

LETTER TO THE EDITOR

AUTOLOGOUS SERUM SKIN TEST REACTIVITY AND BASOPHIL HISTAMINE RELEASE TEST IN PATIENTS WITH NASAL POLYPOSIS: PRELIMINARY RESULTS

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An eosinophilic inflammatory process is generally observed in patients suffering from nasal polyposis (NP), however its onset has not yet been defined. It has been suggested that immune activation of inflammatory cells may be the cause. The aim of this study is to verify whether autoantibodies and/or histamine-releasing factors are present in the serum of patients suffering from NP. In fact, we assume that autoantibodies and/or histamine-releasing factors, as already demonstrated in chronic idiopathic urticaria and asthma, may be involved in the pathogenesis of NP. In this case-control analytical study 40 patients with NP and 27 control subjects underwent the in vivo autologous serum skin test (ASST). The sera from 6 patients suffering from NP and 9 control group subjects, who had all been previously studied and randomly selected, underwent basophil histamine release assay from normal donor as a pilot study. The ASST showed positive results in 55% of patients suffering from NP versus 8% of the control group ($p = .00006$), the basophil histamine release test (BHRT) turned out positive in all patients tested and in 11% of the control group. We found a weak positive correlation between the percentage of histamine release and the wheal diameter. ASST reactivity is very frequent in patients suffering from NP, thus suggesting the presence of histamine-releasing factors in the blood stream. The BHRT was positive in the serum of all patients, thus suggesting the presence of anti-FcεRI, anti-IgE autoantibodies and/or other histamine-releasing factors, the presence of which can play a role in triggering and maintaining the eosinophilic inflammatory process in NP.

NP is a chronic inflammatory illness which exhibits a high comorbidity with asthma (1) and in which tissue T lymphocytes show a mixed phenotype with a prevalence of Th2 (2). IL-5, eotaxin, MMP, GATA-3, T-bet and IgE are up-regulated, whereas TGF-β and FOXP3 are down-regulated. Eosinophils accumulated in tissues are activated and show reduced apoptosis. Cytokines and eosinophilic

inflammation increases in NP with IgE specific against the *Staphylococcus aureus* superantigen which induces T and B cell polyclonal activation with IgE formation which may represent a risk factor for NP, regardless of the allergy. The number of mast cells is not higher, however they are most likely IgE-positive and have a degranulated pattern. NP/intrinsic asthma comorbidity may depend on

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some common aspects (3): increase of local IgE and cytokines (from Th1 and Th2); eosinophilic infiltration and activation, cytokine and chemokine expression similarity, and analogous reaction to the superantigen. However, the cause of the eosinophilic inflammation is still uncertain. In patients with chronic urticaria (CU), and recently in patients with intrinsic asthma (4), it has been demonstrated that intradermal injections of autologous serum cause a cutaneous reaction which seems to be due to the presence of autoantibodies against the high-affinity IgE receptor (FcεRI) or against IgE or a histamine release factor specific for mast cells (5). These circulating factors may activate mast cells, starting and maintaining bronchial inflammation in intrinsic asthma patients and, we believe, eosinophilic inflammation in NP patients. The aim of this study is to determine whether similar factors are present in the serum of NP patients.

MATERIALS AND METHODS

At the Allergology Unit of the ENT Department of the University of Rome, 2 groups of subjects were selected and subdivided as follows.

1. A control group consisting of 27 people subdivided into 3 sub-groups:
 - Sub-group 1 was composed of 10 people (7 females and 3 males; average age = 38 ± 12 years) who showed normal general and local conditions
 - Sub-group 2 was composed of 7 patients (4 females and 3 males; average age = 51 ± 13 years) who suffered from non-allergic, non-infectious perennial rhinitis (Naniper)
 - Sub-group 3 was composed of 10 patients (4 females and 6 males; average age = 31 ± 11 years), who suffered from moderate/severe persistent allergic rhinitis (m/s PAR) according to the ARIA classification (6).

Inclusion of Naniper and m/s PAR sub-groups was necessary in order to evaluate the presence of these factors in the two most frequent forms of rhinitis.

2. A group of 40 bilateral eosinophilic NP patients (16 females and 24 males; average age = 52 ± 14 years).

Both groups were submitted to a preliminary medical examination in order to check family medical history, with special focus on asthma, rhinitis, NP and immune diseases; personal medical history by gathering information about their present disease, related respiratory diseases (including cystic fibrosis), immune diseases, number of previous NP-related surgeries, and any pseudo-allergic reactions (PAR) or allergies to drugs and food. All patients underwent

respiratory functionality tests before and after a specific bronchial provocation with methacholine (measurement of bronchial hyperreactivity, BH). All asthmatic patients showed a 20% decrease of FEV1 due to inhalation of <8 mmol of methacholine or bronchial obstruction to basal spirometry with reversibility after inhalation of 200 µg of albuterol. Moreover, the daily variation of PEF ($>20\%$) was monitored for as long as 2 weeks. Each patient was prick-tested for respiratory and food allergens, the uncertain cases also being tested for specific IgE (UniCAP RAST, Pharmacia, Uppsala, Sweden). Three weeks before the beginning of the study, all patients suspended antihistaminic, corticoid and anti-leukotriene systemic or local therapies. When the study started, patients did not show any sign of acute respiratory infections. All patients enrolled were submitted to a general otolaryngology medical check-up and nasal endoscopy with rigid fiber optic scope to exclude any anatomical alterations. All patients with such alterations were excluded. The NP group was CT-scanned for clinical purposes to assess the size of NP and paranasal sinus involvement. M/s PAR patients were diagnosed through positive medical history for allergenic exposure symptoms, prick-test positivity for perennial allergens and, in some cases, RAST. Naniper patients were diagnosed based on their positive medical history for symptoms due to exposure to non-specific stimuli (cold, humidity, etc.), presence of symptoms including nasal congestion, rhinorrhoea, sneezing, with two or more symptoms occurring for more than 1 hour and many days, skin prick-test and RAST-negativity, and nasal cytology to exclude any increase in eosinophils, basophils, and neutrophils. Normal controls were checked through medical history and skin prick-test; while NP patients underwent endoscopic examination, with polyps not hindering nasal breathing and showing a significant size (extrameatal, II degree of NP, according to Lund's classification) (7), CT scan of the paranasal sinuses and, finally, nasal cytology to confirm the increase of the eosinophils (1-4+ degrees). The results of all these examinations, together with sex and age, were used to compare the two groups. A second medical check-up was performed 7 days later. A 10-ml blood sample was taken from all patients using sterile vacuum tubes without clotting the accelerator (Venoject, Terumo, Leuven, Belgium). Blood was allowed to clot at room temperature for 2 hours. The serum was obtained by centrifugation at $500 \times g$ for 15 minutes under sterile conditions soon before its use in the ASST. Additional 1-2 ml samples of sterile serum were used to perform BHRT.

The hospital ethics committee approved our research protocol. Patients and control subjects were aware of the purpose of this study and gave their written informed consent.

ASST

In both groups intradermal injections of 0.05 ml of fresh autologous serum or saline solution (0.9% w/v NaCl) were used as a negative control using disposable sterile syringes with 27 needles (Myjector, Terumo, Leuven, Belgium) and were bilaterally performed in the volar area of the forearm, 4 cm from each other. The observer was unaware of the nature of patient status. The skin areas selected showed no signs of urticaria or dermatitis during the 2 previous days. Such tests were repeated after one week to assess their repeatability. Reactions were analysed and interpreted after 30 minutes and always by the same operator. Lack of reaction to the saline control was considered as a prerequisite for continuing this study. ASST reactions were deemed positive when the response after serum injection provoked a wheal formation surrounded by erythema of the same size in both forearms. The wheal measured at least a 3 mm in diameter and was classified as 1+, 2+ or 3+, depending on whether the wheal diameter was in the ranges of 3-4, 4-5 or >5 mm, respectively.

BHRT

In 15 cases (9 control subjects: 3 normal controls, 3 Naniper, and 3 m/s PAR; 6 NP patients without respiratory allergies randomly selected in each group) which had previously been studied through ASST, we began a pilot study on the serum reactivity to BHRT. Simple randomization was obtained by assigning a computer-generated number to each case through casual extraction of 3 numbers for each control sub-group and 6 numbers for the NP group (Randomization Com Program). One adult donor whose basophils responded to anti-IgE with a release >30% was used in the assay. The basophil leukocytes were prepared by dextran sedimentation (5 mL of freshly drawn heparinized blood was added to 1 mL of 5.0% dextran and 0.25 mL of 0.2 mol/L EDTA, pH 7.0) and allowed to sediment at 5°C for 45 minutes. The basophil-containing layer was washed once with piperazine-1,4-bis (2-ethanesulfonic acid) containing 0.036% of human serum albumin (PIPES-HSA) and the cells were resuspended in 2 mL of PIPES-HAS containing 500 pg/mL of recombinant IL-3 (R&D Systems, Minneapolis, Minn). Samples of 40 µL cell suspension were incubated in filter plates (Whatman, Brentford, United Kingdom) at 37°C for 60 minutes with serum diluted 1:4 and 1:8 in duplicate (final reaction volume, 80 µL). After incubation, all samples were added to 200 µL of PIPES-HSA and centrifuged at 2000 * g at 5°C for 5 minutes to remove serum proteins and released histamine. The cell sediment trapped on the filter was lysed by using 20 µL of a 7% HClO₄. Then 200 µL of PIPES buffer were added and the samples were centrifuged to recover the histamine from

the lysed basophils. The histamine content in the filtrate was measured by using the glass fiber method according to the manufacturer (RefLab, Copenhagen, Denmark). Serum-induced histamine release was expressed as a percentage of total histamine content. Spontaneous release in all experiments was less than 10% and was subtracted to determine serum specific release. Serum from healthy individuals induced histamine release as much as 16%, and the BHRT was therefore considered positive only at a release >16.5%.

Statistical analysis

Statistical analysis was performed by using the Number Cruncher Statistical System software 6.0.1 version (NCSS Statistical Software, Kaysville, Utah). A stepwise regression model was applied for the identification of the independent predictors of outcome. Fisher's exact test was used in order to compare occurrence of ASST and BHRT positivity in patients with NP and control subjects. Numbers are reported as mean ± SD. Spearman rank correlation was used to examine linear association between percentage of histamine release of the BHRT and wheal diameter of the ASST. A *p*-value lower than 0.05 was considered significant.

RESULTS

Among various demographic characteristics (Table I), family medical history revealed varying percentages of respiratory diseases in the NP patients and in the control group (m/s PAR and Naniper). NP patient personal history showed the highest incidence of intrinsic asthma as well as the presence of non-allergic rhinitis, autoimmune thyroiditis, CU and other skin diseases. None of the patients had cystic fibrosis. Approximately 27% of the NP patients had polyp reformation following surgery. NP patients also showed an ASA/NSAIDs-hypersensitivity response and a presumable allergic origin in approximately 27% of the cases. Among the demographic and clinical characteristics of the two groups it is interesting to note that two variables were identified, by univariate analysis, as weak NP predictors, that is, intrinsic asthma (*p* < .04) and ASA/NSAIDs-hypersensitivity (*p* < .05). 8% of the control group (1 normal control and 1 m/s PAR patient) and 55% of the the NP group were positive for ASST (Fig. 1), with a prevalence of a 3+ response for this group. In 6 NP cases with ASA/NSAIDs-hypersensitivity, ASST positivity

Table I. Demographic characteristics of control and NP groups.

		control group			NP group
		Normal	Naniper	m/s PAR	
Number of subjects		10	7	10	40
Family history:					
Asthma		a	28.5 %	20 %	15 %
Rhinitis		a	14.28%	80 %	22.5 %
Polypsis		a	a	a	12.5 %
Chronic urticaria		a	a	10 %	a
Angio-oedema		a	a	a	2.5 %
Personal history:					
Asthma:					
	- allergic	a	a	40 %	17.5 %
	- non-allergic	a	14.4 %	a	30 % ^
Rhinitis		a	100 % **	100 % *	27.5 % *
Polypsis		a	a	a	100 %
Autoimmune thyroiditis		a	a	a	2.5 %
Hypothyroidism		a	a	a	2.5 %
Chronic urticaria		a	a	a	7.5 %
Eczema		a	a	a	5 %
Pityriasis		a	a	a	2.5 %
Dermatitis		a	a	a	2.5 %
Psoriasis		a	a	a	2.5 %
Anaphylaxis		a	a	a	2.5 %
Releapse after surgery		a	a	a	27.5 %
Allergies/PAR to:					
ASA/NSAIDs		a	a	a	15 % ^^
Other drugs		a	14.3 %	10 %	2.5 %
Foods		a	a	10 %	7.5 %
Prick test:					
D. Pt.		a	a	100 %	15 %
P.O.		a	a	10 %	17.5 %
Alternaria		a	a	a	2.5 %
Grass		a	a	a	7.5 %

Naniper = non-allergic, non-infectious perennial rhinitis; m/s PAR = moderate/severe persistent allergic rhinitis; NP = nasal polyposis; ASA/NSAIDs = acetylsalicylic acid/non-steroidal anti-inflammatory drugs; D.Pt= dermatophagoides pteronyssinus; P.O.= parietaria officinalis; *= allergic rhinitis; **= non-allergic rhinitis; PAR= pseudo-allergic reaction; ^= $p<0.04$; ^^= $p<0.05$; a=absent.

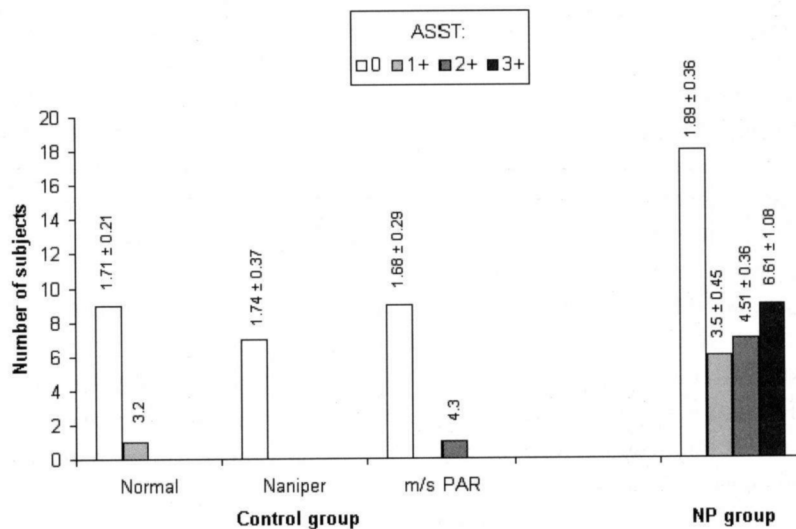
(1+ in 2 cases, 2+ in 1 case and 3+ in 1 case) reached 66.7%. Comparison between the NP group and the control sub-groups (Table II) demonstrated significative p -values for ASST in all cases, with the exclusion of NP vs Naniper for BHRT. The tests were repeated after 1 week, allowing the patients to be classified in a rather analogous manner, thus confirming their intrinsic reproducibility. In the 15 randomly selected cases, BHRT (Fig. 2) was positive in all 6 cases of NP (mean histamine release

= $21.3 \pm 2.87\%$), in 1 control group case (1 Naniper) and negative in the other cases. Furthermore, we observed a weak correlation between the wheal diameter (ASST) and the percentage of histamine released from basophils (BHRT). Finally, a low discrepancy was detected among the results: BHRT was positive when ASST was negative in the NP group and in 1 case of the Naniper sub-group, and was negative in the 2 ASST-positive cases of the 2 controls.

Table II. Comparison of the ASST and BHRT between NP group and control sub-groups (Fisher's exact test).

ASST:
NP vs Naniper p = .007
NP vs m/s PAR p = .01
NP vs Normal p = .01
NP with ASA/NSAIDs-hypersensitivity vs Normal p = .035
NP with ASA/NSAIDs-hypersensitivity vs Naniper p = .021
NP with ASA/NSAIDs-hypersensitivity vs m/s PAR p = .035
NP vs control p = .00006
BHRT:
NP vs Normal p = .01
NP vs Naniper p = n.s.
NP vs m/s PAR p = .01
NP vs control p = .001

Naniper = non-allergic, non-infectious perennial rhinitis; m/s PAR = moderate/severe persistent allergic rhinitis; NP = nasal polyposis; ASA/NSAIDs = acetylsalicylic acid/non-steroidal anti-inflammatory drugs; ASST = autologous serum skin test; BHRT = basophil histamine release test; n.s. = not significant.

**Fig. 1.** Values of the ASST in control and NP groups. ASST values (mean ± S.D.) are given in mm at the top of the bars.

DISCUSSION

ASST positivity was first described in CU (8) and successively in multiple drug allergies (94% of acute urticaria patients) (9), in NSAIDs-hypersensitivity (10) (30% with CU), in intrinsic asthma (11), (45% of the cases, 5% with CU) of which 28%

was correlated with BH (12), in allergic rhinitis (29%) and in patients with non-allergic respiratory symptoms (47% of the cases, 58% with CU) (13). There was a 55% incidence in our NP group. There was a weak ASST/BHRT correlation and the discrepancy observed in some cases may depend on variables which may be attributed to the two

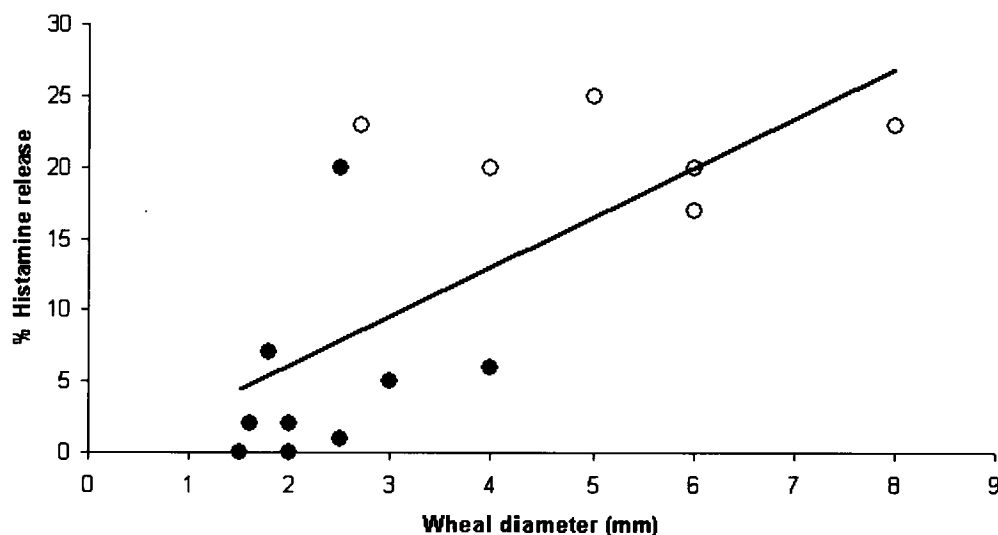


Fig. 2. Linear correlation between percentage of histamine release and wheal diameter.

(● = Control group; ○ = NP group).

Sperman rank correlation, $r=0.69$; $p<0.01$

tests: skin test on previously damaged skin which is incapable of reacting; low title of autoantibodies or their specificity for mast cells rather than circulating basophils (4); use of a functional assay, when there is an absence of a standardized immunoassay procedure, also suggesting the need for an optimal cell source. Western blot analysis was not used since autoantibodies are detectable in the serum of heterogeneous individuals. However, the low BHRT response does not allow us to perform a valid statistical analysis. BHRT and ASST identify two phenomena: the first shows the presence of autoantibodies against IgE or FcεRI *in vitro*, the second shows that the serum is capable of releasing histamine from mast cells, but whether this is due to autoantibodies in all cases or not is not known (14). ASST positivity in NP does not seem to be a non-specific phenomenon since there is a significant difference between this group and the control group, which is also confirmed in NP with ASA/NSAIDs-hypersensitivity. Lastly, ASST may be used as a predictive test. Our results show that 7.5% of NP patients had CU, 47.5% had asthma and it has been recently shown that 77% of CU patients show BH (15). We believe that an autoimmune mechanism acts as a link between these diseases and that skin and upper and lower airway symptoms probably occur

within a single framework of mast cell activation in different tissues. Our investigation has been the first to use ASST and BHRT in the study of NP; however, further research is required in order to verify the role of these autoantibodies in concomitant autoimmune diseases, such as immunologic screening, in order to also evaluate the risk of relapse following surgery, and to better define their role in the eosinophilic inflammatory process.

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