Original Paper

International Archives of Allergy and Immunology

Int Arch Allergy Immunol 2006;141:265–275
DOI: 10.1159/000095296

Received: October 27, 2005
Accepted after revision: April 26, 2006
Published online: August 23, 2006

Nitration Enhances the Allergenic Potential of Proteins

Y.K. Gruijthuijsen[a] I. Grieshuber[a] A. Stöcklinger[a] U. Tischler[a] T. Fehrenbach[b] M.G. Weller[b] L. Vogel[c] S. Vieths[c] U. Pöschl[b] A. Duschl[a]

[a]Department of Molecular Biology, University of Salzburg, Salzburg, Austria; [b]Institute of Hydrochemistry, Technical University of Munich, Munich, and [c]Paul Ehrlich Institute, Langen, Germany

Key Words
Air pollution · Bet v 1 · Epitopes · Immunoglobulin E · Nitration · Nitrotyrosine · Ovalbumin

Abstract
Background: Recent investigations have shown that proteins, including Bet v 1a, are nitrated by exposure to polluted urban air. We have investigated immunogenic and allergenic properties of in vitro nitrated allergens in in vivo models. ***Methods:*** Untreated and nitrated samples of ovalbumin or Bet v 1a were compared for their ability to stimulate proliferation and cytokine secretion in splenocytes from DO11.10 or from sensitized BALB/c mice, and for their ability to induce specific immunoglobulin (Ig)G1, IgG2a and IgE in sensitized mice. Additionally, sera from birch pollen-allergic individuals were analysed for IgE and IgG specific for nitrated Bet v 1a. ***Results:*** Upon splenocyte stimulation with nitrated as compared with unmodified allergens, proliferation as well as interleukin 5 and interferon-γ production were enhanced. Sera of mice sensitized with nitrated allergens showed elevated levels of specific IgE, IgG1 and IgG2a, compared with sera from mice sensitized with unmodified allergens. Moreover, cross-reactivity of antibodies against unrelated, nitrated allergens was observed in mice. We also found higher amounts of functional, specific IgE against nitrated than against untreated Bet v 1a in sera from birch pollen-allergic patients. ***Conclusions:*** Our findings suggest that nitration enhances allergic responses, which may contribute to an increased prevalence of allergic diseases in polluted urban environments.

Introduction

Immune responses can be affected by air pollutants including combustion-generated particles, semi-volatile hydrocarbons and exhaust gases [1–4]. The components of photochemical smog, NO_2 and O_3, induce inflammatory airway responses, enhance immediate- as well as late-phase responses to allergens, and reduce resistance against infections [1, 4, 5]. Recently, it has been demonstrated that NO_2 and O_3 in polluted urban air are also capable of modifying airborne proteins [6]. Under urban smog conditions, in situ nitration of tyrosine residues of various proteins, including the major birch pollen allergen Bet v 1a, were observed. Moreover, nitrated proteins were detected in dust samples from various urban environments [6]. In vitro, the rate of protein nitration was dependent on the abundance of both nitrogen oxides and ozone in air mixtures [6].

Post-translational modifications, including glycosylation, phosphorylation and cysteinylation, can affect the

KARGER

Fax +41 61 306 12 34
E-Mail karger@karger.ch
www.karger.com

1018–2438/06/1413–0265$23.50/0

Accessible online at:
www.karger.com/iaa

Correspondence to: Prof. Dr. Albert Duschl
Department of Molecular Biology, University of Salzburg
Hellbrunner Strasse 34
AT–5020 Salzburg (Austria)
Tel. +43 662 8044 5731, Fax +43 662 8044 5751, E-Mail albert.duschl@sbg.ac.at

immunogenicity of proteins and might play a role in the development of autoimmune responses [7]. In addition, single amino acid residue changes in allergens may alter their allergenicity as illustrated by point mutations in Bet v 1a which dramatically affected immunoglobulin (Ig)E binding [8, 9]. Nitration of tyrosine residues may alter immunogenicity and allergenicity of proteins in a similar fashion. Nitration of proteins occurs naturally during inflammation, oxidative stress and ageing, and may optimize immune responses [10]. Nitration of self-antigens may also be detrimental by promoting the development of autoimmune reactions. Autologous antigens containing 3-nitrotyrosine residues are able to evade immunological self-tolerance [11], and nitration of autologous IgG induces formation of cross-reactive antibodies against single-stranded DNA [12].

The consequences of ex vivo nitration of allergens have so far not been investigated. The recent finding that proteins, including allergens, are nitrated by ambient urban air [6], has received broad interest [13], in particular because it was speculated that nitration may enhance the allergenicity of proteins and in this way might contribute to an increased prevalence of allergic diseases due to traffic-related air pollution [6, 13]. In this study, we compared the in vivo immunogenicity of in vitro nitrated and unmodified samples of the food allergen ovalbumin (Ova) and the birch pollen allergen Bet v 1a.

Material and Methods

Nitration of Allergens

Ova (Sigma, Steinheim, Germany) and recombinant Bet v 1a (Biomay, Vienna, Austria) were nitrated in PBS by reaction with tetranitromethane dissolved in methanol (Merck, Darmstadt, Germany), and the nitrated proteins were purified by size exclusion chromatography (PD-10 columns, Pharmacia, Uppsala, Sweden). The amounts of 3-nitrotyrosine residues in the protein samples were determined by spectrophotometry at 428 nm, after dilution in 0.05 M NaOH. The spectrophotometer was calibrated with free 3-nitrotyrosine. The average number of 3-nitrotyrosine residues per protein molecule was calculated by division of the total of 3-nitrotyrosines through the molar protein content of the sample. Details and validation of these methods have been published elsewhere [6, 14–16]. Mock-nitrated samples of allergen were prepared by the same procedure but without adding tetranitromethane.

Mouse Models

Female BALB/c mice, 6–8 weeks old, were from Charles River Laboratories (Sulzfeld, Germany). Transgenic DO11.10 mice [17] were from in-house breeding stocks. All mice were housed and treated at the animal care facility of the University of Salzburg in agreement with local guidelines for animal care and experiments. BALB/c mice were sensitized at days 1 and 11, by intraperitoneal injection of 5 μg antigen adsorbed to 100 μl $Al(OH)_3$ in a total volume of 200 μl sterile PBS. Mock sensitization was carried out by injecting 100 μl $Al(OH)_3$ in a total volume of 200 μl sterile PBS. Sera were obtained at days 0 and 42. At the end of the experiments, mice were sacrificed by cervical dislocation and spleens were taken for further examination.

Detection of Specific Murine Serum IgE, IgG1 and IgG2a

Sera were analysed for specific antibody titers as previously described [18], with minor modifications. Briefly, functional antigen-specific IgE was determined by the rat basophil leukaemia (RBL) mediator release assay. RBL-2H3 cells (ACC 312) were obtained from DSMZ GmbH (Braunschweig, Germany) and subjected to diluted sera of sensitized animals (1:30 and 1:100 for sera from Bet v 1a and Ova sensitization experiments, respectively). Mediator release in the presence of 0.3 μg/ml antigen was performed subsequently, and values for mediator release in the presence of antigen-specific IgE were normalized to values obtained for cells treated with 1% Triton X-100, equalling maximum mediator release (100%). For IgG1 and IgG2a determinations, sera were diluted 1:1,000 and 1:100, respectively, and the assays were developed using the horseradish peroxidase substrate kit (Biorad, Calif., USA) and 3,3′,5,5′-tetramethylbenzidine liquid substrate supersensitive for ELISA (Sigma), respectively, according to the manufacturers' protocols. Light absorption at 405 and 450 nm was quantified using an optical density microplate reader (Spectrafluor, Tecan, Grödig, Austria).

Murine Spleen Cell Proliferation

Proliferation assays with spleen cells from either naïve, 8-week to 1-year-old DO11.10 and BALB/c mice, or sensitized mice sacrificed at day 56, were essentially carried out as described [18]. Four replicate wells with spleen cell preparations were stimulated with 20 μg/ml antigen for 3 days. Medium alone and 0.25 μg/ml of the immunostimulatory lectin concanavalin A (Con A, Sigma) served as negative and positive controls, respectively. Proliferation levels were normalized to values obtained for cells cultured in the absence of antigen (= 1).

Cytokine ELISAs

Quantities of interferon (IFN)-γ and interleukin (IL)-5 in proliferating spleen cell culture supernatants were determined using sandwich BD OptEIA mouse cytokine ELISA sets (BD Biosciences Pharmingen, San Diego, Calif., USA) according to the manufacturers' protocols. Sensitivity limits were 31.3 and 15.6 pg/ml, respectively.

Detection of Specific Human Serum IgE, Competition Assay

Sera from patients suffering from birch pollen allergy were obtained with informed consent through the Allergy Ambulance of the Salzburger Landeskliniken (SALK) (Salzburg, Austria). Sera were tested positive for Bet v 1a-specific IgE binding, radioallergosorbent test (RAST) 4. Multiwell plates were coated overnight with 4 μg/ml recombinant Bet v 1a or Nitro-(3.4)-Bet in PBS (pH 7.4). Wells were washed with TBS and blocked for 2 h with TBS/0.05% Tween-20 at room temperature. Sera were diluted 1:50 in blocking buffer and pre-incubated for 2 h at room temperature in multiwell plates with Bet v 1a at a dilution series from 10 to 0.1 μg/ml. Pre-incubated sera were transferred to blocked wells

and incubated overnight at 4°C. Binding of IgE was detected using a monoclonal anti-human IgE antibody conjugated with alkaline phosphatase (BD Bioscience Pharmingen) in a dilution of 1:3,000 in TBS. Wells were washed with TBS, and bound IgE was determined with 10 mM PNPP (4-nitrophenyl phosphate disodium salt hexahydrate; Fluka, Austria) in substrate buffer (10 mM diethanolamin, 1 mM $MgCl_2$, 100 mg NaN_3, pH 9.8). Light absorption at 405 and 495 nm was quantified using an optical density microplate reader.

Basophil Leukaemia Cell Mediator Release with Human Bet v 1a-Specific IgE

RBL-2H3 (RBL-703/21) transfected with cDNA coding for the human high-affinity IgE receptor chain [19] cells were plated in 96-well tissue culture plates (1×10^5/well). Passive sensitization was performed by incubating cells with human sera containing Bet v 1a-reactive IgE at a final dilution of 1:100 overnight at 37°C and 7% CO_2. Unbound antibodies were removed by washing the cell layer 3 times in Tyrode's buffer (137 mM NaCl, 2.7 mM KCl, 0.5 mM $MgCl_2$, 1.8 mM $CaCl_2$, 0.4 mM NaH_2PO_4, 5.6 mM D-glucose, 12 mM $NaHCO_3$, 10 mM HEPES and 0.1% w/v BSA, pH 7.2). RBL cell mediator release was induced by cross-linking the receptor-bound Bet v 1a-specific IgE with 0.3 μg/ml Bet v 1a or Nitro-(3.4)-Bet, respectively. The release of β-hexosaminidase from activated RBL cells was measured as described [20].

Detection of Cross-Reactive Antibodies

Sera from patients suffering from mugwort, grass, cat, dog or house dust mite allergy were obtained with informed consent through the Allergy Ambulance of the SALK (Salzburg, Austria). Sera were tested positive for specific IgE binding (RAST 2.7–5). Multiwell plates were coated overnight with 4 μg/ml recombinant Bet v 1a or Nitro-(3.4)-Bet in PBS (pH 7.4). Wells were washed with TBS and blocked with TBS/0.05% Tween-20 for 2 h at room temperature. Sera were diluted 1:50 in blocking buffer and incubated overnight at 4°C. Binding of IgE was detected using a monoclonal anti-human IgE antibody conjugated with alkaline phosphatase (BD Bioscience Pharmingen) in a dilution of 1:3,000 in TBS. Wells were washed with TBS and bound IgE was determined with 10 mM PNPP (Fluka, Austria) in substrate buffer (10 mM diethanolamin, 1 mM $MgCl_2$, 100 mg NaN_3, pH 9.8). Light absorption at 405 and 495 nm was quantified using an optical density microplate reader.

Detection of Specific Human Serum IgG1 and IgG4

Sera from patients suffering from birch pollen allergy were obtained with informed consent through the Allergy Ambulance of the SALK (Salzburg, Austria). Multiwell plates were coated overnight with 4 μg/ml recombinant Bet v 1a or Nitro-(3.4)-Bet in PBS (pH 7.4). Wells were washed with TBS and blocked for 2 h with TBS/0.05% Tween-20 at room temperature. Sera were diluted 1:50 in blocking buffer and incubated overnight at 4°C. Binding of IgG1 and IgG4 was detected using monoclonal anti-human IgG1 and IgG4 antibodies conjugated with alkaline phosphatase (Southern Biotech, UK) in a dilution of 1:1,000 in TBS. Wells were washed with TBS, and bound IgG1 and IgG4 was determined with 10 mM PNPP (Fluka, Austria) in substrate buffer (10 mM diethanolamin, 1 mM $MgCl_2$, 100 mg NaN_3, pH 9.8). Light absorption at 405 and 495 nm was quantified using an optical density microplate reader.

Statistical Analyses

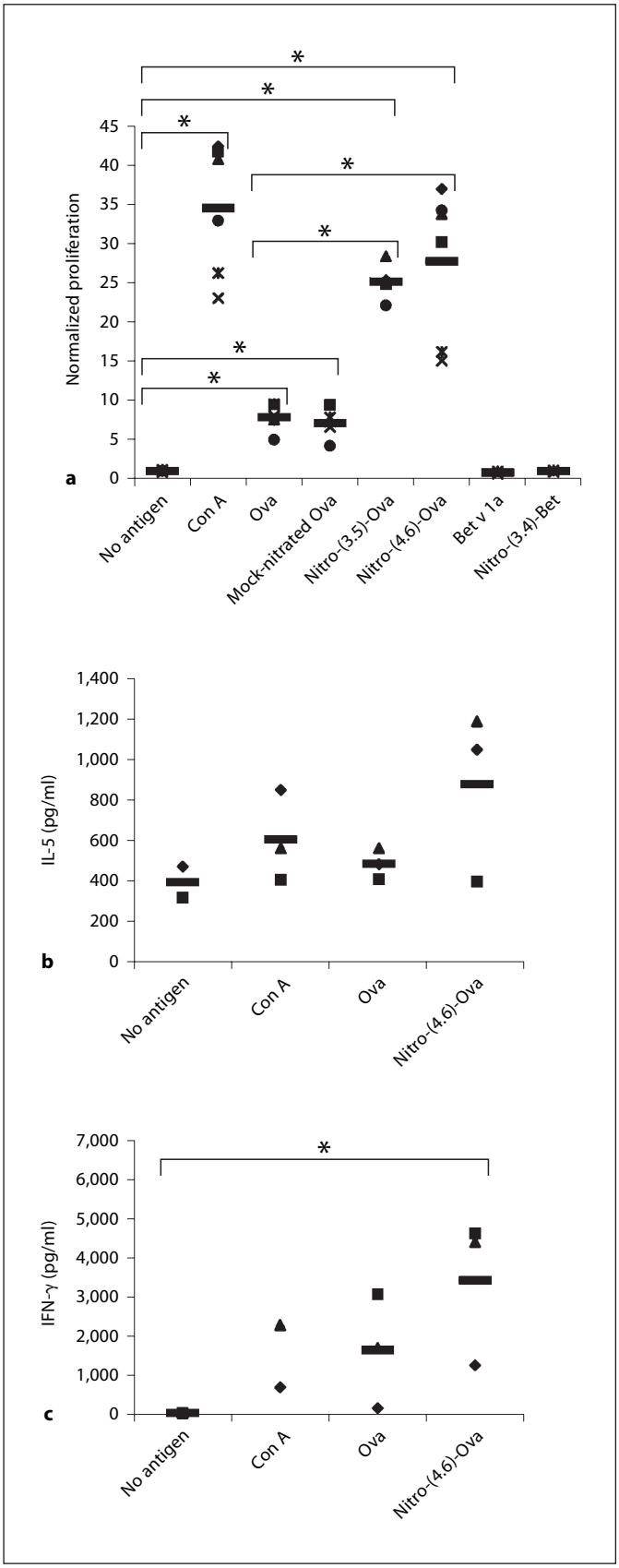
Proliferation assays, cytokine determinations, ELISAs of human serum antibodies and RBL tests were performed at least three times, and representative experiments are illustrated in figures 1–7 discussed below. Data are shown as arithmetic means. Error bars indicate standard errors of the arithmetic mean. When appropriate, significance between experimental groups was calculated by the Mann-Whitney rank sum test or Student's t test. Significant differences are represented by p values <0.05.

Results

Nitrated Ova Enhances Proliferation of DO11.10 Mice-Derived Splenocytes

The immunogenic potential of nitrated allergens was first evaluated in vitro by comparing proliferation-inducing capacities of nitrated and unmodified Ova. Spleen cells from DO11.10 BALB/c mice which carry a transgenic T cell receptor specific for a major histocompatibility complex class II restricted peptide sequence from Ova [17] were stimulated either with unmodified Ova, mock-nitrated Ova or nitrated Ova samples. Ova was used at two different levels of nitration, with an average of 3.5 or 4.6 nitrotyrosine residues per protein molecule, Nitro-(3.5)-Ova and Nitro-(4.6)-Ova, respectively. As negative controls, DO11.10 cells were cultured in the absence of allergen, or in the presence of either recombinant Bet v 1a or nitrated Bet v 1a which contained an average of 3.4 nitrotyrosine residues per protein molecule, Nitro-(3.4)-Bet.

As expected, all Ova samples stimulated the proliferation of DO11.10 cells (fig. 1a). Mock-nitrated Ova induced a similar level of proliferation as unmodified Ova. On average, both nitrated Ova samples induced a more than 3-fold higher normalized proliferation than unmodified or mock-nitrated Ova (fig. 1a). Bet v 1a and Nitro-(3.4)-Bet did not stimulate proliferation of DO11.10 cells (fig. 1a), indicating that the enhanced proliferation triggered by nitrated Ova samples was allergen specific. In addition, spleen cells derived from naïve BALB/c mice did not proliferate in response to any of the antigens used (data not shown). In these and all other experiments using Ova, Nitro-(3.5)-Ova consistently gave comparable results with Nitro-(4.6)-Ova, and mock-nitrated samples consistently gave similar results as allergen samples which were not nitrated. For reasons of clarity, these data are omitted in subsequent figures. Compared with the control, the levels of IL-5 and IFN-γ tended to be higher in cell culture supernatants of DO11.10 splenocytes stimulated with either Con A, Ova or Nitro-(4.6)-Ova. On av-



1

erage, concentrations of both IL-5 (not statistically significant) and IFN-γ were higher when cells had been stimulated with nitrated instead of unmodified allergen (fig. 1b, c). Taken together, these data indicate that nitrated Ova is superior to Ova as a stimulus for DO11.10 cells.

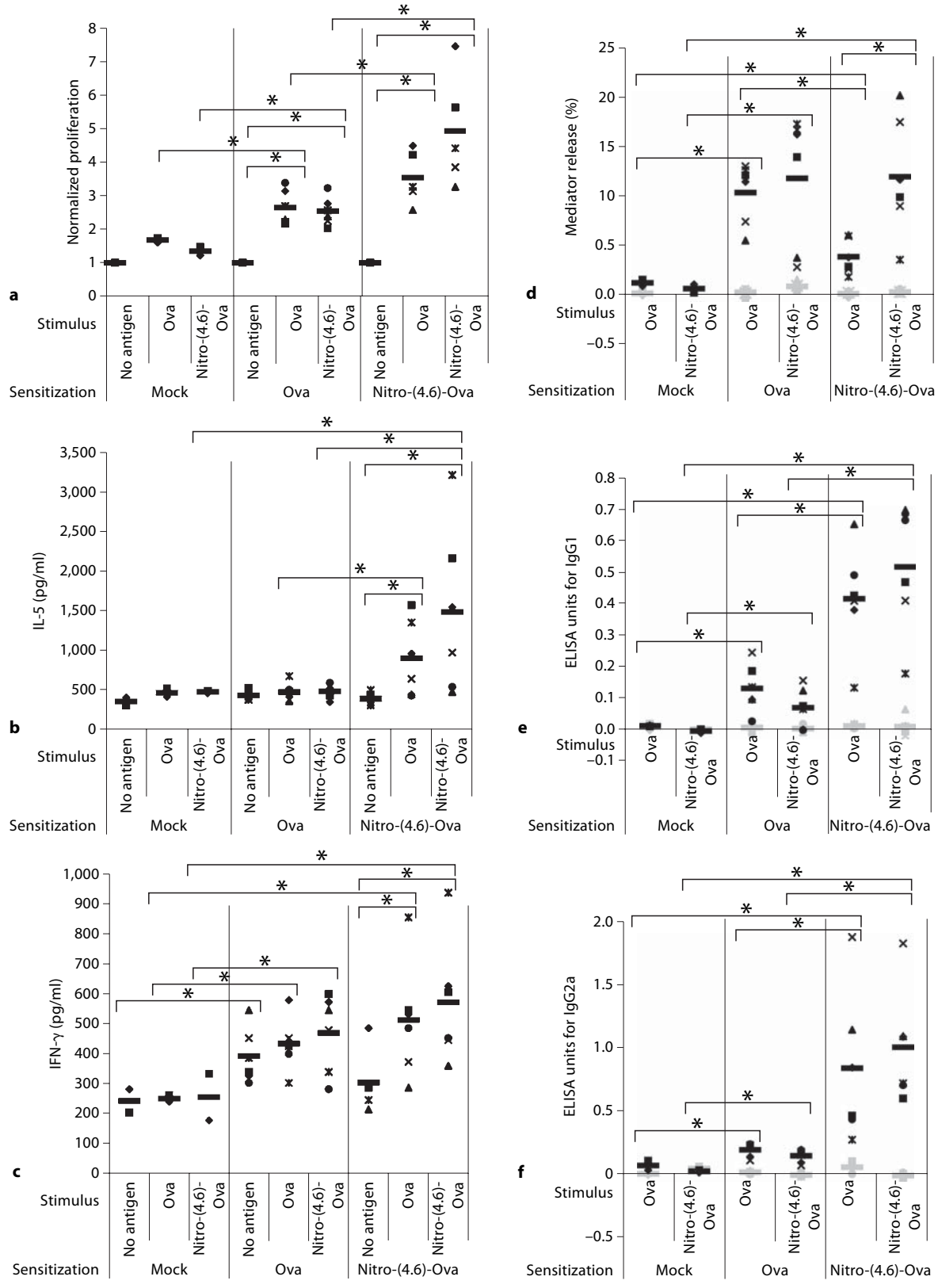
Nitrated Ova Induces a Stronger Response in BALB/c Mice than Unmodified Ova

To compare the allergenicity of nitrated and unmodified Ova in vivo, BALB/c mice were sensitized by intraperitoneal injection of the investigated proteins. Spleen cells from mice sensitized with Ova proliferated much stronger in the presence of antigen than those from mock-sensitized animals, but no significant differences in cell proliferation were found between stimulation with Ova or Nitro-(4.6)-Ova (fig. 2a). However, cells from mice sensitized with Nitro-(4.6)-Ova showed significantly higher mean and maximum values of antigen-induced proliferation than those from Ova-sensitized mice, and the highest proliferation was observed upon stimulation of cells from Nitro-(4.6)-Ova-sensitized mice (fig. 2a). Moreover, antigen stimulation of spleen cells from mice sensitized with Nitro-(4.6)-Ova resulted in a significant production of the T helper cell 2 associated cytokine IL-5, in contrast to cells derived from Ova-sensitized animals (fig. 2b). The highest concentrations of IL-5 were observed in supernatants of Nitro-(4.6)-Ova-stimulated cells (fig. 2b). Production of the T helper cell 1 associated cytokine IFN-γ was generally low in this model, and no significant differences were found between Ova and Nitro-(4.6)-Ova for this parameter (fig. 2c). As expected, no splenocyte proliferation or cytokine production was detected upon stimulation of cells with either Bet v 1a or Nitro-(3.4)-Bet (data not shown).

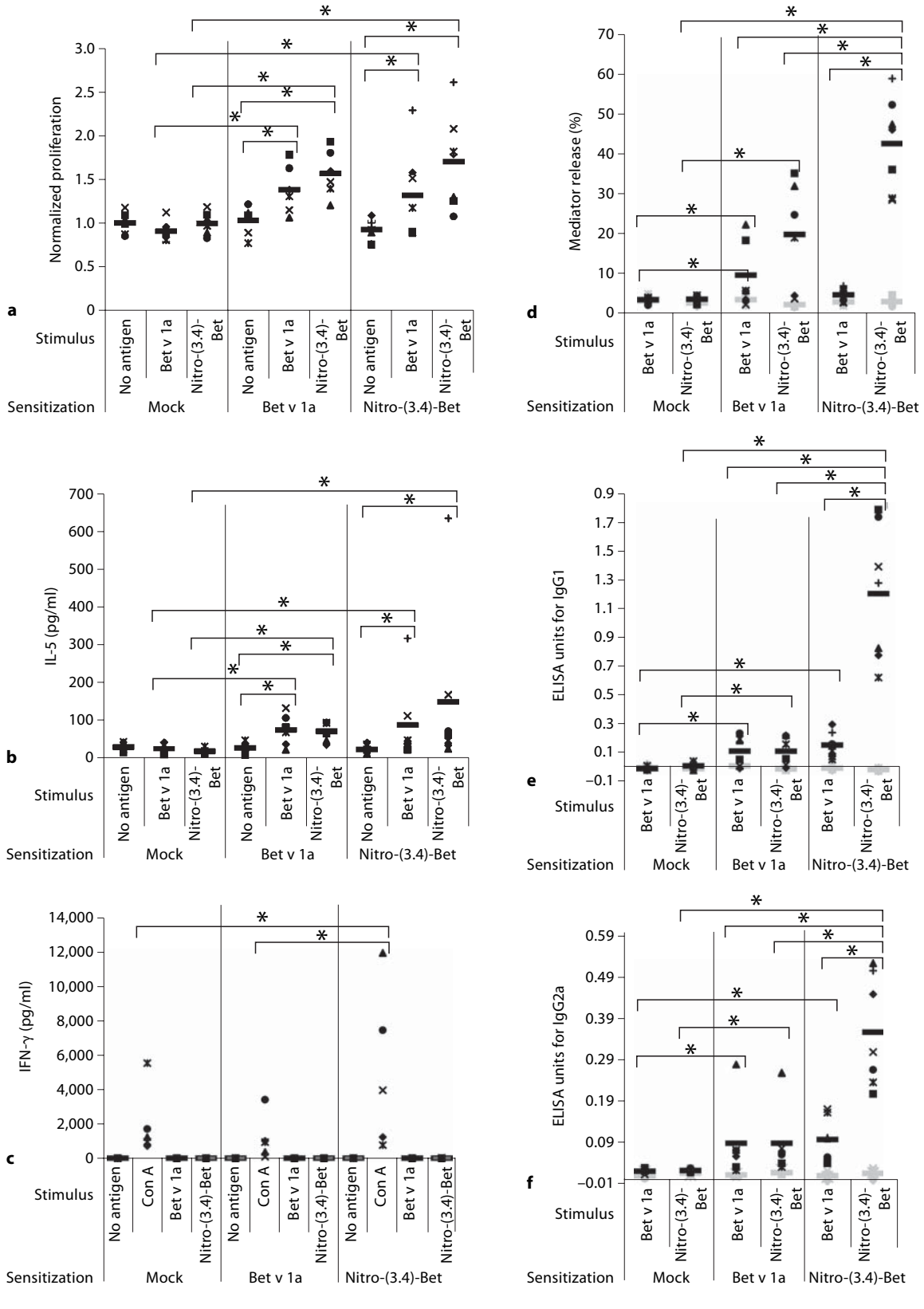
In sera of Ova- and Nitro-(4.6)-Ova-sensitized mice, functional IgE recognizing both Ova forms was present

Fig. 1. Nitro-(4.6)-Ova stimulation of DO11.10 splenocytes. Antigen-specific proliferation of DO11.10 splenocytes (**a**), and IL-5 (**b**) and IFN-γ (**c**) in corresponding culture supernatants. Symbols represent arithmetic means of four replicates of a single animal. Bars indicate arithmetic group means. * $p < 0.05$.

Fig. 2. Enhanced immunogenicity of Nitro-(4.6)-Ova. Antigen-specific proliferation of splenocytes from sensitized mice (**a**), and IL-5 (**b**) and IFN-γ (**c**) in corresponding supernatants. Symbols represent means of four replicates of a single animal. **d–f** Antigen-specific IgE (**d**), IgG1 (**e**) and IgG2a (**f**) in sera of sensitized mice on days 0 (grey symbols) and 42 (black symbols). Bars indicate group means. * $p < 0.05$.


a
Normalized proliferation
b
IL-5 (pg/ml)
c
IFN-γ (pg/ml)
d
Mediator release (%)
e
ELISA units for IgG1
f
ELISA units for IgG2a
Stimulus
No antigen
Ova
Nitro-(4.6)-Ova
Sensitization
Mock
Ova
Nitro-(4.6)-Ova

2


a
Normalized proliferation
3.0
2.5
2.0
1.5
1.0
0.5
0
Stimulus
No antigen
Bet v 1a
Nitro-(3.4)-Bet
Sensitization
Mock
Bet v 1a
Nitro-(3.4)-Bet
d
Mediator release (%)
70
60
50
40
30
20
10
0
b
IL-5 (pg/ml)
700
600
500
400
300
200
100
0
e
ELISA units for IgG1
0.9
1.7
1.5
1.3
1.1
0.9
0.7
0.5
0.3
0.1
–0.1
c
IFN-γ (pg/ml)
14,000
12,000
10,000
8,000
6,000
4,000
2,000
0
Con A
f
ELISA units for IgG2a
0.59
0.49
0.39
0.29
0.19
0.09
–0.01

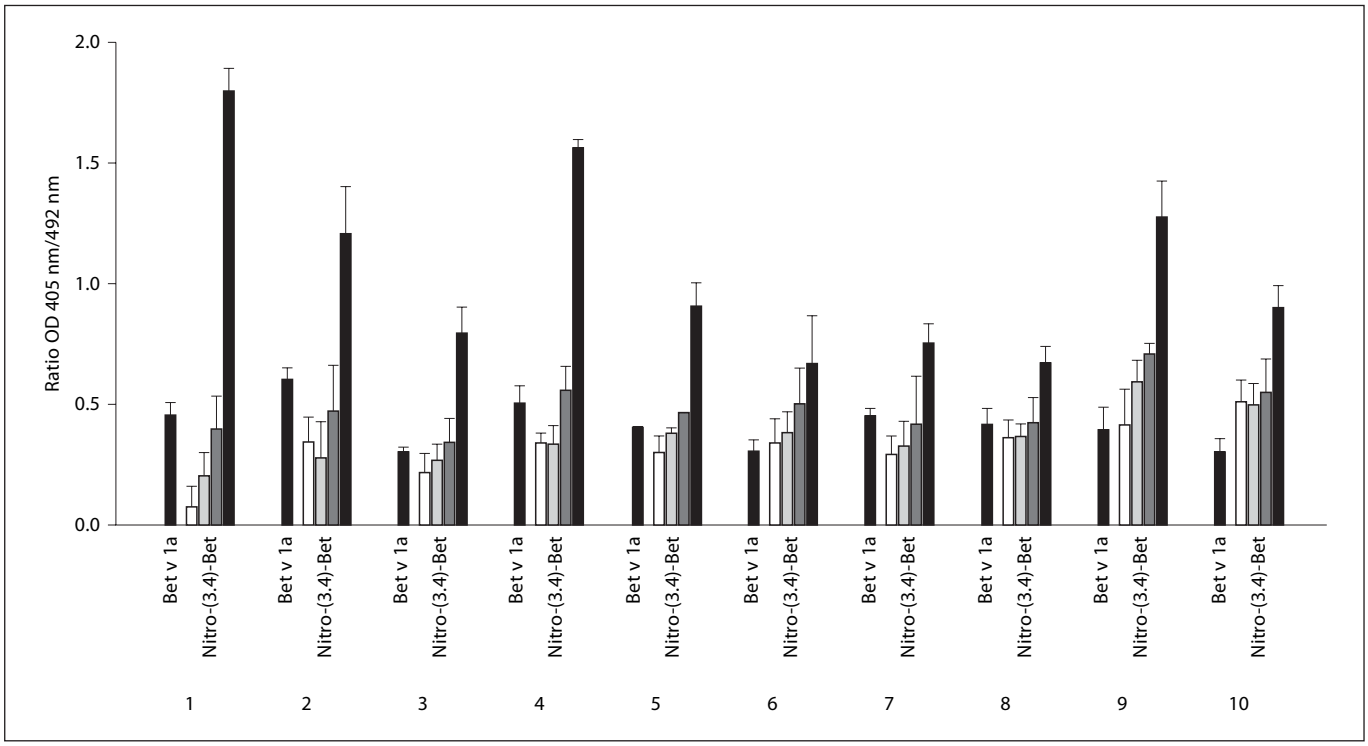


Fig. 4. IgE from 10 randomized birch pollen-allergic patients. Bars represent means of relative IgE binding to Bet v 1 a and Nitro-(3.4)-Bet. Bet v 1a-specific epitopes were blocked with 10 μg/ml Bet v 1a (white bars), 1 μg/ml Bet v 1a (light grey bars) and 0.1 μg/ml Bet v 1a (dark grey bars), or were not blocked at all (black bars). In the incubation experiments with Bet v 1a, inhibition was complete, resulting in signals equal to the baseline.

(fig. 2d). Significantly, IgE of mice sensitized with Nitro-(4.6)-Ova responded much more efficiently to the nitrated form (fig. 2d), indicating that higher amounts of Nitro-(4.6)-Ova-specific IgE were present. Sera of sensitized mice also contained antigen-specific IgG1 and IgG2a. Mean and maximum levels for both classes of antibodies were significantly higher when mice were sensitized with Nitro-(4.6)-Ova compared with the unmodified antigen (fig. 2e, f). Moreover, serum antibodies derived from several mice sensitized with Nitro-(4.6)-Ova recognized Nitro-(3.4)-Bet as an antigen: 3 out of 6 mice had cross-reactive IgG1, 2 out of 6 mice had cross-reactive IgG2a, and 3 out of 6 mice had cross-reactive IgE (table 1). Taken together, nitration made Ova more immunogenic.

Fig. 3. Enhanced immunogenicity of Nitro-(3.4)-Bet. Antigen-specific proliferation of splenocytes from sensitized mice (**a**), and IL-5 (**b**) and IFN-γ (**c**) in corresponding supernatants. Symbols represent means of four replicates of a single animal. **d–f** Antigen-specific IgE (**d**), IgG1 (**e**) and IgG2a (**f**) in sera of sensitized mice on days 0 (grey symbols) and 42 (black symbols). Bars indicate group means. * $p < 0.05$.

Nitrated Bet v 1a Induces a Stronger Response in BALB/c Mice than Bet v 1a

Ova is a food allergen which is not likely to be encountered in ambient urban air. Therefore, we evaluated the same immunological parameters as we did in the Ova model for BALB/c mice sensitized with Bet v 1a or its nitrated derivative Nitro-(3.4)-Bet. Splenocyte proliferation and IL-5 production of sensitized mice were higher in response to Nitro-(3.4)-Bet than to Bet v 1a (fig. 3a, b), without reaching significance. Interestingly though, splenocytes from one particular Nitro-(3.4)-Bet-sensitized mouse produced high levels of IL-5 (fig. 3b). IFN-γ production could not be detected for any of the antigen-stimulated cells in this model (fig. 3c).

Sera from allergen-sensitized mice contained elevated levels of antigen-specific IgE, IgG1 and IgG2a compared with those of mock-sensitized mice (fig. 3d–f). Signifi-

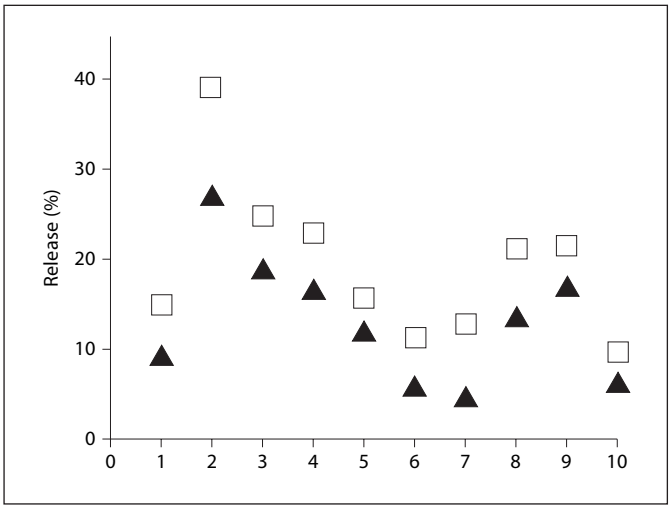


Fig. 5. RBL release assay. Ten randomized sera from birch pollen-allergic patients were tested for their anaphylactic activity by measuring the release of β-hexosaminidase. Cells were passively sensitized with sera diluted 1:100, and mediator release was assessed after cross-linking the human high-affinity IgE receptor chain with Bet v 1a (▲) or Nitro-(3.4)-Bet (□). Data are representative of three independent experiments. Statistical significance was calculated by paired Student's t test ($p = 0.000027$).

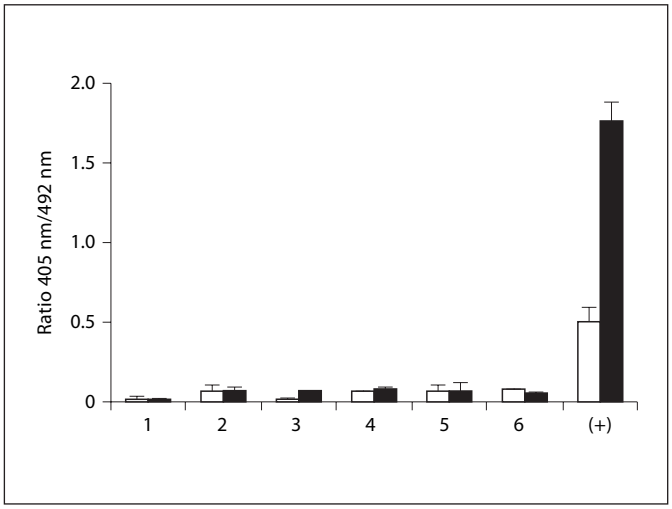


Fig. 6. Binding to unspecific IgE. Bet v 1a (□) and Nitro-(3.4)-Bet v 1a (■) were tested for binding to serum IgE using sera from 6 allergic patients with high IgE titers and 1 birch pollen-allergic patient (+) shown as a control. Sera were from patients with RAST 2.7–5, but without responsiveness to Bet v 1a.

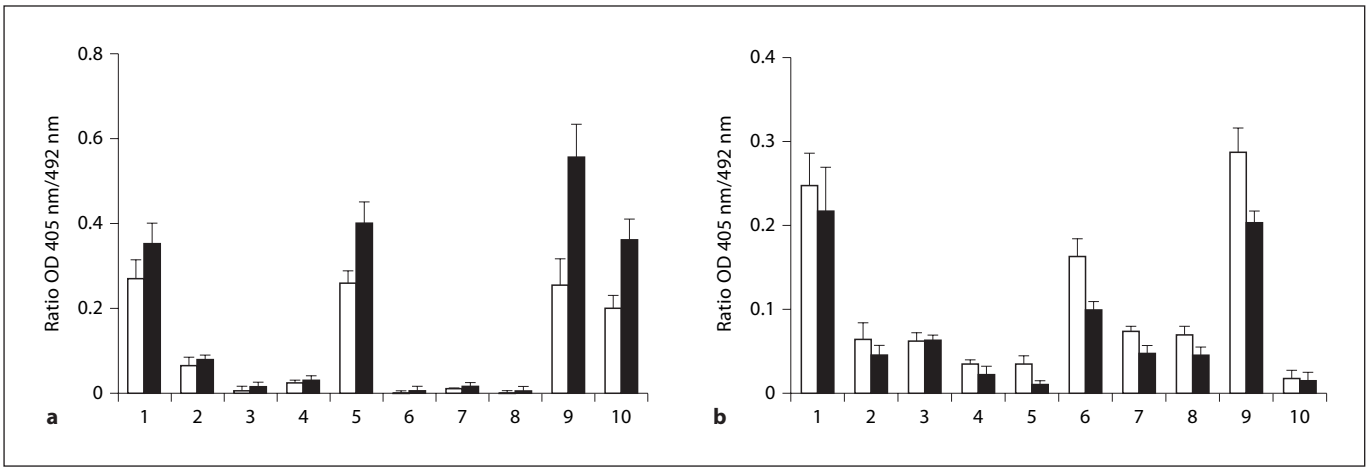


Fig. 7. IgG1 (**a**) and IgG4 (**b**) binding from 10 out of 56 randomized birch pollen-allergic patients. Bars represent means of relative IgG1 and IgG4 binding to Bet v 1a (□) and Nitro-(3.4)-Bet (■).

cantly, the highest levels of all three isotypes were observed in Nitro-(3.4)-Bet-sensitized mice (fig. 3d–f). IgG1 and IgG2a in sera of Bet v 1a-sensitized animals bound equally well to Bet v 1a and Nitro-(3.4)-Bet (fig. 3e, f). IgE of these mice induced more efficient induction of basophil degranulation in the presence of Nitro-(3.4)-Bet compared with regular Bet v 1a, although this difference did not reach statistical significance (fig. 3d). Nitro-(3.4)-Bet-sensitized mice presented a different picture: IgE, IgG1 and IgG2a bound unnitrated antigen similarly well as in Bet v 1a-sensitized mice, but very high binding to nitrated Bet v 1a was apparent (fig. 3d–f). In particular,

Table 1. Number of sensitized mice exhibiting serum antibodies against Ova, Nitro-(4.6)-Ova, Bet v 1a and Nitro-(3.4)-Bet

Sensitization antibody against	Ova	Nitro-(4.6)-Ova	Bet v 1a	Nitro-(3.4)-Bet
IgE				
Ova	6/6	6/6	0/6	0/7
Nitro-(4.6)-Ova	6/6	6/6	0/6	0/7
Bet v 1a	0/6	0/6	3/6	1/7
Nitro-(3.4)-Bet	0/6	**3/6**	4/6	7/7
IgG1				
Ova	5/6	6/6	0/6	0/7
Nitro-(4.6)-Ova	4/6	6/6	0/6	**7/7**
Bet v 1a	0/6	0/6	3/6	7/7
Nitro-(3.4)-Bet	0/6	**3/6**	3/6	7/7
IgG2a				
Ova	6/6	6/6	0/6	0/7
Nitro-(4.6)-Ova	6/6	6/6	0/6	**3/7**
Bet v 1a	0/6	0/6	4/6	7/7
Nitro-(3.4)-Bet	0/6	**2/6**	4/6	7/7

Values are given as n^+/n in which 'n^+' represents the number of animals found positive for the presence of antibodies against the indicated antigen, and 'n' represents the total number of animals in the indicated sensitization group. Values for the presence of cross-reactive antibodies are indicated in bold type.

IgE in sera of these mice was highly specific for the nitrated Bet v 1a form, as it completely failed to induce basophil degranulation upon addition of the unmodified antigen for all mice (fig. 3d). Serum antibodies derived from several mice sensitized with Nitro-(3.4)-Bet recognized Nitro-(4.6)-Ova as an antigen: 7 out of 7 animals contained cross-reactive IgG1, and 3 out of 7 animals had cross-reactive IgG2a. No cross-reactivity was detected for IgE (table 1). Similar to the Ova model, nitration rendered the allergen Bet v 1a more immunogenic in vivo.

IgE Specific for Nitrated Bet v 1a in Sera of Birch Pollen-Allergic Patients

Since Bet v 1a is clinically a highly relevant allergen, we investigated whether sera from birch pollen-allergic patients contained antibodies against nitrated Bet v 1a. Sera from 10 non-atopic healthy individuals and from 56 patients suffering from birch pollen allergy were analysed for the presence of Bet v 1a- and Nitro-(3.4)-Bet-binding IgE. Bet v 1a-specific IgE was absent in sera from healthy individuals (data not shown), but present in sera of all allergic individuals. Significantly higher amounts of Nitro-(3.4)-Bet-binding IgE than regular Bet v 1a-binding IgE were found for almost all sera of allergic patients (52 out of 56 sera). A competition assay with a representative number of sera is shown in figure 4. Human sera were preincubated with Bet v 1a and then tested for binding to either Bet v 1a or Nitro-(3.4)-Bet. Binding to Bet v 1a was completely blocked, even by pre-incubation with the lowest dose of Bet v 1a used, 0.1 μg/ml. In contrast, binding to Nitro-(3.4)-Bet, which was inherently stronger than binding to the unmodified form, was reduced but not abolished in a concentration-dependent fashion. No IgE binding to Nitro-(4.6)-Ova was detected for any of the sera of these allergic patients (data not shown). These findings suggest that birch pollen-allergic patients may possess some IgE specifically recognizing nitrated epitopes of Bet v 1a. The IgE detected in the patient sera was functional, since Bet v 1a and Nitro-(3.4)-Bet induced mediator release from RBL-2H3, stably transfected with the human high-affinity IgE receptor chain (fig. 5). Among sera from 10 birch pollen-allergic patients, all showed higher degranulation upon stimulation with the nitrated form.

The high level of binding by serum antibodies from birch pollen-allergic patients to Nitro-(3.4)-Bet was not in an unspecific way due to the high serum IgE levels found in these cases. Sera with high IgE levels from 6 patients allergic to a variety of allergens, but not Bet v 1a, did not show enhanced binding to Nitro-(3.4)-Bet (fig. 6).

Sera from birch pollen-allergic patients were also tested for binding of human IgG. Data for IgG1 and IgG4 are shown in figure 7. In contrast to IgE, there was no general enhanced binding to the nitrated protein. Despite some enhanced (IgG1) or decreased (IgG4) binding to Nitro-(3.4)-Bet, the overall picture was clearly different to IgE (compare fig. 4 and 7).

Discussion

Various types of post-translational modifications have been shown to affect T cell immunoreactivity, including glycosylation, phosphorylation and cysteinylation [7, 21–23]. This study represents the first report on the immunogenic potential of allergens modified to contain 3-nitrotyrosine. Nitration increased the allergenic potential both of Ova and Bet v 1a in murine allergy models. Nitrated Ova was a more potent T cell stimulus than native Ova and increased levels of antigen-specific IgG1 and IgG2a in sera of nitrated Ova-sensitized mice. Although IgE-inducing and -binding abilities of Ova did not change by nitration, other allergy-associated parameters (IL-5 and IgG1) were upregulated. Nitration of Bet v 1a also

enhanced allergenicity as serum levels of IgG1 and anaphylactic IgE increased dramatically upon sensitization. Like for Ova, nitration of Bet v 1a increased immunogenicity in general, as shown by higher serum IgG2a in sensitized animals. Both, in the case of nitrated Bet v 1a as well as nitrated Ova, these levels were still 10- to 100-fold lower than those of IgG1, which is in agreement with an allergic phenotype.

Increased T cell immunoreactivity upon sensitization with nitrated Ova might be due to improved T-cell-dependent antigen recognition of epitopes containing nitrotyrosine. Alternatively, nitration might facilitate uptake, processing and presentation of Ova by antigen-presenting cells, and thus, enhance the efficiency of T cell stimulation. The possibility that nitrotyrosine might function like a hapten has been suggested by Ohmori et al. [12], who recognized the structural similarity of nitrotyrosine with a well-known hapten, 4-hydroxy-3-nitrophenylacetyl.

Neither enhanced nor reduced T cell reactivity was observed for nitrated Bet v 1a. Some of the known Bet v 1a T cell epitopes contain tyrosine residues [24–27], but it is unknown if these tyrosines might become nitrated. Tyrosine nitration in proteins is selective, since some tyrosine residues are highly susceptible for nitration whereas others cannot be nitrated at all [10]. However, Bet v 1a is known as a very poor T cell antigen with high IgE-binding activity and allergenicity [28]. It might not be a suitable antigen to study effects of tyrosine nitration on T cell epitopes. In this respect, it might be useful to include Bet v 1d in further studies, as this Bet v 1 isoform combines strong T cell antigenicity with low/no IgE-binding capacity [28].

Nitration did not mask epitopes important for humoral immunity. Antibodies raised in mice sensitized with unmodified antigens were equally efficient in binding nitrated and unmodified forms of Ova and Bet v 1a. Since nitrated antigens resulted in enhanced humoral immunity, nitration may even provide additional new epitopes. This hypothesis was strenghtened by the presence of cross-reactive antibodies for the nitrated form of the otherwise unrelated proteins Ova and Bet v 1a. The most striking finding indicating new epitope formation through nitration were high titers of highly specific IgE in nitrated Bet v 1a-sensitized mice. This IgE bound nitrated Bet v 1a, but neither unmodified Bet v 1a nor nitrated Ova. The other antibody classes in most of these mice bound unmodified Bet v 1a, but with much lower affinity than the nitrated form, and surprisingly, they recognized nitrated Ova. Together, these findings suggest that nitration of Bet v 1a generates new epitopes. This hypothesis was strengthened by the finding that pre-incubation with unmodified Bet v 1a can reduce but not obliterate binding of human IgE to Nitro-(3.4)-Bet, which argues that the nitrated form contains at least some new epitopes not shared with the unmodified antigen. In principle, it is possible that nitration induces structural changes which expose new epitopes not containing nitrotyrosine somewhere else in the molecule, but we consider it unlikely that this happens both in Bet v 1a and in Ova. Further studies will aim at identifying the binding sites of antibodies raised against nitrated allergens.

The presence of IgE preferentially binding to nitrated Bet v 1a in human sera suggests that these patients may have previously been exposed to the antigen in nitrated form. It is tempting to speculate that this previous exposure might have been the result of inhalation of ex vivo nitrated birch pollen allergen. It will have to be investigated whether similar findings can be made for other airborne allergens, and whether an association with exposure to pollution can be established. An experiment with sera from 6 patients allergic to various other allergens did not uncover cross-reactive binding to Nitro-(3.4)-Bet, but this matter will have to be studied in more detail once nitration sites in allergens have been localized. A preferential association of nitration with the promotion of allergy may be suspected from the finding that IgG from Bet v 1a-allergic patients did not display the clear increase in binding to the nitrated form encountered with IgE. However, the present investigation cannot rule out that other branches of immunity may also be affected by nitration of antigens.

In mouse models, nitrated allergens induce high amounts of anaphylactogenic IgE, increase allergenicity and induce cross-reactive antibodies. Since nitrated allergens are present in urban environments [6], exposure to ex vivo nitrated allergens might seriously influence the onset and course of allergic disease in humans as well. This mechanism may promote allergies and might contribute to adverse health effects of air pollution.

Acknowledgements

We thank T. Hawranek and M. Wallner for supplying human sera and human IgE antibodies, P. Hammerl and R. Weiss for their help with the mouse models, and R. Niessner for the support. This work was partly financially supported by the European Union, MAAPHRI QLK-CT-2002-02357 (to A.D.), and the European commission, FP6 Contract No. 003956 (to A.D.), by the priority programme 'Biosciences and Health' of the University of Salzburg (to A.D.), and by the BMBF-AFO2000 project CARBAERO (to U.P.).

References

1 Bernstein JA, Alexis N, Barnes C, Bernstein IL, Nel A, Peden D, Diaz-Sanchez D, Tarlo SM, Williams PB: Health effects of air pollution. J Allergy Clin Immunol 2004;114:1116–1123.

2 Bömmel H, Li-Weber M, Serfling E, Duschl A: The environmental pollutant pyrene induces the production of IL-4. J Allergy Clin Immunol 2000;105:796–802.

3 D'Amato G: Urban air pollution and plant-derived respiratory allergy. Clin Exp Allergy 2000;30:628–636.

4 Saxon A, Diaz-Sanchez D: Air pollution and allergy: you are what you breathe. Nat Immunol 2005;6:223–226.

5 Brunekreef B: NO_2: the gas that won't go away. Clin Exp Allergy 2001;31:1170–1172.

6 Franze T, Weller MG, Niessner R, Pöschl U: Protein nitration by polluted air. Environ Sci Technol 2005;39:1673–1678.

7 Doyle HA, Mamula MJ: Posttranslational modifications of self-antigens. Ann NY Acad Sci 2005;1050:1–9.

8 Ferreira F, Ebner C, Kramer B, Casari G, Briza P, Kungl AJ, Grimm R, Jahn-Schmid B, Breiteneder H, Kraft D, Breitenbach M, Rheinberger HJ, Scheiner O: Modulation of IgE reactivity of allergens by site-directed mutagenesis: potential use of hypoallergenic variants for immunotherapy. FASEB J 1998;12:231–242.

9 Spangfort MD, Mirza O, Ipsen H, Van Neerven RJ, Gajhede M, Larsen JN: Dominating IgE-binding epitope of Bet v 1, the major allergen of birch pollen, characterized by X-ray crystallography and site-directed mutagenesis. J Immunol 2003;171:3084–3090.

10 Greenacre SA, Ischiropoulos H: Tyrosine nitration: localisation, quantification, consequences for protein function and signal transduction. Free Radic Res 2001;34:541–581.

11 Birnboim HC, Lemay AM, Lam DK, Goldstein R, Webb JR: Cutting edge: MHC class II-restricted peptides containing the inflammation-associated marker 3-nitrotyrosine evade central tolerance and elicit a robust cell-mediated immune response. J Immunol 2003;171:528–532.

12 Ohmori H, Oka M, Nishikawa Y, Shigemitsu H, Takeuchi M, Magari M, Kanayama N: Immunogenicity of autologous IgG bearing the inflammation-associated marker 3-nitrotyrosine. Immunol Lett 2005;96:47–54.

13 Peplow M: Traffic pollution revs up allergens. News@Nature.com 2005;DOI:10.1038/news050131-16.

14 Franze T, Weller MG, Niessner R, Pöschl U: Enzyme immunoassays for the investigation of protein nitration by air pollutants. Analyst 2003;128:824–831.

15 Franze T, Weller MG, Niessner R, Pöschl U: Comparison of nitrotyrosine antibodies and development of immunoassays for the detection of nitrated proteins. Analyst 2004;129:589–596.

16 Walcher W, Franze T, Weller MG, Pöschl U, Huber CG: Liquid- and gas-phase nitration of bovine serum albumin studied by LC-MS and LC-MS/MS using monolithic columns. J Proteome Res 2003;2:534–542.

17 Murphy KM, Heimberger AB, Loh DY: Induction by antigen of intrathymic apoptosis of CD4+CD8+TCRlo thymocytes in vivo. Science 1990;250:1720–1723.

18 Hochreiter R, Stepanoska T, Ferreira F, Valenta R, Vrtala S, Thalhamer J, Hartl A: Prevention of allergen-specific IgE production and suppression of an established Th2-type response by immunization with DNA encoding hypoallergenic allergen derivatives of Bet v 1, the major birch-pollen allergen. Eur J Immunol 2003;33:1667–1676.

19 Hoffmann A, Kaul S, Fotisch K, Danz N, Luttkopf D, Hatahet L, Kastner B, Vogel L, Holtz G, Vieths S: Mediator release assays based on human and murine IgE: potential and limitations in allergen standardization. Arb Paul Ehrlich Inst Bundesamt Sera Impfstoffe Frankf A M 2003:76–85; discussion 85–86.

20 Hartl A, Weiss R, Hochreiter R, Scheiblhofer S, Thalhamer J: DNA vaccines for allergy treatment. Methods 2004;32:328–339.

21 Backlund J, Carlsen S, Hoger T, Holm B, Fugger L, Kihlberg J, Burkhardt H, Holmdahl R: Predominant selection of T cells specific for the glycosylated collagen type II epitope (263–270) in humanized transgenic mice and in rheumatoid arthritis. Proc Natl Acad Sci USA 2002;99:9960–9965.

22 Pierce RA, Field ED, den Haan JM, Caldwell JA, White FM, Marto JA, Wang W, Frost LM, Blokland E, Reinhardus C, Shabanowitz J, Hunt DF, Goulmy E, Engelhard VH: Cutting edge: the HLA-A*0101-restricted HY minor histocompatibility antigen originates from DFFRY and contains a cysteinylated cysteine residue as identified by a novel mass spectrometric technique. J Immunol 1999;163:6360–6364.

23 Zarling AL, Ficarro SB, White FM, Shabanowitz J, Hunt DF, Engelhard VH: Phosphorylated peptides are naturally processed and presented by major histocompatibility complex class I molecules in vivo. J Exp Med 2000;192:1755–1762.

24 Breiteneder H, Friedl-Hajek R, Ebner C, Schenk S, Fischer G, Kraft D, Scheiner O: Sequence comparisons of the CDR3 hypervariable loops of human T cell receptors specific for three major T cell epitopes of the birch pollen allergen Bet v 1. Mol Immunol 1996;33:1039–1048.

25 Ebner C, Szepfalusi Z, Ferreira F, Jilek A, Valenta R, Parronchi P, Maggi E, Romagnani S, Scheiner O, Kraft D: Identification of multiple T cell epitopes on Bet v I, the major birch pollen allergen, using specific T cell clones and overlapping peptides. J Immunol 1993;150:1047–1054.

26 Friedl-Hajek R, Spangfort MD, Schou C, Breiteneder H, Yssel H, Joost van Neerven RJ: Identification of a highly promiscuous and an HLA allele-specific T-cell epitope in the birch major allergen Bet v 1: HLA restriction, epitope mapping and TCR sequence comparisons. Clin Exp Allergy 1999;29:478–487.

27 Jahn-Schmid B, Radakovics A, Luttkopf D, Scheurer S, Vieths S, Ebner C, Bohle B: Bet v 1142–1156 is the dominant T-cell epitope of the major birch pollen allergen and important for cross-reactivity with Bet v 1-related food allergens. J Allergy Clin Immunol 2005;116:213–219.

28 Ferreira F, Hirtenlehner K, Jilek A, Godnik-Cvar J, Breiteneder H, Grimm R, Hoffmann-Sommergruber K, Scheiner O, Kraft D, Breitenbach M, Rheinberger HJ, Ebner C: Dissection of immunoglobulin E and T lymphocyte reactivity of isoforms of the major birch pollen allergen Bet v 1: potential use of hypoallergenic isoforms for immunotherapy. J Exp Med 1996;183:599–609.