

A Hypoallergenic Derivative of the Major Allergen of the Dust Mite *Lepidoglyphus destructor*, Lep d 2.6Cys, Induces Less IgE Reactivity and Cellular Response in the Skin than Recombinant Lep d 2

Marianne Kronqvist^{a,b} Eva Johansson^b Paul Whitley^{b,d} Susanna Olsson^b
Guro Gafvelin^b Annika Scheynius^c Marianne van Hage-Hamsten^b

^aDepartment of Medicine, Danderyd Hospital and Department of Medicine, Divisions of ^bClinical Immunology and Allergy and ^cClinical Allergy Research, Karolinska Institutet and Hospital, Stockholm, Sweden;

^dDepartment of Biology and Biochemistry, University of Bath, UK

Key Words

Skin prick test · Skin biopsies · RAST · *Lepidoglyphus destructor* · Recombinant allergens · rLep d 2 · rLep d 2.6Cys · Hypoallergenic derivative · Immunohistochemical staining

Abstract

Background: The major allergen of the dust mite *Lepidoglyphus destructor*, Lep d 2, has been produced as a recombinant allergen (rLep d 2) with IgE reactivity both in vivo and in vitro. A modified form of rLep d 2 (rLep d 2.6Cys) obtained by site-directed mutagenesis has been shown to have a reduced IgE reactivity in vitro. In this study we have compared the ability of rLep d 2 and rLep d 2.6Cys to elicit positive skin prick tests and cellular responses among *L. destructor*-sensitized subjects.

Methods: Seventeen subjects were skin prick-tested with rLep d 2, rLep d 2.6Cys, histamine and negative controls and 17–20 h later skin biopsy specimens were taken from the skin prick-tested sites. The biopsy specimens were stained immunohistochemically for EG2+, CD3+, CD1a+, mast cell tryptase+, and IgE+ cells. Dermal cell infiltrates were judged in hematoxylin and eosin staining. Total IgE

and allergen-specific IgE were determined by CAP-RAST.

Results: Compared to rLep d 2, rLep d 2.6Cys induced significantly smaller and fewer skin prick test reactions ($p < 0.001$) and dermal cell infiltrates ($p < 0.05$). Further, rLep d 2.6Cys induced fewer EG2+ cells ($p < 0.001$) but more tryptase+ cells ($p < 0.05$) than rLep d 2. A positive RAST to rLep d 2 was obtained for 88.2% of the subjects, while only 35.2% displayed a positive RAST to rLep d 2.6Cys. **Conclusion:** This study demonstrates that rLep d 2.6Cys is less able to evoke IgE-mediated reactions and cellular responses, as measured both in skin and in serum, than rLep d 2. In the future this hypoallergenic derivative may be a promising candidate molecule for immunotherapy of *L. destructor*-allergic patients.

Copyright © 2001 S. Karger AG, Basel

Introduction

During the last decade molecular biology techniques have made it possible to produce recombinant allergens from a variety of different allergen sources [1, 2]. These allergens are pure, well defined and therefore easy to standardize, in contrast to natural extracts, which contain a

KARGER

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

© 2001 S. Karger AG, Basel
1018–2438/01/1261–0041\$17.50/0

Accessible online at:
www.karger.com/journals/iaa

Correspondence to: Dr. Marianne Kronqvist
Department of Medicine, Division of Clinical Immunology and Allergy
Karolinska Hospital
S-171 76 Stockholm (Sweden)
Fax +46 8 335724

variety of both allergenic and nonallergenic proteins. A number of recombinant allergens have been shown both in vivo and in vitro to evoke an IgE reactivity that is analogous to its natural counterpart [3, 4]. Several studies have demonstrated that a combination of only a few recombinant allergens is needed to achieve the same diagnostic accuracy as that provided by the corresponding natural extract [5–7]. For instance, only one recombinant allergen of birch pollen, rBet v1, is required to identify more than 90% of birch pollen-sensitized subjects [7]. Hence, recombinant-produced major allergens can serve as good tools for the diagnosis of allergic diseases.

Allergen-specific immunotherapy is the only curative approach for type I allergy, but is limited by its IgE-mediated side effects. Since IgE epitopes are often nonlinear and conformational, it is feasible to reduce the IgE-binding capacity by altering the three-dimensional structure of the allergens. Using this approach, it is possible to bypass the risk of IgE-mediated adverse effects of allergen-specific immunotherapy. Disruption of the three-dimensional structure will have no, or limited, effect on the linear T cell epitopes and thus the T cell reactivity will be conserved. Such modifications of allergens can be achieved, e.g. by fragmentation, oligomerization or site-directed mutagenesis [8]. As a consequence, immunotherapy treatment with modified allergens will allow administration of higher allergen doses that will favor a Th1-dominated cytokine response [9, 10]. These hypoallergenic molecules would increase the safety of immunotherapy treatment by a clearly reduced risk of anaphylactic reactions [11].

Major allergens of the dust mite *Dermatophagoides pteronyssinus*, cow, birch and timothy grass have been modified and in vitro shown to have a significantly reduced ability to evoke an IgE response while antigen-specific T cell response remained [12–17]. Furthermore, several modified allergens have in vivo induced significantly smaller skin reactions than the corresponding unaltered recombinant allergen [13–15, 18].

We have earlier shown that skin prick test (SPT) with the recombinant major allergen of the nonpyroglyphid dust mite *Lepidoglyphus destructor* (rLep d 2) identifies almost all subjects sensitized to the corresponding native allergen [19]. Nonpyroglyphid mites are a frequent cause of IgE-mediated sensitization in rural populations (range 5.3–17.1%) [20–22], but are also known to be sensitizers in urban populations (range 6.3–14%) [23, 24]. *L. destructor* is one of the most common mite species in Europe [25]. We have modified the major allergen of *L. destructor* by site-directed mutagenesis, replacing the 6 cysteine resi-

dues of Lep d 2 with serine residues [26]. In wild-type Lep d 2 the 6 cysteine residues form 3 disulfide bonds which stabilize the three-dimensional structure of the protein [27–29]. Consequently, lack of these disulfide bonds results in a disrupted three-dimensional structure and loss of IgE epitopes. This modified allergen (rLep d 2.6Cys) was shown to possess biological activity by eliciting a T cell response [26, 30]. The aims of the present study were to compare in vivo rLep d 2.6Cys and rLep d 2, with respect to SPT reactivity as well as the cellular response in the skin.

Material and Methods

Study Population

The study comprised 17 atopic farmers, 16 men and 1 woman (mean age 43 years, range 31–56), that 1 year prior to the present study were shown to be positive in SPT to rLep d 2 [19]. Fifteen subjects reported symptoms of asthma and/or rhinoconjunctivitis and 2 were asymptomatic (table 1). The control group was composed of 8 nonatopic farmers, 7 men and 1 woman (mean age 52 years, range 34–64). All individuals participated in an investigation which included SPT, a medical interview and venous blood sampling for serological analysis. Skin biopsies were taken from the atopic farmers. None of the farmers were on medication such as oral steroids, long-acting antihistamine or had previously received immunotherapy, and treatment with short-acting antihistamine was withdrawn at least 5 days prior to the investigation. None of the subjects suffered from clinically active atopic dermatitis. The examination was performed on Gotland, an island in the Baltic Sea, in April 1999.

Allergens

rLep d 2.01 (identical to Lep d 1.0101 in Schmidt et al. [31]) and rLep d 2.6Cys were produced in *Escherichia coli* BL21(DE3)pLysS expression strain (Novagen, Madison, Wisc., USA), using the pET expression system (Novagen) as previously described [26, 32]. rLep d 2.6Cys was derived from rLep d 2.01 by site-directed mutagenesis, exchanging all six cysteine residues in Lep d 2 to serines [26]. Both rLep d 2 and rLep d 2.6Cys were expressed as C-terminal His₆-tagged proteins and purified by metal affinity chromatography [26, 32]. The purity of the recombinant proteins was verified by SDS-PAGE giving single protein bands [26, 31]. Protein concentration determinations were based on the optical density of CBB-stained proteins on SDS-PAGE and calculated by comparison to a standard as described by Olsson et al. [32]. The allergens were then diluted in 0.9% NaCl to 100 µg/ml, aliquoted and stored at –20°C until used. The selected allergen concentration was based on previous SPT data [19, 26].

Serological Analysis

Total serum IgE was determined using the Pharmacia CAP RAST System, IgE FEIA (Pharmacia & Upjohn, Diagnostics, Uppsala, Sweden). The cutoff was 2 kU/l. IgE against rLep d 2 and rLep d 2.6Cys were quantified by the Pharmacia CAP RAST System, RAST FEIA (Pharmacia & Upjohn). ImmunoCAPs with rLep d 2 and rLep d 2.6Cys were prepared by Pharmacia Research Forum. The recombinant allergens were immobilized at the same protein concentration

Table 1. Characterization of the farmers, specific and total serum IgE

Subject No.	Symptoms	Age years	Sex	Serum IgE, kU/l		
				rLep d 2 ^a	rLep d 2.6Cys ^a	total IgE ^b
Atopic						
1	A	33	M	84	1.4	770
2	R	37	M	24	0.55	320
3	A+R	45	M	12	0.45	740
4	A	42	M	8.3	<0.35	240
5	R	39	M	8.0	1.5	1,100
6	R	56	M	5.6	1.2	420
7	R	49	M	5.2	0.5	57
8	R	39	M	3.4	<0.35	44
9	R	37	M	3.4	<0.35	220
10	R	41	M	3.2	<0.35	150
11	N	47	M	2.4	<0.35	300
12	R	53	M	1.3	<0.35	140
13	R	35	M	0.87	<0.35	210
14	R	45	M	0.76	<0.35	70
15	A+R	42	F	0.47	<0.35	240
16	A+R	48	M	<0.35	<0.35	15
17	N	43	M	<0.35	<0.35	120
Median value		43		3.4	<0.35	210
Non-atopic						
18	N	64	M	<0.35	<0.35	<2
19	N	63	M	<0.35	<0.35	39
20	A	60	M	<0.35	<0.35	4.8
21	A	58	M	<0.35	<0.35	20
22	N	51	F	<0.35	<0.35	24
23	N	44	M	<0.35	<0.35	12
24	N	42	M	<0.35	<0.35	48
25	N	34	M	<0.35	<0.35	33
Median value		54		<0.35	<0.35	22
A = Asthma; R = rhinoconjunctivitis; N = none.						
^a Specific IgE values <0.35 kU/l are considered negative.						
^b The reference range for total serum IgE is <2–122 kU/L.						

onto the cellulose solid phase (ImmunoCAP) by covalent binding as described earlier [33]. Coupling concentration was adjusted to attain a linear measurement range and a background below 0.35 kU/l, which is the established cutoff value for a positive result in the RAST CAP System.

Skin Testing

SPT was performed on the volar region of the forearms by a trained nurse. Each drop of rLep d 2 and rLep d 2.6Cys consisted of 15 µl (100 µg/ml) and was pricked with a sterile lancet (ALK, Hørsholm, Denmark). Histamine dihydrochloride 10 mg/ml (ALK) was used as a positive control, 0.9% NaCl constituted a negative control for the recombinant allergens in SPT and albumin diluent (ALK) for the skin biopsies. rLep d 2 was tested only once on each subject, since its ability to elicit an SPT response had previously been demonstrated [19]. The modified allergen, rLep d 2.6Cys, was tested in duplicates.

SPT results were recorded after 15 min and the wheals were outlined with a fine-tipped pen and transferred to paper with transparent tape. The diameter of the wheal was calculated according to the formula $(D1 + D2)/2$, where D1 represents the largest longitudinal diameter and D2 the maximal transverse diameter. The results of rLep d 2.6Cys were expressed as the mean diameter of the two wheals. An SPT was considered positive if it was at least 3 mm in diameter and 2+ (wheal at least half the size of the histamine wheal).

When no SPT reaction was recorded, a circumference was drawn around the test site in order to be able to identify the test area at the time of the skin biopsies. The subjects were informed in detail not to remove the SPT labelling until the biopsies had been performed.

Skin Biopsies

Punch biopsies were obtained from all 17 atopic subjects, using a 4-mm sterile disposable biopsy punch (Miltex Instruments Compa-

ny, Lake Success, N.Y., USA). Biopsies were taken from the center of the SPT sites after induction of local anesthesia (lidocaine 5 mg/ml with epinephrine 5 µg/ml, Astra, Södertälje, Sweden). Biopsies were taken 17–20 h after the SPT had been performed from the following four SPT sites: histamine, albumin diluent (ALK), rLep d 2 and rLep d 2.6Cys. Due to difficulties to identify the labelled SPT area, one biopsy specimen could not be obtained from 2 subjects (1 from the histamine wheal and 1 from the rLep d 2.6Cys site).

Processing and Staining of the Biopsy Specimens

The skin biopsies were immediately frozen on dry ice and thereafter kept at -70°C . Acetone-fixed 6-µm-thick cryostat sections were prepared for routine hematoxylin and eosin staining and for PAP (peroxidase-antiperoxidase) staining [34]. Prior to the PAP staining, endogenous peroxidase activity was blocked with 0.3% H_2O_2 and 0.1% NaN_3 in PBS. The following mouse monoclonal antibodies (mAb) were used for immunohistochemical staining: Leu-4 (specificity CD3, T cells, dilution 1/32) and Leu-6 (CD1a, Langerhans cells, 1/20) from Becton Dickinson (San Jose, Calif., USA), EG2 (activated eosinophils, 1/100) from Pharmacia & Upjohn (Uppsala, Sweden), AA1 (mast cell tryptase, 1/75) from DAKO (Glostrup, Denmark), and B3102E8 (IgE, 1/500) from Southern Biotech (Birmingham, Ala., USA).

Evaluation of Skin Biopsy Specimens

The examination was performed under coded conditions by the same investigator (M.K.) and at least two sections per mAb and biopsy specimen were examined at magnification $\times 400$. A semiquantitative scale from 0 to 3 (0 = no, 1 = small, 2 = moderate, and 3 = large cell infiltrates) was used for the evaluation of dermal cell infiltrates in the hematoxylin and eosin staining and for the evaluation of the CD3+ dermal cell infiltrates. In the epidermis all positive cells were counted in at least three fields of view in three sections and the results expressed as the mean value per field of view. For EG2+ cells, dermal CD1a+ cells, and dermal IgE+ cells, the total number of cells was counted in at least three fields of view in three sections of each biopsy specimen and tryptase+ cells were counted in at least five fields of view in three sections per specimen. The results are expressed as the mean value per field of view.

Statistical Analysis

Statistical analysis was performed with Statistica 5.5 (Stat Soft, Uppsala, Sweden). SPT and serological results are presented as medians with the range. Correlation coefficients (r_s) were determined with the Spearman rank correlation analysis. The Mann-Whitney U test was used for comparison of serological results between atopic and nonatopic farmers, and Wilcoxon's sign rank test was used for comparisons within the groups. Differences between dermal cell infiltrates were calculated with Friedman's ANOVA. For comparison of the number of IgE+ cells, EG2+ cells, tryptase+ cells and CD1a+ cells ANOVA repeated measures were used. A p value < 0.05 was considered statistically significant.

Ethical Considerations

The study was approved by the Ethics Committee of the Karolinska Institute, Stockholm.

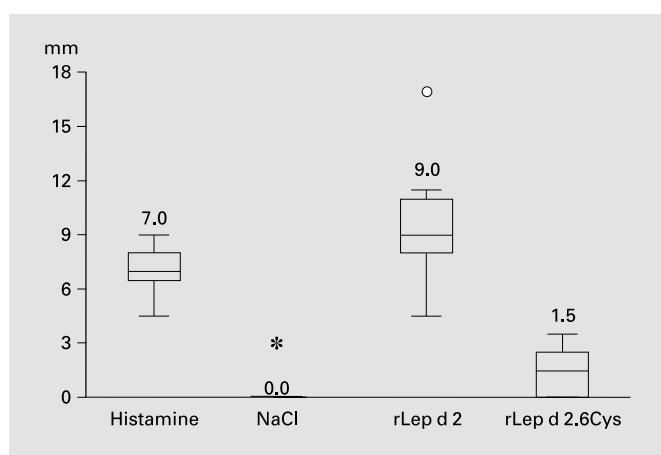


Fig. 1. SPT wheals (mm) induced by rLep d 2.6Cys are significantly smaller ($p < 0.001$) than the wheals induced by rLep d 2 in 17 atopic subjects. Fifty percent of the values are presented within the boxes and numbers given are median values. O = Outliers ($n = 1$); * = extreme outliers ($n = 1$).

Results

Serological Results

A positive RAST against rLep d 2 was found among 15 out of 17 dust mite-sensitized subjects while only 6 had a positive RAST against rLep d 2.6Cys (table 1). The IgE levels against rLep d 2.6Cys were significantly lower than against rLep d 2 ($p < 0.001$). Positive RAST values were not found among the nonatopic controls. The total IgE values among the nonatopic controls were significantly lower ($p < 0.001$) than the ones obtained in the mite-sensitized group (table 1).

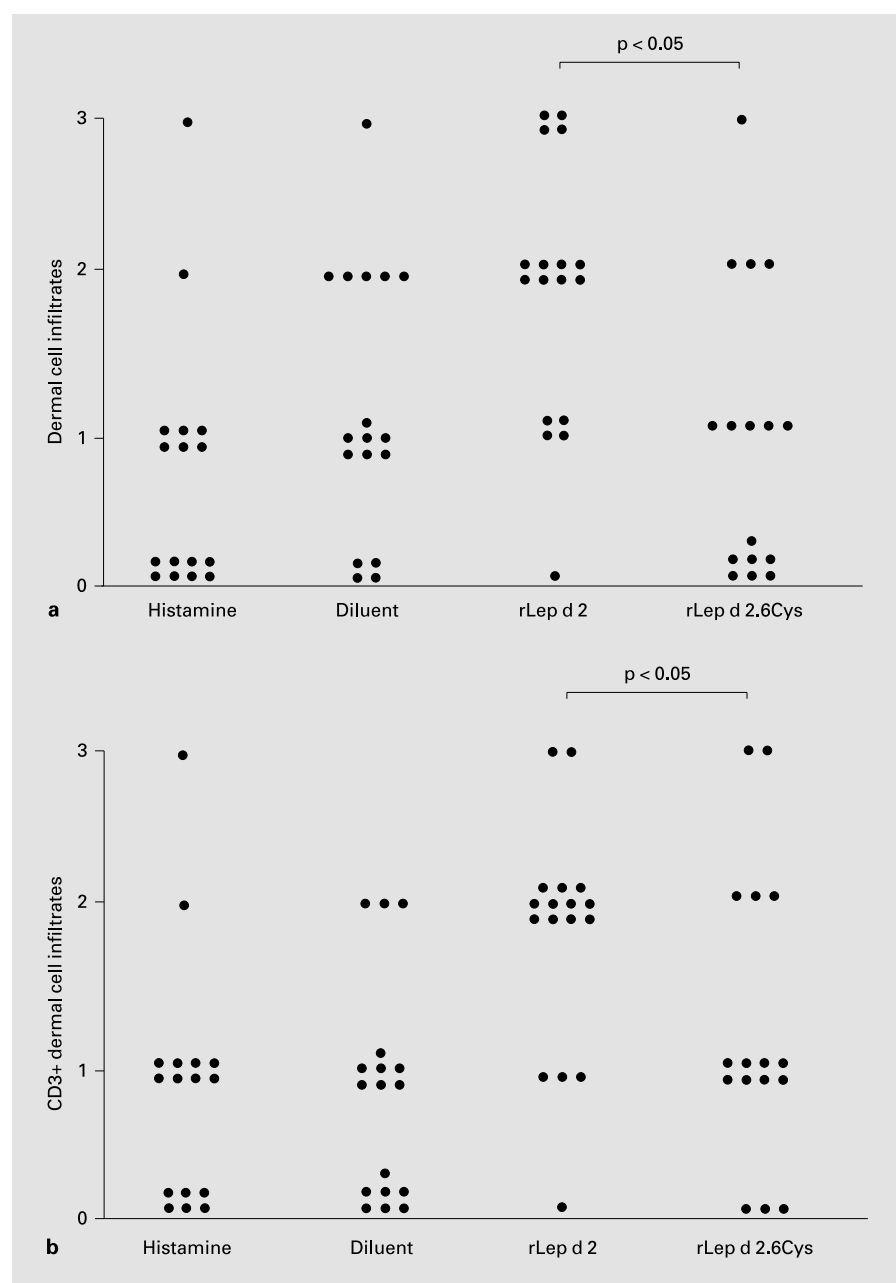
rLep d 2.6Cys Induces Fewer and Smaller Positive SPT

All the 17 dust mite-sensitized subjects had a positive SPT to rLep d 2, while only 3 displayed a positive SPT to rLep d 2.6Cys. The wheals induced by rLep d 2.6Cys were significantly smaller than the ones obtained with rLep d 2 ($p < 0.001$) (fig. 1). None of the nonatopic controls displayed a positive SPT to either of the allergens tested.

Evaluation of Skin Biopsy Specimens

rLep d 2.6Cys Induces Smaller Dermal Cell Infiltrates and Dermal CD3+ Cell Infiltrates than rLep d 2. As judged from the hematoxylin and eosin staining, rLep d 2.6Cys induced significantly smaller dermal cell infiltrates than rLep d 2 ($p < 0.05$). In contrast, no significant difference was obtained between rLep d 2.6Cys and the

Fig. 2. a Size of the dermal cell infiltrates induced by rLep d 2.6Cys was significantly smaller than of those induced by rLep d 2 determined in the hematoxylin and eosin stainings. A semiquantitative scale was used for the estimation: 0 = no, 1 = small, 2 = moderate and 3 = large cell infiltrates. **b** The CD3+ dermal cell infiltrates were evaluated semiquantitatively as 0 = no, 1 = small, 2 = moderate, 3 = large cell infiltrates. **a, b** Significant differences between the specimens are indicated. Each dot represents one subject.



controls (fig. 2a). Dermal cell infiltrates with CD3+ cells were significantly smaller in biopsies from SPT sites tested with rLep d 2.6Cys than from the rLep d 2 sites ($p < 0.05$, fig. 2b). On the other hand, no significant difference was recorded between rLep d 2.6Cys and the controls (fig. 2b).

rLep d 2.6Cys Induces Significantly Fewer Activated (EG2+) Eosinophils but More Tryptase+ Cells than rLep d 2. The number of EG2+ cells induced by rLep d 2.6Cys

were significantly decreased compared with the number obtained at the rLep d 2 site ($p < 0.001$) (fig. 3a). No statistically significant difference was recorded between the number of EG2+ cells in biopsies from rLep d 2.6Cys or the two control sites (fig. 3a).

Dermal tryptase+ mast cells were seen in significantly higher numbers in biopsies from rLep d 2.6Cys sites compared to rLep d 2 sites ($p < 0.05$). Furthermore, the number of tryptase+ cells did not differ significantly between

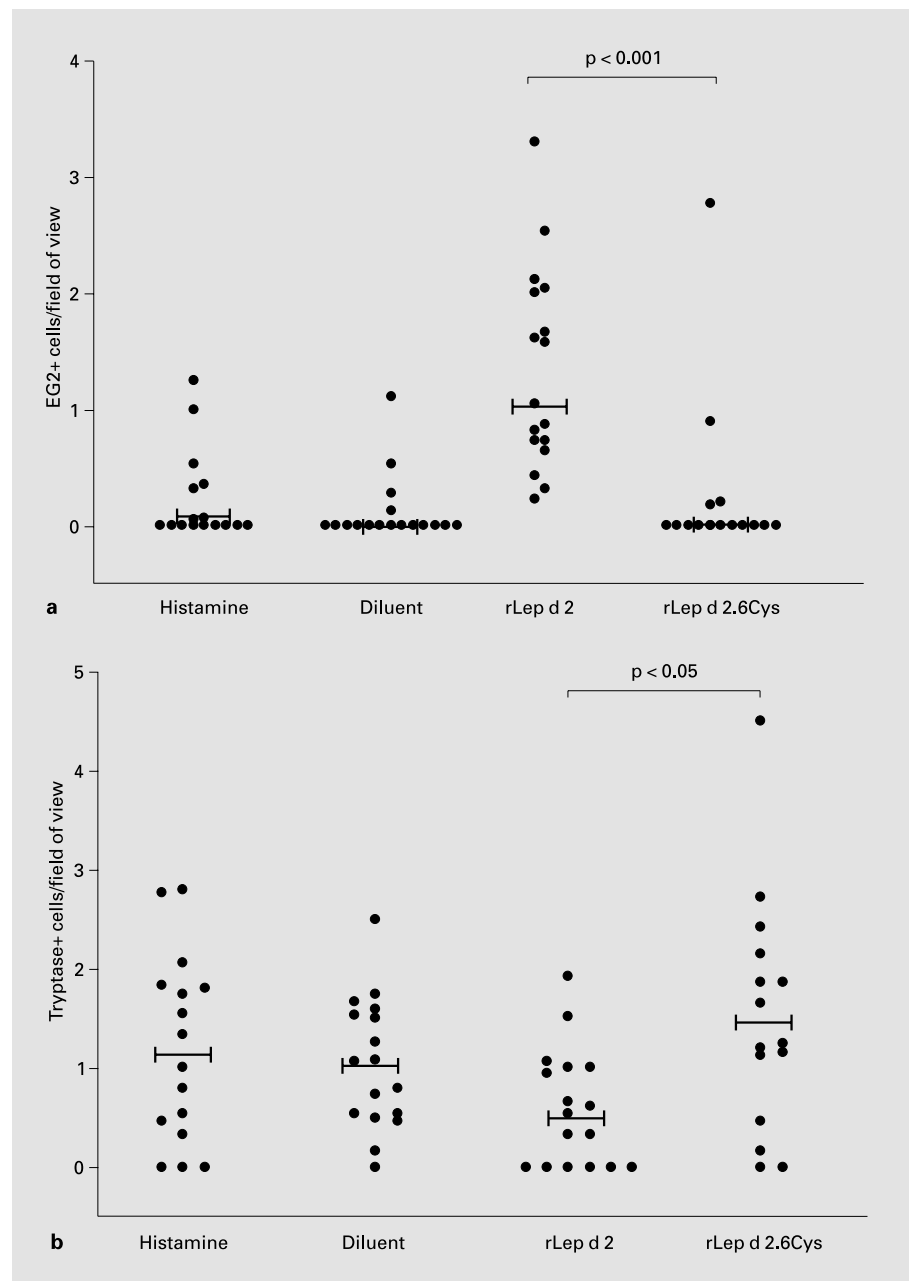


Fig. 3. Compared to rLep d 2, rLep d 2.6Cys induced a significantly lower number of EG2+ cells (**a**) but a higher number of tryptase+ cells (**b**). The number of stained cells is expressed as cells/field of view. The median values are indicated as horizontal bars and each dot represents 1 subject.

biopsies obtained from the rLep d 2.6Cys and the control sites (fig. 3b).

No Difference in the Number of CD1a+ and IgE+ Cells between rLep d 2.6Cys and rLep d 2. The number of epidermal as well as dermal CD1a+ cells did not differ significantly between the various test sites (table 2). In addition, no significant difference in the number of epidermal IgE+ cells was found between biopsy specimens from the different SPT sites (table 2). Even though the median val-

ue of dermal IgE+ cells in biopsy specimens from rLep d 2.6Cys sites was significantly higher compared to the control sites ($p < 0.05$), the range of dermal IgE+ cells between the challenged sites did not differ. No significant difference in the number of dermal IgE+ cells was found between the two allergen-challenged sites (table 2). The number of IgE+ cells at the different SPT sites in epidermis correlated significantly ($p < 0.05$) with the corresponding number in dermis (rLep d 2: $r_s = 0.66$,

Table 2. CD1a+ cells and IgE+ cells at the different biopsy sites

Biopsy site	CD1a+ cells		IgE+ cells	
	epidermal	dermal	epidermal	dermal
Albumin diluent	4.8 (2.2–8.0)	0.3 (0–2.9)	0 (0–3.0)	0.7 (0–4.9)
Histamine	3.2 (2.1–6.0)	0.3 (0–2.2)	0.1 (0–2.3)	0.6 (0–3.2)
rLep d 2	4.0 (2.2–7.0)	0.5 (0–2.5)	0 (0–2.5)	1.3 (0–4.2)
rLep d 2.6Cys	5.6 (1.6–8.4)	0.8 (0–2.1)	0 (0–2.3)	1.8 (0–4.1)*

The number of immunoperoxidase-stained cells is given as median value per field of view, the range within parentheses (magnification $\times 400$).

* $p < 0.05$, significantly higher number vs. histamine or albumin diluent.

rLep d 2.6Cys: $r_s = 0.70$, albumin diluent: $r_s = 0.79$ and histamine: $r_s = 0.82$).

Comparison of SPT, Total IgE and Immunohistochemical Results

Except for a significant correlation between the wheal size and number of EG2+ cells induced by rLep d 2 ($r_s = 0.67$, $p < 0.01$) no significant associations were found between the size of the different SPT wheals and the number of stained cells in the corresponding biopsy specimens. The total serum IgE values were significantly ($p < 0.05$) correlated with the number of epidermal as well as dermal IgE+ cells in all the different biopsy sites (rLep d 2 $r_s = 0.63$ and $r_s = 0.78$, rLep d 2.6Cys $r_s = 0.77$ and $r_s = 0.75$, histamine $r_s = 0.77$ and $r_s = 0.88$ and diluent $r_s = 0.79$ and $r_s = 0.73$, respectively).

Discussion

In this study we have compared the capacity to induce IgE-mediated reactions of the major allergen of *L. destructor* produced as a recombinant protein, rLep d 2 with that of a modified variant rLep d 2.6Cys in SPT and skin biopsy specimens in 17 *L. destructor*-sensitized subjects. The SPT reaction was used to reflect the early phase-allergic reaction, and the cellular events in skin biopsies, obtained after 17–20 h, represent the late phase-allergic reaction.

This is, to our knowledge, the first study that has investigated the cellular events in skin biopsies taken from SPT sites challenged with recombinant allergens as well as with their modified derivatives. The SPT wheals reflect the immediate early phase-allergic reaction characterized by local edema (wheal) which is caused by the rapid release of preformed mast cell mediators, such as histamine and tryptase [35–38]. At the time of the skin biopsies (17–20 h

later) the signs of the early phase reaction (i.e. SPT wheals) have vanished. It has been demonstrated by Frew and Kay [39], among others, that the size of the wheals induced by intradermal allergen injections does not correlate with the late phase-allergic reaction.

This is in line with our results where, with the exception of EG2+ cells induced by rLep d 2, we did not find an association between the wheal size and the number of stained cells in the corresponding biopsy specimen.

Allergen challenges in the skin give rise to an influx of T cells and an activation of eosinophils [40]. Skin biopsies taken between 6 and 48 h after intradermal tests with extracts of timothy grass and house dust mite *D. pteronyssinus* have shown that the number of CD3+ cells as well as EG2+ cells are significantly elevated compared to the controls [41, 42]. In the present study, the dermal cell infiltrates and the dermal CD3+ cell infiltrates induced by rLep d 2.6Cys did not differ from the ones observed in the controls and they were significantly smaller and fewer than the ones induced by rLep d 2. In addition, we found a significantly lower number of activated eosinophils in biopsies, as measured by the number of EG2+ cells, from rLep d 2.6Cys sites than from rLep d 2 sites, which probably is due to a reduced ability of the modified allergen not only to cause a cellular response but also to activate the cells at the site compared to rLep d 2. This is in line with our earlier observation when in a skin chamber model we found that modified recombinant birch pollen allergens activate eosinophils to a significantly lower degree than the corresponding unmodified recombinant allergen [43].

An interesting finding is that we obtained a significantly higher number of tryptase+ mast cells from the sites tested with rLep d 2.6Cys than from the sites tested with rLep d 2. A likely explanation is that rLep d 2.6Cys, compared to rLep d 2, has a reduced IgE-binding capacity

which causes less mast cell degranulation than the modified allergen. The finding is also in line with the SPT results, where rLep d 2.6Cys had a reduced capacity to activate mast cells in the skin. Furthermore, it has previously been demonstrated that it takes more than 24 h after an allergen-induced degranulation until the content of the mast cell granules is fully restored [35]. A recent study by Macfarlane et al. [44] has shown that, compared to diluent sites, the number of tryptase+ cells in skin biopsies obtained from allergen-challenged sites is significantly reduced 6–48 h after intradermal allergen challenge.

The number of epidermal and dermal CD1a+ cells did not differ between the different biopsy sites, which is in concordance with earlier findings by Ying et al. [42] who did not observe any difference in the number of CD1a+ cells in skin biopsies from allergen-induced late phase reactions compared to control sites. We found no significant difference in the number of epidermal IgE+ cells between the different biopsy sites but a significant correlation between the number IgE+ cells and the serum IgE levels.

Significantly fewer subjects had a positive RAST to rLep d 2.6Cys compared to rLep d 2. The RAST values against rLep d 2.6Cys were also significantly lower than to rLep d 2, which indicates that rLep d 2.6Cys, although it has a clearly reduced IgE binding ability, does not completely lack IgE reactivity. It could be that subjects with high levels of specific IgE against rLep d 2 also recognize minor or hidden epitopes that probably still may be present in rLep d 2.6Cys. Furthermore, rLep d 2.6Cys generated significantly smaller and less positive SPT than the unmodified rLep d 2, which confirms our previous in

vitro data, where rLep d 2.6Cys was shown to have less IgE reactivity than the unmodified rLep d 2 [26]. The present observations are in accordance with several previous studies, where modified allergen derivatives, even though produced by different strategies, are shown to elicit a clearly reduced IgE reactivity. For example, the recombinant modified allergens (rBet v 1 fragment mix and rBet v 1 trimer) were shown to elicit fewer and smaller SPT reactions than their unmodified counterpart [16, 18, 45] and similar findings have been reported for recombinant modified allergens of house dust mites, timothy grass and cow [12, 14, 17, 46].

In conclusion, this study demonstrates that the modified allergen, rLep d 2.6Cys, not only induces less IgE reactivity as indicated by SPT reactions and mast cell degranulation, but also is less capable of inducing dermal cell infiltrates and activating eosinophils compared to rLep d 2. These data suggest that rLep d 2.6Cys in the future may be a promising candidate molecule for immunotherapy of *L. destructor* allergy.

Acknowledgements

We thank Annika Lindstedt for professional medical assistance and Anne Svensson for excellent technical assistance. Financial support for this study was received from the Swedish Medical Research Council (grants No. 7924 and 13497), the Swedish Council for Work Life Research, the Swedish Foundation for Health Care, Sciences and Allergy Research, the Swedish Asthma and Allergy Association, the King Gustaf V 80th Birthday Foundation, the Swedish Society of Medicine, the Trygg-Hansa Foundation Fund, the Magnus Bergvall, the Hesselman and the Konsul Th.C. Bergh's Foundations and Karolinska Institutet.

References

- 1 Scheiner O, Kraft D: Basic and practical aspects of recombinant allergens. *Allergy* 1995; 50:384–391.
- 2 Chapman MD, Smith AM, Vailes LD, Aurruda LK, Dhanaraj V, Pomés A: Recombinant allergens for diagnosis and therapy of allergic disease. *J Allergy Clin Immunol* 2000;106:409–418.
- 3 Valenta R, Vrtala S, Laffer S, Spitzauer S, Kraft D: Recombinant allergens. *Allergy* 1998; 53:552–561.
- 4 Laffer S, Spitzauer S, Susani M, Pairleitner H, Schweiger C, Grönlund H, Menz G, Pauli G, Ishii T, Nolte H, Ebner C, Schon AH, Kraft D, Eichler HG, Valenta R: Comparison of recombinant timothy grass pollen allergens with natural extract for diagnosis of grass pollen allergy in different populations. *J Allergy Clin Immunol* 1996;98:652–658.
- 5 Lynch NR, Thomas WR, Garcia NM, Di Prisco MC, Puccio FA, López RI, Hazell LA, Shen HD, Lin KL, Chua KY: Biological activity of recombinant Der p 2, Der p 5 and Der p 7 allergens of the house-dust mite *Dermatophagoides pteronyssinus*. *Int Arch Allergy Immunol* 1997; 114:59–67.
- 6 Niederberger V, Laffer S, Fröschl R, Kraft D, Rumpold H, Kapiotis S, Valenta R, Spitzauer S: IgE antibodies to recombinant pollen allergens (Phl p 1, Phl p 2, Phl p 5 and Bet v 2) account for a high percentage of grass pollen-specific IgE. *J Allergy Clin Immunol* 1998;101: 258–264.
- 7 Pauli G, Oster JP, Deviller P, Heiss S, Bessot JC, Susani M, Ferreira F, Kraft D, Valenta R: Skin testing with recombinant allergens rBet v 1 and birch profilin, rBet v 2: Diagnostic value for birch pollen and associated allergies. *J Allergy Clin Immunol* 1996;97:1100–1109.
- 8 Akdis CA, Blaser K: Regulation of specific immune responses by chemical and structural modifications of allergens. *Int Arch Allergy Immunol* 2000;121:261–269.
- 9 Akdis CA, Blaser K: Mechanisms of allergen specific immunotherapy. *Allergy* 2000;55:522–530.
- 10 Secrist H, DeKruyff RH, Umetsu DT: Interleukin 4 production by CD4+ T cells from allergic individuals is modulated by antigen concentration and antigen-presenting cell type. *J Exp Med* 1995;181:1081–1089.

- 11 Valenta R, Vrtala S, Focke-Tejkl M, Bugajska-Sretter A, Ball T, Twardosz A, Spitzauer S, Grönlund H, Kraft D: Genetically engineered and synthetic allergen derivatives: Candidates for vaccination against type I allergy. *Biol Chem* 1999;380:815–824.
- 12 Smith AM, Chapman MD, Taketomi EA, Platts-Mills TAE, Sung SJ: Recombinant allergens for immunotherapy: A Der p 2 variant with reduced IgE reactivity retains T-cell epitopes. *J Allergy Clin Immunol* 1998;101:423–425.
- 13 Zeiler T, Taivainen A, Rytönen M, Rautiainen J, Karjalainen H, Mäntylä R, Tuomisto L, Virtanen T: Recombinant allergen fragments as candidate preparations for allergen immunotherapy. *J Allergy Clin Immunol* 1997;100:721–727.
- 14 Kauppinen J, Zeiler T, Rautiainen R, Rytönen-Nissinen M, Taivainen A, Mäntylä R, Virtanen T: Mutant derivatives of the main respiratory allergen of cow are less allergenic than the intact molecule. *Clin Exp Allergy* 1999;29:989–996.
- 15 Vrtala S, Hirtenlehner K, Vangelista L, Pastore A, Eichler HG, Sperr WR, Valent P, Ebner C, Kraft D, Valenta R: Division of the major birch pollen allergen, Bet v 1, into two non-anaphylactic fragments. *Int Arch Allergy Immunol* 1997;113:246–248.
- 16 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.
- 17 Schramm G, Kahlert H, Suck R, Weber B, Stüwe H-T, Müller W-D, Bufer A, Becker WM, Schlaak MW, Jäger L, Cromwell O, Fiebig H: 'Allergen engineering': Variants of the timothy grass pollen Allergen Phl 5b with reduced IgE-binding capacity but conserved T cell reactivity. *J Immunol* 1999;162:2406–2414.
- 18 van Hage-Hamsten M, Kronqvist M, Zetterström O, Johansson E, Niederberger V, Vrtala S, Grönlund H, Grönneberg R, Valenta R: Skin test evaluation of genetically engineered hypoallergenic derivatives of the major birch pollen allergen, Bet v 1: Results obtained with a mix of two recombinant Bet v 1 fragments and recombinant Bet v 1 trimer in a Swedish population before the birch pollen season. *J Allergy Clin Immunol* 1999;104:969–977.
- 19 Kronqvist M, Johansson E, Magnusson CGM, Olsson S, Eriksson TLJ, Gafvelin G, van Hage-Hamsten M: Skin prick test and serological analysis with recombinant group 2 allergens of the dust mites *L. destructor* and *T. putrescentiae*. *Clin Exp Allergy* 2000;30:670–676.
- 20 van Hage-Hamsten M, Johansson SGO, Höglund S, Tüll P, Wren A, Zetterström O: Storage mite allergy is common in a farming population. *Clin Allergy* 1985;15:555–564.
- 21 Iversen M, Pedersen B: The prevalence of allergy in Danish farmers. *Allergy* 1990;45:347–353.
- 22 Kronqvist M, Johansson E, Pershagen G, Johansson SGO, van Hage-Hamsten M: Increasing prevalence of asthma over 12 years among dairy farmers on Gotland, Sweden: Storage mites remain dominant allergens. *Clin Exp Allergy* 1999;29:35–41.
- 23 Gislason D, Gislason T: IgE-mediated allergy to *Lepidoglyphus destructor* in an urban population – An epidemiologic study. *Allergy* 1999;54:878–883.
- 24 Luscynska CM, Griffin P, Davies RJ, Topping MD: Prevalence of specific IgE to storage mites (*A. siro*, *L. destructor* and *T. longior*) in an urban population and crossreactivity with the house dust mite (*D. pteronyssinus*). *Clin Exp Allergy* 1990;20:403–406.
- 25 Fernández-Caldas E: Mite species of allergologic importance in Europe. *Allergy* 1997;52:383–387.
- 26 Olsson S, van Hage-Hamsten M, Whitley P: Contribution of disulphide bonds to antigenicity of Lep d 2, the major allergen of the dust mite *Lepidoglyphus destructor*. *Mol Immunol* 1998;35:1017–1023.
- 27 Mueller GA, Benjamin DC, Rule GS: Tertiary structure of the major house dust mite allergen Der p 2: Sequential and structural homologies. *Biochemistry* 1998;37:12707–12714.
- 28 Ichikawa S, Hatanaka H, Yuuki T, Iwamoto N, Kojima S, Nishiyama C, Ogura K, Okumura Y, Inagaki F: Solution structure of Der f 2, the major mite allergen for atopic diseases. *J Biol Chem* 1998;273:356–360.
- 29 Gafvelin G, Johansson E, Lundin A, Smith AM, Chapman MD, Benjamin DC, Derewenda U, van Hage-Hamsten M: Cross-reactivity studies of a new group 2 allergen from the dust mite *Glycyphagus domesticus*, Gly d 2, and group 2 allergens from *Dermatophagoides pteronyssinus*, *Lepidoglyphus destructor* and *Tyrophagus putrescentiae*, using recombinant allergens. *J Allergy Clin Immunol* 2001;107:511–518.
- 30 Eriksson TLJ, Gafvelin G, Elfman LHM, Johansson C, van Hage-Hamsten M, Olsson S: T-cell responses to recombinant isoforms, synthetic peptides and a mutant variant of Lep d 2, a major allergen from the dust mite *Lepidoglyphus destructor*. *Clin Exp Allergy*, in press.
- 31 Schmidt M, Olsson S, van der Ploeg I, van Hage-Hamsten M: cDNA analysis of the mite allergen Lep d 1 identifies two different isoallergens and variants. *FEBS Lett* 1995;370:11–14.
- 32 Olsson S, van Hage-Hamsten M, Whitley P, Johansson E, Hoffman DR, Gafvelin G, Schmidt M: Expression of two isoforms of Lep d 2, the major allergen of *Lepidoglyphus destructor*, in both prokaryotic and eukaryotic systems. *Clin Exp Allergy* 1998;28:984–991.
- 33 Johansson E, Eriksson TJL, Olsson S, Kronqvist M, Whitley P, Johansson SGO, van Hage-Hamsten M: Evaluation of specific IgE to the recombinant group 2 mite allergens Lep d 2 and Tyr p 2 in the Pharmacia CAP System. *Int Arch Allergy Immunol* 1999;120:43–49.
- 34 Sternberger LA: Immunohistochemistry. New York, Wiley, 1979, pp 104–169.
- 35 Dvorak AM, Schleimer RP, Lichtenstein LM: Human mast cells synthesize new granules during recovery from degranulation. In vitro studies with mast cells purified from human lungs. *Blood* 1988;71:76–85.
- 36 Naclerio R: Clinical manifestations of the release of histamine and other inflammatory mediators. *J Allergy Clin Immunol* 1999;103:S382–S385.
- 37 Durham SR: The inflammatory nature of allergic disease. *Clin Exp Allergy* 1998;28(suppl 6):20–24.
- 38 Petersen LJ, Mosbech H, Skov PS: Allergen-induced histamine release in intact human skin in vivo assessed by skin microdialysis technique: Characterization of factors influencing histamine releasability. *J Allergy Clin Immunol* 1996;97:672–679.
- 39 Frew AJ, Kay AB: The relationship between infiltrating CD4+ lymphocytes, activated eosinophils, and the magnitude of the allergen-induced late phase cutaneous reaction in man. *J Immunol* 1988;141:4158–4164.
- 40 Frew AJ, Kay AB: Eosinophils and T-lymphocytes in late-phase allergic reactions. *J Allergy Clin Immunol* 1990;85:533–539.
- 41 Barata LT, Ying S, Meng Q, Barkans J, Rajakulasingam K, Durham SR, Kay AB: IL-4- and IL-5-positive T lymphocytes, eosinophils, and mast cells in allergen-induced late-phase cutaneous reactions in atopic subjects. *J Allergy Clin Immunol* 1998;101:222–230.
- 42 Ying S, Barata LT, Meng Q, Grant JA, Barkans J, Durham SR, Kay AB: High-affinity immunoglobulin E receptor Fc(εR1)-bearing eosinophils, mast cells, macrophages and Langerhans' cells in allergen-induced late-phase cutaneous reactions in atopic subjects. *Immunology* 1998;93:281–288.
- 43 Nopp A, Halldén G, Lundahl J, Johansson E, Vrtala S, Valenta R, Grönneberg R, van Hage-Hamsten M: Comparison of inflammatory responses to genetically engineered hypoallergenic derivatives of the major birch pollen allergen Bet v 1 and to recombinant Bet v 1 wild type in skin chamber fluids collected from birch pollen-allergic patients. *J Allergy Clin Immunol* 2000;106:101–109.
- 44 Macfarlane AJ, Kon OM, Smith SJ, Zeibecoglou K, Khan LN, Barata LT, McEuen AR, Buckley MG, Walls AF, Meng Q, Humbert M, Barnes NC, Robinson DS, Ying S, Kay AB: Basophils, eosinophils, and mast cells in atopic and nonatopic asthma and in late-phase allergic reactions in the lung and skin. *J Allergy Clin Immunol* 2000;105:99–107.
- 45 Pauli G, Purohit A, Oster JP, de Blay F, Vrtala S, Niederberger V, Kraft D, Valenta R: Comparison of genetically engineered hypoallergenic rBet v 1 derivatives with rBet v 1 wild-type by skin prick test and intradermal testing. Results obtained in a French population. *Clin Exp Allergy* 2000;30:1076–1084.
- 46 Takai T, Mori A, Yuuki T, Okudaira H, Okumura Y: Non-anaphylactic combination of partially deleted fragments of the major dust mite allergen Der f 2 for allergen-specific immunotherapy. *Mol Immunol* 1999;36:1055–1065.

Copyright: S. Karger AG, Basel 2001. Reproduced with the permission of S. Karger AG, Basel. Further reproduction or distribution (electronic or otherwise) is prohibited without permission from the copyright holder.