

Comparative analysis of biological activities of *Der p I*-derived peptides on Fcε receptor-bearing cells from *Dermatophagoides pteronyssinus*-sensitive patients

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SUMMARY

The ability of four uncoupled synthetic peptides (p52–71, p117–133, p176–187, p188–199) derived from *Der p I*, a major allergen from the house dust mite *Dermatophagoides pteronyssinus* (*Dpt*) to stimulate FcεR⁺ cells from *Dpt*-sensitive patients was comparatively analysed. Each free peptide may specifically stimulate basophils (FcεRI⁺ cells) and platelets (FcεRII⁺ cells) from patients with significant levels of anti-*Der p I* IgE antibodies; p52–71 and p117–133 appear the best cell stimulation inducers. Both concentration-dependent biological activities of *Der p I*-peptide on FcεR⁺ cells are enhanced by coupling peptide to a carrier (as human serum albumin). Interestingly each *Der p I*-sensitive patient tested presents an individual pattern of response to peptide. Thus, from our results it appears that different *Der p I* sequences could be involved in the immune response to *Der p I*.

Keywords peptides *Der p I* Fcε receptor-bearing cell biological activity house dust mite allergy

INTRODUCTION

The house dust mite *Dermatophagoides pteronyssinus* (*Dpt*) is considered an important source of aeroallergens involved in allergic diseases such as asthma or rhinitis [1]. *Der p I* molecule, a glycoprotein excreted in mite faeces, represents with *Der p II* the major allergenic molecules isolated from *Dpt* [2–4]. Indeed, about 50% of sera from *Dpt*-sensitive patients contain a high proportion (more than 50% of total anti-mite IgE antibodies) of anti-*Der p I* IgE [5,6]. Different approaches have been carried out to map the T and B cell epitopes of *Der p I*. First, monoclonal and polyclonal antibodies were used to define B cell epitopes [7,8]. Then the determination of the sequence of *Der p I* (a 222 amino acid cysteine protease) and the production of the recombinant *Der p I* molecule either in *Escherichia coli* [9,10] or in yeast [11] led to the use of peptides in such approaches [10,12–14]. Recently, by using *Der p I*-derived peptides expressed as fusion proteins, potential IgE- and IgG-binding sequences were identified [10,14]. However, until now the biological activities associated with these sequences have been poorly explored.

In a previous study, using thermodynamic and statistic criteria, *Der p I* amino acid sequences were selected as potential B cell continuous epitopes and the IgE-dependent biological activity of the corresponding synthetic peptides analysed. We demonstrated that the uncoupled peptides, as well as *Der p I*

antigenic extract, specifically stimulate, but at a variable degree, FcεRII-bearing platelets from *Dpt*-sensitive patients [15]. In complementary separated experiments we observed that the peptides induced histamine release from basophils (FcεRI⁺ receptor-bearing cells) from patients allergic to house dust mite [16]. We extend here our analysis of the biological activities of the four *Der p I*-derived peptides (p52–71, p117–133, p176–187, p188–199) by comparing simultaneously their ability to stimulate *in vitro* basophils and platelets from *Dpt*-sensitive patients. Moreover, the effect of coupling of peptide to a carrier was evaluated. Results indicated that only *Dpt*-sensitive patients specifically responded to *Der p I*-peptides, that each *Dpt*-sensitive patient exhibits a typical pattern of response, and that a significant biological activity could be obtained for lower responders when peptides were coupled to a carrier protein such as human serum albumin (HSA).

PATIENTS AND METHODS

Patients

For this study, 32 patients (age 18–59 years) sensitized to *Dpt* were tested for their reactivity to the four peptides either by using histamine release and/or platelet stimulation. All of them presented asthma and/or perennial rhinitis, positive cutaneous prick tests towards dust mite allergens (diameter >9 mm). However, among the patients exhibiting significant anti-*Der p I* IgE antibodies and radioallergosorbent test (RAST; Pharma-

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cia) to *Dpt* ≥ 15 U/ml, only 18 patients have been tested simultaneously with both methods and with the four *Der p I*-derived peptides.

The group of negative controls ($n = 15$) consisted of patients allergic to grass-pollen or exhibiting anaphylactic reactions to hymenoptera venom, and healthy donors without any allergic disorder or atopy.

Allergen and peptides

Dpt extract. A standardized *Dpt* antigen extract (Laboratoire des Stallergènes, Fresnes, France) corresponding to 100 IR (biological units)/ml and containing 2 μ g/ml of *Der p I* (estimated by a two-site MoAb immunoassay) was used throughout this study [16].

Synthetic peptides. Conventional predictive methods were used to select continuous antigenic determinants on the globular protein *Der p I* as described [16]. Four amino acid sequences were selected and the corresponding peptides synthesized following a Boc-benzyl strategy [17], using an automated synthesizer (Applied Biosystems model 430A) [16]. The homogeneity of each peptide was checked by analytical reversed phase-HPLC and the amino acid composition analysed after total acid hydrolysis. The amino acid sequences of four selected peptides were: NQSLDLAEQELVDCASQHGC for p52–71; CQIYPP-NANKIREALAQ for p117–133; VRNSWDTNWGDN for p188–199; (acm)CGYSNAQGVVDYWIC(acm) for p176–187; indeed, two cysteine residues were added to this last peptide and acetamidomethylated to avoid its cyclization.

Non-relevant peptides, VPNLRGDLQVLAQAVARLP (from the protein VP1 of foot-and-mouth disease virus) and KPQEEKEKITKEILNGK (from the protein P28 of helminth *Schistosoma mansoni*) were used as negative controls throughout this study.

Through their thiol groups, p52–71 and p117–133 were covalently linked to human serum albumin using 6-maleimido-caproic acyl-N-hydroxysuccinimide as heterobifunctional cross-linking agent [18]. The approximate amount of peptide coupled to the carrier protein in the complex was 1/1.5; these conjugates were sterilized by filtration on 0.22- μ m membrane (Sartorius, Göttingen, Germany).

Platelet stimulation

Venous blood (6 vol) collected in ACD (1 vol) (citric acid-trisodium citrate dextrose) was centrifuged for 15 min at 120 g. After collection and centrifugation (200 g) of the platelet-rich plasma, the pellet was washed three times in saline buffer supplemented with citric acid (36 mM), glucose (5 mM), calcium (2 mM), magnesium (1 mM), bovine serum albumin (0.35%) and PGE1 (100 mM).

Platelets (15×10^7) (from *Dpt*-sensitive patient), suspended in Eagle's minimum essential medium (MEM) were incubated in flat-bottomed microplates (Nunc, Roskilde, Denmark) with 50–100 *S. mansoni* schistosomula (as targets), 10% autologous serum from *Dpt*-sensitive patient and in the presence of the related allergen (*Dpt* or peptide) in a final volume of 200 μ l. The stock *Dpt* antigen preparation (corresponding to 100 IR/ml) was used at the dilution of 1×10^{-2} to 10^{-4} and peptides at concentrations varying from 0.01 to 100 μ g/ml. After a 24-h incubation at 37°C and 5% CO₂ in air, motionless and dark dead larvae were counted by optical microscopy. The calculated percentage of cytotoxicity was a good correlate of platelet

stimulation [19]. Background, which never exceeded 15%, was deducted from each presented value.

Histamine release

Histamine release from human basophils was quantified by a specific radioimmunoassay (Immunotech, Luminy, France) as described [20]. Briefly, venous blood diluted (1:4) in histamine release buffer was incubated for 30 min at 37°C with different concentrations of the peptides (as described for platelet stimulation) and the amount of histamine was determined in the supernatants collected at 4°C and stored at –20°C. Data were deducted from the standard curve and expressed as a percentage of the total releasable histamine (obtained after cell lysis). The sensitivity of the assay was 0.1 nM. Background, which never exceeded 5–7%, was deducted from each presented value.

Detection of specific anti-*Der p I* IgE

Specific anti-*Der p I* IgE serum level was quantified by ELISA [21].

RESULTS

Comparison of biological activities of *Der p I*-derived peptides

In previous independent experiments we observed that the capacity of basophils from *Dpt*-sensitive patients to respond to four synthetic peptides (p52–71, p117–133, p176–187 and p188–199) was highly dependent on the anti-*Der p I* IgE serum level and also on the value of the RAST to *Dpt*. Thus, taking in account this observation and with the aim of extending our analysis concerning the potential antigenicity of *Der p I*-derived peptides, we comparatively analysed the peptide-dependent basophil and platelet biological reactivity for a series of 18 *Dpt*-sensitive patients with RAST to *Dpt* ≥ 15 U/ml.

As expected, each *Der p I*-derived peptide, as well as *Der p I*-containing *Dpt* allergen, may stimulate basophils and platelets from *Dpt*-sensitive patients. Indeed, in our experimental conditions, platelets appeared more sensitive to synthetic peptides than basophils, and interestingly, a discrepancy was observed between the platelet response and the basophil response to individual *Der p I* peptides (Fig. 1a–f). However, though a simple relationship ($r^2 = 0.70$) was noticed between the platelet reactivity and the histamine release when *Dpt* antigen was used in both tests, no relationship was observed when *Der p I* peptides were tested as potential Fc ϵ R⁺ cell stimulators. Nonetheless, though no peptide could be considered as immunodominant and peptide-induced cell stimulation seemed dependent on several factors, p52–71 and p117–133 appeared to be the most effective inducers of cell activation with the two biological parameters used (Fig. 1b, c). Incubation of these Fc ϵ R⁺ cells with an unrelated peptide (T) (Fig. 1f) or with allergens unrelated to mite (such as hymenoptera venom or grass pollen) (data not shown) did not lead to significant biological response. Thus, though to a variable degree, the four synthetic *Der p I*-derived peptides expressed biological activity only on Fc ϵ R⁺-bearing cells from some *Dpt*-sensitive patients, suggesting that various sequences of *Der p I* could be involved in the immune response to *Der p I*.

Individual pattern of Fc ϵ R⁺ cell response to peptides

Six typical profiles representative of various patterns of response to *Der p I* peptides are shown in Fig. 2.

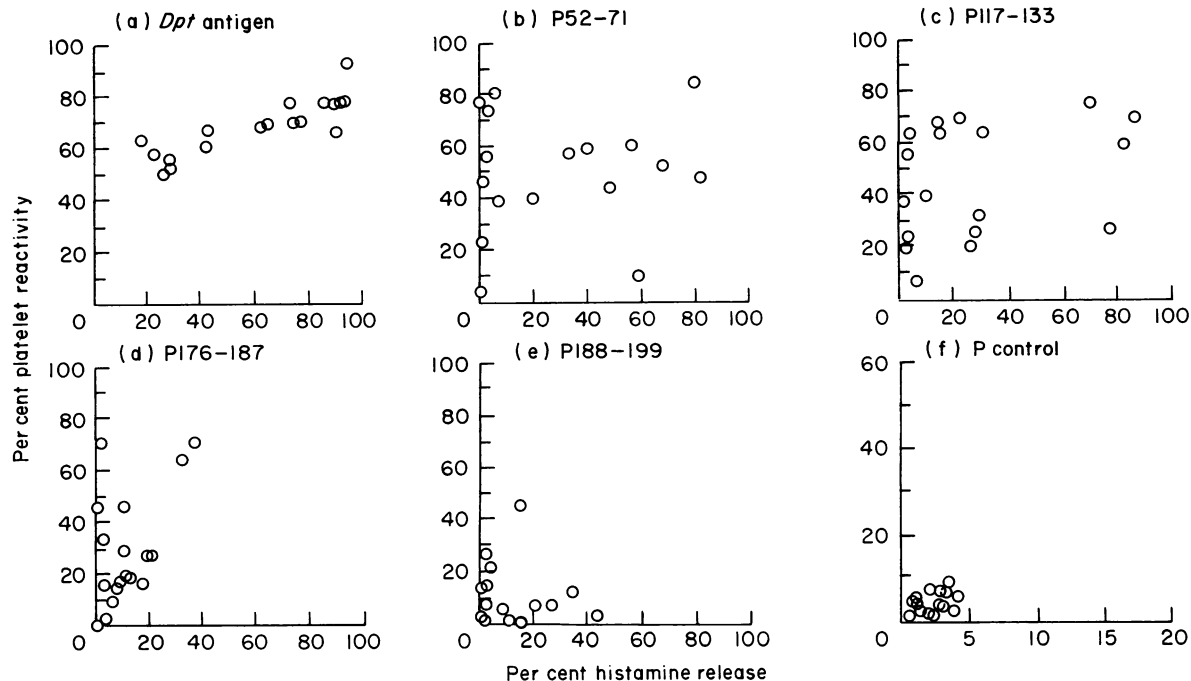


Fig. 1. Biological activity of *Der p I* peptides on $Fc\epsilon R^+$ -bearing cells. $Fc\epsilon R^+$ -bearing cells (basophils and platelets) from *Dermatophagoides pteronyssinus* (*Dpt*)-sensitive patients ($n=18$) were incubated with *Dpt* extract (positive control) (a) (dilution from 10^{-1} to 10^{-4}) or with each *Der p I*-derived peptide (p52-71, p117-133, p176-187, p188-199) (b-e) or with an unrelated peptide (f) (negative control) (from 10^{-2} to 10^2 $\mu\text{g/ml}$). For each patient tested the highest percentage of histamine release and the highest percentage of platelet stimulation obtained in the presence of the optimal concentration of reagent were plotted.

First, it appeared for each patient tested that the reactivity of basophils and platelets was different according to the peptide used. In our study the profiles obtained for patients 1, 4, 5 and 6 were more frequently observed than the others; p52-71 and p117-133 stimulated basophils and platelets more intensively than p176-187 and p188-199. The intensity of biological responses was highly variable according to the patient tested (i.e. patients 1 and 5 *versus* patients 4 and 6). Indeed, as demonstrated for patients 1, 4, 5 and 6, the two $Fc\epsilon R^+$ -bearing cells may be sensitive to the same peptides, and these peptides were associated with higher biological reactivities (histamine release and platelet stimulation) (for example p52-71 for patients 1 and 6 and p117-133 for patient 4). However, in some cases a discrepancy in sensitivity to one peptide was detected; i.e. a high platelet reactivity was accompanied with a lower histamine release, as shown with p117-133 for patients 1 and 6 and with p52-71 for patient 5. In other cases, as illustrated with patient 2, only platelet reactivity could be induced in the presence of some peptides, without any detectable histamine release. Intermediate situations between profile 5 and profile 2 (low histamine release and medium platelet stimulation) were also observed. In contrast, the observation of an intense histamine release without platelet reactivity, as illustrated with patient 3, is an infrequent situation.

Second, the $Fc\epsilon R^+$ -bearing cells from the patients tested did not exhibit the same sensitivity to each *Der p I* peptide. For example, whereas basophils from patients 4, 5 and 6 were stimulated by p117-133 and p52-71, basophils from patient 3 were reactive to p117-133 and p188-199. Patient 1 showed only an intense response to the p52-71 and patient 2 exhibited only a

weak response to p176-188. Concerning platelet stimulation, similar observations were made (i.e. patients 4 and 6 *versus* 3 and 5). Thus the biological effect of the *Der p I*-derived peptide varied with the nature of the $Fc\epsilon R^+$ cell involved and also with the *Dpt*-sensitive patient tested.

Antigen dependence of biological effects

In both *in vitro* conditions used to appreciate the biological effects of peptides, the intensity of the cell response was demonstrated to be dependent on the peptide concentration used [15,16]. Additional experiments have shown that the coupling of a peptide to a carrier could enhance its biological effect (Fig. 3). Indeed, higher percentages of histamine release (Fig. 3a) and of platelet stimulation (Fig. 3b) were induced by p117-133 when coupled to HSA, in comparison with those induced by the free peptide. Usually the coupled peptide (in comparison with the free peptide) increased the intensity of cell response (histamine release and platelet stimulation) and generally triggered an optimal stimulation at lower concentration (at least one hundred less). Interestingly this enhancing capacity of coupled peptides on biological effect was well observed among *Der p I*-sensitive patients exhibiting a small or no response to *Der p I*-derived peptides (Fig. 3a). In the presence of a similar concentration of coupled peptide, no histamine release or platelet stimulation was induced in non-*Dpt*-sensitive patients; moreover, in the presence of carrier human serum albumin, the background was not significantly modified in each test (data not shown). Thus, coupling peptide to a carrier seems to favour an efficient interaction with the $Fc\epsilon R^+$ -bearing cells.

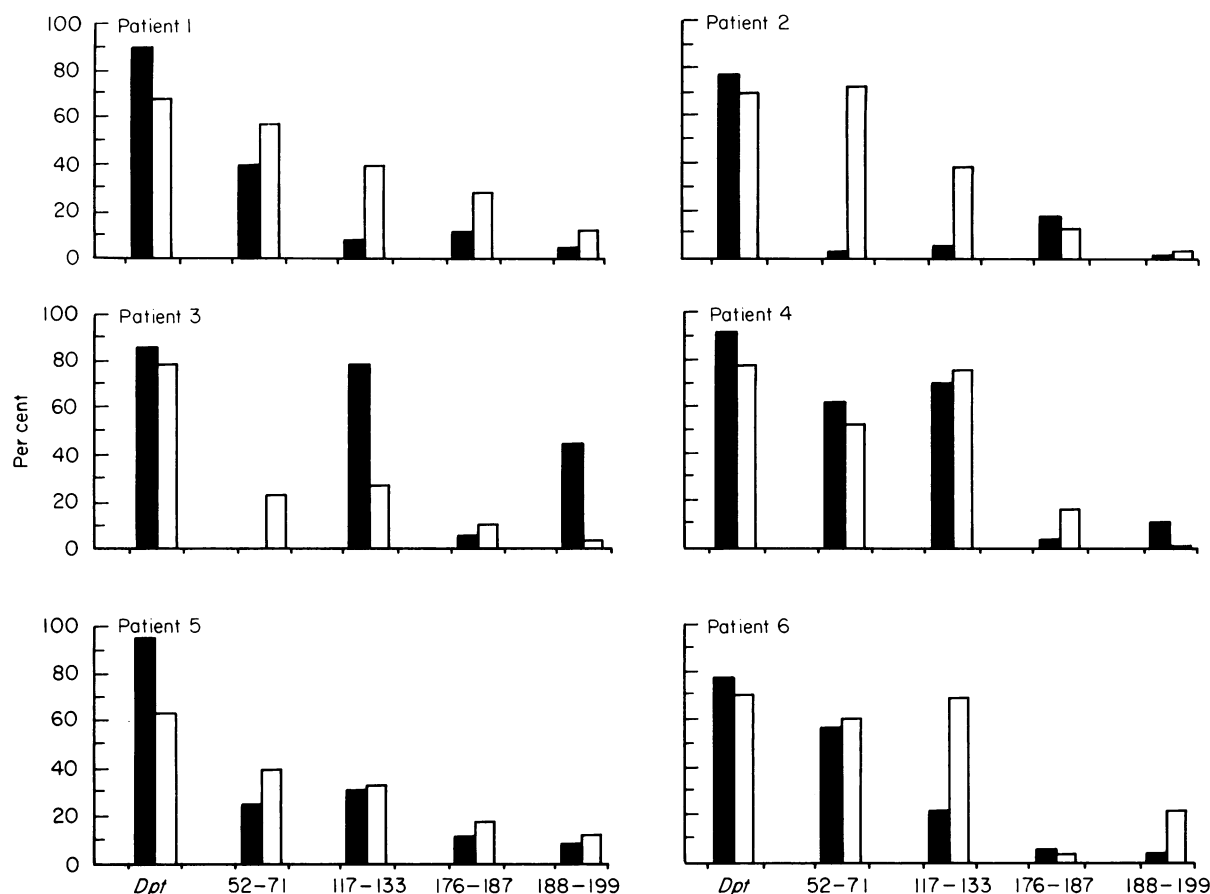


Fig. 2. Individual pattern of the biological response to peptides. Percentages of histamine release (■) and of platelet cytotoxicity (□) obtained for six representative *Dermatophagoides pteronyssinus* (Dpt)-sensitive patients (1 to 6) in response to each *Der p I*-derived peptide or to *Der p I* containing Dpt extract. Different concentrations of peptide were used and only the highest values of FcεR⁺ cell-dependent biological response are shown.

DISCUSSION

In allergic disorders, allergens may trigger various cell populations through an IgE-dependent stimulation. Using *in vivo* and *in vitro* models, basophils and platelets have been reported by several authors as potentially reactive to allergens. Basophils from allergic patients may degranulate following the binding of allergen to basophil-bound IgE [22] and platelets from mite-allergic patients are triggered through their FcεRII in the presence of allergen or of human IgE antibody [23–25].

In this comparative study, we show that four *Der p I*-derived synthetic peptides, selected as potential B cell epitopes, exhibited an *in vitro* biological effect on two FcεR⁺ cells implied in the physiopathology of allergic disorders by specifically stimulating basophils and platelets from *Der p I*-sensitive patients. We have previously demonstrated that platelet stimulation from Dpt-sensitive patients induced by *Der p I*-derived peptides was IgE-dependent [15]. A relationship between circulating specific IgE, basophil cell-bound IgE and histamine release induced by Dpt or *D. farinae* allergen has been established [26,27]. The fact that the anti-*Der p I* IgE level is related to the histamine release induced by *Der p I*-derived peptides suggests that peptides may trigger basophil activation via an IgE-dependent mechanism as demonstrated for platelet stimulation [15,16]. Moreover, the

presence of IgE molecules on platelets and basophils from Dpt-sensitive patients was shown by similar biological effects induced by a specific anti-IgE serum (data not shown).

The way in which *Der p I* peptides stimulate immunocompetent cells was not yet elucidated. Crosslinking of the Fcε receptors by at least divalent ligand was a prerequisite to trigger efficient cell stimulation [28,29]. Thus we have to consider that 12–20 amino acids-long *Der p I* peptides were by themselves bivalent or have been dimerized via an intermolecular disulphide bond due to the presence of cysteine at the end of peptides (i.e. p57–71, p117–133). However, it is noteworthy that p176–187 and p188–199, which do not present a free terminal cysteine residue, may significantly and highly stimulate FcεR⁺ cells (up to 45% for histamine release). Consequently, we cannot exclude the possibility that peptides were more active because they were associated with a protein carrier (i.e. HSA). The fact that FcεR⁺ cells were demonstrated to be more sensitive to the peptide covalently coupled to the HSA (as observed in the penicillin model [28]), reinforced the last hypothesis.

In parallel to our study, by using *Der p I*-derived recombinant peptides in a dot blot IgE immunoassay, Greene *et al.* reported that IgE (and IgG) present in the sera from Dpt-allergic patients preferentially bind to five regions p1–56, p53–99, p98–140, p166–194 and p188–222 [10]. By comparing the sequences

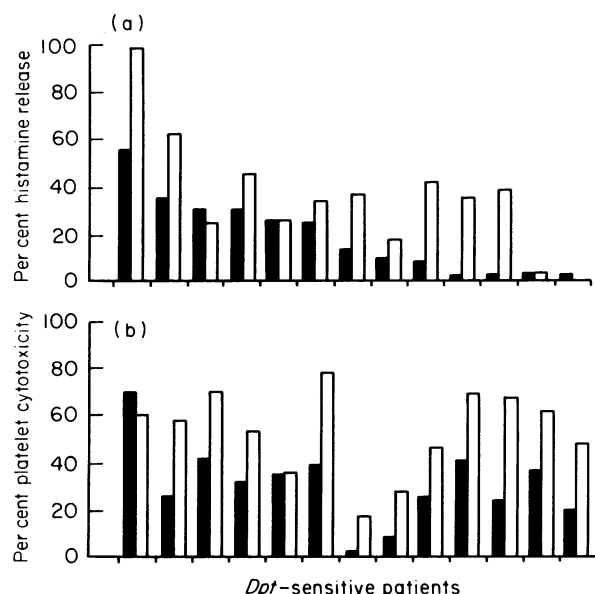


Fig. 3. Comparative analysis of the biological effect of the free or coupled peptide 117–133 on FcεR⁺ cell stimulation. Histamine release (a) and platelet stimulation (b) induced by the peptide 117–133 either free (■) or coupled to human serum albumin (□) are comparatively analysed. Concentrations of free or coupled peptide ranging from 10⁻⁶ to 10¹ μg/ml were used and only the highest response obtained is shown. *Dpt*, *Dermatophagoides pteronyssinus*.

of IgE-binding peptides and our synthetic peptides, it appears that the amino acid sequences of *Der p I*-derived peptides able to specifically stimulate the FcεR⁺-bearing cells from *Dpt*-sensitive patients are included in the highest IgE-binding regions. Recently, we also observed that synthetic *Der p I*-derived p89–104 also express similar biological activities.

Attempts to determine IgE-binding sequences by using synthetic peptides were also described for *Der p II* [30] and for other allergens: hazel (*Cor a I*) [31], ragweed (*Amb III*) [32] and chironomid (*Chi t I*) [33]. Peptides of 10–20 residues were shown to be specifically recognized by human IgE antibodies [31–33]. However, the existence of various IgE determinants generally distributed throughout the molecule as reported for *Der p I* underlines the fact that no particular peptide region was immunodominant [32,33]. Moreover, these epitopes are equally recognized by IgG antibodies [10,32]. Thus for the first time, by analysing the biological reactivity of *Der p I* synthetic peptides, the presence of several B cell epitopes on *Der p I* was evident.

The second main point of our study deals with the observation that each patient exhibits a typical pattern of biological response to each *Der p I* peptide. Indeed, according to the *Der p I*-sensitive patient tested, the intensity and the frequency of response to each peptide were highly variable. By collating all the results, it appears that FcεR⁺ cells from *Dpt*-sensitive patients reacted more intensively and more frequently to p52–71 and p117–133, which could be considered as predominantly reactive as reported [16]. However, high histamine and/or platelet reactivity was observed with p176–187 and p188–199; the size of peptide, therefore, did not seem by itself to be responsible for this heterogeneity of response. From our results we cannot conclude that the reactivity to one peptide was related

with the reactivity or the absence of reactivity to another. The individual patterns of reactivity of FcεR⁺ cells to peptides could be dependent on individual variability in *Der p I* processing (related with variability in MHC antigens or in B and T cell repertoire) and consequently on heterogeneity of IgE binding to *Der p I* as recently described [10]. Indeed, the capacity of Fcε receptor-bearing cells to respond efficiently to various *Der p I* peptides was demonstrated to be highly dependent on the intensity of RAST and on the level of anti-*Der p I* IgE. However, the individual pattern of response to each peptide does not appear to be related to various anti-*Der p I* IgE levels; patients with quite similar levels of anti-*Der p I* IgE may present a different sensitivity to each peptide tested.

One other interesting point was that, though basophils and platelets from one *Dpt*-sensitive patient usually exhibited a similar sensitivity to peptides, a difference in each biological parameter measured for one peptide could sometimes be observed. In particular, p117–133 (or p52–71) may induce a high percentage of platelet stimulation associated or not with significant percentage of histamine release. Indeed, in our comparative study platelets often appeared more sensitive to each tested peptide than basophils. As revealed by the non-parametric data of the scattergram of platelet response *versus* basophil response, peptide-induced cell stimulation seemed dependent on several factors. One explanation could be that basophils and platelets bear different IgE receptors of respectively high (FcεRI) and low (FcεRII) affinity for IgE. Indeed, basophils possess a constant number of FcεRI receptors which could be occupied by IgE molecules [22,26]. The difference in the delay of reaction (30 min for histamine release and 24 h for platelet reactivity) could also explain this difference in reactivity to *Der p I* peptides. In allergic patients, platelets express a variable number of FcεRII receptors and this subclass of IgE receptor has a greater affinity for circulating IgE immune complexes than for serum IgE [23,25]. Then we could hypothesize that an FcεRI-dependent activation could be more difficult to trigger with an antigen of small size as peptide. Nevertheless, further experiments are required to understand fully this difference in functional reactivity.

Synthetic peptides seem useful tools for a better understanding of the interaction of *Der p I* determinants with the different FcεR-bearing cells implied in the immune response to *Dpt*.

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