and AcPh activity, immunohistochemistry preceded enzyme-histochemistry; the colour-enhancement step was omitted. For routine histological screening, sections were stained with toluidine blue.

RESULTS

NALT

The situation at birth; day 0

NALT is visible at birth as a small condensation of lymphoid and non-lymphoid cells; they exist in a few small groups rather than in a single accumulation of cells. B cells are rare at this stage. The T cells present in NALT are mainly of the helper phenotype. Few T cells are present in the epithelium covering NALT; B cells are not found in the NALT-epithelium.

With regard to the macrophages, ED1+ cells are seen unevenly distributed throughout the small NALT. They form a pleiomorphic population with the larger cells located mainly at the periphery of NALT. ED2+ macrophages are rarely detected within NALT, although the border between NALT and the surrounding connective tissue contains several ED2+ cells. These are heavily stained and often bear blunt processes. The ED3+ cell population occurs scattered throughout NALT and comprises lightly stained irregular cells with dendritic processes.

The Ia marker is expressed on both round and branched cells throughout NALT. Ia+ cells are also observed in the NALT-epithelium; some of these cells display cell processes.

Days 2-21

During the first 3 weeks of postnatal life, a gradual but consistent development of NALT is seen. A summary of the main events is presented in Table 2.

T cells remain in abundance in NALT. The overlying epithelium contains a few T cells. Slightly more of the helper phenotype are present than of the suppressor/

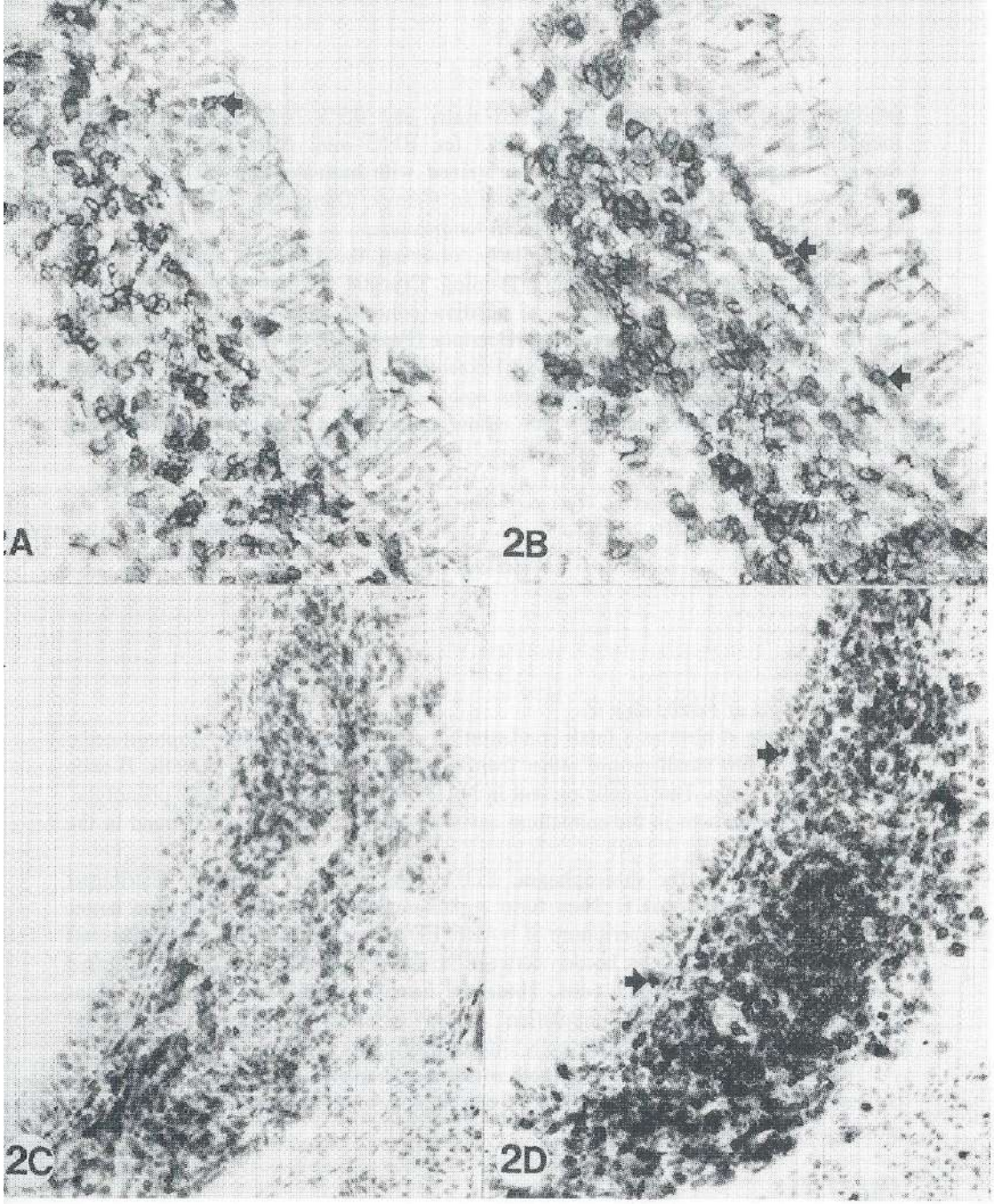


Fig. 2. Serial sections showing B cells (His 14+, **A** and **C**), and T cells (ER-1+, **B** and **D**). Areas of B and T cells on day 10 (**C** and **D**) are clearly shown; they are not distinguished at the age of 7 days (**A** and **B**). Note the presence of intra-epithelial lymphocytes (*arrows*). A and B, x 300. C and D, x160.

cytotoxic phenotype which is similar to the situation within NALT. Intra-epithelial T lymphocytes are more often detected by their subtype antigen than by their T-cell antigen. Serial sections show that distinct areas of T and B cells occur from 10 days after birth (Fig. 2). Few B lymphocytes occur in the epithelium.

With respect to the non-lymphoid cells (Fig. 3), ED1+ macrophages show the same distribution as at birth. From day 2 onwards, some ED1+ cells are seen in the epithelium, mostly in the basal layers. The number of ED2+ macrophages within NALT remains very low and none are seen in the epithelium. Few are present in the subepithelial area, although most of the ED2+ macrophages lie at the border between NALT and the surrounding connective tissue. These are more heavily stained than those within NALT. Some lightly stained ED3+ cells with long processes are observed in NALT. From the end of the first week of age, the ED3+ cells tend to become more restricted to the periphery of NALT, with only a few cells located in the subepithelial area. The peripheral ED3+ macrophages are more heavily stained than those within NALT. A single ED3+ macrophage may be observed within NALT, even on day 21. From day 10, a solitary ED3+ cell is sometimes detected in the epithelium.

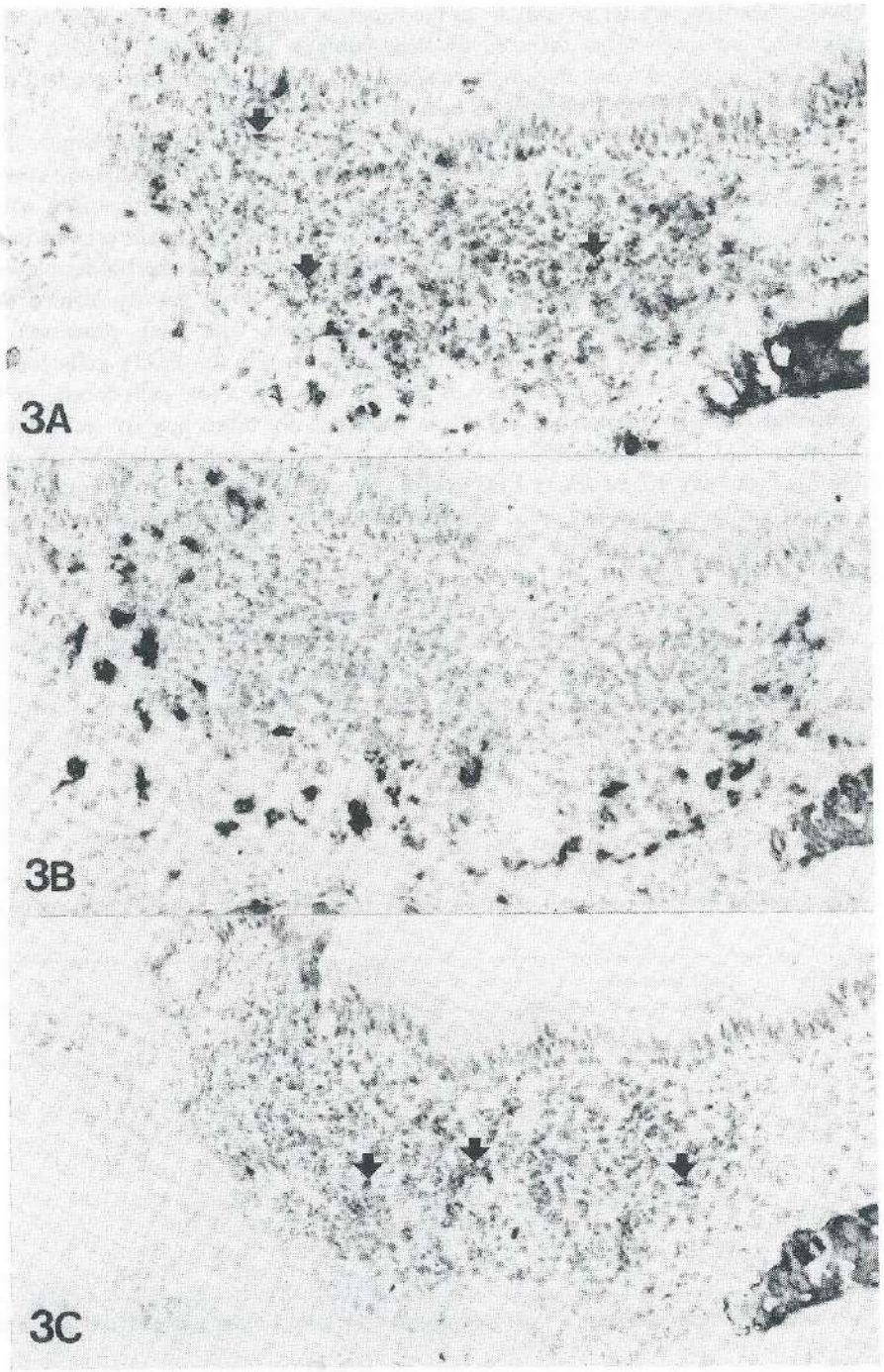
NALT contains many Ia+ cells, which form a pleiomorphic population. Some of these cells show AcPh activity, either diffuse or as a spot. The latter are mostly situated in the T-cell areas. Ia+ non-lymphoid cells are also present in the NALT-epithelium; moreover, some epithelial cells express the Ia antigen.

From 10 days after birth high endothelial venules (HEV) are clearly observed within NALT. They are usually located in the extrafollicular T-cell areas; this becomes more evident as the animal grows older. HEV-like structures are seen by the 7th day.

Table 2. Postnatal development of rat-NALT with respect to lymphoid and non-lymphoid cell types

	Days after birth						
	0	2	4	7	10	14	21
ED1	+	+	+	+	+	+	+
ED2	±	±	±	±	±	±	–
ED3	+	+	+	++	+	–	–
Ia	+	+	++	++	++	+++	+++
Tt	+	+	++	++	+++	+++	+++
Th	+	+	++	++	++	++	++
Ts	±	+	+	+	+	++	++
B	±	±	+	+	++	++	+++

– = not seen; ± = occasionally seen; + = consistently seen. An extra + marks a relative increase in cell number.



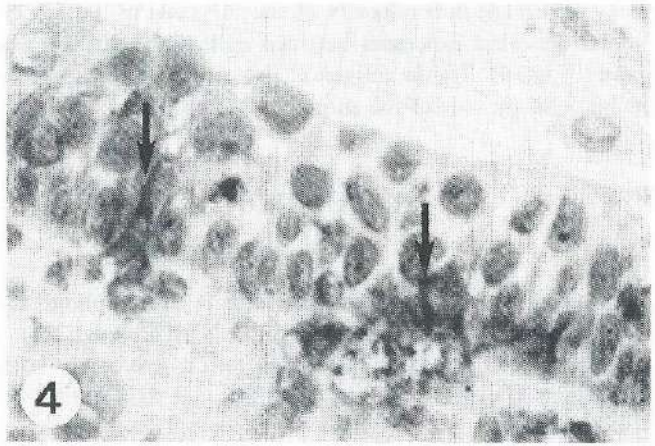


Fig. 4. Ia+ cells extending dendritic processes between the epithelial cells are observed (*arrows*) in the basal layers of the nasal epithelium. x800.

Mucosa

During the first week after birth, the nasal lamina propria shows a gradual increase in the numbers of T cells. In general, the suppressor/cytotoxic T cells slightly outnumber the helper T cells in the early stages of development, but this reverses subsequently. Few T cells are encountered in the epithelial layer; most of these are of the helper phenotype. The monoclonal antibodies ER-1 and ER-3, which react with surface-markers of all T cells and of suppressor/cytotoxic T cells, respectively, also stain the basal layers of the olfactory epithelium in all age-groups. B cells are far less abundant than T cells in the lamina propria and in the epithelium.

Regarding the macrophage-lineage, ED1+ and ED2+ cells are present in all the age-groups studied. The former are either large cells with a granular staining pattern or small cells with dendritic processes. The majority of the latter are strongly stained branched cells. One week after birth, when ED3+ cells tend to migrate out of NALT, few ED3+ macrophages are seen in the lamina propria. Only a few very weakly stained ED3+ cells are seen at earlier stages.

Fig. 3A–C. Distribution of macrophage subpopulations within NALT at the age of 10 days. (**A**) ED1+ cells (*arrows*) are scattered throughout NALT. (**B**) Heavily stained ED2+ cells are mainly situated at the border between NALT and the surrounding connective tissue. (**C**) ED3+ macrophages (*arrows*) are present throughout NALT and display long cytoplasmic processes. x200.

AcPh activity is present in a minority of the Ia+ cells in the mucosa. A few Ia+ cells, which extend dendritic processes between epithelial cells, are observed within the epithelial lining (Fig. 4). The Ia antigen is also expressed on some epithelial cells. More Ia+ epithelial cells are seen as the animal grows older.

DISCUSSION

The various components of MALT (mucosa-associated lymphoid tissue) arise at different stages in ontogeny. The present study on NALT shows that this lymphoid accumulation is present at birth. Previous studies in rats have shown that PP can be seen at birth (Sminia et al. 1983), whereas BALT is not seen until 4 days after birth (Plesch et al. 1983).

Newborn NALT is very small and is predominated by T cells, slightly more of the helper than the suppressor phenotype. These findings are in accordance with the data on PP in the rat (Sminia et al. 1983) and in man (Spencer et al. 1986). A considerable proportion of cells positive for ER-1 or ER-2 are probably macrophages, because of their macrophage-like appearance, and because T-cell markers can occur on macrophages and monocytes (Barclay 1981; Moscicki et al. 1983; Hume et al. 1987). Mainly T lymphocytes are detected in NALT of newborn rats, a finding different from that in BALT. The latter contains exclusively B cells during the first week of life. Furthermore, no areas of T and B cells are seen in BALT until the fourth week of age, whereas these areas are observed in NALT from 10 days after birth. The localization of T and B cells in distinct areas is assumed to be related to the extent of antigenic stimulation (Plesch et al. 1983). Thus, the upper respiratory tract seems to be stimulated earlier than the lower respiratory tract. From 10 days, HEV can also clearly be distinguished by morphological criteria. Lymphocytes then migrate into NALT in great numbers, by which time areas of T and B cells are present. HEV, together with areas of T and B cells, have also been observed in PP around day 10 (Sminia et al. 1983).

With respect to the non-lymphoid cells, similar data have been previously obtained in BALT (Dijkstra et al. 1985; Van der Brugge-Gamelkoorn et al. 1985a). ED1+ cells predominate over the ED2+ and ED3+ subtypes both in BALT and in NALT; ED2+ cells are restricted to the border between the lymphoid tissue and the surrounding connective tissue. The latter cells may be the 'rat-equivalents' of the MOMA-1+ macrophages in murine BALT (Breel et al. 1988). Early in postnatal life, ED2 recognizes few cells within rat NALT; most ED2+ cells are located in the connective tissue, and are considered as resident tissue macrophages (Dijkstra et al. 1985). ED3+ cells have been detected in lymphoid organs, such as spleen and lymph nodes, and have also been observed in BALT and in PP (Dijkstra et al. 1985; Beelen et al. 1987). Early during ontogeny, ED3+ cells are prevalent within the lymphoid accumulation of NALT and its direct surroundings. As the animal grows older, some

cells recognized by ED3 are also found in the lamina propria. The significance of the latter phenomenon remains unclear as long as no functional characteristics of ED3+ macrophages are known. Adult NALT shows few ED3+ cells; this macrophage subtype is found at the border between NALT and the surrounding connective tissue, where ED2+ cells are located. Our finding that ED3+ cells are predominantly seen in NALT of young animals, and in close contact with lymphocytes, suggests a role for ED3+ macrophages in the establishment of the microenvironment in NALT.

AcPh activity is found only in a minority of macrophages; this strongly suggests that the percentage of classical (scavenger) macrophages is very low. The population of Ia+ macrophage-like cells probably includes antigen-presenting cells (Wilders et al. 1983; Sertl et al. 1986). Some Ia+ cells show a spot of AcPh activity, indicative of dendritic cells and their *in vivo* equivalents, the interdigitating cells (IDC), which are found in T-cell compartments (Duijvestijn et al. 1983; Breel et al. 1987). Although T cells surround these Ia+ cells in NALT, electron-microscopic studies are required to provide unequivocal evidence that these are IDC.

The epithelial compartment is of special interest. Since NALT in adult animals is known to penetrate the overlying epithelium, it is not surprising that some lymphocytes are found in the NALT-epithelium, during early ontogeny. In the gut, the initial number of intra-epithelial lymphocytes (IEL) is low (Lyscom and Brueton 1983). IEL in gut-epithelium of both young and adult animals are mainly of the suppressor/cytotoxic phenotype (Selby et al. 1981; Lyscom and Brueton 1983; Van der Heijden 1986; Ernst et al. 1987). In this respect, NALT differs from GALT, as the helper T cells slightly outnumber the T suppressor/cytotoxic cells in NALT-epithelium. Most of the aforementioned studies on GALT refer to the small intestines only. Recently, Smart et al. (1988) have reported that, in the epithelium of human colon, CD4+ (T helper) cells slightly outnumber CD8+ (T suppressor/cytotoxic) cells; these results are similar to our findings in NALT-epithelium. The observation that IEL in NALT often lack the T-cell marker is consistent with findings in GALT (Lyscom and Brueton 1983; Ernst et al. 1987) and in BALT (Plesch 1982).

Despite the fact that Ia expression on epithelial cells is found in nude rats, which have low numbers of IEL (Mayrhofer et al. 1983), IEL are often considered to induce Ia expression (Barclay and Mason 1982; Cerf-Bensussan et al. 1984; Bland 1988). This hypothesis is supported by our findings that the number of both Ia+ epithelial cells and IEL increases as the animal grows older. Ia+ epithelial cells in rat BALT (Van der Brugge-Gamelkoorn et al. 1985b) and similar cells (or M-cells) in human GALT (Owen and Jones 1974; Mayer and Shlien 1987) are involved in antigen handling. Thus, the nasal Ia+ epithelial cells probably participate in antigen uptake and presentation. Furthermore, other cells than Ia+ macrophages and dendritic cells can stimulate lymphocytes. In some recent studies, this capacity has also been ascribed to Ia+ epithelial cells (Mayer and Shlien 1987) and to endothelial cells (Pober et al. 1983; Groenewegen and Buurman 1984.) The basal layer of the nasal epithelium contains some Ia+ cells, which extend dendritic processes between the epithelial cells. These cells may participate in antigen transport from the nasal lumen

to the draining lymph nodes. Such transport has previously been suggested for the epithelium of the trachea (Holt and Schon-Hegrad 1987). Endogenous tissue macrophages in the lung and trachea are thought to down-regulate intra-epithelial T-cell activation in order to prevent tissue damage during the aforementioned passage of antigen to the draining lymph nodes (Holt et al. 1988). Such a function may explain the occurrence of macrophages in the nasal epithelium.

NALT is situated in the floor of the nasal cavity, and extends caudally into the nasopharyngeal duct (Fig. 1). It can be considered as the equivalent of the human adenoid. The term NALT is consistent with previously introduced terms MALT, GALT and BALT. Therefore, the term NALT is used throughout this study, which has demonstrated that the development of NALT has similarities with both BALT and GALT (PP).

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LYMPHOID AND NON-LYMPHOID CELLS IN NASAL-ASSOCIATED LYMPHOID TISSUE (NALT) IN THE RAT.

An immuno- and enzyme-histochemical study

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SUMMARY. Lymphocyte and macrophage subpopulations and the stroma of mucosa-associated lymphoid tissue in the nasal cavity of the rat were examined by application of immunohistochemical and enzyme-histochemical methods to cryostat sections. Nasal-associated lymphoid tissue was composed of a loose reticular network with lymphocytes and macrophages, covered by epithelium. The epithelium was infiltrated with B cells, T helper (W3/13+) and T suppressor/cytotoxic or large granular cells (OX8+), ED1+ macrophages and Ia+ cells. The B-cell areas were populated by B cells, immunopositive for surface IgM or IgG. B cells with surface IgA or IgE were rare. Germinal centres were found infrequently. T helper cells were scattered throughout the B-cell area. A few ED1+ macrophages and ED5+ follicular dendritic cells were observed. Strong Ia staining (mostly of B cells) was found in this area. The T-cell areas contained T helper and T suppressor/cytotoxic cells in about equal numbers, and numerous ED1+ macrophages. ED1 staining was also found in the subepithelial area. Numerous ED1+, ED2+ and ED3+ macrophages were found in the border between the lymphoid mass and the surrounding connective tissue. A few non-lymphoid cells showed weak acid phosphatase or non-specific esterase activity. The morphological observations suggest that nasal-associated lymphoid tissue plays an important role in the first contact with inhaled antigens.

INTRODUCTION

Organized lymphoid tissue in the mucosa of the nasal cavity is present in rats on both sides at the entrance of the pharyngeal duct, closely associated with the respiratory epithelium (NALT; Spit et al. 1989). These lymphoid tissues are the largest and possibly the only mucosa-associated lymphoid tissue in the nasal cavity with a fixed location. The NALT comprises lymphoid cells in complementary T- and B-cell areas; the overlying epithelium is differentiated from the surrounding respiratory epithelium by the presence of specialized non-ciliated cells and slightly modified ciliated cells. The latter are cuboidal instead of cylindrical cells, with the long axis of the nuclei parallel to the basal lamina (Spit et al. 1989). NALT is part of the mucosal immune system to which also belong the mucosal lymphoid tissue in the lower respiratory tract (BALT) and the Peyer's patches in the gut (GALT). The morphology of BALT and GALT, their lymphoid and non-lymphoid cells have been described extensively (Gregson et al. 1979a,b; Tenner-Racz et al. 1979; Bienenstock et al. 1982; Sminia and Plesch 1982; Sminia et al. 1983; Bienenstock and Befus 1984; Van der Brugge-Gamelkoorn et al. 1985a,b, 1986). Functional studies showed that BALT and GALT play a critical role in the initiation of a mucosal immune response, especially to particulate and macromolecular antigens (Asherson et al. 1977; Bienenstock and Befus 1984; Van der Brugge-Gamelkoorn et al. 1985c). Detailed information on the morphology of the cell populations within NALT, and their functions is not available.

The purpose of the present study was to investigate in the rat the lymphoid and non-lymphoid elements and the stroma of NALT by immunohistochemical and enzyme-histochemical methods at the light-microscopical level.

MATERIALS AND METHODS

Animals

Ten young adult male albino Wistar rats (CPB:WU, Wistar random; 15 weeks old; Central Institute for the Breeding of Laboratory Animals, TNO, The Netherlands) were used. They were kept under routine laboratory conditions.

Tissue sampling and preparation

Animals were killed by decapitation. After removal of the lower jaw, the nose was lengthwise split into equal halves. From both halves NALT was collected, snap-frozen in isopentane at -159°C and stored at -80°C.

Immunohistochemistry

Cryostat sections (7 µm thick) were mounted on glass slides, air-dried and fixed for 10 min in pure acetone.

Table 1. Details of mouse anti-rat monoclonal antibodies used in the study

Monoclonal antibody	Directed against	Source; reference
W3/13	All T cells	Seralab (Crawley Down, UK); Williams et al. 1977
W3/25	T helper cells	Seralab; Williams et al. 1977
OX8	T suppressor/cytotoxic cells	Seralab; Williams et al. 1977
IgG clone OX12 F(ab)2	IgG+ B cells	Seralab; Hunt and Fowler 1981
IgM clone MARM-4	IgM+ B cells	Seralab; Bazin et al. 1974
IgA clone MARA-1	IgA+ B cells	Seralab; Bazin et al. 1974
IgE clone MARE-1	IgE+ B cells	Seralab; Bazin et al. 1974
Mas 029c	Ia antigen	Seralab; McMaster and Williams 1979
ED1	Monocytes, macrophages, dendritic cells	Vrije Universiteit (VU), Amsterdam, The Netherlands; Dijkstra et al. 1985
ED2	Tissue macrophages	VU; Dijkstra et al. 1985
ED3	Macrophage subpopulations in lymphoid organs	VU; Dijkstra et al. 1985
ED5	Follicular dendritic cells	VU; Jeurissen and Dijkstra 1986

The fixed cryostat sections were rinsed in phosphate buffered saline (PBS, 0.01 M, pH 7.4) and preincubated with 10% normal rabbit serum for 10 min. Excess serum was removed by rinsing in PBS and consecutive sections were incubated for 60 min with one of the monoclonal antibodies listed in Table 1. The sections were then rinsed in PBS, layered for 30 min with a peroxidase-conjugated rabbit anti-mouse Ig (RAM-PO, Dakopatts, Glostrup, Denmark), diluted in PBS with 4% normal rat serum, rinsed in PBS and TRIS/HCl (0.05 M, pH 7.6) and finally incubated with the chromogen diaminobenzidine-tetrahydrochloride (Sigma, St Louis, Mo, USA) of a concentration of 0.5 mg/ml in TRIS/HCl, pH 7.6 containing 0.01% H_2O_2 for 10 min. The entire staining procedure was carried out at room temperature. Most sections were counterstained with haematoxylin. Control slides were incubated with the conjugated Ig (RAM-PO), PBS or the chromogen only. Peyer's patches, spleen and lymph nodes served as positive controls.

In addition, sections were incubated for 60 min with polyclonal rabbit antiserum against laminin (E.Y Labs, Inc.; Sanbio, Uden, The Netherlands), then for 30 min with swine anti-rabbit antiserum (Dakopatts), followed by rabbit peroxidase-antiperoxidase (Dakopatts). The complex was visualized with the chromogen diaminobenzidine-tetrahydrochloride. Between each incubation step, the sections were rinsed in PBS. The sections were counterstained with haematoxylin.

Enzyme-histochemistry

Acid phosphatase activity was demonstrated according to Burstone (Pearse 1968) with naphthol AS-BI phosphate as the substrate at a concentration of 0.5 mg/ml. Incubation time was 30-60 min at 37°C. The substrate for the demonstration of non-specific esterase was α-naphthyl acetate (Pearse 1972) at a concentration of 0.23 mg/ml. The incubation time was 10-20 min at room temperature. The substrates were purchased from Sigma. For both reactions hexazotized pararosaniline was used as the diazonium salt. The sections were counterstained with haematoxylin for 3-10 sec. Immunohistochemistry preceded enzyme-histochemistry when used in combination on the same section.

Reticulin staining

Frozen sections of NALT were stained for reticulin according to Gomori (Luna 1968).

Quantitative histology

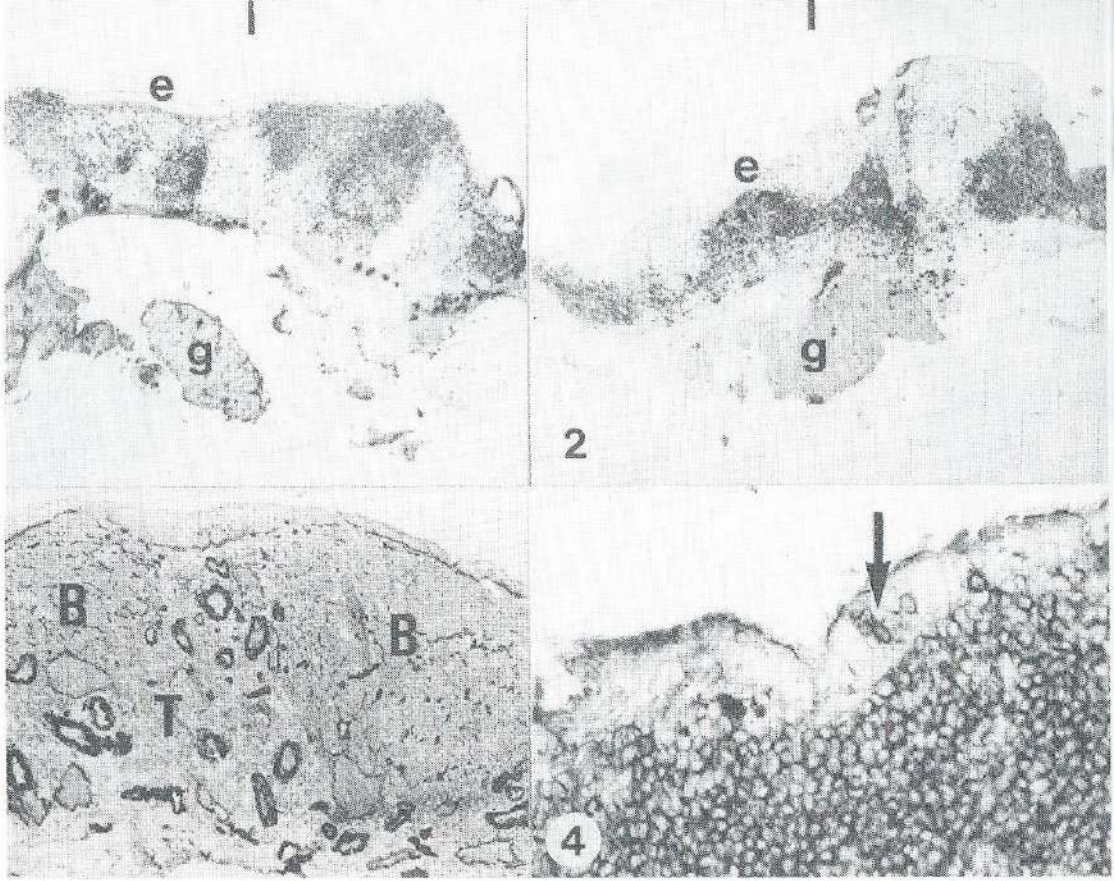
Surfaces of T- and B-cell areas were measured on 2 sets of serial cryostat sections per NALT. Eight NALT tissues were used from 4 rats. In this way, 16 sections per monoclonal were measured. Measurements were performed with the aid of a computerized graphical tablet (MiniMOP, Kontron Messgerät GmbH, Munich, FRG). The results were given as the ratio of T- and B-cell areas (B/T).

Lymphoid cells and ED1+ cells were counted in cryostat sections at 2 sites in both T- and B-cell areas with an ocular square grid (the grid represented 0.03 square mm at 600x magnification). Two sets of serial sections per NALT and 4 NALT tissues from different rats were used. In this way, 8 sections per monoclonal were used. Data were expressed as average cell numbers per square mm.

RESULTS

General features

NALT was composed of a loose reticular network, in which lymphocytes and macrophages were embedded, covered by epithelium. The lymphoid mass was organized into T- and B-cell areas, which appeared not to have a fixed orientation within NALT (Figs. 1 and 2). T- and B-cell areas were approximately of the same size (B/T area mean ± SEM = 0.9 ± 0.05). Some samples of NALT contained a lymphocyte-poor subepithelial area, interrupted by accumulations of B lymphocytes penetrating into the epithelium. The basal lamina of the epithelium was interrupted where lymphocytes infiltrated it. Many lymph and blood vessels were encountered (Fig. 3). Most lymph vessels were found at the base of the B-cell areas and in the T-cell areas. High endothelial venules (HEV) were observed in the T-cell areas, some of them adjacent to the B-cell areas. Small capillaries traversed the B-cell area. A single nerve bundle was found at the border between the lymphoid mass and the submucosa



Figs. 1,2. Consecutive cryostat sections of rat nasal-associated lymphoid tissue. *g* gland; *l* nasal luminal side; *e* epithelium. **Fig. 1.** Anti-B cell staining (IgM clone MARM-4). x40. **Fig. 2.** Anti-T cell staining (W3/13). x40.

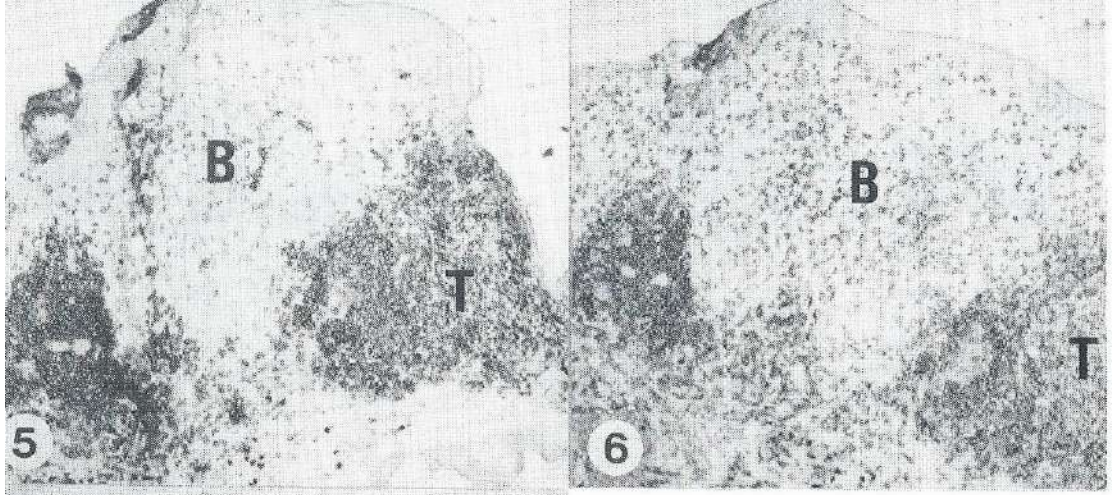
Fig. 3. Cryostat section of rat nasal-associated lymphoid tissue. Staining for laminin. Numerous blood and lymph vessels are present in the T-cell areas (*T*) and at the base of the B-cell areas (*B*). Haematoxylin counterstaining. x160.

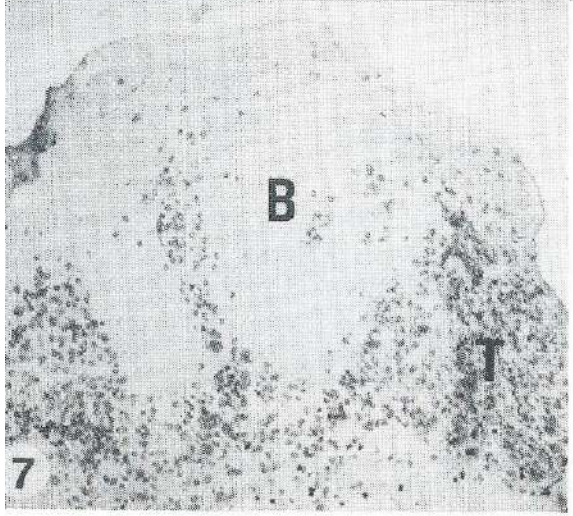
Fig. 4. Intra-epithelial lymphocytes (*arrow*); (IgM clone MARM-4). x400.

near the T-cell area.

Lymphoid cells

The epithelium of NALT was infiltrated by lymphocytes. The majority of these lymphocytes were B cells with IgM or IgG on their surface (Fig. 4); a few were T helper cells (W3/25+) or cells positive for OX8 (suppressor/cytotoxic/large granular cells). Both B and T lymphocytes were found in the lymphocyte-poor subepithelial area. Most B cells in NALT were positive for surface IgM or IgG. Less than 1% of





Figs. 5–7. Consecutive cryostat sections of rat nasal-associated lymphoid tissue. *T* T-cell compartment; *B* B-cell compartment. **Fig. 5.** Anti-T cell staining (W3/13). x160. **Fig. 6.** Anti-T helper cell staining (W3/25). x160. **Fig. 7.** Anti-T suppressor/cytotoxic cell staining (OX8). x160.

the B cells was positive for IgA or IgE. Germinal centres were infrequently found in NALT. T cells were seen scattered in the B-cell areas (Table 2; Fig. 5). They were mainly of the T helper type (W3/25; Table 2; Figs. 6 and 7). The T-cell areas contained T helper and T suppressor/cytotoxic cells; T helper cells outnumbered T suppressor/cytotoxic cells, although the ratio varied within NALT (Table 2). These subpopulations did not show distinct sites of preference. Around the high endothelial venules, B cells as well as T helper and T suppressor/cytotoxic cells were found.

Plasma cells were rare. Single plasma cells were found within NALT and in the surrounding mucosa. The plasma cells contained IgG, IgM or IgA but IgE was

Table 2. Numerical density of lymphoid and macrophage cells (cells/mm^2; mean ± SEM) in B cell- and T cell-rich areas of nasal-associated lymphoid tissue (NALT) in rats

Cell type	B-cell areas	T-cell areas
B cells (MARM-4)	8764 ± 723	1494 ± 62
T cells (W3/13)	983 ± 74	8148 ± 758
T helper cells (W3/25)	822 ± 61	6041 ± 340
T suppressor cells (OX8)	162 ± 45	2742 ± 322
Macrophages (ED1)	40 ± 13	389 ± 45

For each monoclonal, counts were done on 2 sections per NALT from 4 NALT tissues of different rats. The antibody used to define the cell type is given in parentheses.

extremely scarce.

Non-lymphoid cells

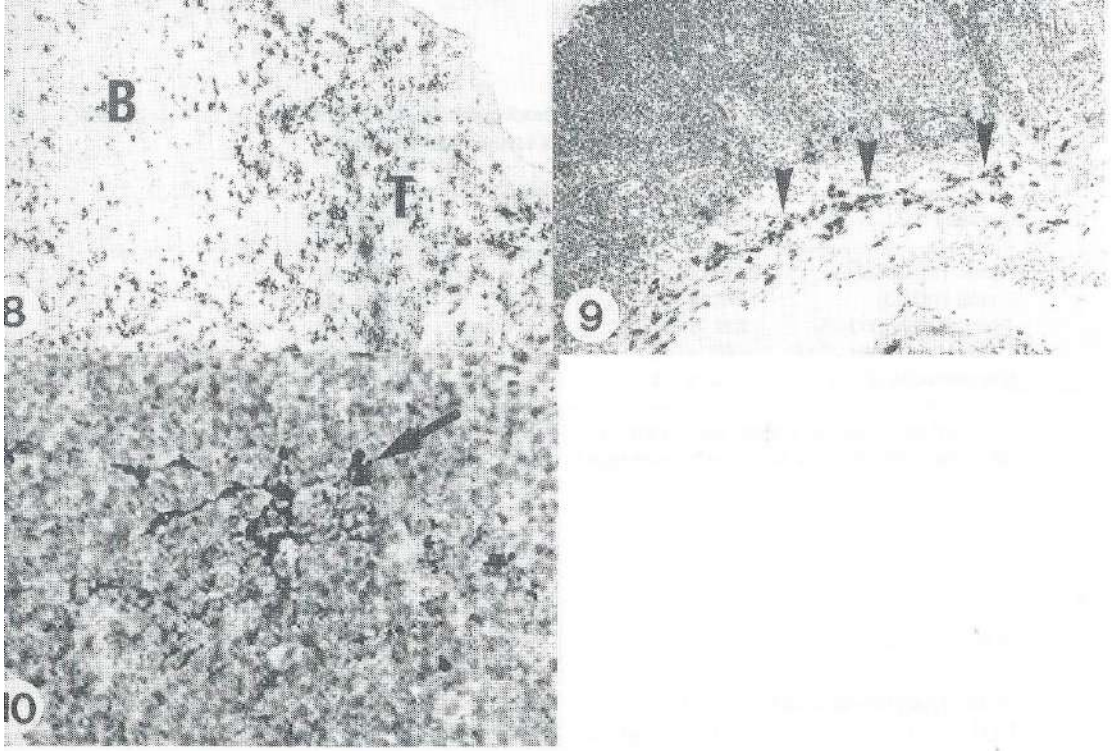
ED1+ cells were observed throughout NALT (Fig. 8). They were found in low numbers between the epithelial cells, in the subepithelial area, and in the B-cell areas. Most of the ED1+ cells were observed in the T-cell areas. In addition, a few were present at the border between NALT and the surrounding mucosa. The ED1+ population was morphologically heterogeneous, including round cells as well as dendritic-like cells.

ED2+ and ED3+ cells were found almost exclusively at the border between NALT and the surrounding mucosa (Fig. 9), where also ED1+ cells were found. They were located in close proximity to mast cells and granulocytes. In addition, a few ED2+ cells were seen in the subepithelial area. ED2+ cells were strongly-marked cells with a dendritic appearance. Only very few ED3+ cells were present.

The majority of ED1+, ED2+ and ED3+ cells was negative for acid phosphatase. Some large acid phosphatase positive cells, which were negative for non-specific esterase, were present in the B-cell areas. Non-specific esterase activity was found almost exclusively in the epithelium and in a few, unspecified, cells directly beneath it.

ED5 showed a web-like (reticular) staining pattern in the B-cell follicles (Fig. 10).

Ia antigen was present throughout NALT, especially in the B-cell areas and in the epithelium above these areas. Most Ia+ cells were round and were considered B cells.



Figs. 8–10. Cryostat sections of rat nasal-associated lymphoid tissue. **Fig. 8.** ED1 staining is found especially in the T-cell compartment. *T* T-cell compartment; *B* B-cell compartment. x160. **Fig. 9.** ED2 staining (*arrowheads*) is restricted to the area adjacent to the lymphoid mass at the non-epithelial side. Positive cells have a dendritic appearance. Haematoxylin counterstaining. x160. **Fig. 10.** ED5 staining (*arrow*) is found in the B-cell areas. Haematoxylin counterstaining. x400.

DISCUSSION

The present study was undertaken to obtain information on lymphoid and non-lymphoid subsets in NALT which is located at both sides of the entrance of the nasopharyngeal duct in rats. General aspects and electron-microscopic features of the epithelial cells of NALT in rat were described by Spit et al. (1989). NALT has also been found in the ventral, dorsal and septal walls of the nasopharynx in Bonnet monkeys (Harkema et al. 1987). NALT may be an equivalent of the Waldeyer's ring in man.

The epithelium that overlies the lymphoid mass was infiltrated by B cells, a few T helper and T suppressor/cytotoxic or large granular cells and macrophages. A lymphocyte-poor subepithelial compartment, as present in BALT (Plesch 1982), was only occasionally observed in NALT. This may be indicative of a difference in the extent of antigenic contact between BALT and NALT in unstimulated rats, since the subepithelial area of BALT in antigen-treated rats contained many lymphocytes (Van der Brugge-Gamelkoorn et al. 1985c) and thus resembled the subepithelial area usually seen in NALT. This hypothesis is supported by the finding that many lymphocytes had infiltrated the epithelium of NALT (this study) as is also found in Peyer's patches epithelium (Sminia and Plesch 1982). However, the epithelium of BALT in the rat contained significant numbers of lymphocytes only upon intratracheal antigen administration (Van der Brugge-Gamelkoorn et al. 1985c, 1986).

NALT contained distinct and complementary T- and B-cell areas, which appeared not to have a fixed location. In contrast to Peyer's patches, where between the large B-cell areas relatively small T-cell areas are present, NALT contained about equally-sized T- and B-cell areas and resembled BALT in this respect (Van der Brugge-Gamelkoorn and Sminia 1985). NALT housed few cells with surface IgA, in contrast to both BALT and Peyer's patches, where far more B cells carry surface IgA (Plesch 1982; Butcher et al. 1982). T helper cells outnumbered T suppressor/cytotoxic cells in the T- and B-cell areas. However, the number of T helper cells may be overestimated since their marker, W3/13, is also found on macrophages (Barclay 1981). These observations suggest that the mucosa-associated lymphoid tissues in the respiratory tract have other or less functions than Peyer's patches in the mucosal immune system.

Non-lymphoid populations were examined by using the monoclonal antibodies ED1, ED2, ED3 (macrophages) and ED5 (follicular dendritic cells). Most ED1+ cells were found in the T-cell areas, which is consistent with the findings in Peyer's patches (Dijkstra et al. 1985). In addition, some were found dispersed in the B-cell areas. In BALT, ED1+ cells were observed throughout the organ (Van der Brugge-Gamelkoorn et al. 1985a). ED2+ cells were located mainly in the periphery of NALT, which is further evidence of the conclusion that ED2 is a marker of tissue macrophages (Dijkstra et al. 1985). In NALT only very few ED3+ cells were found, particularly at the base of the organ. This is consistent with previous observations in Peyer's patches, where small aggregates of ED3+ cells were observed at the base of the interfollicular areas, and in BALT where no positive cells were seen (Dijkstra et al. 1985; Van der Brugge-Gamelkoorn et al. 1985c). ED3 staining is observed predominantly on macrophages in spleen and lymph nodes (Beelen et al. 1987). The pattern of ED3 staining in NALT is possibly age-related. In young rats (age 0-7 days), ED3 staining was observed throughout the NALT (Hameleers et al. 1989), whereas in 4-month-old rats few ED3+ cells were found at the base of the organ (this study). Information on age-related changes in BALT is lacking. In addition to the above mentioned observations on T and B cells, the different distribution patterns with ED1, ED2 and ED3 in BALT, GALT and NALT are further evidence of functional differences between the mucosal lymphoid tissues. The differences in enzyme contents of the macrophages in NALT and BALT also support this hypothesis. Functional differences

between the popliteal and mesenteric lymph nodes have also been suggested because of different cell distribution (Van Rees et al. 1985).

ED5+ cells were seen in NALT but not in BALT. These cells have been described to be follicular dendritic cells (Jeurissen and Dijkstra 1986). This suggests that the B-cell areas in NALT are more developed than those in BALT, which again supports the hypothesis that airborne antigens activate the upper rather than the lower respiratory tract.

Tingible body macrophages with both non-specific esterase and acid phosphatase activity, as described in the spleen (Eikelenboom 1978), were not found in NALT. It is questionable, however, whether non-specific esterase is a prerequisite for these cells since Van der Brugge-Gamelkoorn and coworkers (1985a) found no or only weak non-specific esterase activity in tingible body macrophages in BALT. The observed differences between NALT and BALT on the one hand and Peyer's patches, spleen and lymph nodes on the other, are indicative of a difference in the activity of tingible body macrophages. The latter organs always have well-developed, tingible body macrophage-containing B-cell areas, in contrast to BALT and NALT.

Ample Ia expression was found throughout the NALT. This, in association with only low lysosomal enzyme activities, is suggestive of a role of NALT macrophages in immune initiation and immune regulation. Ia+ macrophages can have antigen-presenting properties (Sertl et al. 1986). In NALT, ED1+ cells probably play such a role, since they were located in significant numbers in the T-cell areas and in the epithelium, where also T helper cells were present. The ED1+ cells might be involved in the primary T-dependent response, since the epithelium also contained surface IgM+ B cells and since these cells occurred in large numbers in the B-cell areas.

The results presented here are ample evidence that NALT is part of the mucosal lymphoid system. The complementary T- and B-cell sites appear to be specific and predestined compartments. The morphological characteristics of NALT are indicative of the following conclusions. In the respiratory tract NALT is the first site of contact for most airborne antigens, since NALT and Peyer's patches, but not BALT, appear activated in healthy, untreated, conventionally housed rats. Because of the presence of several lymphocyte and macrophage subpopulations in the lymphoepithelium of NALT, this epithelium appears to be a site of complex immunological interactions. Furthermore, within a common mucosal immune system NALT appears to have specific functions, as reflected by its cell distribution pattern.

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Chapter 4

EFFECTS OF INTRANASAL IMMUNIZATIONS IN THE RAT

4.1.
Anti-TNP formnig cells in rats after different routes of priming with TNP-LPS followed by intranasal boosting with the same antigen

4.2.
Specific antibody forming cells in the rat after intranasal administration of three different antigens

ANTI-TNP FORMING CELLS IN RATS AFTER DIFFERENT ROUTES OF PRIMING WITH TNP-LPS FOLLOWED BY INTRANASAL BOOSTING WITH THE SAME ANTIGEN

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Taede Sminia, Jeike Biewenga

SUMMARY. To study the reactivity of nasal-associated lymphoid tissue (NALT) and its position in the mucosal immune system, rats were intranasally challenged with 200 µg TNP-LPS. Priming had occurred fifteen days previous to the challenge with the same antigen and dose, either intranasally, intratracheally, subcutaneously in the cheek or intraperitoneally. The number of specific antibody forming cells (AFC) was determined in various tissues using the conjugate TNP-AlPh (AlPh: alkaline phosphatase). Anti-TNP AFC were predominantly found in the posterior cervical lymph nodes, while NALT hardly contained any such AFC. The highest response was induced by subcutaneous priming and intranasal boosting, which evoked a peak response on day 5 in the posterior cervical lymph nodes and spleen. Intraperitoneal priming prior to intranasal boosting resulted in a lower number of AFC in the posterior cervical lymph nodes and spleen, which remained during the period studied. A considerable response was seen in the lung on day 7. Following intratracheal-intranasal immunization a higher response was measured in the posterior cervical lymph nodes on days 3 and 5 than on day 7. Intranasal priming and boosting hardly evoked any specific AFC. Irrespective of the immunization route IgA was the least produced isotype in the spleen, as compared to antigen-specific IgG and IgM. In the posterior cervical lymph node, besides specific IgG and IgM, a considerable proportion of specific IgA was produced. After all four immunization routes anti-TNP antibodies occurred in serum. As for the non-lymphoid cells, the intratracheal-intranasal immunization protocol induced an increase in pulmonary macrophages on days 3 and 5. The immunological role of lung-macrophages is discussed.

INTRODUCTION

Mucosal tissues comprise distinct lymphoid accumulations designated as mucosa-associated lymphoid tissue (MALT). Well-known are the gut-associated lymphoid tissue (GALT) and the bronchus-associated lymphoid tissue (BALT; for a review, see Sminia et al. 1989). In considering a common mucosal immune system as proposed by Bienenstock (1974), several studies have been undertaken to elucidate the role of the various components of MALT and their interrelations. Thus, it was shown that memory cells arising from the gut, migrated to the remote bronchial mucosa (Sminia et al. 1987; Weisz-Carrington et al. 1987) and apparently also to the salivary glands (Dahlgren 1987). Furthermore, subcutaneous priming could induce a mucosal response as shown for BALT after intratracheal boosting (Van der Brugge-Gamelkoorn et al. 1985a) and for saliva after gastric administration of antigen (Cox and Taubman 1984). In man oral vaccination with killed influenza vaccine resulted in specific IgA antibodies in nasal and lacrimal secretions (Bergmann et al. 1987).

Recently, Spit and coworkers (1989) described mucosa-associated lymphoid tissue in the floor of the nasal cavity of rats, denoted NALT. The exact role of NALT and the nasal mucosa in the mucosal immune system as a whole is not yet clear. Therefore, the upper respiratory tract and its draining lymph nodes are subject of the present study in which rats were immunized with the bacterial derived antigen LPS, conjugated to 2,4,6-trinitrophenyl (TNP). TNP-carrier conjugates provide a valuable model to study specific antibody forming cells *in vivo* (Claassen and Van Rooijen 1984).

MATERIALS AND METHODS

Animals

Young adult male Wistar rats (210-240 g) obtained from Harlan Sprague Dawley (Zeist, The Netherlands) were used. The animals were kept under routine laboratory conditions.

Antigen

The thymus-independent antigen trinitrophenylated lipopolysaccharide (TNP-LPS) was kindly donated by dr. Theo Thepen (Vrije Universiteit, Amsterdam, The Netherlands). The LPS-moiety was prepared from *Escherichia coli 055:B5.* Animals were immunized with 200 µg TNP-LPS, dissolved for intranasal administration in 40 µl 0.01M phosphate buffered saline pH 7.4 (PBS) and for intratracheal, subcutaneous and intraperitoneal administration in 200 µl PBS. During the entire procedure TNP-LPS was kept in the dark to avoid photolysis.

Experimental design

One group of 9 animals was primed subcutaneously in the cheek under anaesthesia with Hypnorm: 0.1 ml intramuscularly. The antigen was equally spread over both cheeks. A second group of 9 animals was primed intraperitoneally and a third group (9 rats) received the antigen intratracheally. The latter rats were anaesthetized with 0.2 ml Hypnorm intramuscularly and 0.5 ml 10 % pentobarbitone sodium (Nembutal) intraperitoneally. The antigen was administered according to Van der Brugge-Gamelkoorn and coworkers (1985b), via a nylon tube (Flex nylon tubing, size I, Hythe, Kent, UK) through the larynx into the trachea while the rats were fixed in an upright position. Intratracheal administration of trypan blue showed that an equal distribution over both lungs was achieved by this procedure. In addition, a fourth group of 3 animals received the antigen intranasally by using an Eppendorf pipette, whereby the antigen was equally spread over both nostrils. After 15 days all animals were boosted intranasally. Control animals were 'primed' and 'boosted' with PBS instead of antigen.

Animals were sacrificed by means of an intraperitoneal injection of 0.5-1 ml Nembutal followed by heartpuncture. Animals in groups 1, 2 and 3 were sacrificed on day 0, 3, 5 or 7. The animals in group 4 were sacrificed on day 5. In a separate experiment on PVG rats the response on days 3, 5 and 7 in intranasally primed and boosted animals was determined. PVG rats would provide a model for susceptibility to upper respiratory tract infections. However, hardly any response occurred. Therefore we preferred the use of Wistar rats because of the established expertise in our laboratory on normal morphology of Wistar rats and because they allow determination of anti-TNP antibodies in serum. PVG rats show an extremely high intrinsic anti-TNP titer. The results reported in this study apply to Wistar rats. The following tissues were frozen in liquid nitrogen: spleen, lungs, mesenteric lymph nodes (MLN), superficial cervical lymph nodes (SCLN), facial lymph nodes (FLN), posterior cervical lymph nodes (PCLN), nasal-associated lymphoid tissue (NALT). The nomenclature of lymph nodes is according to Tilney (1971). Tissues were stored at -70°C until used. Frozen sections were cut at 8 µm, mounted on gelatin-coated slides, and used for immunohistochemical stainings or for the detection of specific antibody forming cells.

Immune reagents

The unlabelled monoclonal and polyclonal antibodies used (Table 1) were appropriately diluted in PBS, containing 0.5% bovine serum albumin (BSA). The horseradish-peroxidase labelled conjugates (Table 1), were appropriately diluted in PBS containing 0.5% BSA and 1% normal rat serum.

Staining techniques

The sections were dried above silica gel, fixed in pure acetone for 10 min, dried, rehydrated in PBS, and incubated with the appropriate antibodies for 1 h, rinsed in PBS, and incubated with the corresponding conjugate at 4°C overnight. Thereafter

Table 1. Antibodies used in this study

A. MONOCLONAL ANTIBODIES (moab, anti-rat)		
moab	directed against	source
ED1	monocytes, macrophages, dendritic cells	VU (Dijkstra et al. 1985)
ED3	macrophage subpopulations in lymphoid organs	VU (Dijkstra et al. 1985)
OX4	Ia antigen	Seralab
MARA-2	IgA	Serotec
B. POLYCLONAL ANTIBODIES (poab)		
poab		source
rabbit anti-rat IgG		ICN
goat anti-rat IgM		Nordic
rabbit anti-mouse Ig/HRP labelled		Dakopatts
swine anti-rabbit Ig/HRP labelled		Dakopatts
rabbit anti-goat Ig/HRP labelled		Dakopatts

HRP: horseradish peroxidase; Dakopatts: Dakopatts, Glostrup, Denmark; ICN: ICN Biomedicals, Lisle, IL, USA; Nordic: Nordic Immunological Laboratories BV, Tilburg, The Netherlands; Seralab: Seralab, Crawley Down, UK; VU: Vrije Universiteit, Amsterdam, The Netherlands.

slides were rinsed in PBS and stained for peroxidase activity with 3,3'-diaminobenzidine-tetrahydrochloride (DAB, Sigma, St. Louis, Mo, USA) at a concentration of 0.5 mg/ml in TRIS-HCl buffer pH 7.6, containing 0.025% H_2O_2, for 10 min. The staining of the brown reaction product was enhanced by rinsing the slides in PBS and 0.9% NaCl successively, followed by incubation in 0.5% $CuSO_4$ in 0.9% NaCl for 15 min. After being rinsed in distilled water, the sections were lightly counterstained with haematoxylin, rinsed in tap water, dehydrated, cleared and mounted in Entellan neu (Merck, Darmstadt, FRG). Except for the conjugate incubation the staining procedure was carried out at room temperature. Control slides were treated similarly while omitting the first step antibody.

Acid phosphatase (AcPh) activity was demonstrated according to Burstone (Pearse 1968) using naphthol AS-BI phosphate (Sigma) as the substrate and hexazotized pararosaniline as the diazonium salt. For double staining of Ia antigens and AcPh activity, immunohistochemistry preceded enzyme-histochemistry and the colour-enhancement step was omitted.

Anti-TNP antibody forming cells were detected as described by Claassen and Van Rooijen (1984), using the conjugate TNP-AlPh (TNP coupled to alkaline phosphatase). Sections were lightly counterstained with nuclear fast red (Merck). Double immunohistochemical stainings were performed according to Van Rooijen and Kors (1985). In short, sections were incubated overnight with a mixture of TNP-AlPh and an isotype-specific antibody (Table 1) followed by staining for AlPh-activity. The sections were then incubated for 1 h with the appropriate conjugate containing 1% normal rat serum and finally stained for peroxidase activity with 3-amino-9-ethylcarbazole (AEC, Sigma). Slides were mounted in glycerin-gelatin. Cell-counts were performed independently by two of the authors in a blind fashion.

Routine histological screenings were performed on sections stained with either methylgreen-pyronin or haematoxylin and eosin.

Detection of serum antibodies to TNP

Anti-TNP antibodies in serum were detected by a direct enzyme-linked immunosorbent assay (ELISA) as described previously for murine serum (Delemarre et al. 1989). In short, microtiter plates (Greiner, Alphen aan den Rijn, The Netherlands) were coated with 1 µg/well TNP-ovalbumin. The plates were incubated with the serum samples in twofold serial dilutions, whereafter horseradish peroxidase conjugated rabbit anti-rat Ig (Dakopatts, Glostrup, Denmark) was added. Plates were stained with orthophenylene-diamine-di-hydrochloride (OPD, Sigma). Finally the absorption was determined at 492 nm. Titers were defined as the reciprocal dilution at an optical density of 1.00. All serum samples were tested in duplicate.

RESULTS

General observations

For each immunization route and time point the mean number of antibody forming cells (AFC) was calculated from 3 rats. Although variations within the groups were seen, similar overall patterns were encountered when comparing the various tissues.

Specific AFC were mainly found in the lymph nodes draining the upper respiratory tract, viz. the posterior cervical lymph node (PCLN; Fig. 1, Table 2). The majority of AFC were located in the medulla (Fig. 2). Where specific AFC were found in NALT they were mostly observed adjacent to lymphoid tissue, thus underneath the lymphoid mass and in the relatively cell-poor area under the epithelium (Fig. 3). Similarly, more specific AFC were seen in lung tissue than in the lymphoid mass of BALT. The majority of AFC in the lung were found perivascularly

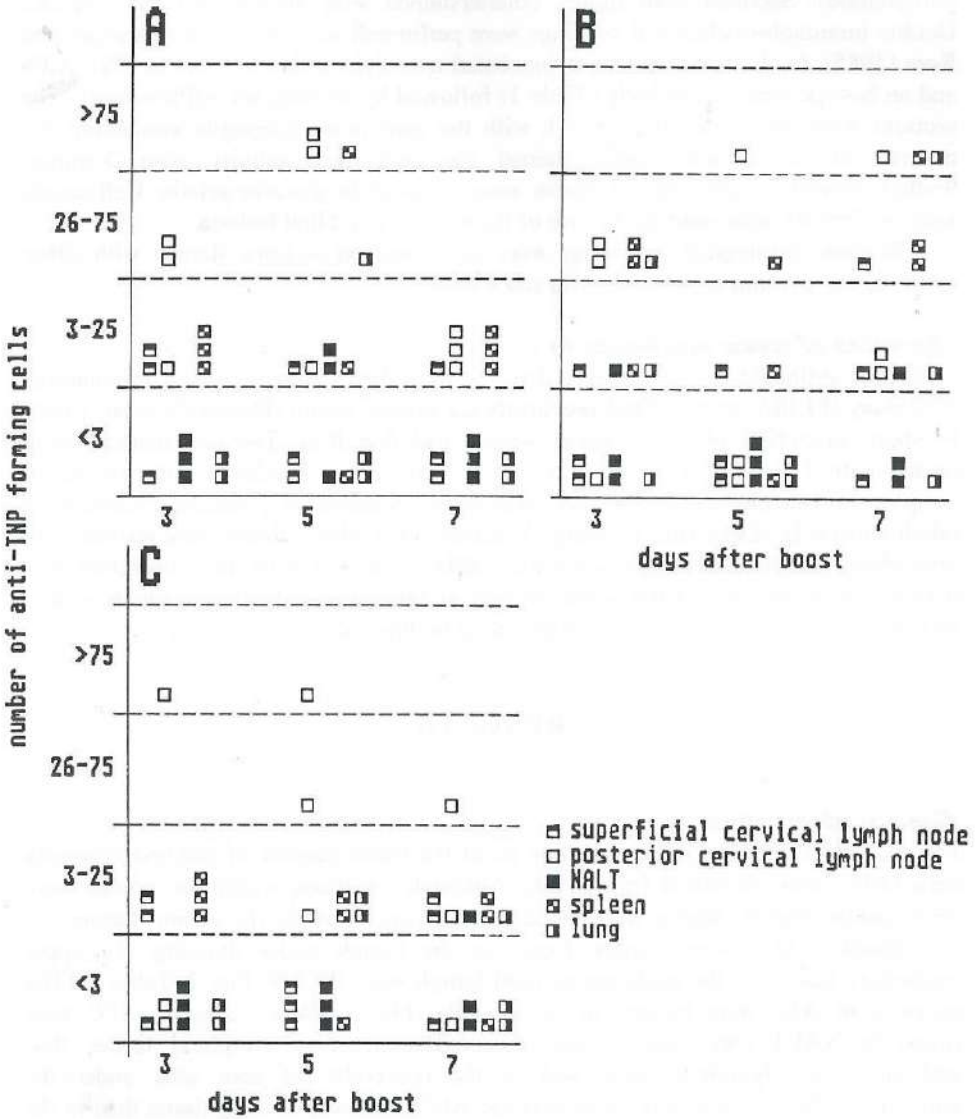

Fig. 1. Individual anti-TNP response in various organs after different priming routes and intranasal boosting with 200 µg TNP-LPS. **(A)** Subcutaneous priming followed by intranasal boosting. **(B)** Intraperitoneal priming followed by intranasal boosting. **(C)** Intratracheal priming followed by intranasal boosting.

Table 2. Specific immune response in various organs after different priming routes and intranasal boosting

	subcutaneous–intranasal			intraperitoneal–intranasal			intratracheal–intranasal		
	day			day			day		
	3	5	7	3	5	7	3	5	7
tissue									
SCLN	±	±	±	–	–	±	±	–	±
FLN	–	–	–	–	±	–	–	–	–
PCLN	+	++	±	+	+	+	+	+	+
NALT	–	±	–	±	–	±	–	–	–
lung	–	±	–	±	–	+	–	±	±
MLN	n.d.	–	–	–	–	–	–	–	–
spleen	±	+	±	+	±	+	±	–	±

At 3, 5 and 7 days after intranasal challenge with TNP-LPS, the number of TNP-specific antibody forming cells (AFC) was determined semi-quantitatively. Each group consisted of 3 animals, which had been primed 15 days earlier, either subcutaneously, intraperitoneally, or intratracheally. The response is expressed as the mean number of AFC per section; –: < 3, ±: 3–25, +: 26–75, ++: > 75 AFC per section. SCLN: superficial cervical lymph node, FLN: facial lymph node, PCLN: posterior cervical lymph node, NALT: nasal-associated lymphoid tissue, MLN: mesenteric lymph node.

and peribronchially in most of the sections.

The mesenteric lymph nodes contained no or neglectible numbers of AFC, as did the tissues from PBS-treated control animals.

Irrespective of the immunization route antigen-specific AFC were mainly of the IgG or IgM isotype. In the PCLN, however, antigen-specific IgG, IgM and IgA plasma cells were observed. The proportion of AFC carrying the IgG isotype was highest at day 7 after boosting. In general, the IgE response was low (2-12%), both in spleen and lymph nodes.

No effect of immunization with TNP-LPS was seen on the distribution of Ia antigen.

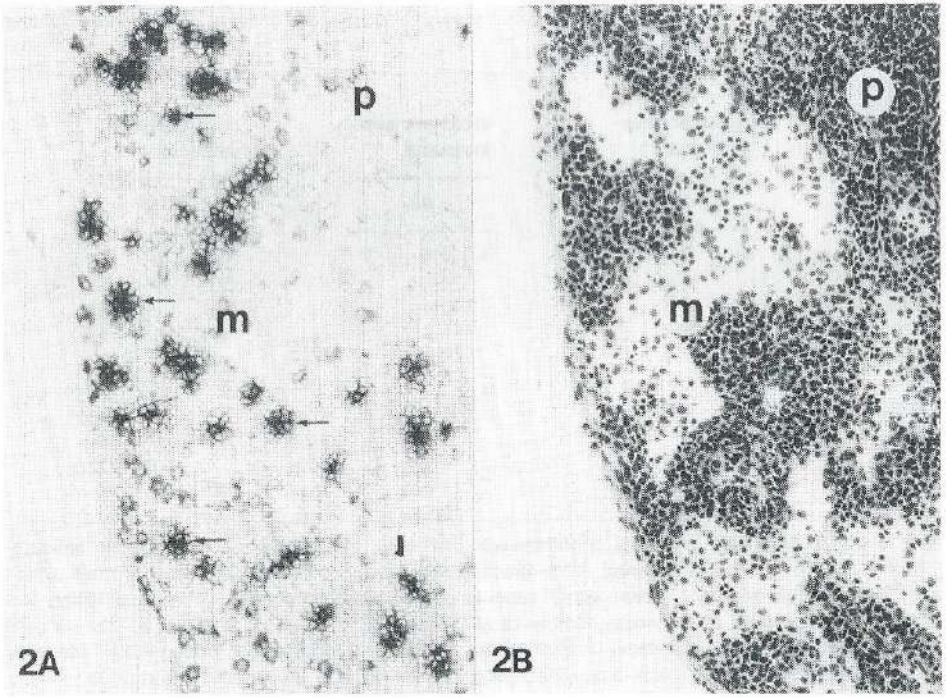

Fig. 2. Posterior cervical lymph node of rat 7 days after intranasal boosting with 200 μg TNP-LPS. Intraperitoneal priming had occurred 15 days previously with the same antigen and dose. Anti-TNP antibody forming cells (AFC) were clearly observed in the medulla. **(A)** Anti-TNP AFC (*arrows*) revealed by staining with the conjugate TNP-alkaline phosphatase. **(B)** Section of the same area stained with methylgreen-pyronin. *m*: medulla, *p*: paracortex. x200.

Higher serum antibody levels were measured at day 7 after the challenge as compared to day 3. Serum titers of PBS-treated animals were always <2.

Subcutaneous priming followed by intranasal boosting

Following this route of immunization specific AFC were already visible on the third day, except in the lung where they occurred on day 5. A distinct peak of AFC was seen in PCLN and spleen on day 5 after the challenge. In SCLN, FLN, NALT and lung only few anti-TNP forming cells were found (Fig. 1, Table 2).

On the peak day the staining pattern of splenic acid phosphatase (AcPh) activity slightly differed from the normal situation. The marginal zone, which normally includes strongly AcPh+ macrophages, showed a slight reduction in enzyme activity. No deviation could be detected, however, in the distribution of the ED1+ and ED3+ macrophages.

At all three time points serum antibodies could be detected and rose from day 3

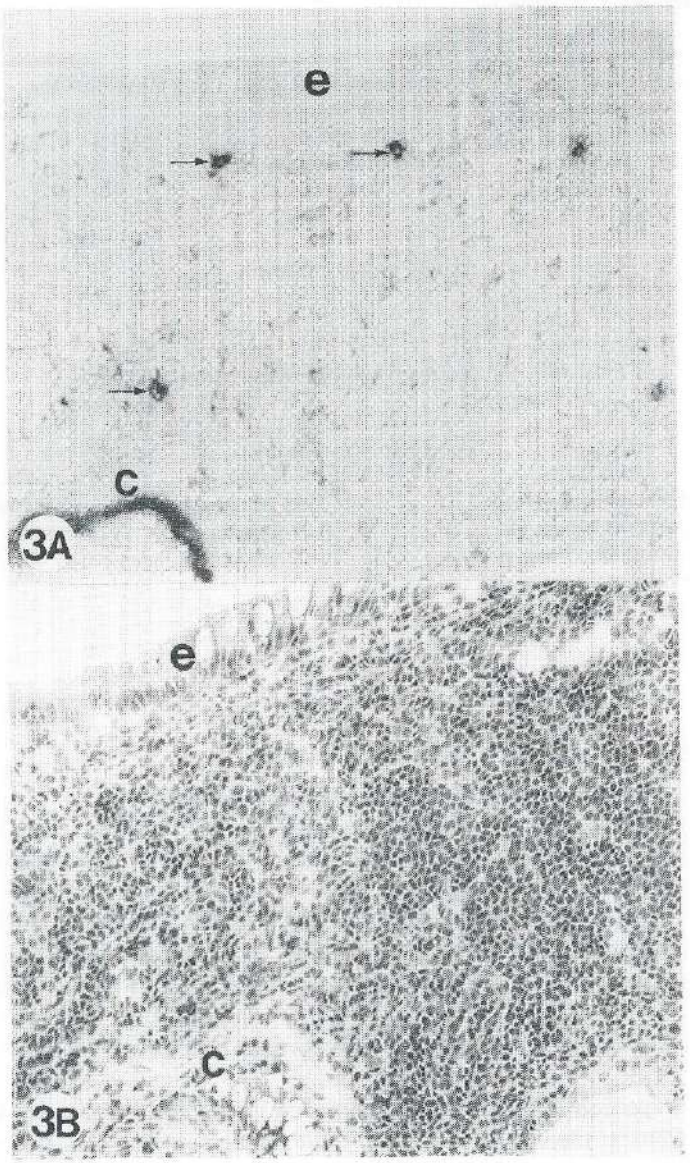

Fig. 3. Rat NALT 3 days after intranasal boosting with 200 μg TNP-LPS. Intraperitoneal priming had occurred 15 days previously, also with 200 μg TNP-LPS. **(A)** Anti-TNP antibody forming cells (*arrows*) revealed by staining with the conjugate TNP-alkaline phosphatase, were scarce; they were predominantly found underneath the epithelium and in the border area between lymphoid and connective tissue. **(B)** Methylgreen-pyronin staining of the same area. *e* epithelium, *c* connective tissue. x200.

Table 3. Mean serum anti-TNP titers

days after boost	immunization	mean anti-TNP titer
3		19 (11)
5	subcutaneous-intranasal	54 (51)
7		91 (50)
3		100 (137)
5	intraperitoneal-intranasal	19 (15)
7		326 (299)
3		6 (3)
5	intratracheal-intranasal	78 (58)
7		61 (27)
5	intranasal-intranasal	24 (18)

Serum anti-TNP response to TNP-LPS, as determined by means of an enzyme-linked immunosorbent assay (ELISA). Serum titers were determined in duplicate and were defined as the reciprocal dilution that corresponded with an optical density at 492 nm of 1.00. Mean values of 3 sera are given (in parentheses: standard deviations between the sera).

to day 7 (Table 3). No correlation with the appearance of AFC in tissue sections could be noticed.

Intraperitoneal priming followed by intranasal boosting

Intraperitoneal priming and intranasal boosting evoked a rather high response in spleen and lung on day 7 after the boost. A lower number of AFC was observed on day 5 as compared to days 3 and 7. The SCLN contained few and the PCLN considerable numbers of antigen-specific AFC. These numbers remained rather constant in both tissues from day 3 to day 7. Few AFC appeared in FLN on day 5 and hardly any on day 7. In NALT some AFC were found on days 3 and 7 (Fig. 1, Table 2).

No visible changes occurred in the number and localization of ED1+ and ED3+ cells.

After intraperitoneal-intranasal immunization the highest serum titers but also the largest variations in titers were observed (Table 3). Serum titers closely corresponded with the number of splenic AFC, including the lower score on day 5 as compared to days 3 and 7.

Intratracheal priming followed by intranasal boosting

When priming occurred intratracheally a considerable response was observed only in the PCLN, on days 3 and 5. The SCLN showed a minor response on days 3 and 7

while the FLN showed no response at all. The splenic reaction remained low with a maximum of 10 antigen-specific AFC per section detected on day 3. In lung tissue few AFC were detected from day 5 onwards and in NALT a low response was seen only on day 7 (Fig. 1, Table 2).

An increase in ED1+ macrophages was observed in the lung from day 3 to day 5. This increase was no longer present after 1 week. A similar pattern was observed with respect to ED3+ cells and cells with AcPh activity.

Considerable antibody levels were observed in the sera from animals sacrificed 5 or 7 days after the boost (Table 3), coinciding with the appearance of few AFC in lung tissue. As after subcutaneous-intranasal immunization, detectable antibodies in serum did not correspond with the number of AFC in tissue sections.

Intranasal priming followed by intranasal boosting

TNP-LPS could hardly elicit anti-TNP antibody forming cells by intranasal-intranasal immunization. Only a few specific AFC appeared in the PCLN. In serum, however, specific antibodies could be detected (Table 3).

DISCUSSION

In the lower respiratory tract specific antibody formation has been studied in the mouse using the antigen ferritin (Weisz-Carrington et al. 1987), and in the rat using the antigens horseradish peroxidase (HRP; Van der Brugge-Gamelkoorn et al. 1985a) and TNP-KLH (Van der Brugge-Gamelkoorn et al. 1986; Sminia et al. 1987). Boosting was beneficial to the response, as primary immune responses either failed to turn up (HRP) or were intensified by boosting (TNP-KLH). In the present study four routes of priming with TNP-LPS, followed by intranasal boosting with the same antigen and dose, were compared. The response in the upper respiratory tract was studied, as was the role of NALT in the mucosal immune system.

The response in the PCLN quantitatively exceeded the response in the other organs tested in the 3 immunization routes that showed marked responses: subcutaneous-intranasal, intraperitoneal-intranasal, and intratracheal-intranasal. The PCLN drain the nasal cavity and all peripheral cervical nodes. The latter include the SCLN which, in turn, drain the skin of the head (Tilney 1971). Thus, subcutaneous priming in the cheek evoked a considerable response in the PCLN as expected. Only a small number of specific antibody forming cells (AFC) was found in the SCLN. The latter lymph nodes play an important role in the suppression of IgE production following aerosol exposure (Sedgwick and Holt 1985). The cervical lymph nodes empty into the subclavian veins. As a consequence of this short lymph to blood tract, splenic AFC may be expected soon after the subcutaneous-intranasal immunization. Indeed, some antigen-specific AFC were seen in the spleen after subcutaneous priming and intranasal boosting. More splenic AFC were detected, however, when animals had

been primed intraperitoneally. Intratracheal-intranasal immunization resulted in the lowest splenic reaction. This observation is in accordance with recent results obtained in mice (Thepen et al. 1989) and supports the statement that the immune response in the respiratory tract is a rather local phenomenon (Sminia et al. 1987, 1989) which hardly expands as far as the spleen, albeit that specific antibodies occurred in the serum. Intratracheal-intranasal immunization resulted in antigen-specific AFC in the PCLN. Considering these data, the AFC are not likely to be recruted from a blood-reservoir of memory cells, although high antibody levels appear in serum from day 5 after the challenge.

Intraperitoneal-intranasal immunization was the most effective to obtain antigen-specific AFC in the lung. This affirms the occurrence of a shared lymphoid drainage of lung and peritoneal cavity. Recently, peritoneal macrophages were shown to migrate across the diaphragm and ultimately enter the pulmonary interstitium (Pitt and Anderson 1988). These macrophages may transport antigen. Also in previous experiments, intraperitoneal administration of antigen proved helpful in priming the mucosa, as shown for the enteric response to cholera toxin (Pierce and Koster 1980). Intraperitoneal priming also enhanced the specific antibody response to intratracheal administration of ovalbumin in sheep (Scicchitano et al. 1984) and porcine (Sheldrake 1989) respiratory tract. In the present study boosting occurred intranasally, and prior exposure to antigen via the trachea again elicited fewer AFC in lung tissue than did intraperitoneal priming. In general, a low response in the lung could well be due to the action of scavenger and suppressing macrophages as proposed (Holt et al. 1985, Holt 1986). This is in accordance with the observed increase in ED1+ monocytes/macrophages in the lung after intratracheal immunization. Suppressor activities of lung-macrophages are further evidenced in a recent finding in mice, in which elimination of alveolar macrophages dramatically enhanced the number of anti-TNP AFC (Thepen et al. 1989).

Especially on day 5 after intratracheal-intranasal immunization the rise in numbers of pulmonary ED1+ cells was parallelled by an increase in ED3+ macrophages. Under normal circumstances ED3 is expressed on a particular subpopulation of macrophages which is confined to distinct compartments of such lymphoid organs as spleen and lymph nodes (Dijkstra et al. 1985; Beelen et al. 1987). Furthermore, ED3 expression has been observed in milky spots present in the omentum of rats (Beelen et al. 1988) and in pathologic tissues (Polman et al. 1986; Verschure et al. 1989). In untreated Wistar rats some ED3+ cells have been found in the periphery of nasal-associated lymphoid tissue (NALT; Kuper et al. 1990). A few ED3+ cells were also found in the periphery of BALT and perivascularly in lung tissue (own observations). As stated above, intratracheal-intranasal immunization induced a transient increase in the number of ED3+ macrophages. The short-term presence of ED3+ cells is in accordance with *in vitro* observations by Damoiseaux and coworkers (1989). They showed an increasing percentage of ED3+ macrophages during the first week of bone marrow cultures, probably due to induction by lymphokines. Addition of Con A supernatant further enhanced the percentage of ED3 expressing cells. Thus, the rise in numbers of ED3+ cells may be ascribed to the lymphokines produced by activated T lymphocytes elicited as a result of the intratracheal antigen exposure and subsequent intranasal challenge. Similarly, in NALT of newborn rats which undoubtedly encounter many unknown aeroantigens, ED3 expression is found throughout the tissue. Later on, ED3+ macrophages are confined to the border area between lymphoid and connective tissue. This suggests a role of ED3+ cells in the establishment of the microenvironment (Hameleers et al. 1989).

The first lymphoid accumulation encountered by intranasally applied or inhaled antigen, is NALT. When plasma cells were seen in NALT, they occurred mainly at the border between lymphoid tissue and the surrounding connective tissue. This corresponds with the localization of ED2+ resident tissue macrophages (Kuper et al. 1990). In NALT, as in other tissues, ED2+ macrophages are observed in the vicinity of plasma cells. This holds true for lymph nodes, where medullary macrophages are ED2+ (Dijkstra et al. 1985), as well as for gut and lung where plasma cells and ED2+ cells are found in the villi and at the periphery of BALT, respectively (Dijkstra et al. 1985, Van der Brugge-Gamelkoorn et al. 1985b, Sminia and Jeurissen 1986). Moreover, in the oral mucosa ED2+ cells, together with plasma cells are observed around the glandular acini (Swart et al. in preparation). Clearly, a role of ED2+ macrophages in the maturation of plasma cells is indicated. Recently, such proposal was made in an elaborate study on the role of macrophages in the intrasplenic immune response (Matsuno et al. 1989).

The immunization routes used in the present study induced very few antigen-specific AFC in and around NALT. Moreover, intranasal-intranasal immunization induced no more than marginal numbers of anti-TNP AFC. It should be kept in mind, however, that the use of particulate antigen instead of the here used soluble antigen may render different results (Cox and Taubman 1984), because of differences in antigen uptake and antigen presentation. Furthermore, although antigen-specific AFC were scarce in NALT they may well exist elsewhere in the nasal mucosa. It is a common feature in the mucosal system that apart from the draining lymph nodes, AFC are elicited and home predominantly in the lamina propria. Only few AFC were reported in the lymphoid accumulations as BALT (Sminia et al. 1987, 1989) and Peyer's patches (PP; Sminia et al. 1983). For PP it was concluded that they have an important function in memory formation (Jeurissen et al. 1987) and in suppressor T cell recruitment (Mattingly and Waksman 1978; MacDonald 1983). Whether NALT plays a similar role in the respiratory tract, has to be determined.

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SPECIFIC ANTIBODY FORMING CELLS IN THE RAT AFTER INTRANASAL ADMINISTRATION OF THREE DIFFERENT ANTIGENS

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SUMMARY. In order to study the role of nasal-associated lymphoid tissue (NALT) in the local nasal immune response, rats were immunized intranasally with either of the following trinitrophenylated (TNP) antigens; the thymus-dependent keyhole limpet haemocyanin (KLH), or the thymus-independent lipopolysaccharide (LPS), or with the particulate (thymus-dependent) sheep red blood cells (SRBC). Primary responses hardly occurred, while only TNP-KLH elicited a considerable secondary response. The major responding organ was the posterior cervical lymph node. Specific antibody forming cells occurred in the medulla and were mainly of the IgA or IgG isotype. Intranasal immunization evoked no antibody response in the lung. Ample anti-TNP antibodies could be detected in the sera of animals, primed and boosted with TNP-KLH or TNP-LPS. After immunization with TNP-SRBC no specific serum antibodies occurred.

The results are discussed in view of the immunological defence in the upper respiratory tract.

INTRODUCTION

The integrity of the respiratory tract mucosa is highly dependent on nasal protection. The nasal filter traps particles larger than 1-3µm (Stuart 1984, Dahl et al. 1988). Inhaled smaller particles and soluble antigens enter the respiratory tract by passing through the nasal cavity. Substances trapped in the mucous blanket are transported towards the pharynx where they can be swallowed. Aerosoled or intranasally instilled antigens are to a large extent recovered in the intestines, as shown by radiotracer studies (Van Hout and Johnson 1972, Willoughby and Willoughby 1977, Holt et al. 1981). The proportion of radiolabel found in the respiratory tract was larger after intranasal administration than after aerosol exposure (Holt et al. 1981). Hence, intranasal inoculation of antigen appears a suitable way of presenting antigens to the respiratory tract. Especially particulate antigens have been used to this end. The effects of intranasal immunization were mostly studied in serum and lungs (Ivanoff et al. 1982, Kumagai et al. 1985, Khavkin and Tabibzadeh 1988, Weisz-Carrington et al. 1987). Recently, nasal secretions were analysed for specific antibodies after intranasal immunization (El Guink et al. 1989, Watanabe et al. 1989).

The predominant isotype in nasal secretions is IgA (Waldman et al. 1973, Morgan et al. 1980), which plays an important role in preventing bacterial adherence (Kurono et al. 1989). Immunoglobulin-producing cells in the respiratory mucosa originate in lymph nodes, tonsils or bronchus-associated lymphoid tissue (BALT; Kaltreider et al. 1987, Brandtzaeg 1984, 1988, Van der Brugge-Gamelkoorn et al. 1986). Whether also the nasal-associated lymphoid tissue (NALT; Spit et al. 1989, Kuper et al. 1990) is directly involved in immune responses in the upper respiratory tract is still unclear.

The aim of the present study is to investigate the role of NALT in local immune reactions. For this purpose intranasal priming and boosting immunizations were carried out, using different forms of antigen, viz. soluble thymus-dependent keyhole limpet haemocyanin (KLH), soluble thymus-independent lipopolysaccharide (LPS) and particulate thymus-dependent sheep red blood cells (SRBC). All of these antigens were used conjugated to 2,4,6-trinitrophenyl (TNP). The localization of specific antibody forming cells *in situ* was studied.

MATERIALS AND METHODS

Animals

Young adult male Wistar rats (210-240 g) were purchased from Harlan Sprague Dawley (Zeist, The Netherlands) and were kept under routine laboratory conditions.

Antigens

The trinitrophenylated antigens keyhole limpet haemocyanin (TNP-KLH) and lipopolysaccharide (TNP-LPS) were kindly donated by dr. Theo Thepen (Vrije Universiteit, Amsterdam, The Netherlands). The LPS-moiety was prepared from *Escherichia coli 055:B5.* Sheep red blood cells (SRBC) were obtained as a sterile suspension in Alsever's solution from the National Institute of Public Health and Environmental Protection (RIVM, Bilthoven, The Netherlands) and conjugated to TNP according to Naor and coworkers (1974). The efficacy of coupling was tested by means of an indirect immunohistochemical staining on cytocentrifuge preparations with murine anti-TNP serum and alkaline-phosphatase conjugated goat anti-mouse Ig (Tago Inc., Burlingame, Ca, USA). The antigens were used in a concentration of 5 mg/ml in 0.01 M phosphate buffered saline (PBS) or $5x10^9$ SRBC/ml in 0.9% NaCl.

Experimental design

To evoke an immune response, 40 µl antigen was inoculated intranasally in small drops with an Eppendorf pipette, whereby the antigen was equally spread over both nostrils. Care was taken that a new drop was only given when the former had been entirely inspired. Each animal thus received 200 µg TNP-KLH (Group A; 6 animals), TNP-LPS (group B; 6 animals) or $2x10^8$ TNP-SRBC (group C; 8 animals). Fifteen days before, 3 or 4 animals of each group had been primed intranasally, with the same antigen and dose as the boost. Control animals received PBS instead of antigen. During the immunization procedure, the animals were under light anaesthesia with Hypnorm: 0.1-0.15 ml intramuscularly.

Five days after the last antigen administration, animals were sacrificed by an intraperitoneal injection of 2 ml Nembutal. Sera were collected via heartpuncture. The following tissues were frozen in liquid nitrogen: spleen, left lung, superficial cervical lymph nodes (SCLN), posterior cervical lymph nodes (PCLN) and nasal-associated lymphoid tissue (NALT). The nomenclature of lymph nodes is according to Tilney (1971). Tissues were stored at -70°C until used. Cryostat sections were cut at 8 µm thickness on gelatin-coated slides and used for immuno- and enzyme-histochemical stainings or for the detection of specific anti-TNP forming cells.

Immune reagents

The unlabelled monoclonal and polyclonal antibodies used (Table 1) were appropriately diluted in PBS, containing 0.5% bovine serum albumin (BSA). The conjugates used (Table 1) were diluted in PBS containing 0.5% BSA and 1% normal rat serum.

Staining techniques

Sections were dried above silicagel and fixed in pure aceton for 10 min, air-dried, rehydrated in PBS and incubated with the appropriate antibodies for 1 h, rinsed in PBS, and incubated with the appropriate conjugate at 4°C overnight. Thereafter slides were rinsed in PBS and stained for peroxidase activity with 3,3'-diaminobenzidine-

Table 1. Antibodies used in this study

A. MONOCLONAL ANTIBODIES (moab, anti-rat)		
moab	directed against	source
ED1	monocytes, macrophages, dendritic cells	VU (Dijkstra et al. 1985)
ED3	macrophage subpopulations in lymphoid organs	VU (Dijkstra et al. 1985)
OX4	Ia antigen	Serotec
OX8	T suppressor cells	Serotec
OX19	T cells	Serotec
W3/25	T helper cells	Serotec
MARA-2	IgA	Serotec
MARM-4	IgM	Serotec
a cocktail of:		
MARG-1	IgG1	Zymed
MARG-2a	IgG2a	Zymed
MARG-2b	IgG2b	Zymed
MARG-2c	IgG2c	Zymed
B. POLYCLONAL ANTIBODIES (poab)		
poab		source
goat anti-rat IgE		Nordic
rabbit anti-mouse Ig/HRP labelled		Dakopatts
swine anti-rabbit Ig/HRP labelled		Dakopatts
rabbit anti-goat Ig/HRP labelled		Dakopatts

HRP: horseradish peroxidase; Dakopatts: Dakopatts, Glostrup, Denmark; ICN: ICN Biomedicals, Lisle, IL, USA; Nordic: Nordic Immunological Laboratories BV, Tilburg, The Netherlands; Serotec: Serotec, Kidlington, Oxford, UK; VU: Vrije Universiteit, Amsterdam, The Netherlands; Zymed: Zymed Laboratories Inc., South San Francisco, Ca, USA.

tetrahydrochloride (DAB, Sigma, St. Louis, Mo, USA) at a concentration of 0.5 mg/ml in TRIS-HCl buffer pH 7.6, containing 0.025% H_2O_2, for 10 min. The staining of the brown reaction product was enhanced by rinsing the slides in PBS and 0.9% NaCl successively, followed by incubation in 0.5% $CuSO_4$ in 0.9% NaCl for 15 min.

After being rinsed in distilled water, the sections were lightly counterstained with haematoxylin, rinsed in tap water, dehydrated, cleared and mounted in Entellan neu (Merck, Darmstadt, FRG). Except for the conjugate incubation the staining procedure was carried out at room temperature. Control slides were treated similarly while omitting the first step antibody.

Acid phosphatase (AcPh) activity was demonstrated according to Burstone (Pearse 1968), using naphthol-AS-BI phosphate (Sigma) as the substrate and hexazotized pararosaniline as the diazonium salt. For double staining of Ia antigens and AcPh activity, immunohistochemistry preceded enzyme-histochemistry and the colour-enhancement step was omitted.

Anti-TNP antibody forming cells (AFC) were detected as described by Claassen and Van Rooijen (1984), using the conjugate TNP-AlPh (TNP coupled to alkaline phosphatase). Sections were lightly counterstained with nuclear fast red (Merck). Double immunohistochemical stainings were performed according to Van Rooijen and Kors (1985). In short, sections were incubated overnight with a mixture of TNP-AlPh and an isotype-specific antibody or, for IgG, a mixture of antibodies (Table 1) followed by staining for AlPh-activity. The sections were then incubated for 1 h with the appropriate conjugate containing 1% normal rat serum and finally stained for peroxidase activity with 3-amino-9-ethylcarbazole (AEC, Sigma). Slides were mounted in glycerin-gelatin. Methylgreen-pyronin stained sections were used for morphological screenings.

The numbers of anti-TNP AFC were determined in a blind fashion and independently by two of the authors.

Detection of serum anti-TNP antibodies

In order to detect anti-TNP antibodies in serum, an ELISA was performed as described previously (Delemarre et al. 1989). In short, twofold serial dilutions of sera were incubated in TNP-ovalbumin coated microtiter plates (Greiner, Alphen aan den Rijn, The Netherlands). After subsequent incubation with horseradish peroxidase conjugated rabbit anti-rat Ig (Dakopatts, Glostrup, Denmark) and orthophenylene-diamine-di-hydrochloride (OPD, Sigma), the absorption at 492 nm was determined. Titers were defined as the reciprocal dilution at an optical density of 1.00.

RESULTS

General observations

Irrespective of the antigen, AFC were mostly found in the medulla of the PCLN (Fig. 1). Rarely, specific AFC were seen in spleen, SCLN, NALT or surrounding mucosa. All lung sections were devoid of anti-TNP AFC, except for the sections of 1 animal. Priming and boosting with TNP-KLH had elicited a moderate number of specific anti-TNP AFC in the lung of that animal. Remarkably, these AFC were

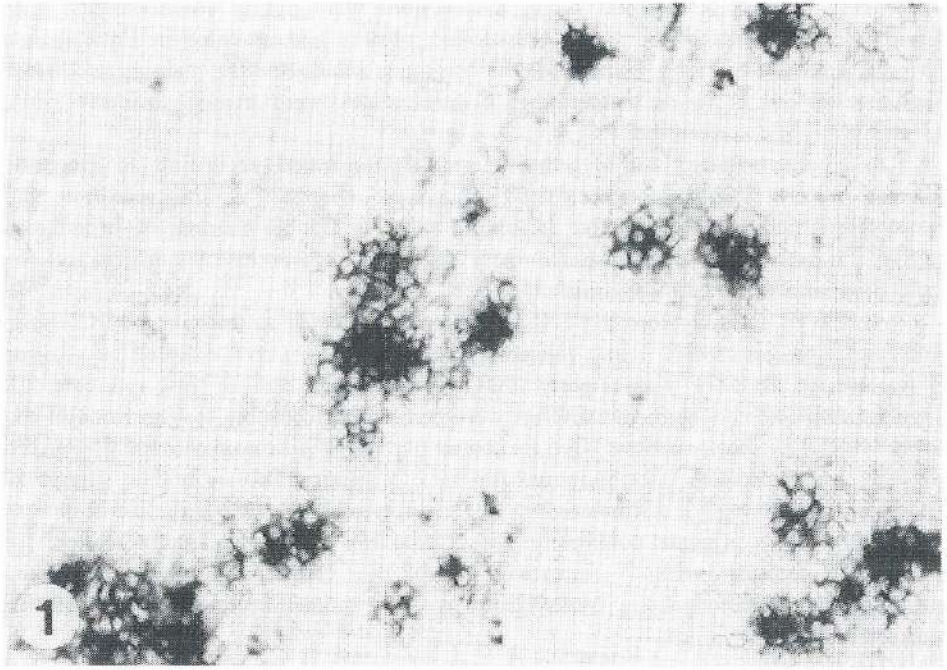

Fig. 1. Anti-TNP antibody forming cells (AFC) in the medulla of the posterior cervical lymph node, 5 days after intranasal priming and boosting with TNP-KLH. Anti-TNP AFC were revealed by staining with the conjugate TNP-alkaline phosphatase. x120.

restricted to one particular site, in the vicinity of large vessels and BALT, in a medial part of the lung. Of these cells 35% produced IgA. This animal also developed a moderate response in the SCLN.

During the time course of the study, no deviations were seen in the distribution of T cells. Except for the lungs, these were neither observed for macrophages or Ia expression. A few animals showed an increase in pulmonary ED1+, ED3+, and Ia/AcPh+ cells, which was unrelated to the antigen administered. To these animals also belonged the one which developed anti-TNP AFC in the lung.

PBS-treated animals showed no or neglectible numbers of anti-TNP forming cells and gave serum titers <3.

Anti-TNP antibody forming cells

A minor reaction occurred after a single intranasal inoculation of TNP-KLH. Boosting

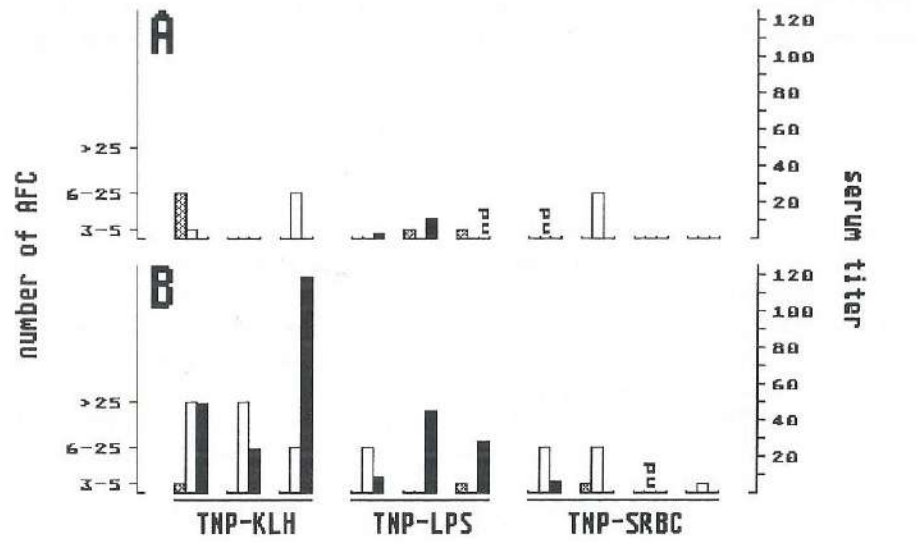

Fig. 2. Individual anti-TNP responses in the spleen (▨), posterior cervical lymph node (□) and serum (■) of rats, 5 days after intranasal immunization with TNP-KLH, TNP-LPS or TNP-SRBC. (**A**) primary immune response, (**B**) secondary immune response. Boosting had occurred 15 days after priming. nd: not done.

enhanced the number of specific AFC considerably, as was especially observed in the PCLN (Fig. 2). Antigen specific AFC were rarely observed in the superficial cervical lymph nodes (SCLN). IgA and IgG were the major isotypes produced, and comprised 15-80% and 35-75% of the anti-TNP AFC, respectively. IgM remained low with 0-15%, as did IgE which made up 5-20% of the anti-TNP AFC.

A single dose of TNP-LPS resulted in very few anti-TNP forming cells in PCLN and spleen. Boosting with the same antigen and dose could not enhance the response (Fig. 2), except for one animal which developed a low response in the PCLN. The proportion of AFC that produced IgA or IgM was similar in this low response as after TNP-KLH administration.

The particulate antigen TNP-SRBC was inappropriate to induce a primary immune response in 3 of 4 animals. Also the secondary response remained very low. The PCLN (Fig. 2) was again the main responding organ.

Serum titers

No titers were observed in the sera of animals immunized once with TNP-KLH or TNP-SRBC. while a single dose of TNP-LPS induced very low serum anti-TNP antibody levels in 2 of 3 animals. After priming and boosting with TNP-KLH or

TNP-LPS considerable titers of specific serum antibodies were detected. Priming and boosting with TNP-SRBC, however, failed to induce such systemic response (Fig. 2).

DISCUSSION

Various mechanisms have been shown to participate in the defence against respiratory infections. The mucociliary clearance is important as a non-immunological way to prevent microorganisms from colonizing the mucosa (Eccles 1983). The auxiliary immunological defence is subject of current investigations. Several studies have revealed the cellular components of such defence, as found in the nasal mucosa and in the local organized lymphoid aggregates (Winther et al. 1987, Hameleers et al. 1990, Kuper et al. 1990, Stoop et al. 1990, Van Nieuwkerk et al. 1990). Moreover, some intranasal immunization studies have been performed to study the local antibody response (El Guink et al. 1989, Watanabe et al. 1989).

The present study shows that after intranasal priming and boosting the great majority of antigen-specific AFC occurs in the posterior cervical lymph nodes. This is in accordance with reports on the lower respiratory tract in which intratracheal immunization elicited specific AFC in the draining lymph nodes in mice (Gerbrandy and Bienenstock 1976; Kaltreider et al. 1987, Thepen et al. 1989) and rats (Van der Brugge- Gamelkoorn et al. 1986, Sminia et al. 1987). Both BALT and NALT, as well as the lamina propria of the upper and lower respiratory tract mucosa, contain only low numbers of AFC. This is in contrast to the situation in the gut, where the lamina propria of the villi contains a vast amount of AFC (Pierce and Koster 1980, Sminia and Plesch 1982).

Particulate antigens, when presented to the mucosa, are expected to be preferentially taken up by M-cells, as has been shown in the gut (Pappo and Ermak 1989). M-cells reside in the epithelium covering lymphoid follicles of MALT and have been shown in the epithelium overlying NALT (Spit et al. 1989). Hence, intranasal immunization with particulate antigen was expected to elicit a considerable response. However, priming with TNP-SRBC failed to induce antigen specific AFC, while boosting only induced a slight response. This phenomenon may be caused by at least two reasons. Firstly, SRBC are non-viable, non-replicating antigens. Live microorganisms may well evoke quite different responses, as the mucosa can probably distinguish between these antigens (Wold et al. 1989). Secondly, the previously mentioned mucociliary system may have cleared the SRBC from the nasal cavity. SRBC are about 7 µm in diameter, and can therefore be retained in the nasal cavity (Stuart 1984, Dahl et al. 1988, Brandtzaeg 1984). Because these antigens can not actively adhere to the epithelium, they are probably trapped in the mucous layer and discharged from the nasal cavity before they can reach immune cells within the mucosa. The latter possibility is sustained by the finding that Cholera toxin is a better mucosal primer than Cholera toxoid. The former can adhere to epithelial cells, while the latter can not (Pierce 1978).

The soluble thymus-dependent antigen TNP-KLH induced a very low number of anti-TNP forming cells in tissues and no serum response when administered once. Boosting had an enhancing effect on the number of anti-TNP forming cells in the tissues and on the amount of antibodies in serum. In contrast, intranasal immunization with the thymus-independent antigen TNP-LPS elicited only a very weak specific antibody response in tissues, either in a primary, or in a secondary reaction. Nonetheless, anti-TNP antibodies occurred in serum, which suggests that the production of serum and secretory immunoglobulins are unrelated. Recent results by Bartholomeusz and coworkers (1990) show that the serum and secretory antibodies to typhoid LPS are produced independently. Both parenteral and oral typhoid vaccination induced IgA antibodies in serum of human volunteers, while an intestinal response could only be induced by oral vaccination. Moreover, a discrepancy between secretory and serum response has been reported with respect to intranasally (Gerbrandy and Van Dura 1972) or intragastrically (Ogawa et al. 1989) immunized mice.

The very low numbers of specific AFC in the tissues after intranasal TNP-LPS immunization might be due to the fact that only a single boost was given, 15 days after priming. Up to 14 days after boosting the response remained low, as observed in similarly treated PVG rats (Hameleers et al. unpublished results). Comparable observations were made on cholera toxoid which appeared a weak mucosal primer (Pierce 1978). Systemic responses, however, may be decreased by frequent mucosal immunization. Repeated aerosol immunization with OVA resulted in a decrease in anti-OVA IgE and IgG in serum (Sedgwick and Holt 1984). In the present experiment the animals were exposed only twice to the antigens. Still, serum titers were much lower after intranasal-intranasal immunization as compared to subcutaneous-intranasal or intratracheal-intranasal immunization (own observations), which is in accordance with the aforementioned study by Sedgwick and Holt (1984). Despite the low serum response after intranasal-intranasal immunization, no visible changes occurred in the number and distribution of T-cell subsets and Ia antigen. Similar results were obtained in spleen, axillary, brachial and mediastinal lymph nodes after aerosol treatment of rats with OVA (Nilsson et al. 1990). Neither was a visible increase of CD8+ cells observed in the superficial cervical lymph nodes, which are the source of suppressor cells in aerosol-induced systemic tolerance (Sedgwick and Holt 1985).

Based on the results presented in this study it appears that the response to intranasally applied antigen is preferentially restricted to the lymph nodes draining the upper respiratory tract. Intranasal-intranasal immunization did not result in specific AFC in pulmonary or nasal mucosa. Moreover, non-viable, non-replicating particulate antigens are presumably discharged rapidly from the nasal mucosa and therefore fail to induce an immune response.

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Chapter 5

LYMPHOCYTES AND NON-LYMPHOID CELLS IN THE HUMAN NASAL MUCOSA

5.1.
Intra-epithelial lymphocytes and non-lymphoid cells in the human nasal mucosa

5.2.
Immunohistochemical characterization of leukocytes in the nasal mucosa of ear, nose and throat patients and controls

INTRA-EPITHELIAL LYMPHOCYTES AND NON-LYMPHOID CELLS IN THE HUMAN NASAL MUCOSA

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SUMMARY. Immunohistochemical staining of biopsy specimens was used to investigate the occurrence of lymphocyte subsets and non-lymphoid cells within the epithelial layer of the human nasal mucosa. The CD19 (B-cell) marker was not expressed on the intra-epithelial lymphocytes, whereas the pan T-cell marker CD2 was varyingly detected. The HLA-DR antigen was abundantly present on epithelial cells, lymphocytes, and non-lymphoid cells. The latter are probably dendritic or Langerhans cells. The findings stated above were the same in patient and control samples. In biopsy sections of 9 ear, nose, and throat patients many CD8+ (T suppressor/cytotoxic) cells and very few weakly stained CD4-expressing (T helper/inducer) cells were present. Quantification on single-cell preparations showed an average of 67% of the lymphocytes to be CD2+, 73% to be CD8+, while only 12% of the lymphocytes expressed the CD4 antigen. In control sections CD8 was similarly present as in patient sections, and, in addition, some clearly stained CD4+ cells were seen.

INTRODUCTION

The mucosal tissue lining the respiratory tract forms the first barrier against penetration of inhaled antigens. With the availability of monoclonal antibodies against human leukocyte determinants, it became possible to recognize cell types and their subsets (Shaw 1987; Zola 1987). However, lymphocyte antigens, e.g., CD4, are sometimes also identified on monocytes/macrophages (Moscicki et al. 1983; Hume et al. 1987). Despite these pitfalls, the monoclonal antibodies are powerful tools in determining the cellular distribution within biological fluids and tissues. So far, studies on nasal mucosa and secretions are merely confined to immunoglobulins and the immunoglobulin-producing cells (Mygind et al. 1975; Nakashima and Hamashima 1980; Brandtzaeg 1985; Kurono and Mogi 1987). With respect to the distribution of the different cell types, most studies on the upper respiratory tract are limited to the tonsils, as reviewed by Brandtzaeg (1984). Recently, Winther et al. (1987) described the lymphocyte distribution in the normal nasal mucosa as detected by immunohistochemistry on tissue sections. The present study deals with the distribution of intra-epithelial lymphocytes in the nasal mucosa of ear, nose, and throat patients. For quantification purposes, cell suspensions were used beside tissue sections.

MATERIALS AND METHODS

Patients and controls

Nasal biopsies were taken from 34 patients visiting the polyclinics of allergology at the Ear, Nose, and Throat Department of the University Hospital of the Vrije Universiteit, Amsterdam. All patients suffered from nasal complaints (e.g., nasal obstruction, rhinorrhoea, sneezing) and underwent an extensive routine screening for allergy (e.g., skin tests, determination of serum IgE, radioallergosorbent test, sinus X-ray). Biopsy specimens of 17 of the patients were of sufficient quality to be evaluated. Of the 17 patients 41% had positive skin tests and serum IgE concentrations over 100 IU/l, 12% had positive skin tests only, and 18% had negative skin tests, but serum IgE concentrations over 100 IU/l; the remaining 29% of the patients scored negative for both tests (see Table 1). None of these 17 patients had used locally applied corticosteroids during at least 6 weeks before entering the study. Twelve volunteers without nasal complaints served as controls.

Tissues

Biopsy specimens were taken from the epithelial compartment of the inferior turbinate by means of a curette. Biopsies obtained from 20 patients and 12 controls were immediately embedded in OCT tissue compound (Miles/Elkhart, Ind, USA) and frozen in liquid nitrogen. Frozen samples were stored at -20°C until used. Biopsies of

Table 1. Data on the patients included in the study

Patient No.	Sex	Age (years)	Main diagnosis	Positive skin test for	Serum IgE (IU/l)	Positive RAST for
1	f	15	NO	–	7	–
2	m	18	AR	house dust mite, meal mite	389	mite
3	m	26	AR	birch, cat dander, house dust mite, meal mite, oak, rye, ryegrass, timothy	867	cat, mite, ryegrass, birch, timothy
4	f	26	AR	–	388	mite
5	f	19	AR	meal mite, horse dander, house dust mite	150	mite, ryegrass, timothy
6	f	19	NO	–	11	cat, timothy
7	m	39	AR	cat dander, house dust mite	16	dog, mite, timothy
8	m	30	NO	–	23	–
9	m	18	AR	cat dander, grey alder, rye, ryegrass, timothy, Yorkshire fog	261	ryegrass, timothy
10	m	46	AR	cat dander, house dust mite, meal mite, mugwort	373	cat, dog, mite, mugwort, timothy
11	m	27	CR	–	6	–
12	f	39	AR	ryegrass	101	ryegrass, timothy
13	m	29	NO	–	69	–
14	m	10	AR	–	371	milk
15	f	40	AR	birch, grey alder, hazel, oak	216	birch
16	f	28	NO	–	105	–
17	f	23	AR	cat dander	45	mite, ryegrass

AR = Allergic rhinitis; CR = Chronic rhinitis; NO = nose obstruction. Evaluation of the allergy scores: positive skin test: diameter of skin eruption > 2 mm; positive RAST: ≥ 2 IU; raised serum IgE: > 100 IU/l.

the remaining 14 patients were collected in cell-suspending medium (Earl's medium, NPBI, Emmer-Compascuum, The Netherlands) on ice. These biopsies were incubated with collagenase III (1 mg/ml; Cooper Biomedical, Malvern, Pa, USA), under gentle shaking for 30 min at 37°C. The biopsy material was then gently pressed through a metal sieve and suspended in 0.1% bovine serum albumin in Earl's medium. A metal sieve was chosen because the pores of the generally used nylon gauze appeared too small for this mucus-containing tissue, while a longer incubation period implied the risk of cell damage. The cell suspensions were washed two times and centrifuged

Table 2. Mouse monoclonal antibodies against human leukocytes used in this study

Antibody	Manufacturer[1]	Subtype	Specificity
Anti-CD2	CLB	IgG1	all peripheral T cells, 90% of the thymocytes
Anti-CD4	Sanbio	IgG2aκ	helper/inducer T cells and subpopulations of macrophages
Anti-CD8	Sanbio	IgG2a	suppressor/cytotoxic T cells
Anti-CD19	Dakopatts	IgG1κ	precursor and mature B cells (no plasma cells)
Anti-HLA-DR	CLB	IgG1	cells of the monocyte lineage, myeloblasts, promyelocytes, and cells of the B lymphocyte lineage

[1] CLB: Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, The Netherlands; Sanbio: Sanbio Biological Products, Uden, The Netherlands; Dakopatts: Dakopatts, Glostrup, Denmark.

on slides (Shandon Cytospin 2 apparatus) in 2% bovine serum albumin in Earl's medium. Cytotocentrifuge preparations were dried overnight above silica gel.

Immune Reagents

The mouse monoclonal antibodies against human leukocytes used in this study are listed in Table 2. These were appropriately diluted in 0.01M phosphate buffered saline (PBS, pH 7.4) containing 0.5% bovine serum albumin and used in an indirect immunoperoxidase staining.

The conjugate used was horseradish-peroxidase-labelled rabbit anti-mouse Ig (Dakopatts, Glostrup, Denmark). This was diluted 1:300 in PBS containing 0.5% bovine serum albumin and 1% normal human serum.

Staining procedure

Cryostat sections (6-8μm thick) were picked up on slides and allowed to dry overnight above silica gel. The sections were fixed in pure acetone for 10 min and incubated with the appropriate antiserum for 1 h at room temperature. After washing in PBS, the sections were covered with the conjugate for 1 h at room temperature, washed again in PBS and stained for peroxidase activity with 3,3'-diaminobenzidine-tetrahydrochloride (Sigma, St.Louis, Mo, USA) at a concentration of 0.5 mg/ml in TRIS-HCl buffer (pH 7.6) containing 0,02% H_2O_2. To enhance the staining of the brown reaction product, slides were rinsed with subsequently distilled water and 0.9% NaCl, and incubated in 0.5% $CuSO_4$ in 0.9% NaCl for 10-15 min at room temperature. After rinsing in distilled water the sections were counterstained with haematoxylin (3-10 s), dehydrated, and mounted in Entellan (Merck, Darmstadt, FRG).

Tonsil sections were used as positive controls and were mostly mounted onto the same slides as the nasal epithelium sections. Controls for non-specific staining were incubated with 0.5% bovine serum albumin in PBS or with only the second stage conjugate. All stainings were carried out in duplicate.

The cytocentrifuge preparations were fixed and stained similarly to the sections.

For routine histology methylgreen-pyronin staining was performed on all sections, and May-Grünwald-Giemsa staining was used for the cytocentrifuge preparations.

Evaluation

The proportion of positive cells detected in the sections was expressed as no cells (-), few cells (+), many cells (++). The intensity of the staining was expressed in a scale from 0 to 3 (0 = no staining, 3 = extremely dark staining). The number of positive lymphocytes in the cytocentrifuge preparations was counted and given as per cent of the total number of lymphocytes.

RESULTS

The evaluation of the results obtained on the biopsy specimens did not provide reasons to divide the patients in an allergic and a non-allergic group.

Tissue sections

Several tissue sections were difficult to evaluate because of damage by the procedure of biopsy and further preparation of the sections. These specimens were excluded from the study.

Due to endogenous peroxidase activity, neutrophilic granulocytes were easily recognized by strong cytoplasmic staining. Some samples showed substantial non-specific staining of cells other than neutrophils. These specimens were also excluded from the study. Consequently, the biopsies of only 9 out of 20 patients were to be evaluated, as well as 9 samples out of 12 control biopsies. Control staining of the tonsil tissue showed specific staining for each of the monoclonal antibodies used.

As all the biopsies were taken from the epithelial layer of the nasal mucosa, at least 90% of the cells were epithelial cells which were acid phosphatase positive; in addition, infiltrated lymphocytes and acid phosophatase positive non-lymphoid cells were present in the epithelial compartment. The epithelial cells were made visible by staining with anti-keratin, a mouse anti-human epithelium monoclonal antibody (Dakopatts). The HLA-DR antigen was abundantly present on epithelial cells, lymphocytes, and on non-lymphoid cells. These findings refer to both patient and control sections. The CD19 marker was found neither in patient biopsies nor in

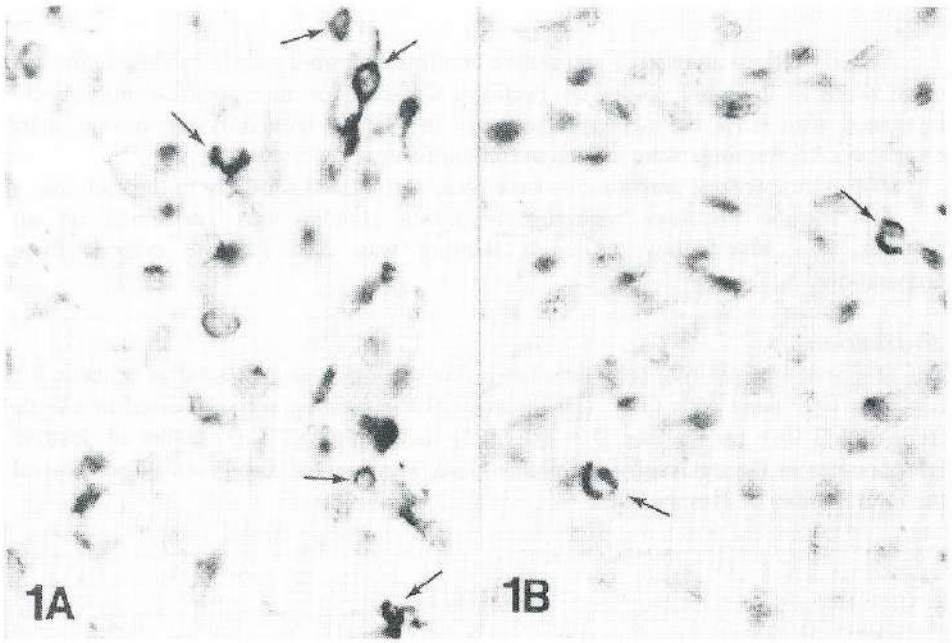

Fig. 1. Cryostat sections of the epithelial layer of the nasal mucosa, stained for CD4 (T helper/inducer) and CD8 (T suppressor/cytotoxic) expression. x 400. Numerous CD8+ cells (*arrows*) were found in patient as well as in control sections (**A**). In patient biopsies CD4+ cells were hardly or not detectable, whereas in control sections (**B**), some CD4+ cells (*arrows*) were clearly detectable.

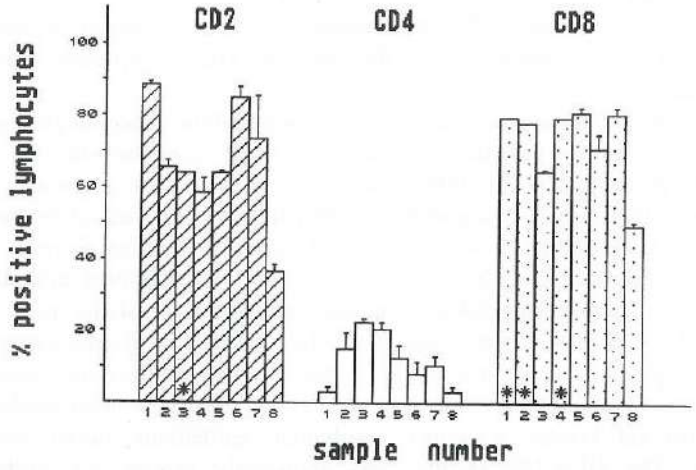

Fig. 2. Quantification of CD2+, CD4+ and CD8+ lymphocytes on eight cytocentrifuge preparations. The bars represent the mean values of duplicates ± SD. Asterisks indicate single determinations. For further details see text.

control samples. This demonstrates that little or no B cells are present within the epithelium. CD2, a pan T-cell marker, was varyingly detected in the tissue sections. In patient as well as in control sections, the CD8 (T suppressor/cytotoxic) marker was detected on the majority of the lymphocytes. In control tissues some CD4 (T helper/inducer) expression could be found (Fig. 1). Patient mucosa, however, showed only very few, weakly stained CD4+ cells.

Cell suspensions

The findings described above were compared with the data obtained from the cytocentrifuge preparations. Eight samples were included in the study, based on the criteria that at least 50 lymphocytes per slide (100 per duplicate slides) could be counted and that non-specific staining of cells other than neutrophils was so weak that it could be neglected.

HLA-DR was found as in the sections: on epithelial cells, non-lymphoid cells, and on lymphocytes. The pan T-cell marker CD2 could be observed on an average of 67 ± 16.3% of the lymphocytes. As observed on tissue sections, the percentage of CD4+ lymphocytes remained far below that of CD8+ lymphocytes. The mean values were 12 ± 7.3% and 73 ± 11.1%, respectively (Fig. 2).

DISCUSSION

The distribution of lymphoid cells within mucosal tissues is different for the various layers of the mucosa. This has been demonstrated for the human nose (Winther et al. 1987) as well as for other tissues (Van der Brugge-Gamelkoorn and Sminia 1985; Jeurissen et al. 1985). We have chosen in this study to investigate exclusively the epithelial layer of the nasal mucosa.

CD19+ cells (B cells) were not found in the epithelium, and plasma cells could not be recognized by routine histology. No screening for immunoglobulin-bearing cells was performed, but it seems reasonable to assume that B cells are hardly or not present within the epithelium. Although epithelial cells play an active role in the transport of soluble sIgA and sIgM into the external secretions, the immunoglobulin-producing plasma cells are particularly present in the lamina propria (Kutteh et al. 1982; Brandtzaeg 1984; Ernst et al. 1987; Mestecky and McGhee 1987).

CD4 expression was only slightly observed in the nasal biopsy specimens of the patients. In contrast, CD8 was present abundantly. As demonstrated on cytocentrifuge preparations, the vast majority of lymphocytes carried the CD8 antigen, thus showing that the infiltrated lymphocytes are mainly of the suppressor/cytotoxic phenotype. The fact that more clearly stained CD4+ cells occur in control tissues suggests an infection-related decrease of the helper population in favour of the suppressor cells. However, it should be considered that biopsies of normal epithelium, which is not

swollen, might contain some of the underlying tissue and thereby CD4+ cells. Recently, Winther et al. (1987) described the distribution of lymphocytes in the normal nasal mucosa. As in our study, they encountered problems in the quality of the epithelial layer in the specimens and also found far more T than B cells between epithelial cells. Furthermore, they found Leu-3+ (T helper/inducer) cells to predominate over Leu-2+ (T suppressor/cytotoxic) cells in the epithelium. They suggest that clinical disorders may lead to changes in the cellular contents of the nasal mucosa. This would be a plausible explanation for the discrepancy between their and our study. Nonetheless, the fact remains that we do not find a predominance of CD4+ cells in our control sections.

Another explanation for the discrepancy between the study of Winther et al. (1987) and the present investigation might be the possible expression of the CD4 (Leu-3a) antigen on human tissue macrophages (Hume et al. 1987) and on human monocytes (Moscicki et al. 1983). Although these findings were obtained on other tissues than the nasal mucosa, they may induce errors in counting CD4+ lymphocytes. It is more likely that these errors occur in counting on sections than in counting on cytocentrifuge preparations, since lymphocytes and macrophages can more easily be distinguished by morphologic criteria on single-cell preparations.

Our finding that T cells of the suppressor phenotype predominate in the epithelial compartment corresponds to the T-cell distribution found in the mucosa of the gut (Selby et al. 1981; Van der Heijden 1986; Ernst et al. 1987). It is known that T cells in the gut epithelium may not bear the pan T-cell markers (Ernst et al. 1987). Likewise, in our nasal biopsy specimens, not all T cells showed expression of CD2 (Fig. 2), which is a pan T-cell marker. As stated above, we found an absence of intra-epithelial B cells. This also is described for the gut mucosa. These findings are another indication for the existence of a common mucosal immune system, as described (McDermott and Bienenstock 1979; Mestecky 1987).

In the epithelial sections a marked staining with anti-HLA-DR monoclonal antibodies was demonstrated, both in samples of patients and controls. HLA-DR molecules play an important role in the immunoregulation (Brandtzaeg 1984). HLA-DR+ macrophages can take up foreign antigens and present them to T helper/inducer cells as well as to B cells. Thus, the population of HLA-DR+ macrophages may include antigen-presenting cells (Sertl et al. 1986). Antigen-presenting cells in the skin are often recognized as Langerhans cells. As demonstrated in animal experiments, they originate in the bone marrow (Frelinger et al. 1979; Katz et al. 1979) and migrate to the epidermis where they can take up antigen (Silberberg-Sinakin et al. 1976). Recently dendritic cells in the human bronchial epithelium have been shown to contain Birbeck granules, which is characteristic of Langerhans cells (Richard et al. 1987). Electron microscopic studies will be needed to determine whether the HLA-DR+ cells in the nasal epithelium also include Langerhans cells. In addition to playing a role in antigen presentation, a subpopulation of antigen-presenting cells, the dendritic cells, can probably directly activate CD8+ cytotoxic cells. In the mouse this was demonstrated by Inaba et al. (1987). Moreover, as described by Mayer and Shlien (1987), human CD8+ suppressor cells can be activated by HLA-DR+ (Ia+) gut epithelial cells. Whether these ways of lymphocyte activation are relevant with respect to our finding of particularly CD8+ lymphocytes in close proximity to HLA-DR+ cells is not known yet. It would be reasonable to assume that HLA-DR+ epithelial cells are involved in antigen uptake, which has been shown for lympho-epithelial tissues in rat bronchus-associated lymphoid tissue (Van der Brugge-Gamelkoorn et al. 1985) and in human gut-associated lymphoid tissue (Owen and Jones 1974; Mayer and Shlien 1987).

We have studied the epithelial layer of the nasal mucosa. In order to obtain a better insight into the immunologic reactions in the nasal mucosa, it is indispensable to investigate the lamina propria as well. Therefore, deeper biopsies will now be studied. This will enable us to compare the epithelial compartment with the deeper layers of the nasal mucosa and to study the relations between the layers.

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IMMUNOHISTOCHEMICAL CHARACTERIZATION OF LEUKOCYTES IN THE NASAL MUCOSA OF EAR, NOSE AND THROAT PATIENTS AND CONTROLS

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T. Sminia, S. van der Baan, J. Biewenga

SUMMARY. The distribution of lymphoid and non-lymphoid cells in the human nasal mucosa of ear, nose and throat patients and controls was studied by immuno- and enzyme-histochemistry as well as by routine histology. In patient samples squamous epithelium instead of respiratory columnar epithelium was regularly seen. CD8+ lymphocytes always outnumbered CD4+ lymphocytes, most strikingly so in the epithelial compartment of patient samples and in both patient and control glandular tissue. B cells were scarce. Both in immunohistochemical and routine histological stainings plasma cells were frequently seen underneath the epithelium and to a larger extent around the glands. The main isotype was IgA. The HLA-DR antigen was found on round cells and cells which extended dendritic processes. Also epithelial cells, both superficial and glandular, were HLA-DR+. The latter were more frequently observed in patient samples. In both patient and control samples clusters of lymphoid cells occurred. High endothelial venules were occasionally seen adjacent to large clusters.

INTRODUCTION

Nasal symptoms may reflect upper, but also lower respiratory tract phenomena. Rhinitis is e.g. frequently associated with asthma (Cole and Stanley 1983). Therefore, nasal defence mechanisms and the various cell populations within the nasal mucosa are important when studying the defence of the respiratory tract.

A wide choice of monoclonal antibodies is available for the recognition of specific cell markers on a variety of cells, among which T and B cells (Shaw 1987; Zola 1987). Leukocyte markers have been used extensively to describe lymphocytes and their subsets in the human intestinal mucosa (Selby et al. 1981; Cerf-Bensussan et al. 1983; Trejdosiewicz et al. 1987; Smart et al. 1988).

Holt et al. (1988) analysed the lymphocytes in lung tissue. Most immunohistological studies on the human nasal mucosa focussed on immunoglobulins and immunoglobulin-producing cells, in healthy and immunodeficient persons (Brandtzaeg 1985; Brandtzaeg et al. 1986, 1987; Karlsson et al. 1987). In 1987, Winther et al. described the distribution of T and B lymphocytes and their subsets in the normal nasal mucosa, while Nishimoto et al. (1988) did so for the inflamed maxillary mucosa. Ganzer and Bachert (1988) studied the lymphocyte populations in atopic nasal mucosa.

The first site of defence against airborne antigens is formed by the nasal epithelium. To apprehend the nasal defence mechanisms lymphocytes and non-lymphoid cells in the superficial nasal epithelium of healthy volunteers and of ear, nose and throat patients were described previously (Hameleers et al. 1989). Subsequently, the connective tissue compartment and the glandular area are described in the present study.

MATERIALS AND METHODS

Subjects

Twenty-three patients (12 women, 11 men; mean age 29 years, range 17-54) visiting the polyclinics of allergology at the Department of Oto-rhino-laryngology of the Academic Hospital of the Vrije Universiteit, Amsterdam, are included in this study. All had nasal complaints (e.g., nasal obstruction, rhinorrhoea, sneezing) and underwent an extensive routine screening for allergy (e.g., skin tests, serum IgE, radioallergosorbent test). Six of them were diagnosed as allergic. None had used locally applied corticosteroids during at least 6 weeks before entering the study. Patient data are listed in Table 1.

Nineteen patients (13 women, 6 men; mean age 27 years, range 17-51) of the Department of Maxillo Facial Surgery, undergoing an osteotomy, served as controls, by consent. None had nasal complaints or had had a common cold within 6 weeks

Table 1. Clinical data on the patients included in the study

Patient No.	Sex	Age (years)	Positive skin test for[a]	Serum IgE (IU/l)	Positive RAST for[b]
1	f	31	cat, dog	48	-
2	f	27	-	32	-
3*	m	19	-	1159	house dust mite, mite
4	f	42	-	15	-
5	m	46	-	19	timothy
6	m	30	-	44	-
7*	f	20	cat	117	cat, mugwort, ryegrass
8	m	23	-	<5	-
9	f	50	-	13	-
10*	m	38	house dust mite, mugwort waybread	451	meal mite, ryegrass, timothy
11	f	19	-	332	-
12	f	54	-	19	-
13*	f	24	house dust mite	127	mite
14	m	20	-	192	-
15	f	20	-	<5	-
16	f	35	-	7	-
17	f	19	-	5	ryegrass
18	m	32	-	36	-
19*	f	20	rye, timothy, Yorkshire fog	312	mite, ryegrass, timothy
20	m	17	-	<5	-
21	m	37	-	150	-
22*	m	18	cat, Cladosporium Herbarum ryegrass, timothy	220	cat, ryegrass, timothy
23	m	21	-	25	-

* diagnosed as allergic; [a] Positive skin test: diameter of skin eruption >2mm; [b] Positive RAST: ≥ 2 IU

prior to the biopsy taking. All were non-smokers and had a normal aspect of the nasal mucosa.

Tissue sections and immune reagents

Biopsy specimens were taken from the lateral part of the inferior turbinate, 1.5 cm behind the anterior part. The biopsies were immediately embedded in OCT tissue compound (Miles, Elkhart, Ind., USA) and frozen in liquid nitrogen. Cryostat sections (8 µm thick) were picked up on gelatin-coated slides and dried above silica gel.

Table 2. Mouse monoclonal antibodies against human cell antigens used in this study

antibody	obtained from	specificity
anti-CD2	1	all peripheral T cells, 90% of the thymocytes
anti-CD4	2	helper/inducer T cells and subpopulations of macrophages
anti-CD8	2	suppressor/cytotoxic T cells
anti-CD19	3	precursor and mature B cells (no plasma cells)
anti-CD22	1	all B cells (no plasma cells)
anti-HLA-DR	1	cells of the monocyte lineage, myeloblasts, promyelocytes and cells of the B-lymphocyte lineage
194-5.1	4	human IgA (Biewenga et al. 1986)
anti-IgG	5	human IgG
anti-IgM	5	human IgM
anti-IgE	5	human IgE

1: CLB; Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, The Netherlands. 2: Sanbio Biological Products, Uden, The Netherlands. 3: Dakopatts, Glostrup, Denmark. 4: Medical Biological Laboratory, Division of Health Research, TNO, Rijswijk, The Netherlands. 5: Nordic Immunological Laboratories, Tilburg, The Netherlands.

The monoclonal antibodies used (Table 2) were appropriately diluted in 0.01M phosphate buffered saline (PBS), pH 7.4, containing 0.5% bovine serum albumin. Horseradish-peroxidase-labelled rabbit anti-mouse Ig (Dakopatts, Glostrup, Denmark) was used as the conjugate. This was diluted in PBS with 0.5% bovine serum albumin and 1% normal human serum.

Immuno- and enzyme-histochemistry

The sections were fixed in pure acetone for 10 min and incubated with the antibody for 1 hr. After thorough washing in PBS, the preparations were incubated with the conjugate for 60 min, washed again in PBS, and stained for peroxidase activity with 3,3'-diaminobenzidine-tetrahydrochloride (DAB, Sigma, St. Louis, Mo, USA) at a concentration of 0.5 mg/ml in TRIS-HCl buffer, pH 7.6, containing 0.03% H_2O_2. The staining was intensified by rinsing the preparations in distilled water and in 0.9% NaCl, followed by incubation for 15 min in 0.5% $CuSO_4$ in 0.9% NaCl. After being washed in distilled water, the sections were lightly counterstained with Mayer's haematoxylin. Finally, the sections were dehydrated, cleared and mounted in Entellan (Merck, Darmstadt, FRG). The entire procedure was carried out at room temperature.

Control slides for non-specific staining were incubated in the same way, omitting the first-step monoclonal antibody. Tonsil sections were used as positive controls. For routine histology methylgreen-pyronin or haematoxylin and eosin stainings were

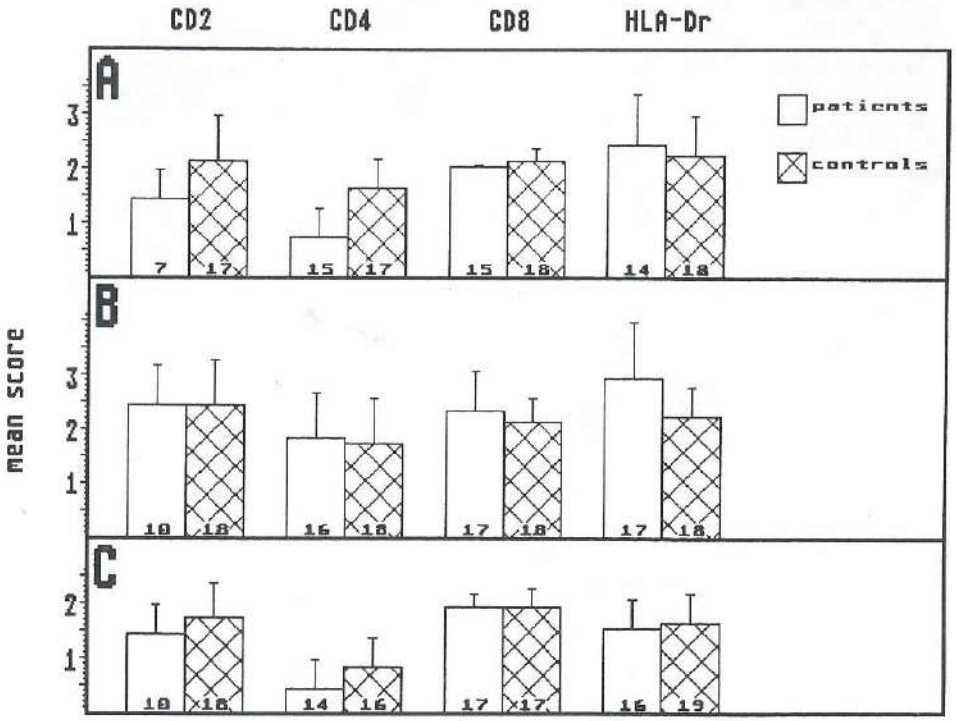

Fig. 1. Mean scores ± SD of CD2, CD4, CD8 and HLA-DR in the nasal mucosa of patients and controls in the superficial epithelium (**A**), the subepithelial connective tissue (**B**) and in the glandular tissue (**C**). The number of biopsy samples is given in the bars.

performed. Acid phosphatase (AcPh) activity was demonstrated according to Burstone (Pearse 1968), using naphthol-AS-BI phosphate (Sigma) as the substrate and hexazotized pararosaniline as the diazonium salt.

Evaluation

The proportion of positive cells detected in the sections was graded on a 0 to 4 scale (0 = no positive cells, 4 = many positive cells). Only the scores of corresponding compartments are comparable. Mean scores were calculated separately for superficial epithelium, subepithelial connective tissue and glandular tissue (Fig. 1). The tonsil sections showed specific staining by each of the monoclonal anibodies. Samples that showed substantial non-specific staining except for granulocytes were excluded from the study.

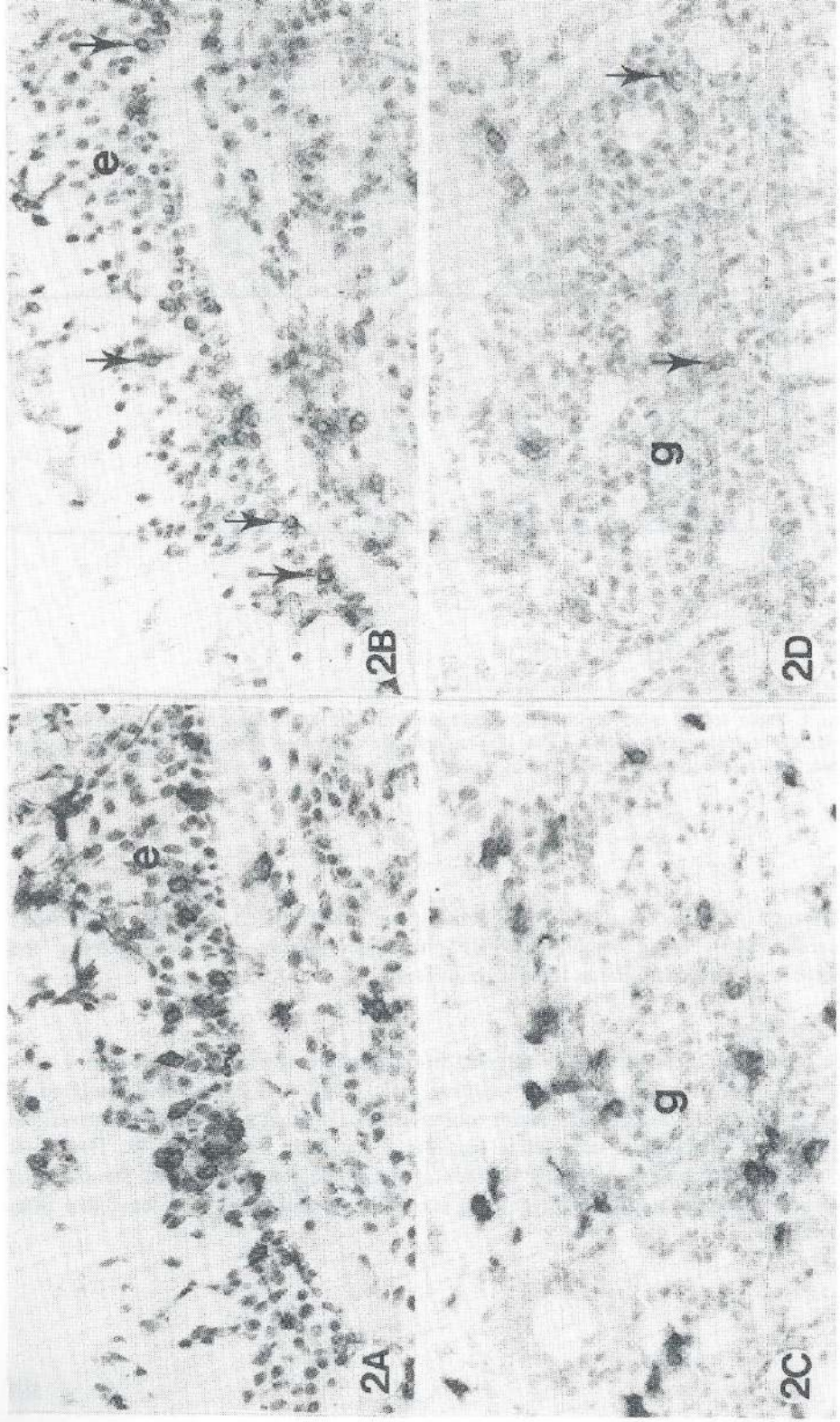

Table 3. Ratios of CD8- and CD4-scores (CD8/CD4) for the various compartments of the human nasal mucosa, in patient and control tissue

compartment	patients	controls
epithelial compartment	2.9	1.3
subepithelial connective tissue	1.3	1.2
glandular tissue	4.8	2.4

RESULTS

In the tissue of 6 patients and 1 control at least the upper part of the epithelium was damaged. Thus, the type of epithelium was not determined. Especially in routine histological stainings it was noticed that 9 of the remaining 17 patients (53%) had epithelium of the non-keratinizing stratified squamous type, whereas the other patient and all control samples showed respiratory columnar epithelium. Biopsies were, however, taken from the same area of the inferior turbinate. Two controls also showed squamous epithelium, in another part of the section. Methylgreen-pyronin staining clearly distinguished patient and control tissues by conspicuously scarlet staining of the abundant mucus in patient tissue.

AcPh+ cells (other than superficial and glandular epithelial cells) occurred throughout the nasal mucosa. They were mainly located in the upper part of the subepithelial area and to a lesser extent deeper in the mucosa. In general the number of lymphoid and non-lymphoid cells decreased from the subepithelium to the deeper layers of the nasal mucosa.

Superficial epithelium

Lymphocytes were found in the epithelial layer of all samples and were varyingly positive for CD2. The intra-epithelial CD8+ (T suppressor/cytotoxic) cells outnumbered CD4+ (T helper/inducer) cells (Fig. 2A,B). In patient epithelia the ratio CD8/CD4 was 2.9 and in control sections this was 1.3 (Table 3). A larger proportion

Fig. 2. Serial sections of the nasal mucosa. CD8+ (suppressor/cytotoxic) T cells (**A**) and (**C**) outnumbered CD4+ (helper/inducer) T cells (**B**) and (**D**), in the superficial epithelium and in the glandular tissue. (**A**) and (**B**): superficial epithelium in control tissue, (**C**) and (**D**): glandular area in patient tissue. Arrows indicate CD4+ cells. *e* epithelium, *g* glandular tissue. x400.

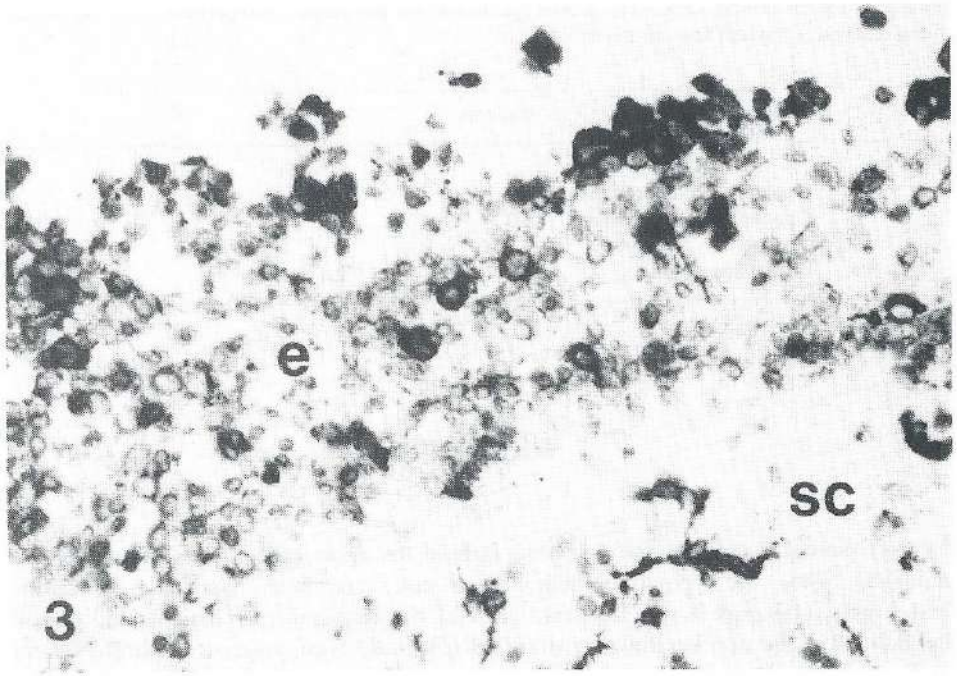

Fig. 3. HLA-DR expression in superficial nasal epithelium of a patient. *e* epithelium, *sc* subepithelial connective tissue. x400.

of the CD4+ than the CD8+ cells was located in the basal layers. The latter were usually found throughout the epithelium. CD19+ B cells were not seen in the epithelium, while hardly any CD22+ B cell was detected. Several epithelial cells stained with the anti-IgA monoclonal antibody. The HLA-DR antigen (Fig. 3) was found within the epithelial layer on round cells, on cells with a dendritic appearance and sometimes also on epithelial cells. In patient sections many round HLA-DR+ cells were seen, while in control tissue relatively more HLA-DR+ cells had a dendritic morphology.

Subepithelial connective tissue

In both patients and controls the subepithelial connective tissue contained many CD2+ (T) cells. Although the difference was small, slightly more CD8+ than CD4+ cells were detected. Some CD22+ B cells were present. However, hardly any CD19+ cell was seen. Plasma cells occurred underneath the superficial epithelium and around the glands, as described below. HLA-DR+ cells were abundantly present, comprising round cells and cells with processes. As in the epithelium, control tissue appeared to contain relatively more of the latter cells. In the subepithelial layer clusters of

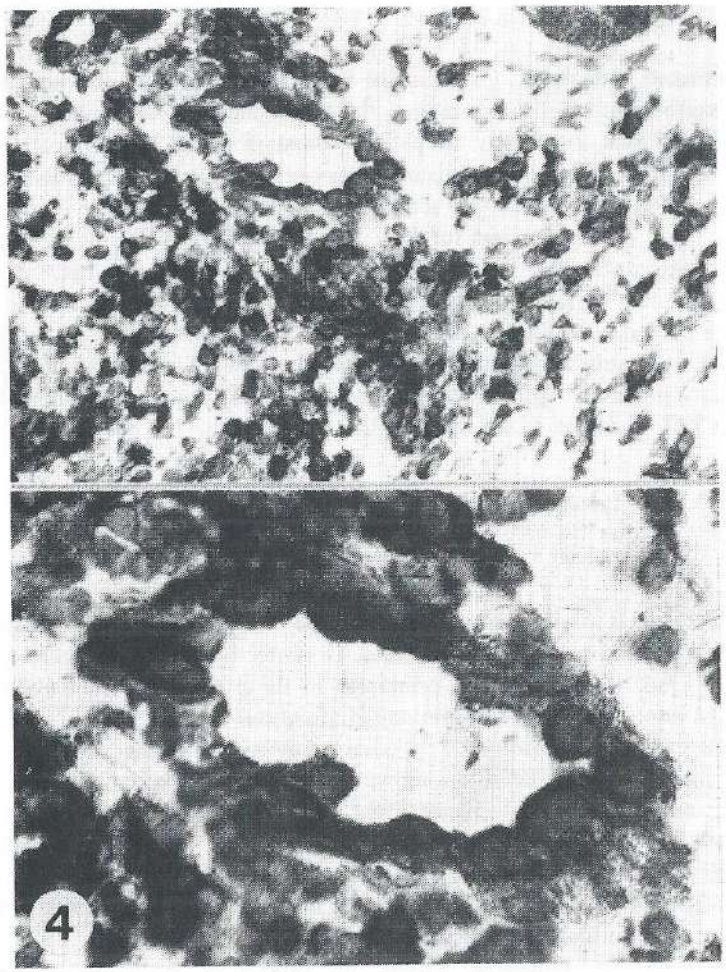

Fig. 4. High endothelial venules (HLA-DR+) were occasionally seen adjacent to large lymphocyte clusters. Patient section. x400 and x1000.

lymphocytes were seen in almost 50% of the samples. This applied to both patient and control tissues, as did the observation that HLA-DR+ high endothelial venules (HEV) were occasionally seen adjacent to large clusters (Fig. 4). In 5 out of 6 clusters in controls CD4 was the predominant surface marker, while in 1 out of 5 clusters in patient tissue CD4 predominated.

Glandular epithelium and adjacent connective tissue

In all samples CD2+ cells occurred in the glandular tissue. Also in this area CD8 was

the predominant phenotype. CD8+ cells were found adjacent to and within the glandular epithelium. Cells expressing the CD4 antigen were scarce within the glandular formations, especially in patient tissue (Fig. 2C,D). Almost no B cells (CD22+) were found. By routine histology as well as immunohistochemistry plasma cells were observed around the glands in higher density than underneath the epithelium. They mostly stained for IgA, but IgG and IgM producing plasma cells occurred as well. In patient tissue but not in control tissue a few IgE+ plasma cells were detected. The surface of glandular epithelial cells regularly stained with the anti-IgA monoclonal antibody. HLA-DR+ cells were sparse, but occurred in the connective tissue around glandular formations. In 16% (3 out of 19) of the control samples many glandular epithelial cells were HLA-DR+, while in patient tissue this occurred in 29% (5 out of 17) of the samples.

DISCUSSION

An important function of the nasal mucosa, in which several immunological and non-immunological mechanisms cooperate, is protection against inhaled pathogens. Brandtzaeg (1984) distinguished 3 principles in these mechanisms, namely immune exclusion, immune regulation and immune elimination.

Immune exclusion is reinforced by ciliary movements. In this respect it should be mentioned that patient tissue often showed squamous epithelium instead of ciliary respiratory epithelium. Most likely, this is a secondary and not a primary phenomenon. Once the epithelium has become squamous, its cleaning capabilities are severely impaired. Penetration of particles and subsequent inflammatory processes probably induce chronic rhinitis, which results in further damage of ciliary respiratory epithelium and the formation of squamous epithelium.

Lymphocytes are numerous within the superficial epithelium of the nasal mucosa. They are mainly of the CD8 phenotype. This is consistent with other studies, concerning the mucosa of the upper respiratory tract (Nishimoto et al. 1988; Hameleers et al. 1989; Stoop et al. 1990) and the gut (Cerf-Bensussan et al. 1983; Van der Heijden 1986; Austin and Dobbins 1988). The population of CD8+ T cells comprises suppressor T cells and cytotoxic T cells. The former have a down-regulating effect and thereby may protect the nasal mucosa from damage by inflammatory reactions. On the other hand, CD8+ cytotoxic T cells are able to kill virus infected epithelial cells. As a result deleterious agents are released and can damage the epithelium. This could be an explanation of the transition of respiratory epithelium into squamous epithelium in the patient tissue. Such mechanism suggests that at least at the onset of chronic rhinitis, the CD8+ population in the nasal epithelium is composed of cytotoxic rather than suppressor T cells.

Winther et al. (1987) found more intra-epithelial T than B cells and a predominance of T helper cells in the superficial nasal epithelium of healthy persons.

More than the previously suggested explanations, viz. the different origin of the tissues and the differences in evaluation (Hameleers et al. 1989), technical factors are likely to account for the discrepancy between their and our results. In this respect, Kootte et al. (1988) reported different results with monoclonal antibodies against identical markers.

The strongly HLA-DR+ cells with cell processes are most likely involved in antigen presentation (Sertl et al. 1986). In skin dendritic antigen presenting cells are recognized as Langerhans cells which originate in the bone marrow (Frelinger et al. 1979; Katz et al. 1979). They contain characteristic Birbeck granules and have also been recognized in human bronchial (Richard et al. 1987), buccal (Cruchley et al. 1987) and nasal mucosa (Fokkens et al. 1989, Fokkens et al. submitted), where they simultaneously expressed the CD1 and HLA-DR markers.

The round HLA-DR+ cells present in the superficial epithelium are not likely to be B cells, as no CD19 and hardly any CD22 expression is found. Under certain circumstances HLA-DRw antigens are detected on human T cells (Metzgar et al. 1979). The round HLA-DR+ cells could reasonably be classified as activated T cells or also as epithelial cells. The presence of HLA-DR+ epithelial cells is in accordance with previous observations (Hameleers et al. 1989).

The few B cells seen in the present study were mainly located in the subepithelial connective tissue. Plasma cells were clearly present, underneath the epithelium but to a larger extent around the glands. Thus, of the B-cell lineage the plasma cell prevails. This suggests that activated B cells migrate from distant tissues into the nasal mucosa. They possibly originate from the lungs (BALT) or from the tonsillar tissue (Brandtzaeg 1985; Brandtzaeg et al. 1986). The immunoglobulins produced and required in immune exclusion (Brandtzaeg 1984, 1985), are predominantly of the IgA isotype, but IgG and IgM are produced as well (Brandtzaeg 1984, 1985; Brandtzaeg and Korsrud 1984). Of the nasal IgA producing cells, 96% forms IgA1 (Kett et al. 1986). Since IgA in secretions has been transported through glandular or superficial epithelial cells, it is not surprising that these cells stain with the anti-IgA monoclonal antibody. Immunoglobulins are preferentially secreted via the serous glandular cells (Brandtzaeg 1984). The observation that patient tissue shows relatively more mucous secretion, might imply a decrease in the contribution of serous cells. Immunoglobulin concentrations in secretions will then be diminished, and the humoral defence will be impaired. As mentioned for the epithelial transition, this again is a self-enhancing phenomenon, since defective humoral immunity is followed by inflammatory reactions, e.g. mucous secretion.

HLA-DR expression on glandular epithelial cells has been described in Sjögren's syndrome (Lindahl et al. 1985). HLA-DR+ glandular cells are also observed in the nasal specimens. A correlation with chronic inflammatory disease is suggested, as glandular HLA-DR expression occurs almost twice as much in patient tissue as in control tissue, 29% and 16% respectively. Inhaled antigen rarely reaches the glandular acini. Therefore, the HLA-DR antigen must be induced by cells of the nasal mucosa or by their products. A possible candidate would be interferon-gamma (IFNγ),

produced by sensitized T lymphocytes after antigen exposure. IFNγ induced HLA-DR expression has been shown for colonic epithelial cells (Pallone et al. 1988) and endothelial cells (Pober et al. 1983). Thus the HLA-DR expression on high endothelial venules, adjacent to lymphoid clusters, may also be due to IFNγ.

It has been reported that the epithelium in allergic rhinitis is more permeable than in chronic sinusitis (Inagaki et al. 1985). Based on clinical and (immuno)histological findings of our patients no reasonable subgroups can be distinguished. In conclusion, the major differences between patients and controls are that in patient material:

- The epithelium is often of the squamous instead of the respiratory type.
- A smaller number of CD4+ T cells is observed in the superficial and glandular epithelium.
- The glandular activity is augmented and presumably more mucous secretion occurs.
- HLA-DR expression on glandular cells occurs more frequently.

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Chapter 6

GENERAL DISCUSSION

The internal milieu is separated from the external milieu by skin or mucosal membranes. These surfaces fulfil different functions. The gut-mucosa is primarily involved in the uptake of nutrients from the gut lumen and the function of the respiratory tract mucosa is merely to exchange gases. Potentially harmful substances must be excluded from the interior body. Whereas the skin provides a firm physical line of defence, the mucosae can not count on their physical barrier, despite the tightly joined epithelial cells. Therefore, the mucosal lining is supplied by auxiliary mechanisms. These can be divided into an aspecific system, such as cilia and mucous blanket on top of the mucosa and a specific system composed of lymphoid as well as non-lymphoid immunocompetent cells in the mucosal tissues. At several sites these cells form more or less structured accumulations of mucosa-associated lymphoid tissue (MALT). In the respiratory tract bronchus-associated lymphoid tissue (BALT; Bienenstock et al. 1973a, 1973b) occurs in the lungs as reviewed by Sminia et al. (1989). The upper respiratory tract contains lymphoid accumulations mainly in the nasopharyngeal region. In the rat well-defined lymphoid tissue occurs at the entrance of the nasopharyngeal duct (NALT; Spit et al. 1989; chapter 3). It is regarded as an equivalent of Waldeyer's ring in man (WRE; De Jong et al. 1985, Koornstra et al. 1987, 1989). The latter is more extended by comprising a ring of lymphoid accumulations in the nasopharyngeal area. Furthermore, the nasal mucosa is provided with dispersed lymphoid and non-lymphoid cells. Clusters of lymphocytes occur frequently.

In this chapter the experimental data obtained in the separate studies of this thesis on rat and human nasal mucosa will be discussed in relation to relevant literature data.

6.1. Structure and development of rat NALT

6.1.1. Occurrence and anatomy of NALT

NALT is a paired organ which is situated in the floor of the nasal cavity, at the entrance of the nasopharyngeal duct (chapter 1: Fig. 4). Similar to BALT and Peyer's patches (PP), NALT is situated immediately underneath the epithelium. This thesis concerns NALT in rats, but nasal lymphoid tissue also occurs in other mammalian species, as mentioned above for Waldeyer's ring in man. The equine respiratory tract contains many dispersed nodules of lymphoid tissue in the nasal cavity and pharynx, collectively denoted NALT. Also laryngeal and tracheal-associated lymphoid tissues occur, whereas bronchus-associated lymphoid tissue (BALT) is infrequently encountered in horses (Mair et al. 1987, 1988). In the nasopharynx of the bonnet monkey also several aggregations of lymphoid cells were observed (Harkema et al. 1987).

Thus, the nasopharyngeal lymphoid accumulations occurring in several species differ in extent. Interstrain differences also exist. In NALT of Wistar rats, used in the presented experiments, few germinal centres are seen, while germinal centres occur more frequently in NALT of Lewis rats (Koornstra, personal communication), and other strains (Brown Norway, PVG; own observations). As for PVG rats, the presence of germinal centres may reflect their susceptibility to middle ear infections, because this may indicate an immunologically active upper respiratory tract mucosa.

6.1.2. Ontogeny of NALT

The components of MALT arise at different stages in ontogeny. The first to be seen are PP which develop before birth (Sminia et al. 1983, Spencer et al. 1986). In the respiratory tract, lymphoid tissue arises at birth when NALT becomes visible. Its postnatal development is described in chapter 3.1. Newborn NALT consists of mainly T cells. This is also true for PP both in man (Spencer et al. 1986) and rat (Sminia et al. 1983). In contrast, BALT arises as a condensation of merely B cells and is first seen in lungs of 4-day-old rats (Plesch et al. 1983). Since the onset of breathing at birth implies a vast antigenic challenge, an immunological role for NALT is thus indicated.

NALT exhibits a unique feature by comprising clearly stained ED3+ macrophages during its development. Normally this marker is seen on a particular macrophage subpopulation in lymphoid organs as spleen and lymph nodes but not in the mucosal tissues BALT and PP (Dijkstra et al. 1985, Van der Brugge-Gamelkoorn et al. 1985a, Beelen et al. 1987). ED3+ macrophages also occur in pathological conditions in the central nervous system (Polman et al. 1986) and in arthritic knee joints (Verschure et al. 1989) and *in vitro* after induction by cytokines, derived from activated T cells (Damoiseaux et al. 1989). In adult NALT the ED3 expression was restricted to cells localized within the border area between the lymphoid tissue and the surrounding connective tissue (chapter 3.2), while in the first postnatal week ED3+ macrophages were observed throughout NALT (chapter 3.1). New antigens are encountered in early postnatal life. These may induce activated T cells. As a consequence cytokines will be released, which can induce ED3 expression. A function of ED3+ cells in the establishment of the microenvironment of NALT has been considered (chapter 3.1).

The aforementioned finding of an earlier development of NALT than BALT is also expressed in the order in which distinct T- and B-cell areas occur. In NALT these were found by 10 days post-partum (chapter 3.1) and in BALT more than 2 weeks later (Plesch et al. 1983). Remarkably, the reverse is seen in the chicken, where distinct T- and B-cell areas arise in BALT before they are seen in the Harderian gland (Jeurissen et al. 1989) which serves the upper respiratory tract. This is probably related to the fact that chickens are no obligatory nose-breathers, as rats are. In horses which are obligatory nose-breathers, NALT precedes BALT in ontogeny. The former is present before birth. Moreover, at all ages equine NALT is far more extended than equine BALT (Mair et al. 1988).

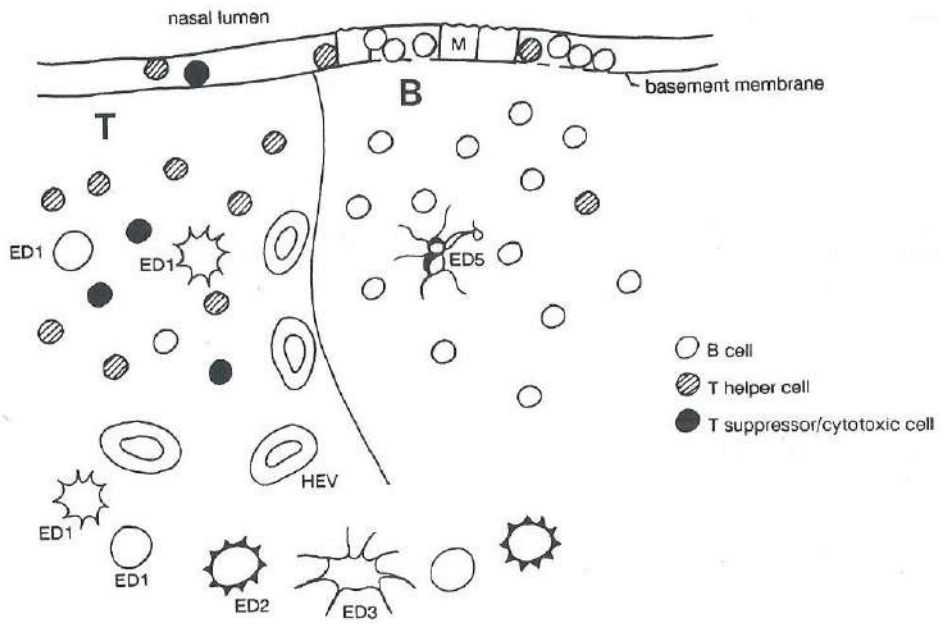

Fig. 1. Cellular distribution in rat NALT. B: B-cell area; T: T-cell area; M: M-cell; HEV: high endothelial venule

6.1.3. Cellular composition of adult NALT

Within the reticular network of NALT, lymphoid and non-lymphoid cells were found (Fig. 1). Once established, NALT contains T and B cells in about equal numbers. The cell ratios as determined on tissue sections of Wistar-rat NALT (chapter 3.2) were confirmed on isolated Lewis-rat NALT cells by FACS analysis (Koornstra et al. submitted), which showed the ratio T/B = 0.9. In this respect NALT and BALT are comparable. The T/B ratio in BALT = 0.7 (Van der Brugge-Gamelkoorn and Sminia 1985). Differences among mucosal lymphoid tissues in the ratios of lymphocyte subpopulations are summarized in Table 1. To compare, the T/B ratios in peripheral lymph nodes and spleen are 1.5 and 0.5, respectively.

Both adult NALT and BALT contain slightly more B than T cells and resemble the mesenteric lymph node (MLN) in this respect, where B and T cells occur in equal numbers. The lymphoid tissues in the respiratory tract are thus different from PP, because the vast majority of PP lymphocytes are B cells. T/B ratios strongly correlate

Table 1. Lymphocyte subpopulations in mucosal lymphoid tissues

	T/B	Th/Ts
NALT	0.9	2.4
BALT	0.7	2.6
PP	0.2	5.0
MLN	1.0	1.7

(Data obtained from chapter 3.2, Jeurissen et al. 1984, Kraal et al. 1985a, Van der Brugge-Gamelkoorn and Sminia 1985)

with the specificity pattern of T-and B-cell adherence to high endothelial venules (HEV). T and B cells show more or less equal affinity for HEV in BALT and MLN, while B cells show more affinity for HEV in PP than T cells (Van der Brugge-Gamelkoorn and Kraal 1985). *In vitro* data on lymphocyte binding to HEV in NALT can be expected soon (Koornstra et al. in preparation). In line with the aforementioned data it seems reasonable to expect that also in NALT the pattern of lymphocyte adherence to HEV can be deduced from the T/B ratio, being about 1.

As for the non-lymphoid cells, attention should be paid to some macrophage subpopulations. As mentioned in section 6.1.2, NALT contains clearly stained ED3+ cells during the first postnatal week. Later these migrate towards the border area of NALT and the surrounding connective tissue. By then the ED3+ macrophages are found in a broad zone where besides plasma cells, also ED1+ and ED2+ cells occur (chapter 3.2). This combination of cells also occurs in the outer periarteriolar lymphoid sheat (PALS) in the spleen. The ED2+ cells in spleen are located at the site where antibody formation takes place. A similar coincidence in localization was seen in the gut and lung. ED2+ macrophages occur in the villi (Dijkstra et al. 1985, Sminia and Jeurissen 1986), in the periphery of BALT and perivascularly as well as peribronchially in the lung (Dijkstra et al. 1985, Van der Brugge-Gamelkoorn et al. 1985a). In the same areas numerous plasma cells are found in the gut (Sminia and Plesch 1982) and lung (Van der Brugge-Gamelkoorn et al. 1985b). Moreover, in the oral mucosa of rats ED2+ cells are observed around glandular acini among, mainly IgA producing, plasma cells (Swart et al. in preparation). Consequently, the preferred localization of ED2+ cells at the periphery of NALT is suggestive of a role of these macrophages in plasma cell maturation (chapter 4.1). Recently such role was suggested for splenic ED2+ macrophages (Matsuno et al. 1989). Probably these ED2+ macrophages act through T cells. A role for macrophages in the maturation of thymocytes has been suggested (Beller and Unanue 1978, Sminia et al. 1986). This concerned cortical macrophages, which appeared to carry the ED2 antigen (Dijkstra et al. 1985, Sminia et al. 1986).

Another striking feature of NALT is the presence of ED5+ cells (chapter 3.2), which are absent in BALT. Since ED5 is a marker of follicular dendritic cells (Jeurissen and Dijkstra 1986), this is a further indication that in the steady state the upper respiratory tract is immunologically more activated than the lower respiratory tract (see also chapter 3.2), despite the fact that germinal centres are infrequent in NALT.

6.2. Functional aspects of rat NALT

6.2.1. Humoral immunity

Humoral immunity in the mucosa is to a large extent supplied by secretory immunoglobulin A. In humans 30-100 mg of secretory IgA is produced daily per kg of bodyweight (Mestecky and McGhee 1987, Russell and Mestecky 1988), whereas the daily production of serum IgA amounts to 18.5-30 mg per kg of bodyweight (Mestecky et al. 1986). Secretory IgA is produced as an 11S dimer, complexed with J-chain, by plasma cells residing in the mucosal tissues adjacent to epithelial cells. The complex of IgA plus J-chain is picked up by secretory component (SC) which acts as a receptor on epithelial cells. The entire complex is then transported through the epithelial cell and excreted into the lumen. This translocation of immunoglobulins across epithelial membranes is reviewed by Brandtzaeg (1987). In the gut and lung plasma cells are present underneath the luminal epithelium, which expresses SC, while in the oral and nasal cavities IgA plasma cells are largely restricted to the area around the glandular acini (Brandtzaeg 1984, Lacasse et al. 1987). The glandular epithelium in the human nasal mucosa as well as the duct epithelium were shown to contain SC and thus participate in the translocation of secretory IgA to the external environment (chapter 1: Fig. 1, Brandtzaeg 1984). Consequently, secretory IgA is a component of the nasal secretion.

Secretory IgA is the predominant immunoglobulin in secretions, whereas serum IgA forms only 15-20% of all serum immunoglobulins. Moreover, serum IgA is synthesized largely in the bone marrow and consists of a 7S monomer. It has been concluded that serum IgA acts anti-inflammatory, e.g. by inhibiting IgG mediated complement activation (Russell et al. 1989). Serum IgA helps to protect the internal milieu from detrimental immunological reactions (Mestecky et al. 1986, Russell et al. 1989). With respect to antibody formation a compartmentalization in mucosal and systemic immunity is justified because of differences in components and in reactivity. Systemic immunization may not lead to specific antibodies in the mucosae and mucosal immunization may not result in serum antibodies (Russell and Mestecky 1988). Moreover, secretory IgA antibody, and not specific serum IgG, was shown in mice to provide mucosal protection against systemic infection with group A *Streptococci*. However, the specific serum IgG displayed more bactericidal activity than the secretory IgA antibodies (Bessen and Fischetti 1988). The importance of sIgA explains the vast amount of IgA producing cells in the mucosae. In the gut the great majority of the plasma cells contain IgA, as shown in rat (Sminia and Plesch 1982) and man (Van Spreeuwel et al. 1982). Underneath the BALT epithelium IgA is the

major produced isotype (Komatsu et al. 1980). In contrast, rat NALT houses few IgA producing lymphocytes. Less than 1% of the B cells bear surface IgA. The majority of Ig bearing cells were found positive for IgM, as shown in chapter 3.2. Although IgM bearing cells can switch to IgA bearing cells, the low proportion of IgA bearing cells in NALT combined with the scarcity of IgA plasma cells suggest that NALT itself does not have a major function in IgA secretion. This would strengthen the suggestion that the various components of MALT fulfil different functions. More evidence with respect to the secretory function of NALT can be obtained by determining whether the epithelium covering NALT expresses SC. The presence of IgA in NALT epithelium, either bound to epithelial cells or intercellularly, is an indirect indication for the presence of SC, since SC acts as a receptor for J-chain containing polymeric IgA. The intra-epithelially detected IgA is not evenly distributed which suggests that the follicle-associated epithelium of NALT is devoid of SC, as has been shown for both PP (Pappo and Owen 1988, Bjerke and Brandtzaeg 1988) and BALT (Gehrke and Pabst 1990). Thus the actual external transport of sIgA is not mediated by the follicle-associated epithelium. Other parts of the epithelium, including the glandular epithelium, will be involved in the transcellular transport of secretory IgA as found in nasal secretions.

6.2.2. Lymphocyte migration

Despite many differences, the components of MALT cooperate in surveying the mucosal membranes. Adequate communication between the tissues is obtained by recirculating lymphocytes. Antigenic stimulation of MALT can lead to the formation of lymphoblasts and memory cells. Lymphocytes of MALT migrate through the lymph and blood stream, respectively, to repopulate preferentially mucosal sites (Bienenstock and Befus 1980, Brandtzaeg 1988, Duijvestijn and Hamann 1989). Specific receptors on lymphocytes as well as on high endothelial cells of specialized post-capillary venules control the preferential extravasation of the cells (Kraal et al. 1987, Streeter et al. 1988, Duijvestijn and Hamman 1989). Koornstra and coworkers (submitted) showed that a higher proportion of lymphoid cells from mesenteric lymph nodes migrate to mucosal lymphoid tissues than do lymphocytes from NALT (called by the authors WRE: Waldeyer's ring equivalent). In turn, a higher proportion of NALT lymphocytes migrate to mucosal lymphoid tissues than do lymphoid cells derived from PP. The finding that cells primed at one mucosal site seed remote mucosal tissues, led in the seventies to the concept of a common mucosal immune system (Bienenstock 1974, McDermott and Bienenstock 1979). Several tissues have since been shown to be part of this common mucosal immune system, whereby a central role has been attributed to PP. Intestinal priming leads to potential memory cells in distant unprimed mucosal tissues (Cox and Muench 1984, Sminia et al. 1987, Weisz-Carrington et al. 1987, Freihorst et al. 1989). However, the concept of a common mucosal immune system does not apply to all mucosal tissues, as bronchial lymph-node cells appear not to show more preference to localize in the gut than do axillary, brachial and inguinal lymph-node cells (McDermott and Bienenstock 1979).

Both lung and gut contain organized lymphoid aggregates, thus participation in a common mucosal immune system is not warranted by the presence of MALT. Moreover, the female genital tract is devoid of similar structures but was shown to take part in the common mucosal immune system (McDermott and Bienenstock 1979). In this respect it is noteworthy that intra-intestinal priming with TNP-KLH elicited anti-TNP antibody forming cells in the intestinal and pulmonary mucosa after intratracheal boosting. After immunizing in the reversed order anti-TNP forming cells were exclusively found in the lung (Sminia et al. 1987). A compartmentalization within the mucosal system is thus likely. Similar results were obtained after immunization with Cholera toxoid (Pierce and Cray 1981) and ovalbumin (Butler et al. 1982), leading to the conclusion that the immune response in the respiratory tract is a rather local phenomenon (Sminia et al. 1987). The results described in chapter 4.1 indicate that intratracheal priming with TNP-LPS even fails to prime the upper respiratory tract. Either pulmonary lymphocytes do not migrate to extrapulmonary tissues, or hardly any memory cell is elicited due to suppression by macrophages or to scavenger macrophages that break down the antigen. Which mechanism is responsible, may depend on the nature of the antigen. On the one hand, intratracheal priming and boosting with TNP-KLH, Cholera toxoid or ovalbumin resulted in elevated numbers of specific antibody forming cells in the lung. On the other hand, after intratracheal-intranasal or intranasal-intranasal immunization with TNP-LPS only few anti-TNP forming cells could be detected in lung tissue. Since an increase in the number of lung-macrophages was seen, the absence of anti-TNP forming cells might be caused by lack of memory formation rather than by lack of migratory capacity of the lymphocytes. Alveolar and interstitial lung macrophages suppress the pulmonary immune response (Holt et al. 1985, Holt 1986, Thepen et al. 1989). Most likely, alveolar macrophages eliminate antigens before they can elicit an immune response, including the formation of memory cells. The cleaning function of alveolar macrophages can be enhanced by IgA-mediated phagocytosis, as Fcα receptors have been detected on murine (Gauldie et al. 1983) and rat (Sibille et al. 1987) alveolar macrophages. The majority, however, do not express Fcα receptors and, thus, will eliminate a broad range of antigens in a non-specific manner. A similar way of surveyance would not be appropriate in the gut, because there the load of presented foreign material also comprises nutrients which must be absorbed. Therefore, a more specific defence is mediated by sIgA, that is secreted in large quantities into the gut lumen via enterocytes and the hepatobiliary IgA pathway (Underdown and Schiff 1986, Russell and Mestecky 1988). The obviously greater need of specificity in the gut than in the respiratory tract might explain why the latter does not have an equally vast IgA production as the intestine, and why in the gut the bacterial IgA1-protease resistant subclass IgA2 predominates, in contrast to the respiratory tract where relatively more IgA1 occurs (Kett et al. 1986).

6.2.3. Immunization of the upper respiratory tract

Despite the apparent importance of the nasal mucosa as a first site of defence against

inhaled antigens, local immune responses have rarely been studied in the upper respiratory tract. Determination of antibody production usually applied to the lung or serum (Ivanoff et al. 1982, McCaskill et al. 1984, El Guink et al. 1989). Several effects of intranasal administration of antigen have been observed. Intranasal inoculation of virus clearly showed that this route of antigen administration is at least as immunogenic as oral immunization (Ivanoff et al. 1982). Moreover, intranasal priming enhanced the DTH reaction to sheep red blood cells (SRBC) when these were administered simultaneously with Cholera toxin. The Cholera toxin needed interaction with the nasal mucosa for the reported enhancement (Tamura et al. 1988). The impact of the upper respiratory tract was also recognized by Sedgwick and Holt (1985). Their study on tolerance induction to inhaled ovalbumin demonstrated that the superficial cervical lymph nodes were the source of suppressor cells to IgE production. These cells were able to induce transferrable tolerance. Inhalation tolerance could not be induced in animals up to 2 weeks of age (Holt et al. 1988). While this might well be an effect of a general immunological immaturity, the authors also suggested an immaturity of a component (or components) of the mucosal immune system, which in view of its development (chapter 3.1) might be NALT. Still, it remained to be explored what would happen locally when an antigen is delivered intranasally. In chapters 4.1 and 4.2 various antigens were used to elucidate this subject.

It appears from chapter 4.2 that intranasal priming and boosting has little immunological effect in the upper respiratory tract or lungs. A single dose of antigen inoculated into the nasal cavity resulted after 5 days in a moderate number of anti-TNP antibody forming cells in the posterior cervical lymph nodes when TNP-KLH was used as the antigen. With TNP-SRBC (sheep red blood cells) a weak primary response and with TNP-LPS hardly any response was seen. The three antigens differ in the nature of their carrier molecules. They are T dependent, particulate as well as T dependent and T independent, respectively. Thus, even immunization with particulate antigen did not induce a major reaction. Particles with the size of red blood cells do not pass the nasal filter and, thus, remain some time in the nasal cavity in contact with the nasal mucosa. Particulate antigen may be taken up by M-cells which are present in NALT (Spit et al. 1989). Some animals were boosted 15 days after priming and the response was determined 5 days later as the number of anti-TNP forming cells. The finding that a second inoculation with TNP-SRBC and TNP-KLH, respectively, resulted in a slightly enhanced response indicates that memory formation had occurred, and was mainly effected in the posterior cervical lymph nodes. The cervical lymph nodes are thus likely to be part of the mucosal lymphoid system. This is in accordance with observations by Fritz et al. (1989) who reported that cervical and mesenteric lymph node cells migrate equally well to the male genital tract mucosa.

Intra-intestinal priming followed by repeated boosting with Cholera toxoid resulted in specific antibodies in jejunal washings (Pierce and Reynolds 1975). The fact that TNP-LPS did not induce specific antibody forming cells in the nasal mucosa or in the cervical lymph nodes, might thus be overcome by repeated antigen administration or by longer intervals between priming and boosting. TNP-LPS was shown to induce memory cells in the upper respiratory tract region after subcutaneous or intraperitoneal administration (chapter 4.1). TNP-KLH induced the highest response in the draining posterior cervical lymph nodes after intranasal priming and boosting, as compared to TNP-LPS and TNP-SRBC (chapter 4.2).

Even after intranasal immunization, few germinal centres were seen in NALT, which is in contrast to PP where numerous and large germinal centres occur. Germinal centres are important in antigen specific B-cell differentiation and proliferation (Kraal et al. 1982) and in memory formation (Kraal et al. 1985b). Presumably, NALT does not have a major function in memory formation, despite the previous assumption that the upper rather than the lower respiratory tract is activated (chapter 6.1.3).

Intraperitoneal administration of antigen has been shown an effective route for priming of mucosae. Intraperitoneal priming with Cholera toxin resulted in augmented numbers of anti-toxin producing cells in jejunal mucosa (Pierce and Koster 1980). The respiratory mucosa in sheep could also be primed by intraperitoneal injection of antigen (Scicchitano et al. 1984). In sheep an indirect route via PP was suggested, but in rodents a more direct route may exist from the intraperitoneal cavity to the lungs. In mice macrophages were shown to migrate from the intraperitoneal cavity to the lung interstitium (Pitt and Anderson 1988). By injection of Indian ink or trypan blue into the peritoneal cavity in rats this route was confirmed in our laboratory. After 24 hours these dyes could be detected in the lungs. Accordingly, intraperitoneal priming with TNP-LPS and subsequent intranasal boosting with the same antigen elicited anti-TNP forming cells in the lungs, but hardly any anti-TNP forming cell was detected in NALT or in the surrounding mucosa (chapter 4.1). Apparently intraperitoneal priming does not result in memory cells seeding the nasal mucosa or NALT.

It should be reminded that different results may be obtained when viable or replicating microorganisms are used for immunizations, instead of the soluble and particulate antigens used in our experiments. In this respect it is noteworthy that mucosal immunity is able to 'distinguish' between food and bacterial antigens (Wold et al. 1989).

6.3. Hypothesis on the role of NALT within the rat nasal mucosa

Based on morphological and functional data, as presented in this thesis, the following hypothesis on NALT may be considered. Particulate antigens instilled in the nasal cavity will be taken up by M-cells, present in the epithelium overlying NALT (Spit et al. 1989). Repeated antigen exposure or exposure to viable, replicating antigens, will induce lymphocyte proliferation and differentiation in the posterior cervical lymph nodes, which drain NALT (Koornstra et al. in preparation). The elicited lymphoblasts might migrate towards NALT and extravasate through the HEV in NALT. The majority of the cells will then locate at the border area between the lymphoid mass of NALT and the surrounding connective tissue, where they may further differentiate.

When developed into mature plasma cells they might migrate towards the glandular acini to produce immunoglobulins which are secreted. In addition, it is possible that lymphoblasts migrate directly from the draining lymph nodes to the acinar area. In this way the nasal mucosa and NALT could deal adequately with particulate antigens such as bacteria and viruses. The specific antibodies in the nasal secretion may combat further invasion of these antigens. Some plasma cells remain underneath the epithelium or at the mucosal periphery of NALT and may deal with antigens that have penetrated the NALT epithelium. Different from particulate antigens, soluble antigens most likely penetrate the nasal mucosa away from NALT. The superficial cervical lymph nodes directly drain the nasal mucosa, as was shown by injection of Indian ink (own observations), and was more elaborately studied by Koornstra and coworkers (in preparation). According to aerosol exposure experiments, soluble antigens can suppress systemic IgE but also IgG responses (Sedgwick and Holt 1984, 1985, Holt et al. 1987). The responsible T suppressor cells were induced in the superficial cervical lymph nodes (Sedgwick and Holt 1985). As a consequence, intranasal administration of soluble antigen may result in systemic tolerance. High doses of antigen, however, may cause antigenic material to directly reach the next lymphoid station, being the posterior cervical lymph nodes (Tilney 1971). The balance between the activating effect by the posterior cervical lymph node and the suppressing effect by the superficial cervical lymph node will determine the eventual response.

In short, this hypothesis proposes that the nasal mucosa and NALT are directed to a specific defence against microorganisms in the lumen (secretion) and to dampening of the reaction to soluble antigens which have penetrated the nasal mucosa.

6.4. Antigen handling in the human nasal mucosa

As mentioned, lower respiratory tract homeostasis is maintained largely by rapid elimination of aeroantigens. In the upper respiratory tract the mucus blanket in which particles larger than 1-3 μm are trapped (Brandtzaeg 1984, Dahl et al. 1988, Stuart 1984) certainly leads to efficient elimination. The role of the mucociliary system will not be discussed here, but attention will be given to the underlying epithelium. More and more functions and differentiations are described nowadays for intra-epithelial cells. Antigens reaching the mucosal epithelium may be endocytosed by M-cells, originally described in the epithelium overlying PP (Owen and Jones 1974). Since then similar cells have been detected in the epithelium covering the appendix (Rosner and Keren 1984), BALT (Fournier et al. 1977, Van der Brugge-Gamelkoorn et al. 1986), tonsils (Pappo and Owen 1988) and NALT (Spit et al. 1989). Antigen is taken up from the lumen by M-cells and subsequently discharged into the intercellular space. During the intracellular transport the antigen is not digested by lysosomal enzymes. After release into the baso-lateral intercellular space, the antigen can be picked up by infiltrated lymphocytes, macrophages or dendritic cells. Controversy exists about M-cells bearing Ia/HLA-DR antigens, essential in antigen presentation. Most likely they are Ia/HLA-DR negative (Russell and Mestecky 1988, Brandtzaeg

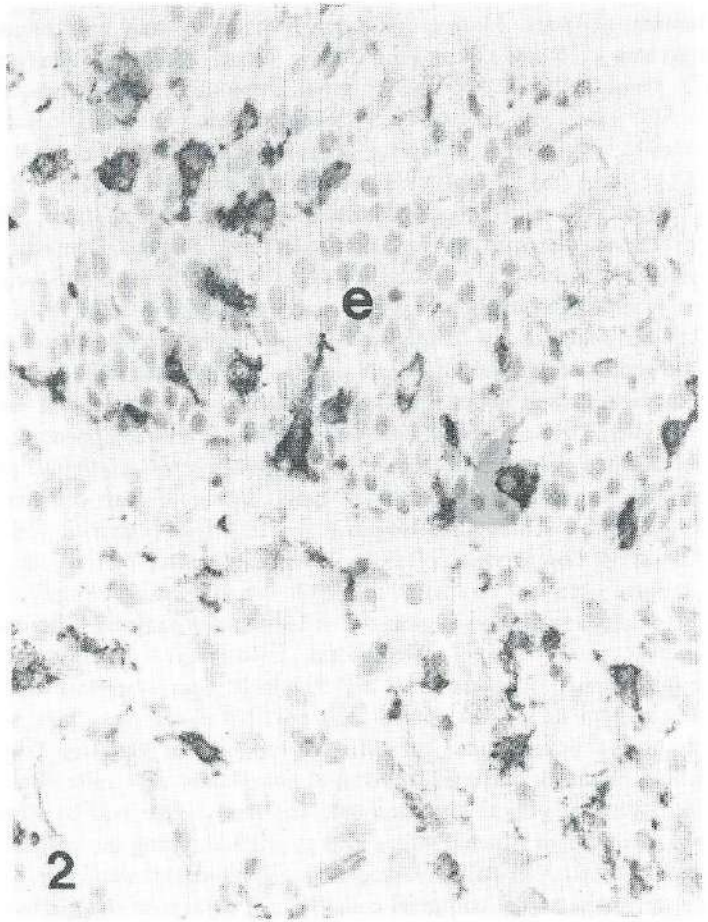

Fig. 2. HLA-DR expression in the normal nasal mucosa of the human inferior turbinate. *e* epithelium. x400.

and Bjerke 1989).

The localization of M-cells is restricted to the epithelium overlying lymphoid aggregations in the mucosae, but they are not found in the villi, tracheal, bronchial or nasal epithelium. In these areas dendritic cells may be present in the epithelium. Dendritic cells with antigen presenting capacities are found throughout the respiratory mucosa (Sertl et al. 1986). In pseudostratified epithelia as found in the respiratory tract (chapter 1: Fig.2), the extension of dendritic processes clearly has a function in

capturing luminal antigens. Among the antigen presenting cells are Langerhans cells. Hence, Langerhans cells have been described in human tracheal epithelium (Richard et al. 1987), nasal epithelium (Fokkens et al. submitted) and tonsils (Brandtzaeg 1984). HLA-DR+ cells with a dendritic appearance were seen in the nasal epithelium of human middle and inferior turbinates (Fig. 2) as reported in chapter 5.2 and by Stoop et al. (1990), and in polyp epithelium, where also CD1+ cells, probably Langerhans cells, have been observed (Stoop et al. in preparation). Most likely, Langerhans cells are also present in the human oral mucosa. Dendritic cells with simultaneous expression of CD1 and HLA-DR have been noticed but the presence of the characteristic Birbeck granules was not reported (Cruchley et al. 1987, Van Loon et al. 1989). When taken up by Langerhans cells, antigens are transported towards the draining lymph nodes. Regarding the nasal mucosa, Langerhans cells will thus migrate towards the cervical lymph nodes. In human nasal biopsy specimens of ear, nose and throat patients HLA-DR+ intra-epithelial cells with a dendritic appearance were less frequently seen than in control tissue. In patient tissue relatively more round HLA-DR+ cells, presumably activated T cells and epithelial cells, were found (chapters 5.1 and 5.2). Although a decrease in HLA-DR+ dendritic cells does not prove a decrease in Langerhans cells, the possibility exists that in the mucosa of patients the antigen transport by Langerhans cells towards the draining lymph nodes is impaired. This could result in an augmented inflammatory response within the mucosa with subsequent tissue damage. Microscopic examinations of the nasal mucosa confirm this. However, Fokkens et al. (1989) have suggested that in atopici the number of Langerhans cells is increased. This possible discrepancy may be explained by different patient populations and different periods of sampling. The patients described in this thesis all suffered from nasal complaints with miscellaneous causal factors, excluding nasal polyps and tumours. The study described by Fokkens et al. concerned patients with an isolated pollen allergy studied during the pollen season.

Apart from M-cells (in follicle-associated epithelia) and dendritic cells antigen may be taken up by the actual epithelial cells. Recent studies on the gut have provided evidence that enterocytes are capable of presenting antigen to T cells of the suppressor phenotype (Bland and Warren 1986a, Mayer and Shlien 1987). Such mechanism might explain the predominance of CD8+ T cells in the human nasal epithelium, as reported in chapters 5.1 and 5.2. A preponderance of CD8+ over CD4+ cells is not exclusive for the nasal epithelium. Similarly, more CD8+ than CD4+ cells were detected in the epithelium of the lower respiratory tract by Fournier and coworkers (1989) and in the nasal lamina propria in our studies (Fig. 3, chapter 5.2, Stoop et al. 1990). CD8+ T suppressor cells generally exhibit a downregulating effect, which might be advantageous in maintaining mucosal integrity. Similar to M-cells, antigen presenting epithelial cells do not desintegrate the ingested antigen (Bland and Whiting 1989). They present it intact. Ia expression is a prerequisite for antigen presentation, also by epithelial cells (Bland and Warren 1986b). In the upper respiratory tract HLA-DR+ epithelial cells occur in the human nasal mucosa (chapters 5.1 and 5.2) and adenoid (Van Nieuwkerk et al. 1990). The experiments with antigen presenting

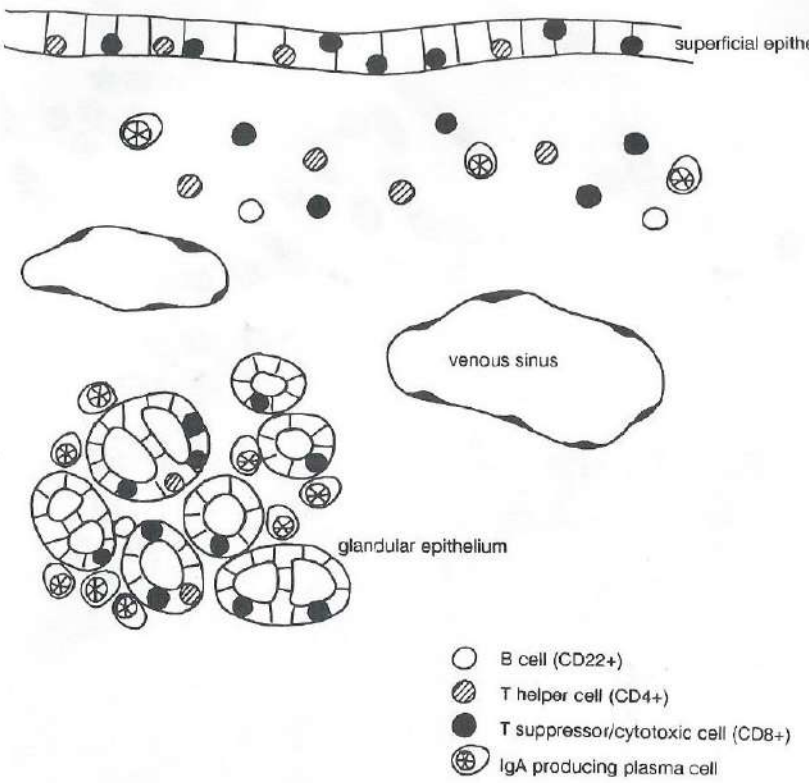

Fig. 3. Distribution of lymphocytes in the human nasal mucosa

epithelial cells were performed with soluble antigens, viz. Tetanus toxoid. M-cells, however, preferentially take up particulate antigen (Jeurissen et al. 1985, Pappo and Ermak 1989) and presumably do not present antigen.

The CD8+ IEL (intra-epithelial lymphocyte) population comprises suppressor but also cytotoxic T cells. Cytotoxic T lymphocyte precursors have been detected in the murine intestinal epithelium. These may develop into mature cytotoxic cells after stimulation by luminal antigens (Ernst et al. 1986). Cytotoxic cells were observed in similar frequencies among IEL and spleen cells (Flexman et al. 1983). Considerable cytotoxic activity has been reported for IEL (Arnaud-Battandier et al. 1978, Davies and Parrott 1981, Flexman et al. 1983) but the degree of cytotoxicity described varies among authors, presumably depending on the experimental design used.

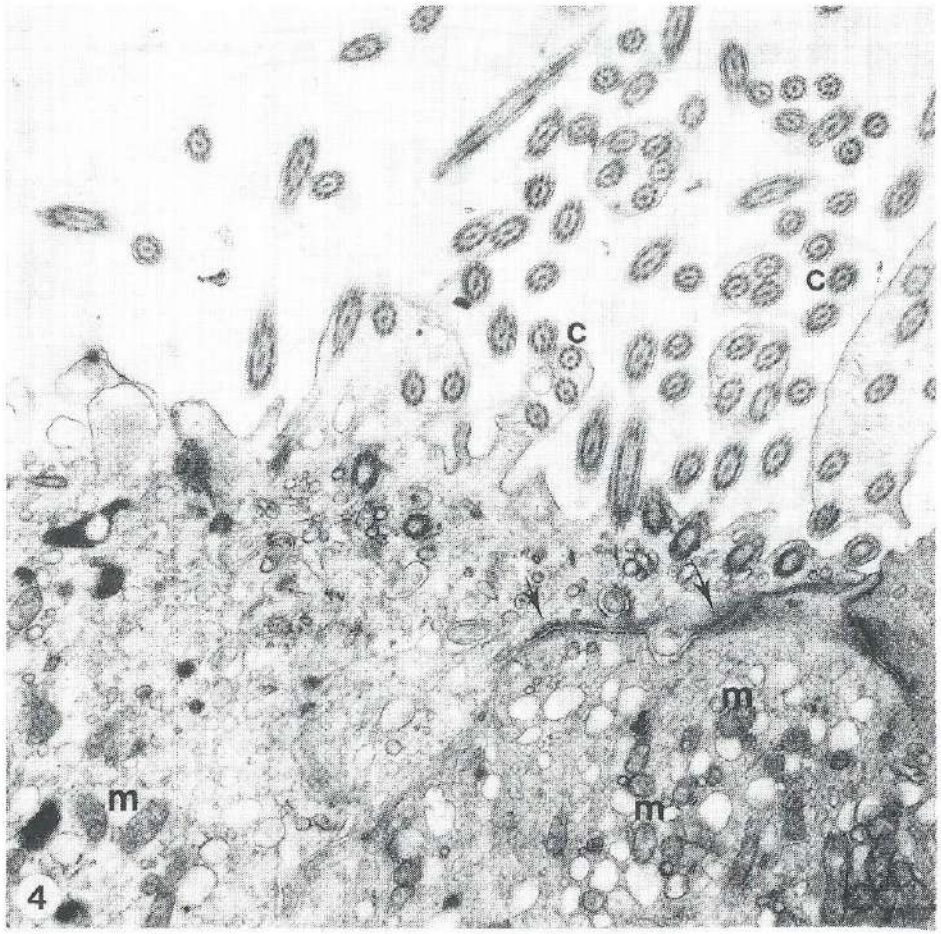

Fig. 4. Electron micrograph of the normal human nasal epithelium. A ciliated cell (right) is situated next to a non-ciliated cell (left). *c* cross-sections through cilia, *m* mitochondria. Note the cell junctions (*arrows*). x28,000.

If virus infected epithelial cells in the human nasal mucosa are cleared by CD8+ intra-epithelial cytotoxic T cells, tissue damage will undoubtedly follow. In an attempt to restore the epithelial integrity, the respiratory epithelium which contains ciliated cells (Fig. 4, Davis and Smallman 1988) will be replaced by squamous epithelium. The observations on patient tissue described in chapter 5.2 confirm this. Replacing respiratory type epithelium by the squamous type will severely impair the mucociliary clearance, resulting in an enhanced proneness to infection. Reinfection will cause further tissue damage, followed by further repair with squamous type epithelium, enhanced susceptibility to infection and so on. A state of chronic rhinitis will be the unfortunate result.

Antigen penetration in the nasal mucosa is prevented by components in the nasal secretion, such as specific secretory IgA, mucus and the ciliary movements. If penetration occurs, the consequence of an intranasal antigenic challenge is limited to the epithelial compartment as much as possible, whether mediated by cytotoxic T cells or by suppressor T cells. It is tempting to draw a parallel to the pulmonary defence, where alveolar macrophages eliminate foreign material and thus help to prevent penetration, while also alveolar and endogenous macrophages suppress the immune response (Holt et al. 1985, Holt 1986). Apparently, both upper and lower respiratory systems aim to avert the induction of an immune response.

The differences between the ear, nose and throat patients and controls were most obvious in the epithelial compartment, as described in chapters 5.1 and 5.2. Firstly, tissue sections of patient mucosa frequently showed squamous instead of the usually seen respiratory epithelium, as mentioned above. Secondly, in both luminal and glandular epithelia the ratio CD8+/CD4+ cells was higher in patient material than in control biopsies. Moreover, the glandular tissue of patient mucosa showed more mucous secretion than the glandular tissue in control mucosa. Since secretory IgA is preferentially secreted through serous acini, this suggests a decrease in the activity of serous glands and thus in IgA secretion. As a consequence, humoral immunity, including the protective function of secretory IgA, could be impaired (chapter 5.2). Recent preliminary results, obtained by Swart and coworkers (in preparation), showed that the amount of secretory IgA in nasal secretions of patients with an IgE mediated allergy was increased when compared to controls. This would indicate that despite the increased CD8/CD4 ratio and despite the abundance of mucous glands, the secretory activity is augmented. Remarkably, the glandular epithelium in patient tissue more frequently expresses HLA-DR than control glandular epithelium, as described in chapter 5.2.

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SUMMARY

The study described in this thesis concerns the immunological role of the upper respiratory tract mucosa in both rat and man. Immuno- and enzyme-histochemical staining techniques were applied to study the distribution of lymphocytes and non-lymphoid cells. In the rat, special attention was paid to the nasal-associated lymphoid tissue (NALT). Moreover, some *in vivo* immunization experiments were performed.

Chapter 1 provides an overview of the literature concerning morphology and function of the mucosae in general and the nasal mucosa in particular.

An introduction to the subsequent studies on the nasal mucosa and NALT, as well as the purpose of the experiments are provided in **chapter 2.**

In **chapter 3** morphological aspects of rat NALT are reported. The development of NALT during the first 3 weeks of postnatal life is described in chapter **3.1.** NALT is already present at birth as a small condensation of mainly T cells and non-lymphoid cells. Most T cells are of the helper phenotype. B and T lymphocytes increase in numbers during ontogeny. By the age of 10 days distinct T- and B-cell areas occur and also high endothelial venules (HEV) are clearly present by then. The non-lymphoid cells were studied, using the anti-macrophage monoclonal antibodies ED1, ED2 and ED3. ED1+ cells, comprising monocytes, macrophages and dendritic cells, are unevenly distributed throughout NALT at all ages. From day 2 after birth some ED1+ macrophages are detected in the epithelium overlying NALT. ED3+ mononuclear cells, which comprise a particular subpopulation of macrophages in lymphoid organs, can be observed throughout NALT of young animals as irregular cells with dendritic processes. From the end of the first week of age they tend to become more restricted to the border area between NALT and the surrounding connective tissue. In the same area and at all ages heavily stained ED2+ cells with blunt processes, comprising resident tissue macrophages, are seen. The Ia marker is expressed throughout NALT on both round and dendritic cells, which are present from birth and increase in number as the animal grows older. Ia+ cells can always be observed within the epithelium, while also some epithelial cells express Ia antigen. Intra-epithelial B cells are rarely seen, but T cells regularly occur, slightly more of the T helper subtype.

In adult NALT, as described in **chapter 3.2.**, the majority of the intra-epithelial lymphocytes have become B cells with IgG or IgM as surface immunoglobulins. Within NALT, T- and B-cell areas are approximately of the same size (B/T = 0.9), with T helper cells predominating over T suppressor/cytotoxic cells. With respect to the B cells, IgA or IgE carrying cells comprise less than 1% of the B cells. Most B

cells in NALT bear surface IgM. The distribution of non-lymphoid cells has not changed as compared to the situation on day 21 after birth. ED1+ cells occur throughout NALT, mainly in the T-cell areas and some ED1+ macrophages are seen within the epithelial compartment. Several ED1+, ED2+ and ED3+ cells are found in the border area between the lymphoid mass of NALT and the surrounding connective tissue. ED3+ cells are the least in number. Few of the non-lymphoid cells show acid phosphatase or non-specific esterase activity. ED5, which is a marker of follicular dendritic cells, can occasionally be observed in NALT. Germinal centres, however, are infrequently seen. Ia antigen is abundantly present in NALT, mostly on B cells. The data are discussed in view of the position of NALT in the mucosal immune system.

After having studied the structure and cellular composition of the developing and adult NALT, immunization studies were performed, as reported in **chapter 4.** Animals were primed and boosted with the antigen TNP-LPS. Priming occurred intranasally, subcutaneously in the cheek, intraperitoneally or intratracheally. Fifteen days later the animals were boosted intranasally with the same antigen and dose. The number of anti-TNP forming cells was determined *in situ* in a semiquantitative way. In NALT very few anti-TNP forming cells occur, located in the periphery of NALT. The response in the posterior cervical lymph node quantitatively exceeds the response in the other organs tested for all 4 immunization routes. Antibody forming cells are located in the medulla. Intranasal-intranasal immunization hardly evokes any specific antibody forming cells. Obviously, the highest response occurs after subcutaneous-intranasal immunization with a peak response on day 5 in the posterior cervical lymph node and spleen. The four isotypes IgG, IgA, IgM and IgE are produced, as determined on tissue sections. In this lymph node a considerable proportion of the anti-TNP antibodies is of the IgA subclass, while in spleen IgA is produced by few cells. IgG and IgM are abundantly produced in lymph node and spleen. Intraperitoneal-intranasal immunization evokes a marked response on day 7 in lungs and spleen. After intratracheal-intranasal immunization a considerable response is observed only in the posterior cervical lymph nodes on days 3 and 5. This response is parallelled by an increase in pulmonary macrophages, as detected by the ED1 and ED3 markers and by acid phosphatase activity. In connection herewith the immunological role of lung macrophages is discussed. The superficial cervical and facial lymph nodes show minor responses in all experiments and the mesenteric lymph node contains no or neglectible numbers of anti-TNP forming cells. All four immunization routes induce anti-TNP antibodies in the serum. No correlation is seen between serum titers and the number of anti-TNP forming cells in the tissues. Except that after intraperitoneal priming and intranasal boosting, the serum and splenic response closely corresponded.

The study in **chapter 4.1** showed that specific reactions can be induced in the upper respiratory tract. Subsequently, the effect of various intranasally applied antigens was investigated. It is shown in **chapter 4.2** that intranasal priming followed by a single intranasal boost with TNP-KLH evokes a clear response in serum. The

isotypes of the anti-TNP forming cells in tissue sections of posterior cervical lymph nodes were determined. Again, specific IgA, IgG, IgM and IgE is produced, with IgE produced by the lowest number of plasma cells. When TNP-LPS or TNP-SRBC are used as antigens, the responses remain very low. TNP-LPS can induce some serum antibodies, whereas TNP-SRBC fails to do so. Primary responses to either of the antigens hardly occur. Deviations in the distribution of T cell subsets, macrophages or Ia are not observed, except in some animals in which an increase in pulmonary macrophages occurs. A similar increase had previously been seen after intratracheal-intranasal immunization with TNP-LPS (chapter 4.1). Based on the results described in chapter 4, a role of NALT in the mucosal immune system is considered.

Studies on the human nasal mucosa are reported in **chapter 5.** Data on the nasal epithelial compartment of ear, nose and throat patients and healthy subjects are given in **chapter 5.1.** In both patient and control tissue CD8+ (suppressor/cytotoxic) T cells predominate over CD4+ (helper/inducer) T cells, while CD19+ B cells are absent. In control sections but not in patient sections some CD4+ cells are observed. Consequently, the ratio CD8+/CD4+ cells is greater in patient tissue than in control tissue. The percentages of CD4+ and CD8+ lymphocytes in patient tissue are 12% and 73% respectively, whereas 62% of the lymphocytes are CD2+ (pan T-cell marker). The HLA-DR antigen is abundantly present on epithelial cells, lymphocytes and non-lymphoid cells. Possible implications of the observations on patient and control nasal epithelium are considered.

The study was extended to the deeper layers of the human nasal mucosa, as reported in **chapter 5.2.** Apparently, the predominance of CD8+ cells over CD4+ cells applies to both luminal and glandular epithelium. The predominance of CD8+ lymphocytes is most striking in patient epithelia. In the subepithelial connective tissue CD22+ B cells are scarce, but plasma cells occur frequently. They are seen underneath the epithelium and to a larger extent around the glands. The predominant isotype is IgA. In both patient and control samples clusters of lymphocytes are observed in the subepithelial layer. HLA-DR+ HEV are occasionally found adjacent to large clusters. The HLA-DR antigen is observed on round cells and cells with dendritic processes. In patient tissue relatively more of the round cells are found. Luminal and glandular epithelial cells are also HLA-DR+. HLA-DR+ glandular epithelium is more frequently observed in patient material. Routine histological staining with methylgreen-pyronin clearly distinguishes between patient and control sections. The abundant mucus within the glands in patient tissue stains conspicuously scarlet with methylgreen-pyronin. A striking feature is the frequently seen squamous epithelium instead of respiratory epithelium in patient samples. The defence of the nasal mucosa is discussed in view of the results.

Finally, in **chapter 6**, the obtained results are discussed in view of relevant literature. Moreover, a possible role of NALT and the nasal mucosa is considered, as are clinical implications of the human data.

SAMENVATTING

Dit proefschrift handelt over de immunologische afweer in de bovenste luchtwegen, met name in de neus. Deze vormt de 'porte d'entrée' van het ademhalingsstelsel. Tegelijk met de geïnhaleerde lucht worden allerlei lichaamsvreemde stoffen (antigenen) ingeademd die mogelijk schadelijk en in ieder geval ongewenst zijn voor de longen. In de neusholte wordt een begin gemaakt met de verwijdering of het onschadelijk maken van deze stoffen. Naast bestudering van het neusslijmvlies (neusmucosa) bij de mens, werd in het onderzoek speciale aandacht gegeven aan het neus-geassocieerde lymfoïde weefsel (NALT) in de rat. De ratte NALT is een ophoping van lymfoïd weefsel in de bodem van de neusholte.

In **hoofdstuk 1** wordt een overzicht gegeven van de literatuur betreffende de morfologie en funktie van slijmvliezen (mucosae) in het algemeen en van het neusslijmvlies in het bijzonder. Slijmvliezen hebben een gespecialiseerde funktie, bijvoorbeeld de uitwisseling van zuurstof tegen koolzuurgas via de mucosa van de longen en de opname van voedsel via de darmmucosa. Tegelijkertijd moeten de slijmvliezen zoveel mogelijk voorkómen dat schadelijke stoffen het lichaam binnendringen. Hiertoe beschikken ze over zowel niet-immunologische als immunologische afweersystemen. Tot de eerste behoren bijvoorbeeld de trilharen welke deeltjes verwijderen die 'gevangen' zijn in de slijmlaag die de slijmvliezen bekleedt, en allerlei anti-microbiële eiwitten (bijvoorbeeld enzymen). Het immunologische afweersysteem omvat lymfoïde cellen, zoals T en B cellen, niet-lymfoïde cellen, zoals macrofagen ('vreetcellen') en antigeen-presenterende cellen welke nodig zijn om een immuunrespons op gang te brengen, en verder specifieke afweerstoffen (antilichamen = immunoglobulinen = Ig). Deze worden geproduceerd door uit B cellen ontwikkelde plasma cellen. Er zijn 5 isotypen (soorten) immunoglobulinen, namelijk IgM, belangrijk in de vroege reakties; IgE, dat een rol speelt in allergische reakties en in parasitaire infekties; IgD, het minst voorkomende immunoglobuline (minder dan 1%) in plasma; en tenslotte IgA en IgG, de meest voorkomende immunoglobulinen. In de sekretoire produkten van slijmvliezen is IgA verreweg het belangrijkste. Het komt daar in een bijzondere vorm, het sekretoir IgA (sIgA) voor, opgebouwd uit 2 molekulen (dimeer) IgA plus een J-keten (joining-chain), plus een sekretoire component. De plasma cellen die het dimeer IgA met J-keten produceren liggen met name onder het slijmvlies-epitheel en rondom klierstrukturen. Dit vergemakkelijkt de uitscheiding van het sIgA via de sekretoire component (SC) op deze (klier)epitheel cellen (zie hoofdstuk 1: Fig. 1).

Op diverse plaatsen direkt onder het epitheel, in het bindweefsel van de slijmvliezen worden georganiseerde aggregaten van lymfoïd weefsel aangetroffen. De algemene benaming hiervoor is mucosa-geassocieerd lymfoïd weefsel (mucosa-associated lymphoid tissue: MALT). In de darm bevindt zich het darm-geassocieerde

lymfoïde weefsel (gut-associated lymphoid tissue: GALT) en in de longen het bronchus-geassocïeerde lymfoïde weefsel (bronchus-associated lymphoid tissue: BALT). Bij verschillende diersoorten is ook in de neusholte MALT aangetoond. Al in 1947 werd MALT in de ratteneus beschreven, maar het duurde tot ver in de tachtiger jaren alvorens er structureel onderzoek aan werd gedaan. Dit neus-geassocïeerde lymfoïde weefsel (NALT) wordt wel beschouwd als het equivalent van de neusamandel (adenoïd) bij de mens.

Na de algemene inleiding geeft **hoofdstuk 2** een toelichting op het motief en het doel van de opeenvolgende experimenten. Een steeds terugkerende techniek in de experimenten is de immunohistochemie. Deze techniek berust op het feit dat cellen kenmerkende antigenen dragen op hun oppervlak of in hun cytoplasma. Tegen diverse van deze antigenen zijn antilichamen opgewekt. Na incubatie van weefselcoupes (een plakje weefsel van meestal 8/1000 mm dik) met deze antilichamen, is te zien of de betreffende antigenen en dus de betreffende celtypen, in de coupe aanwezig zijn. Draagt een cel namelijk een bepaald antigeen, dan bindt het op dat antigeen passende antilichaam en dit is vervolgens zichtbaar te maken via een kleurreaktie. De aangekleurde cel noemt men positief voor dat antigeen.

In **hoofdstuk 3** wordt de normale struktuur en ontwikkeling van de neusmucosa inklusief de NALT beschreven, omdat de morfologie en ontogenie van een weefsel belangrijke informatie kunnen verschaffen over de funktie van dat weefsel. **Hoofdstuk 3.1** schetst de ontwikkeling van de NALT vanaf de geboorte van een rat tot drie weken daarna. Aanvankelijk bestaat de NALT voornamelijk uit T cellen en niet-lymfoïde cellen. Gedurende de ontwikkeling nemen T en B cellen in aantal toe en gaan zich groeperen waardoor na 10 dagen afzonderlijke T- en B-cel gebieden zijn te onderscheiden. Dan worden ook hoog endotheel venulen (HEV) waargenomen. Dit zijn kleine veneuze bloedvaten met een gespecialiseerd hoog, kubisch, endotheel. HEV zijn de vaatjes van waaruit T en B cellen de bloedbaan verlaten om het lymfoïde weefsel in te gaan.

De niet-lymfoïde cellen werden bestudeerd met behulp van 3 verschillende determinanten, herkend door de (monoklonale) antilichamen ED1, ED2 en ED3. ED1-positieve (ED1+) cellen omvatten monocyten, macrofagen en dendritische cellen. Deze komen op alle leeftijden ongelijkmatig verspreid in de NALT voor en vanaf de tweede dag na de geboorte wordt een enkele ED1+ cel ook in het epitheel aangetroffen. ED3+ cellen vormen een bepaalde subpopulatie macrofagen in lymfoïde organen en komen in de NALT van jonge dieren voor als onregelmatige cellen met dendritische uitlopers. Na een week raken ze steeds meer geconcentreerd in het grensgebied tussen de NALT en het omringende bindweefsel. In dat gebied worden op iedere leeftijd ook sterk aangekleurde ED2+ cellen gezien, welke stompe uitlopertjes hebben. De subpopulatie van ED2+ cellen omvat vaste, niet-mobiele weefselmacrofagen. Het Ia antigeen, een determinant die normaal voorkomt op B cellen, geactiveerde T cellen, en antigeen-presenterende cellen, wordt door de hele NALT gezien, zowel op ronde cellen als op cellen met uitlopers. Het aantal cellen positief voor Ia neemt toe met de leeftijd van het dier. Ook in het epitheel komen Ia+ cellen voor, zelfs enkele

epitheel cellen brengen zelf het Ia antigeen tot expressie. Intra-epitheliale B cellen worden zelden gezien, maar T cellen komen regelmatig voor in het epitheel. Een krappe meerderheid van de intra-epitheliale T cellen is van het T helper subtype. Dit type T cel heeft in het algemeen een aktiverende invloed op het verloop van immunologische reakties.

In de volwassen NALT, welke beschreven wordt in **hoofdstuk 3.2**, blijkt het merendeel van de lymfocyten in het epitheel boven de NALT uit B cellen te bestaan. Anders dan in het epitheel, komen dieper in de NALT de T en B cellen in ongeveer gelijke hoeveelheden (B/T = 0.9) voor. Helper T cellen zijn groter in aantal dan suppressor/cytotoxische T cellen. Suppressor T cellen hebben een onderdrukkend effekt op het verloop van immunologische reakties, en cytotoxische T cellen kunnen bepaalde doelwit cellen onschadelijk maken. B cellen kunnen, zoals eerder vermeld, uitrijpen tot immunoglobuline producerende plasma cellen. Al vóór het stadium van plasma cel zijn immunoglobulinen aanwezig op het oppervlak van B cellen. Van de B cellen in de NALT draagt minder dan 1% IgA of IgE op het oppervlak; verreweg de meeste dragen IgM. De verdeling van de niet-lymfoïde cellen is in de volwassen NALT niet gewijzigd in vergelijking met de situatie op dag 21 na de geboorte. ED1+ cellen komen door de hele NALT voor, met name in het T-cel gebied, terwijl ook enkele ED1+ cellen in het epitheel voorkomen. ED1+, ED2+, èn ED3+ macrofagen komen voor in het grensgebied tussen het lymfoïde deel van de NALT en het omringende bindweefsel. Hiervan zijn de ED3+ cellen het geringst in aantal. Enkele van de niet-lymfoïde cellen vertonen zure fosfatase of niet-specifieke esterase activiteit. Zure fosfatase is een verteringsenzym dat met name in de klassieke (vreet)macrofagen aktief is. Niet-specifieke esterase komt minder verbreid voor; dit enzym wordt onder andere waargenomen in zogeheten 'tingibele körper macrofagen', die brokstukken van 'opgeruimde' cellen bevatten. Er is nog een andere monoklonaal antilichaam gebruikt, namelijk ED5, dat een determinant herkent op 'folliculair dendritische cellen'. Deze cellen met dendritische uitlopers komen voor in lymfoïde follikels (bolvormige opeenhopingen van meestal B cellen). De aanwezigheid van ED5+ cellen geeft een zekere mate van aktivatie van het betreffende lymfoïde weefsel aan. Desondanks worden weinig kiemcentra gezien. Ook kiemcentra wijzen op aktivatie van het weefsel. Het Ia antigeen is ruimschoots aanwezig in de NALT, voornamelijk op B cellen. De morfologische kenmerken van de NALT suggereren dat dit lymfoïde weefsel onder normale, gezonde omstandigheden, in een meer geaktiveerde toestand is dan de BALT in de lagere luchtwegen.

Hoewel morfologie en funktie nauw aan elkaar verbonden zijn, bleef de vraag bestaan: 'wat gebeurt er wanneer antigenen via de neusholte aan het lichaam worden aangeboden?' Daarom zijn immunisatiestudies uitgevoerd. Deze staan beschreven in **hoofdstuk 4**. In de eerste studie, **hoofdstuk 4.1**, kregen ratten het antigeen TNP-LPS toegediend. TNP-LPS bestaat uit een groot drager-molekuul, LPS, en een zogeheten hapteen, TNP. LPS, een bakteriëel antigeen, is een T cel onafhankelijk (thymus-onafhankelijk) antigeen. Dit betekent dat LPS B cellen kan aanzetten tot de

produktie van antilichamen, zonder tussenkomst van T helper cellen. In een immuunrespons tegen TNP-LPS zullen ook antilichamen tegen TNP gevormd worden. De anti-TNP antilichaam vormende cellen kunnen in weefselcoupes worden aangetoond middels een kleurreaktie. Het aantal anti-TNP antilichaam vormende cellen werd als maat voor de optredende reaktie gebruikt.

Alle dieren werden in de neus (intranasaal) geïmmuniseerd, nadat ze 15 dagen eerder reeds een dosis TNP-LPS ontvangen hadden, en wel op 4 verschillende manieren, namelijk a. intranasaal, zodat het neusslijmvlies en de NALT twee keer met het antigeen in kontakt komen, b. in de wang onder de huid (subcutaan), waarbij dezelfde lymfeklieren het antigeen verwerken als na intranasale toediening, c. in de buikholte (intraperitoneaal), aangezien deze manier van immuniseren geschikt is om een reaktie in de slijmvliezen op te wekken, en d. in de luchtpijp (intratracheaal) hetgeen aanwijzingen zou kunnen opleveren over een mogelijke communicatie op immunologisch gebied tussen de longen en de neus. De resultaten laten zien dat slechts weinig anti-TNP vormende cellen in de NALT ontstaan. Deze zijn gelokaliseerd in de periferie, hetgeen overeenkomt met de lokalisatie van antilichaam vormende cellen in BALT. Daar worden antilichaam vormende cellen ook met name rondom het lymfoïde weefsel waargenomen. De respons in de diep cervicale lymfeklieren (deep cervical lymph nodes; zie hoofdstuk 1, Fig. 4) is groter dan in de andere onderzochte organen, ongeacht de wijze van immuniseren. De hoogste respons treedt op na subcutaan-intranasaal immuniseren, er is dan een piek te zien op dag 5 na de laatste immunisatie. De meest voorkomende isotypen van de immunoglobulinen (IgM, IgG, IgA, en IgE) worden alle vier geproduceerd. In de lymfeklieren wordt veel IgA gemaakt, terwijl in de milt IgA juist in heel geringe hoeveelheden wordt geproduceerd. Dieren die intraperitoneaal geprimed en intranasaal geboosterd zijn, ontwikkelen een aanzienlijke respons op dag 7 in long en milt. Intratracheaal-intranasaal immuniseren induceert vrijwel uitsluitend in de diep cervicale lymfeklieren een respons. Opvallend bij deze laatste immunisatie route was de toename aan long-macrofagen. De mogelijkheid bestaat dat deze macrofagen het antigeen hebben opgeruimd voordat er antilichaam vormende cellen geïnduceerd zijn, en dat daardoor een geringe reaktie optreedt. Bovendien is bekend dat long-macrofagen een immuunrespons ter plekke kunnen onderdrukken. Het twee keer aanbieden van antigeen in de neus veroorzaakt slechts geringe aantallen anti-TNP antilichaam vormende cellen. Alle vier de immunisatie routes leiden tot anti-TNP antilichamen in het bloed. In het algemeen wordt geen verband gevonden tussen het aantal anti-TNP antilichaam vormende cellen in de weefsels en de reaktie in het bloed.

Nu gebleken was dat specifieke antilichaam vormende cellen in de bovenste luchtwegen opgewekt kunnen worden, zij het met name in de lymfeklieren van dat gebied, werd vervolgens het effekt van drie verschillende soorten antigenen bestudeerd. Weer werden ratten intranasaal geïmmuniseerd. De drie gebruikte soorten antigenen waren weer koppelingen van TNP en een drager molekuul. Het drager-molekuul bepaalt de aard van de reaktie. Er werd geïmmuniseerd met: TNP-LPS, TNP-KLH en TNP-SRBC. TNP-KLH is een T-afhankelijk antigeen. Het

kan geen antilichaam vormende cellen opwekken zonder de hulp van T helper cellen, dit in tegenstelling tot TNP-LPS. Deze twee antigenen zijn oplosbare antigenen. TNP-SRBC (SRBC = schape rode bloedcellen), is een deeltjes-antigeen. Rode bloedcellen zijn relatief groot (7/1000 mm), en zouden in de neusholte verwerkt moeten worden. Een aantal dieren kreeg het antigeen nu slechts eenmalig toegediend, de andere kregen het wederom twee keer, met een tussenpoos van 15 dagen. De resultaten in **hoofdstuk 4.2** laten zien dat het eenmalig toedienen van deze antigenen niet leidt tot specifieke antilichamen in het bloed en nauwelijks tot antilichaam vormende cellen in de weefsels. Van de drie gebruikte antigenen geeft TNP-KLH na twee keer toedienen de hoogste respons in de diep cervicale lymfeklier. TNP-LPS en TNP-SRBC geven een hele lage respons. TNP-SRBC leidt, met of zonder boosten, niet tot anti-TNP antilichamen in het bloed. De resultaten van de uitgevoerde immunisatie-experimenten suggereren dat de respons op intranasaal toegediende antigenen bij voorkeur beperkt blijft tot de lymfeklieren die de bovenste luchtwegen draineren. Bovendien blijken deeltjes antigenen, zoals SRBC, geen immuunrespons te veroorzaken, waarschijnlijk omdat ze in de slijmlaag gevangen en vervolgens door de werking van trilharen van de neusmucosa verwijderd worden. Hoewel bakteriën ook deeltjes zijn, kunnen deze tot andere resultaten leiden omdat ze levend zijn, zich vermenigvuldigen en aktief kunnen hechten aan het slijmvlies.

Op basis van de gevonden resultaten, gecombineerd met literatuurgegevens wordt de volgende suggestie gedaan: de NALT en de neusmucosa zijn gericht op een specifieke verdediging tegen microorganismen in het lumen van de neus, en tegelijkertijd op het onderdrukken van de immuunrespons tegen oplosbare antigenen welke in de neusmucosa zijn doorgedrongen. Het is bekend dat het herhaald aanbieden van antigenen via de slijmvliezen de respons in het bloed kan verminderen.

De resultaten die verkregen zijn in dierexperimenteel onderzoek, zijn niet zonder meer toepasbaar op de humane situatie. De mens heeft immers geen NALT als zodanig, hoewel de NALT beschouwd wordt als het ratte equivalent van het humane adenoïd. Daarnaast is de neusmucosa zelf bezaaid met lymfoïde en niet-lymfoïde cellen. In **hoofdstuk 5** is de neusmucosa van KNO-patiënten en van mensen met een gezonde neus onderzocht. **Hoofdstuk 5.1** richt zich met name op het epitheel dat de neusmucosa bedekt. Hierin blijken veel meer T dan B cellen voor te komen, waarbij de suppressor/cytotoxische (CD8+) T cellen veruit in de meerderheid zijn. Dit komt het sterkste naar voren bij patiënten. Van de lymfoïde cellen in het neusepitheel is bij patiënten 12% T helper (CD4+) en 78% T suppressor/cytotoxische cel. Het HLA-DR antigeen (het humane equivalent van het Ia antigeen) komt in ruime mate voor, op epitheelcellen, op lymfoïde en op niet-lymfoïde cellen.

Deze studie werd uitgebreid naar de overige lagen van de mucosa, waarover in **hoofdstuk 5.2** gerapporteerd wordt. Niet alleen in het bedekkende epitheel, maar ook in het klierepitheel komen meer CD8+ cellen dan CD4+ cellen voor. Ook in de klieren is de verhouding CD8+/CD4+ het hoogste in weefsel van patiënten. De indruk bestaat dat de CD8+ cellen behoren tot de groep cytotoxische T cellen. Deze kunnen

bijvoorbeeld virus-geïnfekteerde cellen opruimen. In het bindweefsel onder het epitheel zijn B cellen schaars, maar plasma cellen komen veelvuldig voor. De plasma cellen zijn onder het epitheel gelokaliseerd en in grotere aantallen rondom de klieren (zie hoofdstuk 6: Fig. 2). Het meest geproduceerde isotype is IgA. Zowel in patiënten als in controle weefsel worden regelmatig clusters van lymfoïde cellen gevonden in het subepitheliale bindweefsel. Soms worden naast een dergelijk cluster HLA-DR+ HEV waargenomen. Dit is opmerkelijk omdat HEV onder normale omstandigheden uitsluitend in lymfoïde organen voorkomen. Voorts komt het HLA-DR antigeen voor op ronde cellen en op cellen met uitlopers. Relatief gezien komen meer ronde HLA-DR+ cellen in het weefsel van patiënten voor, en meer HLA-DR+ cellen met uitlopers in het weefsel van controle personen. De ronde HLA-DR+ cellen zijn naar alle waarschijnlijkheid geaktiveerde T cellen. In geaktiveerde toestand kunnen T cellen namelijk het HLA-DR antigeen tot expressie brengen.

In standaard histologisch gekleurde coupes is patiënten materiaal duidelijk van controle weefsel te onderscheiden. De toegepaste 'methylgroen-pyronine' kleuring laat zien dat de klieren in de neusmucosa van patiënten rijkelijk gevuld zijn met mukeus (slijmerig) sekreet. Een opvallend verschijnsel is ook het veelvuldig voorkomen van plaveisel epitheel bij patiënten. Deze vorm van epitheel, die bestaat uit afgeplatte cellen welke laagsgewijs zijn gestapeld, komt normaal niet voor in het onderzochte gedeelte van de neusmucosa. Hier wordt doorgaans ademhalingsepitheel aangetroffen, waarin slijm producerende cellen en trilhaardragende cellen aanwezig zijn (zie hoofdstuk 1: Fig. 2). Ademhalingsepitheel is van belang bij het vangen en afvoeren van deeltjes. Vervanging van ademhalingsepitheel door plaveisel epitheel maakt deze basale funkties onmogelijk. Ten gevolge van de verminderde reinigingsfunktie ontstaat een verhoogde kans op (re)infektie, met als gevolg dat ontstekingsreakties optreden, leidend tot weefselbeschadiging. Herstel van deze weefselbeschadigingen resulteert in verdere vervanging van ademhalingsepitheel door plaveisel epitheel, waarmee de ongunstige kring gesloten is.

Tenslotte worden in **hoofdstuk 6** de beschreven resultaten besproken in het licht van relevante literatuur. In deze samenvatting komen enkele discussiepunten bij het betreffende onderdeel ter sprake.

CURRICULUM VITAE

De schrijfster van dit proefschrift werd geboren in oktober 1960 te Maastricht. Na het eindexamen atheneum-B in juni 1979 aan het Stedelijk Lyceum te Maastricht, begon zij in datzelfde jaar met de studie geneeskunde aan de Rijksuniversiteit te Utrecht. Na het kandidaatsexamen in juni 1982 werd de opleiding vervolgd als vrij doctoraal studie geneeskunde. Deze studie werd in december 1984 cum laude afgesloten, met als hoofdvak immunologie en als bijvakken ziekenhuiswetenschappen en sociale geneeskunde, met name gezondheidsvoorlichting en -opvoeding (GVO). Aansluitend aan het laatste bijvak werd aan de Rijksuniversiteit Utrecht, bij de vakgroep Algemene Gezondheidszorg en Epidemiologie, meegewerkt aan een literatuurstudie betreffende de medische en maatschappelijke aspekten van passief roken. Van augustus 1985 tot augustus 1986 was de schrijfster in dienst van ZWO verbonden aan de Rijksuniversiteit van Leiden, bij de afdeling Fysiologie en Fysiologische Fysica, werkzaam aan de ontwikkeling van een microscopische fluorescentietechniek ter bestudering van metabole inhomogeniteiten in de hartspier. Vervolgens kwam zij in augustus 1986 in dienst van de Vrije Universiteit te Amsterdam; vakgroep Celbiologie, sectie Histologie. Aldaar werd de immunologische afweer in de bovenste luchtwegen bestudeerd, hetgeen resulteerde in de totstandkoming van dit proefschrift. Het onderzoek werd gefinancierd door het EMGO (Extramuraal Geneeskundig Onderzoek) instituut.

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STELLINGEN

behorende bij het proefschrift:

Immunology of the upper respiratory tract:
studies on rat nasal-associated lymphoid tissue (NALT) and human nasal mucosa

1. De struktuur en ontwikkeling van het neus-geassocieerde lymfoïde weefsel (NALT) laten het belang van de bovenste luchtwegen in de eerste afweer tegen geïnhaleerde antigenen zien.
   Dit proefschrift

2. De aanduiding 'perifere lymfeklier' als zelfstandige benaming is onduidelijk, wordt vaak onjuist gebruikt, en dient derhalve vermeden te worden.

3. De relatie tussen bovenste en onderste luchtweg pathologie (Cole & Stanley 1983) dient weerspiegeld te worden in een nauwe samenwerking tussen KNO- en longarts.
   Cole & Stanley, 1983, Eur J Respir Dis 64 Suppl 126:145-148

4. Het hanteren van een 'mac-waarde' is in het geval van formaldehyde zinloos en kan leiden tot ongezonde situaties.

5. De combinatie van veel zout in het eten en het gebruik van de magnetron-oven kan ongunstig zijn, zowel voor de bloeddruk als voor het risico op voedselvergiftiging.
   Dealler & Lacey, 1990, Nature 344:496

6. De gevolgen van atmosferische depositie ('zure regen') zijn algemener dan veelal wordt aangenomen, hetgeen blijkt uit de enorme achteruitgang in soortdiversiteit in natuurterreinen gelegen op bodems die als niet-verzuringsgevoelig worden aangemerkt.
   Bobbink, 1989, Brachypodium pinnatum and the species diversity in chalk grassland. Proefschrift, Rijksuniversiteit Utrecht

7. Vanuit het oogpunt van rechtvaardigheid dienen geluidswallen eerder om natuurgebieden dan om woonwijken geplaatst te worden.

8. Het aantal vrouwen in de immunologie is aanzienlijk groter dan de adresseringen doen vermoeden.

9. De puntentelling in het badmintonspel werkt zelfoverschatting van mannen in de hand.

10. Het op elkaar afstemmen van noden en behoeften op het gebied van ontwikkelingshulp maakt te vaak een onderontwikkelde indruk.

11. Het neusje van de zalm is niet echt uitstekend.

Amsterdam, 29 juni 1990
Dona Hameleers