

Original article

Are basophil histamine release and high affinity IgE receptor expression involved in asymptomatic skin sensitization?

Background: Immunoglobulin (Ig)E-sensitized persons with positive skin prick test, but no allergy symptoms, are classified as being asymptomatic skin sensitized (AS). The allergic type 1 disease is dependant on IgE binding to the high affinity IgE-receptor (FcεRI) expressed on basophils and mast cells. However, a relationship between the AS status and FcεRI has not been investigated. We aimed to characterize basophils from AS by looking at histamine release (HR) (sensitivity and reactivity) and the FcεRI molecule, and compare it with nonatopic (NA) or allergic (A) persons.

Methods: Blood was obtained from NA ($n = 14$), grass and/or birch A persons ($n = 17$) and mono-sensitized grass or birch pollen AS ($n = 12$). The basophil sensitivity and reactivity were examined by anti-IgE triggered HR. Surface expression of FcεRI and IgE were measured by flow cytometry. FcεRIα protein was identified using a radioimmunoassay and Western blot. mRNA coding for the classic FcεRIβ-chain and the truncated form (FcεRIβ_T) were determined by real-time PCR.

Results: The AS group was less reactive than NA or A persons when triggered by anti-IgE and had a significant higher number of nonresponders. However, there was no difference in sensitivity among the three groups and furthermore; the groups did not vary in FcεRI- and IgE-surface expression, FcεRIα-protein level or β/β_T ratio.

Conclusion: Basophils from AS persons are less reactive and include more nonresponders than basophils from NA and A persons, but do not differ regarding the FcεRI molecule.

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Allergic type 1 disease is dependent on immunoglobulin (Ig)E binding to its high affinity receptor (FcεRI) expressed on basophils and mast cells. Cross-linking of FcεRI activates these effector cells to release inflammatory mediators (1). However, some IgE-sensitized persons with positive skin prick test (SPT) do not show any allergic symptoms. Despite these asymptomatic skin sensitized (AS) persons being frequently encountered in epidemiological studies (2, 3), the mechanism(s) underlying this AS phenomenon has still not been clarified.

Mast cells are responsible for the early inflammatory events after allergen challenge, and the basophil a key participant in the late-phase allergic reaction (4, 5). It is known that mast cells from different tissues can vary in reactivity (6, 7). Asymptomatic skin sensitized persons

could have a difference in their mast cell responsiveness, i.e. allergen induces only activation of their skin mast cells (SPT) and not of mast cells from other tissues, but this has not been investigated. The reactivity of the basophil in the AS persons is not known either, but it is possible that their reactivity also differs compared with nonatopics (NA) or allergic (A) persons.

A study of mast cell reactivity would be complicated by the high risk of modifying and activating the cells during purification. In contrast the responsiveness of basophils are more easily analysed in blood samples.

FcεRI is central in the induction and maintenance of an allergic response. Since 1977 it has been known that there is a correlation between the serum level of IgE and the cell surface expression of FcεRI (8, 9). The mechanism behind this IgE controlled FcεRI-expression was in 2001 described to be a stabilization of FcεRI (10–12). This was later shown to be an effect of IgE protecting an unstable FcεRIα-protein from degradation (13).

Basophil and mast cells express FcεRI as a tetrameric complex (αβγ₂) (14). The β-chain is found to play more

Abbreviations: FcεRI, High affinity IgE receptor; FcεRIα, High affinity IgE receptor α-chain; β, High affinity IgE receptor β-chain; β_T, Truncated β-chain; SPT, Skin prick test; HR, Histamine release; s-IgE, Specific IgE; RIA, Radio immunoassay.

than one role in the FcεRI induced response. It amplifies FcεRIα maturation, cell surface expression and signalling (15, 16).

Recently it has been reported that a truncated form of the β-chain (β_T) exists, both in human basophils and the cell lines KU812 (basophil) and LAD2 (mast cell) (17, 18). It prevents FcεRI surface expression by inhibiting α-chain maturation and competes with the classic β-chain to control FcεRI-expression (19).

We aimed to characterize basophils from grass or birch pollen mono-sensitized AS persons by looking at histamine release (HR) and the FcεRI parameters: surface FcεRI- and IgE-expression, serum IgE level, FcεRIα-protein level and the β/β_T-chain ratio, and compare it with grass and/or birch pollen A persons and an age-matched NA control group.

Materials and methods

Participants

Forty-three persons, recruited from a longitudinal investigation initiated in 2002, participated in this study. Based on anamnestic reports, daily diary cards and SPT the persons were divided into three groups:

1. Fourteen NA control persons with no clinical history of allergy and with negative SPT to a panel of aeroallergens.
2. Twelve AS persons with a negative clinical history of allergy, but with a reproducible positive SPT to birch or grass pollen. Only mono-sensitized persons were included to assure as well characterized persons as possible.
3. Seventeen birch and/or grass A persons, with a positive clinical history of birch and/or grass pollen allergy and positive SPT to birch and/or grass pollen.

Blood samples were drawn out of season. None of the participants had taken glucocorticosteroids or anti-histamines 3 months prior to the study period. For each patient group age, total serum IgE, symptom scores, SPT and specific IgE (s-IgE) levels are shown in Table 1.

Symptom registration

Symptoms from eyes, nose and lungs were graded from 0 to 3 points (0 = none, 1 = mild, 2 = moderate, 3 = severe symptoms). The symptom score was recorded as maximum symptom score registered by each person during the season. Symptoms were considered as birch or grass pollen allergy when lasting ≥7 days, or when symptoms were repeatedly elicited when pollen counts exceeded a certain individual level (2;2).

Skin prick test, specific IgE and total serum IgE protein

Skin prick test was performed in duplicates on the volar surface of the antebrachium using a commercially available panel of 10 aeroallergens [birch (*Betula verrucosa*), grass (*Phleum pratense*), mugwort (*Artemisia vulgaris*), horse, dog, cat, house dust mites (*Dermatophagoides pteronyssinus*, *D. farinae*) and molds (*Alternaria alternata*, *Cladsporium herbarum*) (Soluprick, ALK-Abello, Hørsholm, Denmark)] according to EAACI guidelines (SPT > 3 mm) (20). Histamine dihydrochloride (10 mg/ml) and diluent were used as positive and negative controls respectively. There was no difference in positive and negative skin reactions among the three groups, but AS group had smaller skin reaction compared with the A group when using allergens.

Specific IgE in serum was measured using the CAP Systems standard inhalation allergen panel (Pharmacia Diagnostics, Uppsala, Sweden). CAP class >1 was considered positive. Serum IgE protein level in patient serum was determined using the radioimmuno-metric method described by Poulsen and Weeke (21). As regards aeroallergens other than grass and birch all persons in NA and AS groups were negative.

Basophil enrichment

The basophils in anticoagulated blood were enriched using a Percoll-based double density gradient centrifugation technique. Six milliliters of diluted blood was layered onto 3.5 ml Percoll (Amersham Biosciences, Uppsala, Sweden) with a density of 1.071 g/ml on top of 3.5 ml Percoll with density 1.079 g/ml. After centrifugation at 700 × g for 20 min at 22°C, basophils were collected from the Percoll 1.071–1.079 interface. Basophil purity ranged from 0.7% to 54% (mean 16%, SD 10.8, *n* = 43) as determined by microscopic counts using Alcian blue staining.

Table 1. Characteristics for each study group

Group	Age (years)*	Serum IgE (ng/ml)†	Birch			Grass		
			Symptoms‡	SPT§	Specific IgE (KU/l)¶	Symptoms‡	SPT§	Specific IgE (KU/l)¶
NA	29.43 ± 8.0	52 (13 to 205)	0/14, 0 (0)	0/14	<0.35 (<0.35 to <0.35)	0/14, 0 (0)	0/14	<0.35 (<0.35 to <0.35)
AS Birch	25.5 ± 2.16	35 (26 to 59)	0/4, 0 (0)	4/4	<0.35 (<0.35 to 0.56)	0/4, 0 (0)	0/4	<0.35 (<0.35 to <0.35)
AS Grass	26 ± 2.59	144 (16 to 3330)	0/8, 0 (0)	0/8	<0.35 (<0.35 to <0.35)	0/8, 0 (0)	8/8	0.46 (<0.35 to 9.11)
A Birch	27 ± 7.11	104 (62 to 1120)	6/6, 2 (1 to 2)	6/6	11.15 (0.61 to 34.8)	0/6, 0 (0)	2/6	1.34 (1.19 to 1.49)
A Grass	25 ± 3.0	81 (3 to 790)	0/11, 0 (0)	1/11	9.57 (9.57 to 9.57)	11/11, 2 (2 to 3)	11/11	15.3 (<0.35 to 78)

NA, nonatopic; AS, asymptomatic; A, allergic.

*Values are given as mean ± SD.

†Values are given as median (min to max).

‡Patients with symptoms/total no. of patients in that particular group, symptom score median (min to max).

§Skin prick test (SPT) patients with a positive test/total no. of patients in that particular group.

¶Values are given as median (min to max).

Flow cytometry

Contaminating erythrocytes were removed by lysis (0.15 M NH_4Cl , 10 mM KHCO_3 , 0.1 mM Na_2EDTA , pH 7.4) before analysing the enriched basophil suspensions for expression of Fc ϵ RI α , IgE, CD203c and CD14 by flow cytometry. Cells were incubated with primary antibodies [10 $\mu\text{g}/\text{ml}$ mouse anti-human Fc ϵ RI α chain antibody CRA 1 (Cosmo Bio Co., Ltd., Tokyo, Japan), 1 $\mu\text{g}/\text{ml}$ biotin-conjugated mouse anti-human IgE (PharMingen, BD Biosciences, San Diego, CA), 10 μl mouse anti-human CD203c-Phycoerythrin conjugated antibody (Immunotech, Marseille, France) or 2.5 μl CD14-R-Phycoerythrin conjugated (Dako, Horsholm, Denmark)] for 30 min on ice and secondary antibodies [1 $\mu\text{g}/\text{ml}$ Fluorescein Isothiocyanate-conjugated rat anti mouse IgG2b and 0.5 $\mu\text{g}/\text{ml}$ streptavidin-phycoerythrin conjugate (both conjugates from PharMingen)] for 20 min on ice, and then analysed by flow cytometry (FACScan, BD Immunocytometry systems, San Jose, CA). Ten micrograms per millilitre mouse IgG2b (Dako), 1 $\mu\text{g}/\text{ml}$ biotin-conjugated mouse IgG2a (PharMingen) and 2.5 μl mouse IgG1-R-Phycoerythrin (Dako) were used as negative controls. The data on basophils were obtained from a basophil region defined as CRA1 positive, CD203c positive and CD14 negative. The extent of Fc ϵ RI expression or surface IgE expression are represented as the geometric mean fluorescence intensity (geoMFI) of CRA 1 or anti-IgE, respectively.

Histamine release

Heparinized blood from each patient was used for the HR experiments according to the method described by Skov et al. (22). In brief, washed blood cells were incubated (37°C, 60 min) in the presence or absence of birch or grass (Soluprick, ALK-Abello, Hørsholm, Denmark) dilutions (1 : 350, 1 : 1225 and 1 : 4288) or anti-IgE (rabbit anti-human IgE, Dako) dilutions (1 : 200, 1 : 600, 1 : 2000, 1 : 6000, 1 : 20 000 and 1 : 60 000). Released histamine bound to glass fibre coated microtitre plates (Reflab, Copenhagen, Denmark) was coupled to *o*-phthalaldehyde, stabilized by HClO_4 and measured fluorometrically. A release of $\geq 10\%$ was considered significant. Cells releasing $< 10\%$ histamine were considered as nonresponders. The HR results were evaluated by two parameters: *Basophil reactivity*; the maximal percentage of HR in the range of the anti-IgE dilution.

Basophil sensitivity; the minimum concentration of anti-IgE required for a 10% HR. The sensitivity was graded from 1 to 6, where 1 is basophils only reacting to the strongest anti-IgE concentration and 6 is basophil reaction to the lowest.

The histamine content per basophil was measured by dividing the histamine content in a lysed basophil-enriched cell suspension by the number of basophils in the sample.

Detection of Fc ϵ RI α using a radioimmunoassay

The level of Fc ϵ RI α protein from cell suspensions including 40 000 basophils [basophil purity $> 8\%$ was needed to standardize sample volume (mean purity $19.2 \pm 10.2\%$, $n = 34$ (11 NA, 10 AS, 13 A), mean content of CD14 $^+$ cells was $6.8 \pm 7.9\%$] was obtained by lysis (10^7 total cell/ml) and determined by using the radioimmunoassay previously described (13). The amount of Fc ϵ RI α -protein in the lysate was validated in arbitrary ' α -chain units'.

Western blotting

Lysate from basophil cell suspensions including 40 000 basophils was analysed on 10% SDS-polyacrylamide gels, prepared according

to the Laemmli protocol (23). In order to standardize sample volume only basophil preparations $> 13\%$ purity [$n = 22$ (10 NA, 5 AS, 7 A), mean purity of basophils was $24.3 \pm 9.2\%$, mean content of CD14 $^+$ cells was $7.9 \pm 9.5\%$] were used. The Fc ϵ RI α protein was analysed by Western blot as previously described (18).

For controlling differences in Western blots, each blot included a standard lane that consists of KU812 cell lysate. Comparisons of scanned blots were made by expressing the digitized band intensities relative to the 50 kDa Fc ϵ RI α protein band in the KU812 standard within the same blot and expressed in 'Wb units'.

RNA isolation, reverse transcription and real-time PCR

Total RNA from whole blood was isolated by RNA blood Mini Kit (Qiagen GmbH, Hilden, Germany). For quality and quantity, RNA samples were measured using an Agilent 2100 bioanalyser (Agilent Technologies Denmark A/S, Naerum, Denmark) according to the manufacturers instruction. First strand synthesis and expression of β - and the truncated β_T -chain determined by real-time PCR were performed as reported in Jensen et al. (18). The C_T -values for β and β_T were found to be between; 27.1–32.2 and 32.0–36.5 respectively. The level of β - and β_T -chain in each patient is expressed as a β/β_T ratio.

Statistical analysis

Statistical software was used for data analysis (SAS system for Windows V8; SAS Institute, Cary, NC, USA). Wilcoxon signed rank test and Spearman correlation test were used when comparing groups or correlating data respectively. Fishers exact test was used for table analysis. P -values ≤ 0.05 were considered significant.

Results

Basophil reactivity and sensitivity measured by histamine release

Analysis of the basophil reactivity and sensitivity demonstrated that the AS persons were less reactive than the other two groups (Fig. 1A). There was a significant difference between the NA and AS group, $P = 0.024$, and between the A and AS group, $P = 0.018$. There was no significant difference between the NA and A group. Regarding basophil sensitivity no significant differences were found between the three groups (Fig. 1B).

Interestingly, five AS persons did not respond to anti-IgE, which is significantly more compared with the other two groups ($P = 0.005$, Fishers exact test). Only one person in the NA group was a nonresponder, and none were found among the A persons (Fig. 1A).

All subjects in the A group and 5 AS-individuals had a positive HR to the relevant (SPT-positive) allergen. No controls reacted to allergens in HR.

Expression of the Fc ϵ RI-molecule

To study the Fc ϵ RI molecule we first compared the groups regarding Fc ϵ RI surface expression, IgE surface expression and IgE serum level. No difference was found between NA and AS persons, or between A and AS persons. However, a significant difference between NA

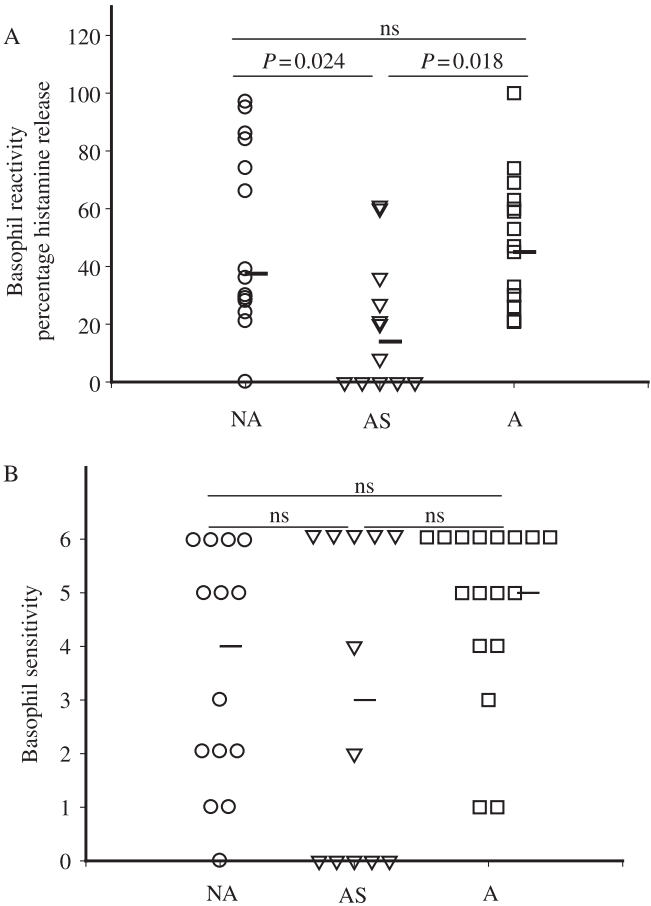


Figure 1. (A) Basophil reactivity measured by anti-IgE triggered histamine release. The results are given as maximal percentage histamine. The CV for each group is; 84.9 (NA), 162.9 (AS) and 50.6 (A). (B) Basophil sensitivity measured by anti-IgE triggered histamine release. The sensitivity is graded from 1 to 6, where 1 is basophils reacting to the strongest anti-IgE concentration and 6 is basophils reacting to the lowest anti-IgE concentration. The CV for each group is; 56.8 (NA), 96.4 (AS) and 33.6 (A). Anti-IgE stimulation was used as the NA and most AS basophils did not respond to allergens. NA, nonatopic; AS, asymptomatic; A, allergic; NS, not significant.

and A persons was found ($A > NA$; $P = 0.027$, $P = 0.0012$ and $P = 0.05$ respectively), which could be ascribed to a significant correlation between the three parameters as shown in Fig. 2. Regarding the AS persons, Fig. 2 illustrates that their data, in all three cases, ranged from the lowest to the highest value, which explains why no difference was found between the AS persons and the other two groups.

The FcεRIα protein level

Western blot (qualitative) and radioimmunoassay (RIA, quantitative) were used to further investigate possible differences regarding the FcεRIα protein. The insert in Fig. 3A shows an example of a Western blot where seven

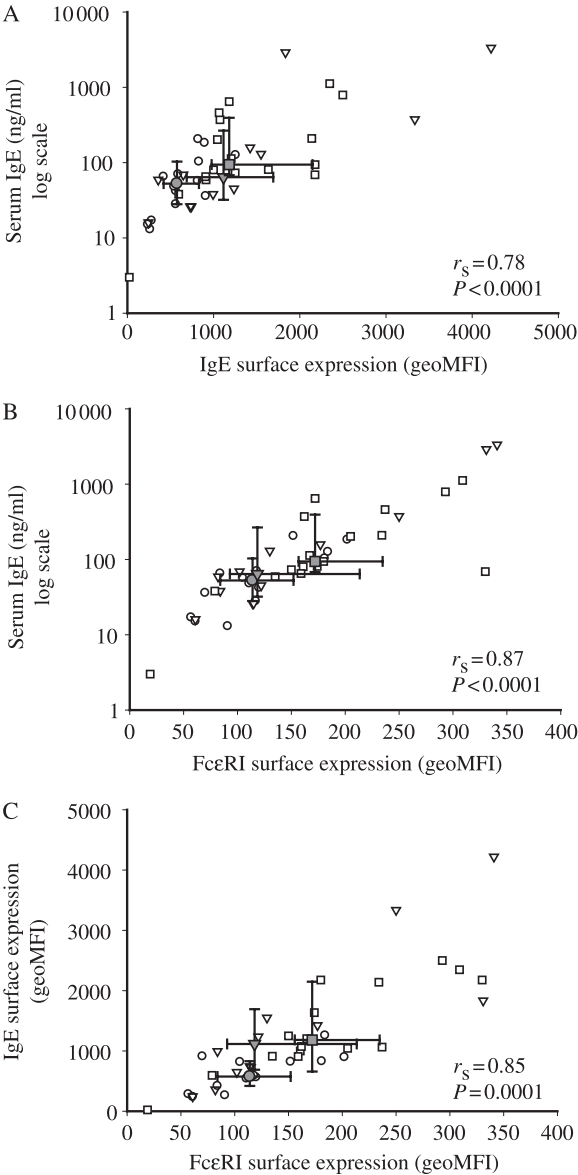


Figure 2. (A) Basophil cell surface expression of IgE measured by flow cytometry expressed relative to the serum IgE value ($r_s = 0.78$, $P < 0.0001$). (B) Basophil cell surface expression of FcεRI measured by flow cytometry expressed relative to the serum IgE value ($r_s = 0.87$, $P < 0.0001$). (C) The relation between the cell surface expression of IgE and FcεRI ($r_s = 0.85$, $P < 0.0001$). The correlation is found using Spearman rank correlation. Median and quartile are given for each patient group. ○, nonatopic; ●, median; ▽, asymptomatic; ▽, median; □, allergic persons; ■, -median; †, quartile.

persons' FcεRIα proteins are visualized. Each lane include two bands: a 66 and a 50 kDa protein, i.e. the surface and the intracellular FcεRIα protein, respectively (24). Neither of these two proteins differed in amount between the three groups (Fig. 3A). Additionally, when using the RIA no difference was found between the groups (Fig. 3B). The results of the two techniques were

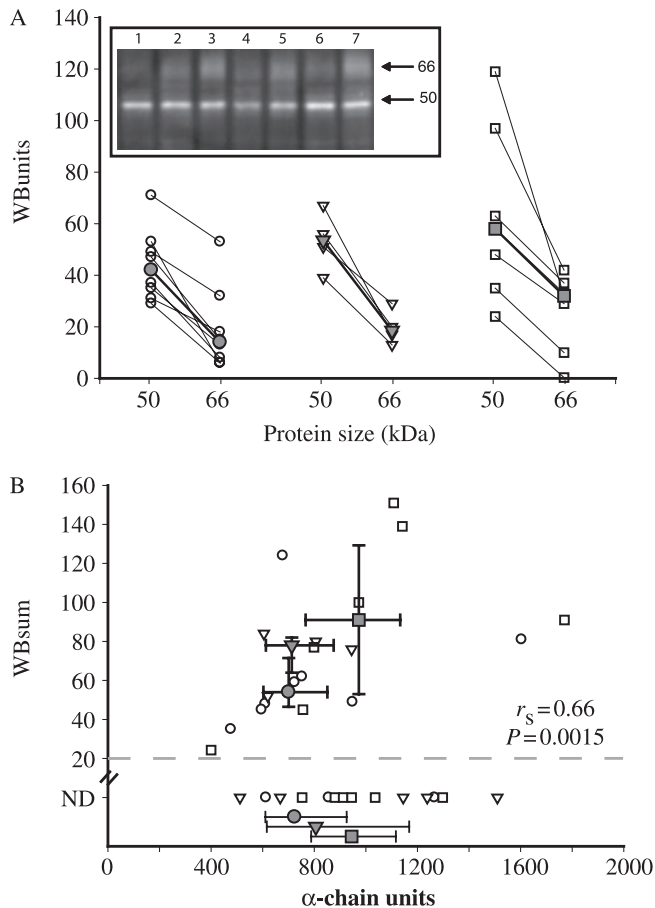


Figure 3. (A) The level of 50 and 66 kDa FcεRIα protein from 22 persons [10 NA (1 nonresponder), 5 AS (2 nonresponders), 7 A] given in Wb units. Insert; Western blot illustrating the 66 and 50 kDa FcεRIα-protein bands from seven persons [lane 1; NA, lane 2 (nonresponder), 3, 4 and 6 (nonresponder); AS, lane 5 and 7; A]. (B) The FcεRIα protein amount found using the RIA (α-chain units) expressed relative to the sum (Wbsum) of band intensities of the 50 and 66 kDa protein. Only basophil suspensions with a purity >13% were analysed by Western blot. The correlation was $r_s = 0.66$, $P = 0.0015$. Median and quartile are given for each group. The RIA data from individual lacking Western blot results are listed as 'Wbsum ND'. Median and quartile are given for the entire RIA data for each group. ○, nonatopic; ●, median; ▽, asymptomatic; ▽, median; □, allergic persons; ■, -median; †, quartile.

supportive of each other as a correlation between the RIA results and the Western blot (50 and 66 kDa sum) was found. Only samples with a basophil purity >13% were analysed by Western blot and therefore samples lacking Western blot data are listed as 'Wbsum ND' in the figure.

The ratio of FcεRIβ and β_T and its relation to FcεRI surface expression

We have measured the level of β and β_T mRNA in whole blood from all 43 persons. Figure 4A shows that the ratio

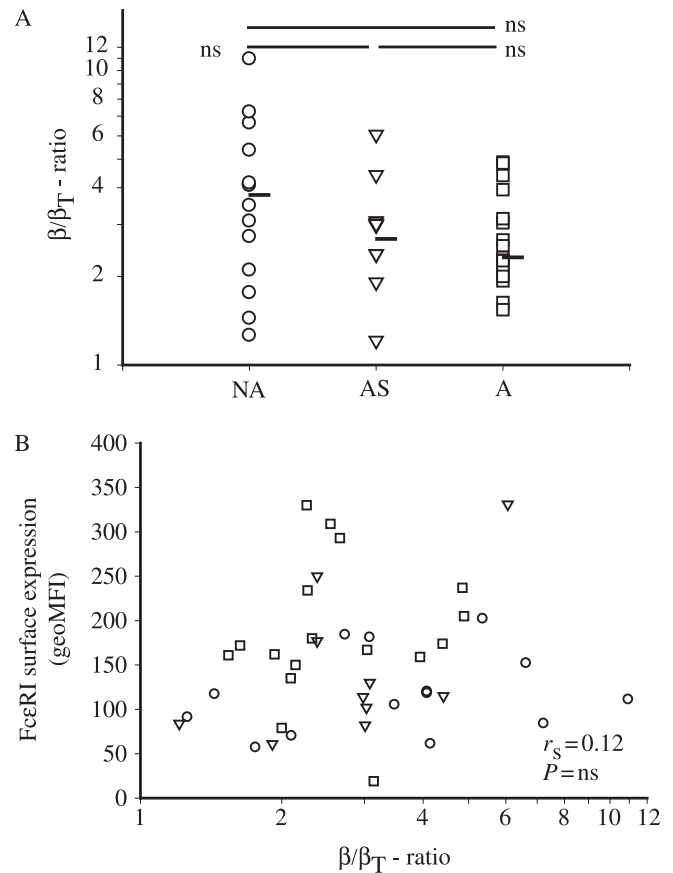


Figure 4. (A) The β/β_T ratio in basophils from the three persons group measured by real-time PCR. (B) The β/β_T ratio expressed relative to the basophil FcεRI cell surface expression measured by flow cytometry. The correlation is $r_s = 0.12$, $P = ns$. ○, nonatopic; ▽, asymptomatic; □, allergic persons.

of β/β_T in the three groups was similar. Furthermore; the β/β_T ratio did not correlate with the FcεRI surface expression found on the basophils (Fig. 4B).

Differences between responder and nonresponder regarding FcεRI

We found a difference in reactivity but not in any of the FcεRI parameters when comparing AS with NA or A. In order to investigate the reactivity more carefully we focussed on the nonresponders. Figure 5 illustrates how the responders and nonresponders relate regarding FcεRIα or IgE surface expression, serum IgE level, FcεRIα protein, histamine content pr. basophil or the β/β_T ratio. There was no significant difference between the responders and nonresponders, and therefore the nonresponder phenomenon cannot be explained by these parameters.

Discussion

The allergic type 1 response requires IgE. However, IgE-sensitization does not always coincide with clinical

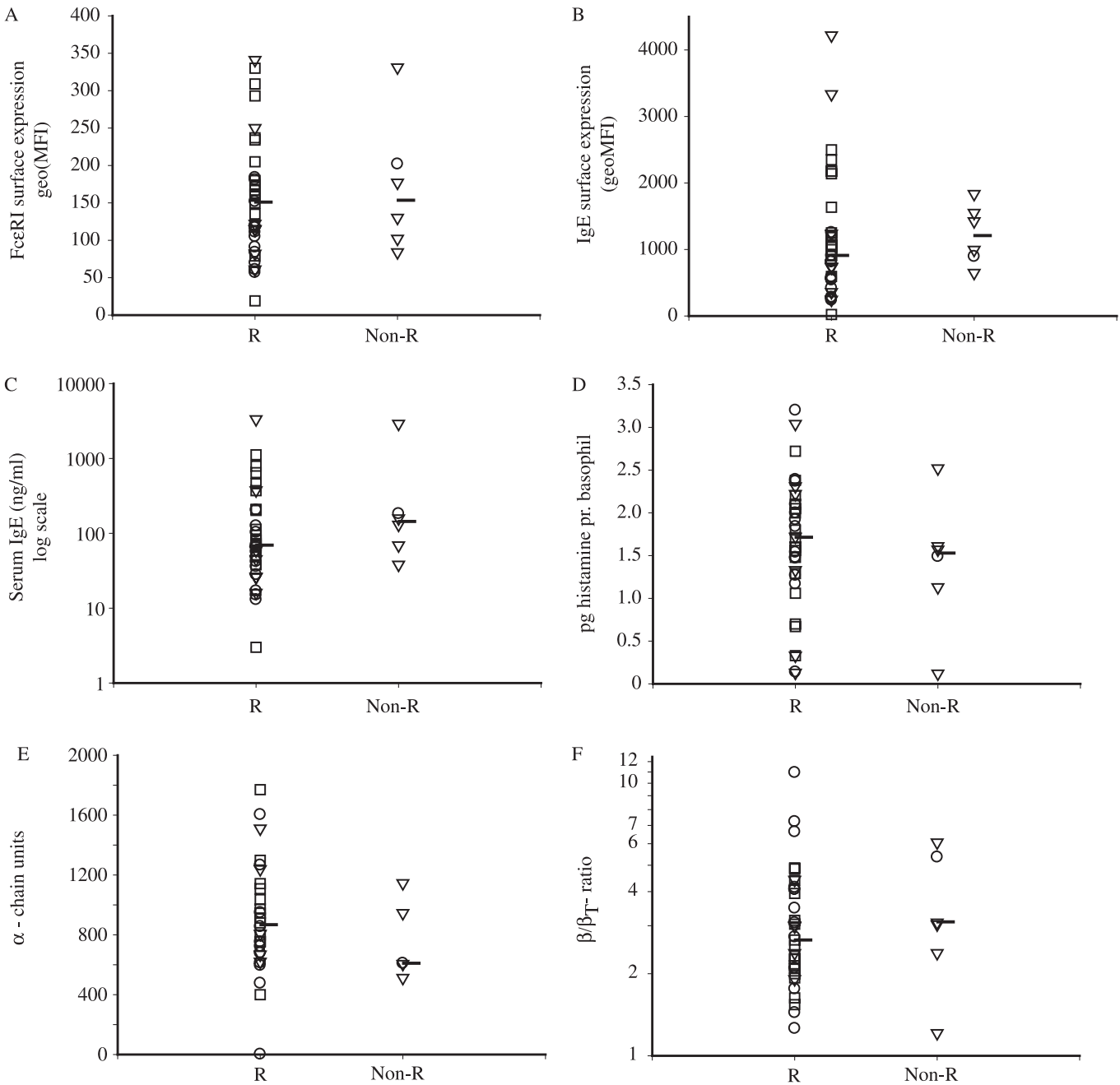


Figure 5. The responders and nonresponders are compared regarding: (A) basophil cell surface expression of FcεRI, (B) basophil cell surface expression of IgE, (C) IgE serum level, (D) histamine content pr. basophil, (E) FcεRIα-protein (α-chain units) and (F) the basophil β/β_T ratio. ○, nonatopic; ▽, asymptomatic; □, allergic persons; –, median.

allergic manifestation. Indeed, some individuals with detectable levels of s-IgE to aeroallergens do not develop any symptoms (2, 25). We investigated the basophil HR in a group of AS mono-sensitized birch- or grass pollen persons, and found that these persons reacted significantly less compared with NA or A persons. It has been reported that age affects the reactivity (26, 27), but as seen in Table 1, the three groups were age-matched.

The AS group included a high number of nonresponding basophils which strengthens the difference in reactivity. Moreover; there is a trend among the AS responding basophils to react more poorly. It is interesting, however, that no difference in sensitivity was found among the groups, despite the nonresponders. This indicates that the responders in the AS group were as sensitive (as easy to trigger to release histamine) as basophils from NA and A. Nevertheless, regarding reactivity (the amount of

histamine released) there seems to be a difference, even though the histamine amount per basophil did not vary among the three groups (data not shown).

We analysed the three groups regarding different FcεRI parameters to investigate whether or not the AS group differed. The surface expression of FcεRI and IgE, and the IgE serum level was not different, but the A group had a significantly higher value regarding all three parameters compared with NA, which supports the fact that there is a correlation between the IgE serum concentration and the FcεRI expression (9, 28).

We additionally examined semi-purified basophils from the three groups for differences in their expression of FcεRI protein, but no difference was found when either using Western blot or RIA.

The FcεRIα protein could originate from cells other than basophils, as FcεRI is expressed on antigen presenting cells [e.g. monocytes, Langerhans cells, peripheral blood dendritic cells (14)]. This could reduce possible difference between the groups, but as RIA and Western blot data were found to be correlated with the basophil FcεRI expression, it indicates that the detected FcεRIα protein primarily originates from the basophils (data not shown).

The β-chain is important for amplification of the FcεRI expression and signalling, meaning that the level of β-chain expression could influence basophil activity regarding intracellular signals. We examined the β/β_T ratio in basophils from the three groups by real-time PCR. Although previous reports of the β-chain in human basophils, have been made on only semi-purified basophils (purity of 2–5%), it is important to remember that other cells can express and thereby contribute to the β-chain amount in the sample (17, 24). In a pilot study we observed that β- and β_T-chain levels correlated with the basophil number and that the β/β_T ratio was constant regardless of purity (~2–67%, results not shown). This strongly suggests that the main part of the β-chain originates from basophils.

We did not find any difference in the β/β_T ratio between the three groups. In addition, the increase in FcεRI expression seen in the A group is not, besides the IgE effect, a result of a decrease in the β_T-level. Moreover, we were unable to find an effect of the β/β_T ratio on the

FcεRI surface expression. However, this does not necessarily mean that β_T is without influence on the FcεRI expression. The study done by Donnadieu et al. (17) of β_T was done in αβ_Tγ transfectants, where there was no competition from the classic β-chain, but because basophils have both β and β_T, our results could simply be an effect of β overruling β_T.

Only the reactivity and the number of nonresponders distinguish AS persons from the other two groups. In order to explore the nonresponsiveness we compared the total number of responders with nonresponders regarding the parameters used, but were unable to explain the nonresponder phenomenon. It is reported that the density of surface IgE found on both releasing and nonreleasing basophils are equal, which supports our findings concerning the FcεRI and IgE surface expression (29). Furthermore, recent findings illustrate that the nonresponder phenomenon is not associated with mutations in the FcεRIβ (30).

It has been reported that basophil unresponsiveness is related to a deficiency in expression of the tyrosine kinase Syk (31, 32). This could explain the nonresponder phenomenon found in the AS and NA group. However; other kinases like Fyn and Lyn have been reported to be key kinases in mast cell degranulation (33), which also could be the case in basophils. Moreover, factors like cell maturity and cytokine milieu could play additional roles regarding activity, as IL-3 has been reported to affect basophil responsiveness (31, 34).

We examined basophils from three groups of persons: NA, AS and A. We found that the AS persons reacted more poorly when stimulated with anti-IgE. Additionally, the number of nonresponders was significantly higher. It is tempting to speculate that the asymptomatic phenomenon is a reflection of the nonresponder phenomenon.

Acknowledgments

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