Staphylococcus aureus-derived superantigens in nasal polyp disease

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Abbreviations used

AERD	aspirin-exacerbated respiratory disease
ASNP	aspirin sensitive nasal polyps
ATNP	aspirin tolerant nasal polyps
CRSsNP	chronic rhinosinusitis without nasal polyps
CRSwNP	chronic rhinosinusitis with nasal polyps
CysLT	cysteinyl leukotrienes
ECP	eosinophil cationic protein
IFN-γ	interferon-gamma
IL	interleukin
MHC	major histocompatibility complex
NP	nasal polyps
SA	*Staphylococcus aureus*
SAE-IgE	IgE antibodies to SAE
SAE	*Staphylococcus aureus* enterotoxin-like toxins
SEA - SEU	Staphylococcal enterotoxin A - U
TCR	T cell receptor
TGF-β	transforming growth factor beta
Th	T helper
TNF-α	tumor necrosis factor alpha
TSST-1	toxic shock syndrome toxin-1

Core messages

- *Staphylococcus aureus* secretes enterotoxins, which can cause an intense polyclonal stimulation of the adaptive immune system and drive it towards a T helper 2 response.
- This superantigen mechanism is involved in the pathogenesis of nasal polyps in at least 50% of the cases.
- The superantigenic effect is hallmarked by immunoglobulin changes in biopsies: high total IgE, polyclonal IgE to multiple allergens, and IgE specific to *S. aureus* enterotoxins. Serum immunoglobulins only partially coincide with biopsy findings.
- Patients with this local IgE pattern have an increased risk of asthma and aspirin-exacerbated respiratory disease.
- Future treatment with topical or systemic antibiotics, and monoclonal antibodies to IgE and interleukin 5 are under investigation.

The discovery of IgE antibodies to *Staphylococcus aureus* enterotoxins A and B in nasal polyp tissue homogenates (Bachert, Gevaert et al. 2001) for the first time indicated that these bacterial products could be involved in the pathogenesis of nasal polyposis. Nasal polyposis, also referred to as chronic rhinosinusitis with nasal polyps (CRSwNP) (Fokkens, Lund et al. 2007), is mostly characterized by an eosinophilic, T helper 2 (Th2) skewed type of inflammation, driven by interleukin-5 (IL-5) and eotaxin which orchestrate chemotaxis, activation and increased survival of eosinophils (Bachert, Wagenmann et al. 1997; Simon, Yousefi et al. 1997; Bachert, Gevaert et al. 2000; Bachert, Gevaert et al. 2001). Based on its local cytokine profile, this disease can be differentiated from chronic rhinosinusitis without nasal polyps (CRSsNP), a T helper 1 (Th1) skewed inflammation with increased levels of interferon-gamma (IFN-γ) and transforming growth factor beta1 (TGF-β1) (Van Zele, Claeys et al. 2006). An important subgroup of nasal polyp patients demonstrates high nasal colonization rates with *Staphylococcus aureus* (SA). These patients have an increased local polyclonal immunoglobulin E (IgE) synthesis, correlating with the degree of eosinophilic inflammation, and an increased prevalence of asthma and aspirin hypersensitivity (Bachert, Gevaert et al. 2001).

There is a wealth of data to support the hypothesis of a modifying role of *S. aureus* enterotoxins in nasal polyposis. We here summarize the current evidence of an active role of *S. aureus* enterotoxins in nasal polyposis and contemplate on the possible clinical implications. After introducing the superantigenic properties of the staphylococcal enterotoxins, we present evidence of an increased nasal colonization with SA in nasal polyps together with specific humoral immune response to these molecules. We provide insight in possible mechanisms eliciting the polyclonal, Th2-skewed, eosinophilic milieu characteristic of nasal polyps and discuss current and future therapeutic approaches directed towards these key events in the pathophysiology of nasal polyp disease.

[insert Figure 1]

SUPERANTIGENIC PROPERTIES OF *STAPHYLOCOCCUS AUREUS* ENTEROTOXINS

Bullet messages:

- *Staphylococcus aureus* secretes enterotoxins, small proteins that act as superantigens because of their potent effect on the immune system.
- The main mode of action of superantigens is the coupling of the major histocompatibility complex (MHC) molecule with the T-cell receptor.

- The effect is a powerful stimulation of the adaptive immune system in a polyclonal (non-antigen-specific) way, resulting in a T-helper-2-biased inflammation.

Since its discovery in the 1880s (Newsom 2008), *Staphylococcus aureus* has been recognized as an important pathogen in human disease, being causative of diseases ranging from minor skin infections and food poisoning to life-threatening infections, septicemia, and toxinoses as the toxic shock syndrome (Lowy 1998). Despite its powerful pathogenic capabilities, around 20% of the population is persistent nasal carrier of *S. aureus*, and up to 60 % carries *S. aureus* intermittently (Wertheim, Melles et al. 2005). In contrast with intermittent carriers, persistent carriers tend to be colonized with the same bacterial strain over time. The versatile virulence is determined largely by its ability to regulate the production of surface proteins and secreted proteins by a set of more than 50 genes known as the virulon (Novick 2003). The secreted proteins include extracellular enzymes, such as catalase and coagulase, and a group of host-damaging proteins known as exotoxins. Of the latter, the enterotoxins have potent gastrointestinal effects and are the cause of staphylococcal food poisoning (Thomas, Chou et al. 2007). An increasing number of staphylococcal toxins is being described. The classical members Staphylococcal enterotoxin A to E are designated SEA-SEE, and newer toxins have been assigned a letter in the order of discovery (SEG-SEJ). However, some toxins lack proof of emetic properties, and they are considered as enterotoxin-like toxins (SElK-SElR, SElU), together with toxic shock syndrome toxin-1 (TSST-1) (Lina, Bohach et al. 2004).

The staphylococcal enterotoxin-related toxins (further referred to as SAE) share the ability to mount a massive inflammatory reaction resulting from a polyclonal activation of T and B lymphocytes nondependent of a specific adaptive immune response, a unique interaction for which they are known as superantigens, as first described by Kappler and Marrack in 1989 (Marrack and Kappler 1990). It has been suggested that the pathogens evolved over time to produce superantigens, disturbing an efficient adaptive immune response of the host, thus aiding in colonization and spread of the organism (Seiberling, Grammer et al. 2005). Superantigens from other bacteria have been described, including *Streptococcus pyogenes, Streptococcus dysgalactiae, Mycoplasma arthritidis, Yersynia pseudotuberculosis, Peptostreptococcus magnus* (Fraser and Proft 2008).

Unlike conventional T-cell activation via specific recognition by the T-cell receptor (TCR) of processed antigen peptides in the MHC molecule, SAE directly activate T-cells via bridging the MHC class II molecule with the TCR in a direct way, without being processed by antigen presenting cells (APC). Superantigens bind to one of the

112 domains of the MHC class II molecules on APCs in a region distant from the peptide-binding cleft, and to the
113 Vβ-domain in the β chain of the TCR, bypassing specific antigen recognition and resulting in a polyclonal
114 activation of T-cells (Fraser and Proft 2008). As there have been only 52 Vβ gene segments described coding for
115 the Vβ domain, it is estimated that SAE are able to stimulate up to 20-30% of the T-cell population, compared
116 to <0,01% by conventional antigen recognition. Staphylococcal enterotoxin-related superantigens show
117 specificity for one or more Vβ domains, linking them to specific T-cell populations and creating a superantigen-
118 specific Vβ signature (Gould, Takhar et al. 2007).

119 [insert Figure 2]

120 There are diverse ways in which the staphylococcal superantigens can exert their function on effector cells of
121 the immune system. T-cell superantigens stimulate T-cells, both CD4+ and CD8+, resulting in polyclonal
122 activation and expansion of specific Vβ subsets of T-cells. SAE are able to induce both a Th1-polarized and a
123 Th2-polarized CD4+ T-cell activation, with subsequent release of IFN-γ, TNF-α or IL-4, IL-5 and IL-13, not only
124 resulting from direct T-cell activation but also from stimulation of antigen presenting cells. The direction of the
125 T-helper response to either Th1 or Th2 cytokines is influenced by concentration of the superantigens, as well as
126 the nature of the APC and the costimulatory molecules. Mandron (Mandron, Aries et al. 2006) showed that SEB
127 activates monocyte derived dendritic cells (DCs) to secrete IL-2 and that these activated DCs polarize naïve T
128 cells to a Th2 type.

129 Despite the polyclonal T-cell expansion that has been observed in acute diseases as toxic shock syndrome,
130 chronic stimulation by superantigens may lead to an oligoclonal T-cell pattern, presumably resulting from the
131 concerted action with the conventional T-cell activation mechanism, where clones recognizing antigens are
132 selected after chronic exposure (Kim, Jacob et al. 2003). Moreover, after polyclonal expansion, superantigen
133 stimulation induces clonal deletion and anergy of remaining T-cell populations (Ivars 2007). The loss of the
134 immunosuppressive effects of naturally occurring regulatory T cells ($CD4^+$ $CD25^+$) has been described in
135 different inflammatory conditions; in atopic dermatitis, SEB has been shown to suppress their activity (Ou,
136 Goleva et al. 2004).

137 A polyclonal humoral immune response is evoked by SAE in a T-cell dependent way by cross-linking MHC class-
138 II molecules on B-lymphocytes and the TCR. In addition SAE enhance the Th2 response by augmenting isotype
139 switching to and synthesis of IgE (Gould, Takhar et al. 2007). Furthermore SEA and SED, together with

Staphylococcal protein A (SpA), may act as a B-cell superantigen by directly binding to VH3 or VH4 domains of the BCR, resulting in enhanced survival of these subsets of B-cells.

The finding of both T-lymphocytes and IgE specific to SAE indicate that SAE are also involved as conventional antigens, in which the SAE are processed into oligopeptides and presented in the antigen-binding groove of the MHC molecule. It is hypothesized that both the superantigenic and the conventional response act in concert, where the polyclonal stimulation of both T- and B-lymphocytes allow for an increased specific humoral or cellular response to SAE (Gould, Takhar et al. 2007). Lastly staphylococcal superantigens may have a direct effect on proinflammatory and other cells, such as eosinophils, macrophages, epithelial cells and fibroblasts. SpA is also able to induce degranulation of mast cells by crosslinking FcεRI molecules via binding to VH3 IgE domains, and is therefore called a superallergen (Marone, Rossi et al. 2007).

[insert figure 3]

INVASION OF NASAL TISSUE BY *STAPHYLOCOCCUS AUREUS*

- Nasal colonization with *Staphylococcus aureus* is increased in nasal polyp patients.
- The bacterium might persist by invading tissue and cells or by forming biofilms, to protect itself from the host immune system or from antibiotics.

Staphylococcus aureus is a frequent colonizer of the nose, with an average persistent colonization rate in 20-30% of individuals (Wertheim, Melles et al. 2005). Although *S. aureus* can frequently be isolated in acute and chronic rhinosinusitis, a disease-modifying role in CRS without NP has never been proven. Microbiology studies of the middle nasal meatus in chronic rhinosinusitis present conflicting results, however, in controlled studies, SA has been isolated in comparable rates in controls and CRS patients (Araujo, Palombini et al. 2003; Damm, Quante et al. 2004).

We reported for the first time an increased colonization rate in middle meatus nasal swabs from patients with nasal polyps but not in CRS without nasal polyps (Van Zele, Gevaert et al. 2004). SA colonization was present in 63.6% in CRSwNP vs. 27.3% in CRSsNP, and even higher rates were detected in NP patients with concomitant asthma (66,7%) and aspirin hypersensitivity (aspirin-exacerbated respiratory disease, AERD) (87.5%), whereas there was no significant difference between CRSsNP and control subjects. Furthermore, repeated swabbing in patients with NP indicated long-term colonization. The colonization rates in these patients were paralleled by IgE antibodies to SAE, total IgE and eosinophil cationic protein (ECP) in nasal tissue homogenates. These

findings were corroborated in a second study by our group, showing a colonization rate of 71% in nasal polyps versus 25% in controls (Gevaert, Holtappels et al. 2005). Conflicting results with our above studies have been reported (Niederfuhr, Kirsche et al. 2008), with detection of staphylococci in nasal lavage samples and in minced biopsies, in comparable levels between CRSwNP, CRSsNP and controls, using conventional culture methods, PCR and FISH.

As above studies used endoscopically guided swabs from the middle meatus, these results do not necessarily reflect the presence of SA within the nasal mucosal tissue. While SA has traditionally been regarded as an extracellular pathogen, there is increasing evidence that *S. aureus* has the ability to invade and survive in non-phagocytic eukaryotic cells such as keratinocytes and respiratory epithelial cells (Clement, Vaudaux et al. 2005). An intracellular reservoir of SA in 3 patients with recurrent/chronic rhinosinusitis undergoing sinus surgery has been shown by confocal immunofluorescence microscopy in nasal epithelial cells, mucous gland cells, myofibroblasts and CD45-positive phagocytes (Clement, Vaudaux et al. 2005). These findings were confirmed in a population of CRS patients undergoing sinus surgery, where intracellular SA could be demonstrated in nasal epithelium of 17 of the 27 patients (Plouin-Gaudon, Clement et al. 2006). Long-term carriage of identical clonal strains in CRS suggests that intracellular invasion presents an escape mechanism for host defense or antibiotic therapy. This finding may point to the involvement of *Staphylococcus aureus* small colony variants (SCV), strains that show a decreased growth rate, decreased hemolytic activity, increased intracellular survival and decreased antibiotic susceptibility; however, evidence of involvement in nasal pathology is lacking (von Eiff, Peters et al. 2006). The role of biofilms in CRS is being studied extensively (reviewed in (Harvey and Lund 2007)), but studies explicitly involving nasal polyps are scarce (Bendouah, Barbeau et al. 2006; Mladina, Poje et al. 2008). However, as biofilms have been shown to be related to protracted disease and antibiotic resistance, their role in the continuous immune stimulation by SA superantigens in nasal polyps is of particular interest.

We recently demonstrated the intraepithelial presence of SA in a subgroup of nasal polyps using immunohistochemistry. Interestingly, SEB could be colocalized to the intracellular *S. aureus*, indicating a potential local intracellular production of SA enterotoxins (Patou J., unpublished). Investigating invasive SA presence in different chronic sinus disease subgroups, we used peptide nucleic acid fluorescence in situ hybridisation (PNA-FISH) technique to stain for SA in nasal tissue samples (Corriveau, Zhang et al. 2009). Intramucosal presence of SA was comparable between control and CRSsNP groups. Although we did not

demonstrate a significantly higher rate of intramucosal presence in nasal polyps per se, we showed for the first time that intramucosal *S. aureus* presence is significantly augmented in aspirin sensitive asthmatic nasal polyp patients compared to polyp patients without comorbidities.

AUGMENTED IMMUNE RESPONSE TO SAE IN POLYPS

- *S. aureus* enterotoxins can be detected in nasal polyps.
- Immunoglobulin E antibodies against *S. aureus* enterotoxins are involved in nasal polyps.

In 2001 we presented the first paper suggesting a role for staphylococcal superantigens in nasal polyps (Bachert, Gevaert et al. 2001). Investigating the relation between atopy, local IgE concentration and parameters of eosinophilic inflammation in nasal polyp tissue, we demonstrated IgE specific to staphylococcal enterotoxins (SAE-IgE) in a subgroup of polyp patients. This subgroup, representing 50% of the nasal polyp patients in the study, had high local IgE concentrations and a local multiclonal IgE pattern, and showed higher concentrations of sCD23, ECP, IL-5, eotaxin, cysteinyl leukotrienes (CysLT) and a higher eosinophil count, compared to control tissue and to polyps with low local IgE. These patients also had a higher prevalence of asthma, and the inflammatory parameters and IgE concentrations in polyps were not related to atopy.

We subsequently reported a higher colonization rate of SA in nasal polyps (63,6%) which was paralleled by an increased presence of SAE-IgE (SEA, SEC, TSST-1) (in 27,8%), total IgE and ECP; observations that further increased in subgroups with asthma and with aspirin-exacerbated respiratory disease (AERD), detecting SAE-IgE in 53,8% and 80%, respectively (Van Zele, Gevaert et al. 2004). These colonization rates always exceeded those of the SAE-IgE rates, indicating that colonization may not necessarily lead to the generation of a humoral immune response. Furthermore ECP and total IgE were increased with the presence of IgE antibodies to SAE, suggesting a role for SA in eosinophilic inflammation and generation of high IgE levels. These results were confirmed in a further study where we detected SAE-IgE (SEA-SEE, TSST-1) in 50% of polyp samples, compared to 0% in control tissue (Gevaert, Holtappels et al. 2005). Total IgE, the ratio of IgE to albumin concentrations, and eosinophil count was higher in tissue of polyps that were positive for SAE-IgE. In line with these findings, of nasal polyps collected in a defined time frame in a South-Chinese hospital, 10/27 were positive for SAE-IgE versus 0/15 controls, although those rates may be lower in other parts of China (Zhang, Holtappels et al. 2006). In a study comparing polyps of patients with aspirin-sensitive versus aspirin-tolerant asthma, Suh et al. found IgE to SEA and SEB in one third of aspirin-sensitive polyps compared to one fifth in aspirin-tolerant polyps (Suh,

Yoon et al. 2004). Both the levels of SEA-IgE and SEB-IgE showed a close correlation with total IgE, ECP and IL-5 concentrations.

Most evidence of the in vivo secretion of enterotoxins is indirect, by demonstration of IgE specific for staphylococcal enterotoxins. One study (Bernstein, Ballow et al. 2003) isolated enterotoxin-producing *S. aureus* strains in 55% of polyp patients; although it is not clear whether and to what extent these organisms secrete superantigens under in vivo conditions. Seiberling (Seiberling, Conley et al. 2005) detected common staphylococcal toxins (SEA, SEB, SEC1-3, SED, TSST-1) using ELISA in 48% of polyp patients and in 7,7% of CRSsNP patients, with 9 of 15 positive patients demonstrating more than one toxin – indeed, it is common for *S. aureus* to produce more than one toxin at a time. In a study in Chinese sinus disease patients, the same superantigens were detected by ELISA in 12 of 22 polyps, compared to none in CRSsNP or control patients (Wang, Shi et al. 2008).

The classical superantigens, SEA through SEE and TSST-1, have been characterized and studied intensively in the past years, and most IgE responses described are directed against one or more of these proteins. Recently the egc gene cluster was identified in *S. aureus*, encoding SEG, SEI, SEM, SEN and SEO (Jarraud, Peyrat et al. 2001). We could identify enterotoxin genes in 75% of *S. aureus* strains detected in middle nasal meatus swabs, and showed an amplification of the egc gene cluster in 67,5% of strains (Van Zele, Vaneechoutte et al. 2008). Interestingly, there were no differences in enterotoxin genes between SA isolated from controls compared with nasal polyposis patients, suggesting that the specific immunological response of the host to SAE rather than the panel of enterotoxin genes produced by the species determines the clinical outcome. As there are no validated tests for the measurement of specific IgE against egc cluster enterotoxins, previous data regarding specific IgE production against SAE might underestimate the impact of enterotoxins.

[insert figure 4]

MECHANISMS LEADING TO POLYPS

- SAE have a specificity for subsets of T cell receptors. This is reflected in the T cell population in polyps.
- SAE drive T cells to a T helper 2 response and elicit a local polyclonal IgE production, only partially reflected in serum.
- Staphylococcal products also have a direct effect on B cells and mast cells.

Evidence of the involvement of a response of T lymphocytes to staphylococcal superantigens has been shown in a series of studies showing proliferation of T lymphocytes bearing specific Vβ domains. Bernstein *et al.* (Bernstein, Ballow et al. 2003) demonstrated in three nasal polyp patients significant clonal expansion of T cells with specific Vβ domains (Vβ skewing). In a further study including 12 polyp patients, Vβ skewing was demonstrated using flow cytometry in polyp lymphocytes of 7 patients whereas this expansion was not detectable in peripheral blood lymphocytes (Tripathi, Kern et al. 2005). Subsequently this group reported expansion of polyp lymphocytes expressing TCRs with specific Vβ domains in all of 18 polyp patients (Conley, Tripathi et al. 2006). The average number of Vβ clones per CRSwNP subject was seven in polyp lymphocytes but only two in peripheral blood lymphocytes. In another study, seven of 20 subjects exhibited skewing in Vβ domains with strong association to SAE (Conley, Tripathi et al. 2006). In Chinese patients, an increased percentage of Vβ-expressing T cells was observed in toxin-positive polyps (Wang, Shi et al. 2008). Many of the clonally expanded Vβ domains found in these studies are known to be associated with specific SAE. Moreover the ratio of Vβ skewing of polyp lymphocytes compared to peripheral blood lymphocytes points to a local expansion of these lymphocytes.

In a recent study we elucidated the modulatory effects of SEB and SpA exposure on nasal polyp cytokine secretion in an *ex vivo* setting (Patou, Gevaert et al. 2008). Nasal polyp and inferior turbinate fragments were suspended in culture medium and stimulated with SEB ad SpA for 30 minutes and 24 hours. Spontaneous release of IL-5, IL-13, TNF-α and IL-10 was greater in polyps than in control tissue. 24-hour stimulation with SEB caused a significant increase of Th1 and Th2 cytokines (IFNγ, IL-2, IL-4, IL-5, IL-10, IL-13) in inferior turbinates and to a greater extent in polyp tissue. By calculation of the ratio of increase in polyps to increase in control tissue it became apparent that the cytokine production was increased predominantly in Th2 cytokines (IL-4, IL-5) but that an increase in T-regulatory cytokine production (IL-10 and TGF-β) was disfavored by SEB stimulation. This study clearly confirmed that SEB can polarize mucosal inflammation to a Th2 pattern. SEB may contribute to persistent inflammation by suppression of T-regulatory lymphocytes, in line with our previous findings, where we showed a decreased FOXP3 and TGF-β1 expression in nasal polyps vs. CRSsNP and controls (Van Bruaene, Perez-Novo et al. 2008).

Of interest, we demonstrated that nasal polyps from South Chinese patients do not share the Th2-biased inflammatory pattern of polyps in European patients, as they where characterized by a neutrophilic

inflammatory pattern and lacked increased IL-5, ECP or IgE concentrations within polyp tissue (Zhang, Holtappels et al. 2006). Further studies revealed that Chinese polyps were characterized by a Th1-Th17 type of inflammation (Zhang, Van Zele et al. 2008). Those polyps may be less susceptible or may respond differently to the same exposure of SAE than European NP.

By detailed analysis of the pattern of increased IgE in nasal polyps and in serum, three groups of nasal polyps can be discerned (Bachert, Gevaert et al. 2001; Gevaert, Holtappels et al. 2005): (i) a group with no detectable specific IgE and low total IgE, (ii) a group with an 'allergic' type of IgE expression characterized by increased concentrations of total IgE and presence of selected specific IgE antibodies to aeroallergens corresponding to those found in serum and to skin prick test positivity and (iii) a group with a polyclonal pattern of IgE expression with specific IgE to a majority of allergens and increased total IgE, reflecting only partially the serum IgE response and independent of skin prick test positivity. The 'allergic' type can overlap with the 'polyclonal' type. The polyclonal pattern was detected in 10 of 20 nasal polyps in our first study and in 16/24 polyps in our second study, and there were IgE antibodies to SAE in respectively 10 and 12 of these polyps, indicating that SAE are most often involved in the polyclonal IgE response but that other than the classical staphylococcal enterotoxins or bacterial products from other organisms might have acted as superantigens in some cases.

Although extravasation of serum proteins has been shown in nasal polyps (Bachert, Gevaert et al. 2000), there is indirect evidence of a local production of IgE rather than a local reflection of a systemic production. Total IgE and SAE-IgE concentrations were in all cases higher in polyp tissue compared to serum (Gevaert, Holtappels et al. 2005); SAE-IgE may be detected in the serum of polyp patients, unrelated to atopic status, especially when asthma coexists (Conley, Tripathi et al. 2004; Tripathi, Conley et al. 2004). Moreover the IgE/albumin ratios in polyp tissue and serum were dissociated, and specific IgE antibodies in polyp tissue only showed a partial relation to serum IgE antibodies, indicating that tissue IgE is rather the result of a local IgE production than of extravasation (Gevaert, Holtappels et al. 2005).

When nasal polyps were analyzed for T and B lymphocytes and for IgE by immunohistochemistry, there were lymphoid accumulations seen in all samples, and lymphoid follicular structures were seen in 25% of polyps, whereas no secondary lymphoid tissue could be shown in control samples (Gevaert, Holtappels et al. 2005). Follicular structures stained positive for B cells (CD20) and T cells (CD3), and for IgE and CD23, whereas FcεRI was found only outside the follicles. Lymphoid accumulations stained positive for plasma cells (CD38), CD3, IgE

and FcεRI but not for CD23. We demonstrated binding of biotinylated SEA to both follicular structures and lymphoid aggregations. These data support the hypothesis of a local organization of secondary lymphoid tissue with polyclonal activation of B cells due to the stimulation by staphylococcal enterotoxins.

Acting as B cell superantigens, there is evidence that SAE can directly alter the B cell repertoire,. By crosslinking MHC class II molecules on B lymphocytes with TCR on T-lymphocytes, SAE can stimulate B cells in a T cell dependent way. SpA, a surface protein of *S. aureus*, can directly induce the proliferation of B cells. Moreover, TSST-1 induces isotype switching and synthesis of IgE, dependent on CD40L expression on B cells (Jabara and Geha 1996). A more recent study provided evidence for a direct effect by demonstrating TSST-1-induced expression of B7.2 on B cells, enhancing the Th2 response and regulating IgE production (Hofer, Harbeck et al. 1999). In mucosal tissue of hay fever patients, mRNA for the ε chain of IgE was found in a significant proportion of B cells using in situ hybridization, supporting the hypothesis of a local IgE synthesis in upper airway mucosa. Coker (Coker, Durham et al. 2003) showed evidence that local clonal expansion of B cells, somatic hypermutation and class switching occur in the nasal mucosa. A significantly biased expression of the VH5 regions of the IgE molecule (Coker, Harries et al. 2005) suggests that superantigens may modulate IgE production.

A high degree of infiltration by plasma cells in nasal polyps had been described earlier, and has been confirmed by our group(Van Zele, Claeys et al. 2006). Immunohistochemically we described increased $CD19^+$ naïve B cells and $CD138^+$ plasma cells but not $CD20^+$ mature B cells in nasal polyps compared to controls (Van Zele, Gevaert et al. 2007), implying a differentiation of memory B cells into plasma cells. In this study, we extended our observations of increased IgE to other immunoglobulin isotypes. Nasal polyps showed increased total IgA, IgG and IgE concentrations compared to CRSsNP and controls which was not the case in the serum of these patients. Of interest, polyps with detectable SAE-IgE had significantly higher concentrations of IgE and IgG, and a larger fraction of the IgG4 subset of the IgG isotype, than SAE-IgE negative polyps. The fraction of IgG4 correlated strongly with IgE concentrations and CD138 counts. These findings were not reflected in the serum of these patients, supporting the hypothesis of the modulation by SAE of the local immunoglobulin production by plasma cells and local isotype switching towards IgG4 and IgE.

Investigating the effect of staphylococcal products on nasal polyp cytokines and effector molecules, Patou (Patou, Gevaert et al. 2008) reported an increased secretion of histamine, cysteinyl leukotrienes, PGD_2 and IL-5

after stimulation with SpA. These results support the view that SpA may be acting not only as a B cell superantigen but may have a direct impact on mast cell and basophil activation. This activity, for which SpA is referred to as a superallergen, is mediated by interaction of SpA with the VH3 region of IgE bound to FcεRI, the antigen-independent crosslinking of FcεRI which it causes resulting in activation of the effector cell (Marone, Rossi et al. 2007).

It has been shown that nasal symptoms and markers of inflammation did not increase in relation to seasonal allergen exposure even in ragweed sensitive patients with nasal polyps, and nasal provocation was largely unsuccessful in NP patients (Keith, Conway et al. 1994). A polyclonal IgE pattern in nasal polyps may however cause a permanent degranulation of mast cell by conventional aeroallergens and superantigens, maintaining polyp growth, but not giving rise to acute allergic symptoms. This hypothesis needs further study, but may be of utmost importance to also explain similar mechanisms in non-atopic, but IgE-positive asthma.

RELATION TO EICOSANOID METABOLISM AND ASPIRIN SENSITIVITY

We reported increased SA colonization rates, total local IgE, specific IgE to SAE and ECP in nasal polyp patients with aspirin sensitivity (aspirin sensitive nasal polyps, ASNP) (Van Zele, Gevaert et al. 2004). In nasal polyp patients with aspirin intolerance we demonstrated increased total IgE, SAE-IgE, IL-5 and ECP compared to aspirin-tolerant nasal polyps (ATNP) (Perez-Novo, Kowalski et al. 2004), suggesting a relation of staphylococcal superantigens to aspirin sensitivity by upregulation of the eosinophilic inflammation. Post-hoc subgroup analysis revealed increased IL-5 and ECP in SAE-IgE-positive ATNP compared to SAE-IgE-negative ATNP, but these differences could not be shown in SAE-IgE-positive vs. SAE-IgE-negative ASNP groups, suggesting that aspirin sensitivity is linked indirectly to SAE by the severity of inflammation rather than via direct mechanisms. Our findings have been confirmed by Suh (Suh, Yoon et al. 2004), reporting increased ECP, IgE and SAE-IgE levels in Korean polyps.

Comparing eicosanoid production in CRSwNP and CRSsNP, concentrations of leukotriene C_4 synthase, 5-lipoxygenase and cysteinyl leukotrienes (CysLT) were increased in different sinus disease subgroups (CRSsNP, ATNP and ASNP) in parallel and in correlation with eosinophilic inflammation severity whereas COX-2 and PGE_2 were inversely correlated (Perez-Novo, Watelet et al. 2005). These data confirmed the notion that changes of eicosanoid metabolism do occur in CRS even in the absence of clinical aspirin sensitivity and appear to be related to the severity of eosinophilic inflammation. We extended our observations by demonstrating that the

production of CysLT, LTB_4, and LXA_4 is upregulated in SAE-IgE-positive NP compared to SAE-IgE-negative NP, and correlates to SAE-IgE, IL-5 and ECP levels (Perez-Novo, Claeys et al. 2006). Taken together these results, staphylococcal enterotoxins have an amplifying role in upper airway disease with aspirin sensitivity, without evidence for a direct causal relationship of SAE with aspirin sensitivity. However we recently isolated inferior turbinate fibroblasts and cultured the cells in presence of different concentrations of SEB (Perez-Novo, Waeytens et al. 2008). After pre-incubation with IFN-γ, SEB significantly downregulated PGE_2, COX-2 and EP2-receptor mRNA expression, pointing to a direct effect of staphylococcal superantigens on eicosanoid metabolism in upper airway tissue.

CLINICAL IMPLICATIONS

- Nasal swab culture or serum immunoglobulin assays are not sensitive enough to demonstrate superantigen-related disease.
- In polyp biopsies, a high total IgE, a polyclonal IgE pattern and IgE specific for SAE indicates superantigen-related disease.
- Nasal polyps may be refractory to local or systemic corticosteroid treatment.
- Future treatments under investigation include antibiotic treatment, and monoclonal antibodies to IL-5 and IgE.

There is accumulating evidence that staphylococcal superantigens may also have a major impact on lower airway disease such as asthma, chronic obstructive pulmonary disease and early wheezing (Bachert, Gevaert et al. 2007). In a patient presenting with nasal polyposis, the clinician could speculate about the activity of SAE, especially if comorbidities are present such as severe non-allergic asthma, aspirin sensitivity, or in corticosteroid-resistant disease. The detection of *S. aureus* by culture of swabs from the nasal middle meatus is a readily available diagnostic tool, but gives only a limited idea about an active immune response to the enterotoxins. Indeed the colonization rates exceeded the levels of SAE-IgE, and it is the latter correlating with severity of inflammation (Van Zele, Claeys et al. 2006). Furthermore, the in vivo ability of *S. aureus* to produce a superantigenic effect in the nasal tissue may vary according to the number and type of strains of the colonizing bacterium, and also depends on individual host factors, such as the genetic makeup and the inflammatory background, affecting the virulence and the interaction of enterotoxins with MHC molecules, T cell receptors and immunoglobulins.

The local immunoglobulin pattern may give a more specific idea about the effect of superantigens; this pattern is only partially reflected in serum. The presence of IgE antibodies to SAE indicates a former or present stimulation of the local immune system by the respective enterotoxin. A locally high total IgE and a polyclonal IgE response, directed to multiple conventional aeroallergens, which may be unrelated to serum IgE specificities, is indicative for a superantigenic effect. In asthmatic patients, the SAE-IgE level in serum is related to disease severity (Bachert, Gevaert et al. 2003).

In contrast to the polyvalent mechanisms of action of superantigens, the therapeutic armamentarium currently mainly consists of topical or systemic glucocorticoids and surgery (Fokkens, Lund et al. 2007). Therapeutic failure and recurrence account for a large part of patients treated with glucocorticoids, and cellular resistance to glucocorticoids is considered a main cause of treatment failure (Pujols, Mullol et al. 2007). Staphylococcal enterotoxins may impair corticosteroid treatment possibilities, as it has been shown that superantigens may alter steroid sensitivity and expression of glucocorticoid receptor beta (Hauk, Hamid et al. 2000).

Having an established role in nasal polyp pathophysiology, eradication of *S. aureus* with antibiotics seems a logical treatment option. This has not been studied extensively yet in nasal polyposis but the benefit of antibiotic and antiseptic treatment has been shown in atopic dermatitis, a disease sharing modifying effects of staphylococcal superantigens. An eradication scheme, consisting of oral antibiotics, topical antiseptics and nasal mupirocin ointment resulted in a significant but temporary improvement of atopic dermatitis patients who were colonized with *S. aureus* (Breuer, S et al. 2002). Nasal mupirocin lavage might be particularly useful in eradicating nasal *S. aureus* because of its potent effect on *S. aureus* in biofilms (Ha, Psaltis et al. 2008). Studies investigating the therapeutic benefit of antibiotic treatment in nasal polyp disease are currently underway. Further studies are needed to suggest other treatment options including long-term treatment with intracellular active antibiotics, *S. aureus* vaccination and specific enterotoxin antagonists. Based on the hypothesis of a continuous mast cell degranulation by an overwhelming polyclonal local IgE, treatment with monoclonal antibodies to IgE could be of relevance in suppressing IgE-mediated effects in analogy to the effect in allergic disorders. A randomized double-blind placebo-controlled trial is currently performed.

In the light of the association of SAE antibodies with a Th2-biased eosinophilic inflammation, treatment strategies antagonizing IL-5 provide an opportunity to prove the hypothesis. We recently reported a double-blind placebo controlled randomized trial evaluating safety and pharmacokinetics of intravenous injection of

420 humanized anti-IL-5 antibody in nasal polyp patients (Gevaert, Lang-Loidolt et al. 2006). We demonstrated that
421 a single injection of anti-Il-5 is safe and well tolerated, and reduced levels of blood eosinophilia and ECP, and IL-
422 5Rα concentrations in both blood and nasal secretions. In half of the patients, polyp scores improved after
423 single injection, and responders could be differentiated by increased levels of IL-5 in nasal secretions.

424 **SUMMARY AND PERSPECTIVES**

425 To summarize, we presented evidence for an at least modifying role of *S. aureus* superantigens in the
426 pathogenesis of chronic rhinosinusitis with nasal polyps by (i) showing an increased colonization rate of nasal
427 polyps with SAE-secreting *S. aureus* strains, (ii) the presence of superantigens in nasal polyps, (iii) evidence of
428 an immune response to SA by showing IgE antibodies to SAE, (iv) in vitro evidence of a modulation of nasal
429 polyp cytokine pattern to a Th2 response by SEB and (v) specific T lymphocyte Vβ-skewing characteristic of an
430 SAE effect.
431 However, data supporting the superantigen hypothesis by these modalities have been shown in approximately
432 50% of nasal polyps only (Bachert, Gevaert et al. 2001; Van Zele, Gevaert et al. 2004; Gevaert, Holtappels et al.
433 2005). One half of nasal polyp patients do not show evidence for a superantigen effect, but shares a similar
434 eosinophilic Th2 type of inflammation, albeit less pronounced in terms of intensity of inflammation. It remains
435 currently unclear and challenging why only a subset of nasal polyps is showing evidence of superantigenic
436 action and why only a part of superantigen-exposed individuals develop nasal polyps.
437 Genetic predisposition by expression of alleles specific to the superantigen interaction with MHC and TCR
438 molecules could explain a part of this observation. Measurement of IgE antibodies to only the classical
439 enterotoxins (SEA-SEE, TSST-1) could mask possible effects of other staphylococcal superantigens or of
440 superantigens produced by different organisms. Furthermore, the observation of the variable possibility of *S.*
441 *aureus* to invade tissue and cells could point to defects in mechanical or innate immunity. In such an immune
442 barrier hypothesis (postulated in (Kern, Conley et al. 2008)), genetic, epigenetic or environmental factors are
443 involved in epithelial antigen passage and processing, and could explain the highly variable immune response
444 to a given staphylococcal load.
445 The above evidence indicates that Staphylococcus aureus enterotoxins with superantigenic activity do play an
446 amplifying role in a subgroup of nasal polyp patients, eventually leading to asthma comorbidity and persistent
447 unified airway disease. The clinical identification of those patients is currently indirect, but the analysis of total

448 and specific IgE antibodies in serum, or better in tissue biopsies, support such diagnosis. First steps in the
449 development of appropriate new therapeutic targets have been made, and will in the near future impact our
450 daily clinical management (Bachert, Van Bruaene et al. 2009).
451

IMAGES

Image 1.

Scanning electron micrograph of a *Staphylococcus aureus* colony under a magnification of 20,000x. Photograph courtesy of J. Carr, Centers for Disease Control and Prevention, 2001.

Image 2.

Stimulation of a T lymphocyte by an antigen presenting cell (APC). Left: conventional antigens are processed by the APC and presented in the peptide binding cleft of the major histocompatibility complex (MHC) class II molecule. Upon recognition by the T cell receptor (TCR), signal is transduced to the T cell. Right: superantigens are not processed by an APC and activate the TCR directly by crosslinking the TCR to the MHC class II molecule, distant from the complementarity-determining regions. Illustration courtesy of Dr. T. Van Zele, 2006.

Image 3.

Effects of *Staphylococcus aureus* enterotoxins on antigen presenting cells, T lymphocytes, B lymphocytes, eosinophils and epithelial cells. Illustration courtesy of Dr. T. Van Zele, 2006.

Image 4.

Suggested model of SAE induced disease modulation of nasal polyps. Illustration courtesy of Dr. T. Van Zele, 2006.

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