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Specific Activation of Platelets from Patients Allergic to *Dermatophagoides pteronyssinus* by Synthetic Peptides Derived from the Allergen *Der p I*

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Key Words

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Abstract

Previous studies have shown that FcεR2-bearing platelets from patients with allergic disorders could be stimulated either by anti-human IgE antibodies or the relevant allergens to generate cytotoxic mediators. In this report, the reactivity of platelets from *Dermatophagoides pteronyssinus* (*D. pt*)-sensitive patients to three *Der p I*-derived synthetic peptides (52-71, 117-133 and 188-199) was investigated. The platelet stimulation observed in *D. pt*-sensitive patients was demonstrated to be allergen-specific and to be mediated by IgE. These *Der p I*-derived peptides failed to stimulate platelets from healthy donors or platelets from non-*D. pt*-allergic patients. *D. pt*-sensitive patients responded more frequently to the peptides 52-71 and 117-133 than to the peptide 188-199. Thus, in this model, synthetic peptides selected for their assumed accessibility on the native antigen could be associated to an IgE-dependent biological activity.

Introduction

Dust mites are now considered as a major cause of human allergic disorders [1]. Several studies have established a strong association between allergy to house dust mite extracts (as defined by skin positive tests and elevated serum IgE antibodies) and symptoms of rhinitis and asthma [2]. Epidemiological and immunological investigations have noticed the prevalence of asthma in patients sensitive to antigens derived from mites of the genus *Dermatophagoides* [3-9]. Attempts to characterize and purify IgE-binding proteins from *Dermatophagoides farinae* and *Der-*

matophagoides pteronyssinus (*D. pt*) extracts led to define, at least, three groups of allergens according to several physicochemical and immunological criteria as reviewed by Chapman [10].

Der p I, a 25-kD protein, was the first major allergen characterized from *D. pt* [11, 12]. By skin tests and radioimmunoassays, the purified *Der p I* protein was shown to react with IgE in about 80% of patients allergic to mites [12, 13]. The use of mouse, rabbit or human polyclonal antibodies and the development of monoclonal antibodies have led to the demonstration of the existence of species-specific epitopes (belonging predominantly to allergens of

the group I, 25 kD) and cross-reactive epitopes (preferentially associated with the group II, 15 kD) [14, 17]. However, despite the evidence of binding sites for IgE and IgG antibodies, corresponding to potential B-cell epitopes, the location of amino acid residues involved in antibody binding is still unknown.

Recently, a complementary DNA clone for the *Der p* I was isolated and the amino acid sequence of the corresponding protein, identified as a cysteine protease, was determined [17], opening thus the possibility of epitope mapping studies. Many physicochemical criteria have been taken into account to select potential B-cell epitopes [18–23]. Following precise mapping of various proteins, it appears that antigenic sites are generally located on the surface of proteins [23] in a region of relatively high segmental flexibility [21, 22] and hydrophilicity [19, 20].

The characterization of epitopes involved in the induction of the immune response could be of potential use in immunotherapy. To this end, we have synthesized different peptides mainly selected for their potential accessibility on the native antigen.

Previous studies have shown that in allergic disorders, platelets could be directly stimulated by the addition of either anti-human IgE antibodies or the relevant allergen [24–26]. Here, at variance with a previous approach investigating the IgE-associated reactivity to octapeptides from *Der p* I of a pool of sera from human mite allergic patients by an Elisa technique [27], we studied the capacity of *Der p* I-derived peptides to induce the biological reactivity of Fcε-receptor-bearing cells associated with the IgE immune response, in patients allergic to house dust mite *D. pt*. Thus, by using the IgE-dependent platelet cytotoxicity, we demonstrated the capacity of selected peptides to specifically stimulate IgE receptor-bearing platelets from patients sensitive to *D. pt*.

Materials and Methods

Patients

Twenty-five patients (age 17–47) sensitive to house dust mite were included in this study. Patients using corticosteroid treatment or under specific desensitization were not included in this study. Theophylline or inhaled β₂-agonists were withheld for at least 24 h before analysis. All patients presented asthma or perennial rhinitis with usual clinical features of house dust mite sensitization, skin prick tests positive towards dust mite allergens (diameter ≥ 9 mm) with dilutions of an allergenic extract corresponding to 100 IR/ml (biological units used by Laboratoire des Stallergènes) and to 2 µg of *Der p* I/ml Alyostal (Laboratoire des Stallergènes, Fresnes, France), positive RAST (Pharmacia, Sweden; class 3 or 4) and positive skin tests to histamine (> 5 mm). Patients sensitive to *Hymenoptera* venom (with a

history of anaphylactic reactions after *Hymenoptera* stings, positive skin tests to *Hymenoptera* venom and specific IgE antibodies to *Hymenoptera* venom) and healthy donors (without any allergic disorder or atopy) were tested as negative controls.

Allergen and Peptides

Der p I-containing extracts of *D. pt* were standardized by Laboratoire des Stallergènes. Allergenic extract of *D. pt* was prepared from mite culture (Allerbio, Varennes, France) by extraction at 10% in 0.1 M NaHCO₃ (pH 9.5) during 18 h at 4 °C. At the end of purification, the extract was centrifuged, filtered on a 0.45-µm membrane and lyophilized. The dilutions of *D. pt* allergen used throughout this study were made from a stock antigenic solution (26 µg of nitrogen/ml) corresponding to 100 IR/ml and to 2 µg of *Der p* I/ml. The presence of *Der p* I was detected by a two-site monoclonal antibody immunoassay [13] and evidenced by Western immunoblotting.

In order to select potential B-cell epitopes, exposed sequences were determined from the primary structure of *Der p* I, mainly by using three criteria: the hydrophilicity estimated by using Hopp and Woods' method [18], the mobility [21] and the accessibility [20]. Solvent-accessible sequences of the protein were initially predicted from the primary structure using the conventional computerized algorithm described by Hopp and Woods [19]. Briefly, the hydrophilicity parameters were averaged over 6 amino acids, and mean values were then attributed to the first residue of each hexamer. The same calculation was performed using different exposure criteria, i.e. surface accessibility [20] and mobility [21]. For each parameter analyzed, a precise profile was obtained (table 1). Three amino acid sequences corresponding to major peaks of these profiles were selected, and the peptides synthesized.

Peptides were synthesized by solid phase methodology according to a Boc-benzyl strategy [28] using an automated synthesizer (Applied Biosystems model 430 A). Side chain protection was as follows: Lys (2-Cl Z); Ser (Bzl) and Thr (Bzl); Asp (OcHex); Glu (OcHex), Arg (Tos), Cys (p-Me Bzl or Acm), Tyr (2,6-diCl Bzl). After hydrogen fluoride treatment, the crude peptides were purified by reverse phase HPLC, using a water-acetonitrile TFA solvent system. The homogeneity of each peptide was checked by thin-layer chromatography and reverse phase HPLC and its composition by amino acid analysis after a total acid hydrolysis. According to the amino acid sequence of *Der p* I shown in table 1, the three peptides selected were the following: A1 amino acids 52–71, situated in the N-terminal part of *Der p* I; A2 amino acids 117–133, situated in the central part of *Der p* I, and A3 amino acids 188–199, situated in the C-terminal part of *Der p* I.

In this study, a nonrelevant peptide, VPNLRGDLQVLAQAVARLP corresponding to the 141–160 sequence of VP1 derived from the foot-and-mouth disease virus was used as a control. *Hymenoptera* venom antigen (standardized to the content of hyaluronidase) was purchased from Laboratoire des Stallergènes and the stock solution (100 µg/ml) used at various dilutions (from 10⁻² to 10⁻⁴).

Platelet Preparation

Venous blood collected in ACD (citric acid/trisodic citrate/dextrose, 1 vol ACD/6 vol blood) was centrifuged for 15 min at 120 g. After the collection and the centrifugation of the platelet-rich plasma (200 g), the pellet was washed three times in saline supplemented with citric acid (36 mM), glucose (5 mM), calcium (2 mM), magnesium (1 mM), bovine serum albumin (0.35%) and PGE1 (100 mM). The last pellet was resuspended, and the platelets counted and adjusted to the appropriate concentration before use.

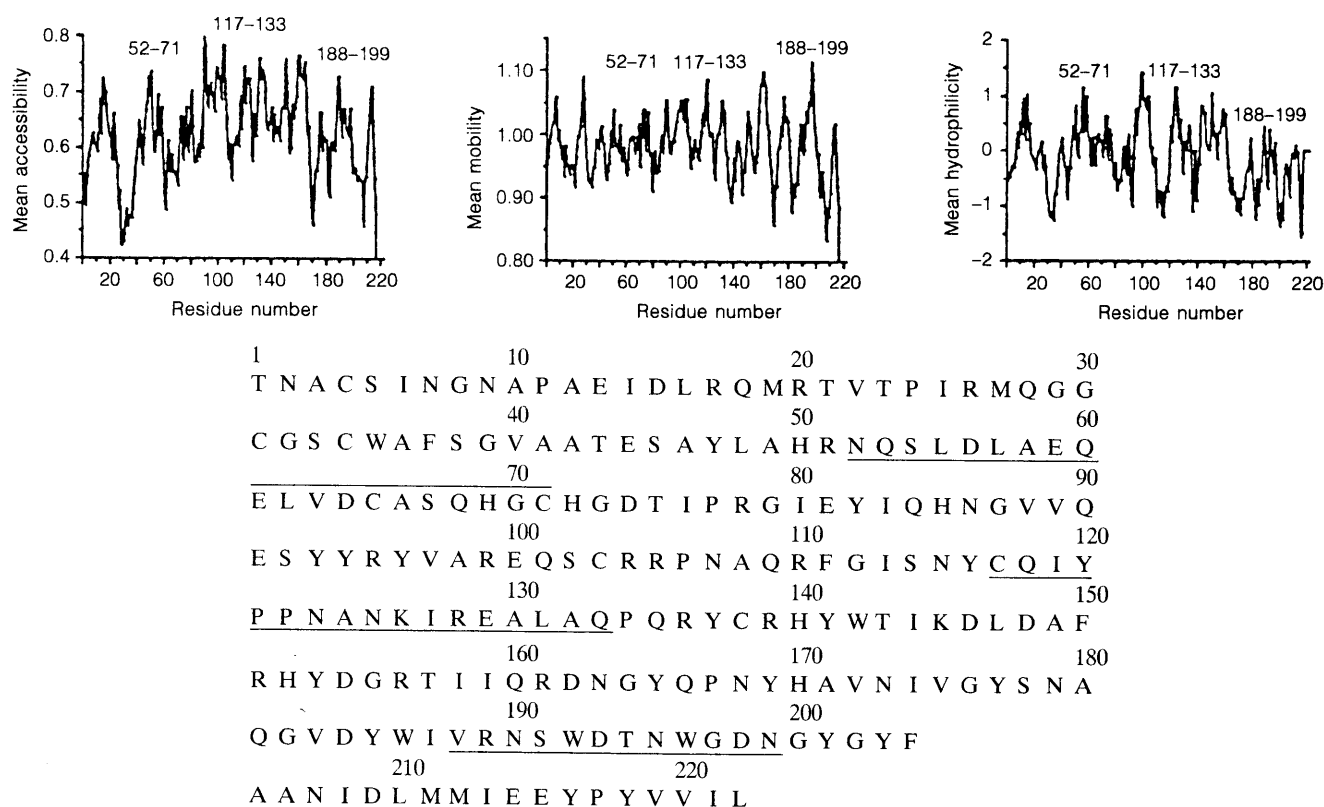


Table 1. Amino acid sequence of the synthetic peptides derived from the *Der p I* protein. The peptides were selected from the sequence described by Chua et al. [17] using conventional algorithm methods [18–21]. Profiles obtained with three criteria are shown at the top, and the selected sequences have been underlined

Test of Platelet Stimulation

Platelets (15×10^7 ; from patients or healthy donors), suspended in Eagle's minimum essential medium were incubated in flat-bottomed microplates (Nunc-Roskilde, Denmark) with 50–100 *Schistosoma mansoni* schistosomula, 10% serum from *D. pt*-sensitive patient, and in the presence of the related allergen (*D. pt* or the peptides) in a final volume of 200 μ l according to the method previously described [25, 29]. The stock *D. pt* antigenic preparation (corresponding to 100 IR/ml) was used at dilutions of 10^{-1} to 10^{-4} and peptides at concentrations varying from 0.01 to 50 μ g/ml. After 24 h at 37°C and 5% CO_2 in air, motionless and dark dead larvae were counted under an optical microscope, and to evaluate the level of the platelet stimulation the percentage of cytotoxicity was calculated.

Immunoabsorption

Sepharose 4B preactivated with CNBr (Pharmacia) was incubated in HCl 10^{-3} M and washed in bicarbonate buffer. Antibodies against human IgE (Miles, USA) were coupled to the preactivated Sepharose following a 2-hour agitation at 20°C. After washing, the remaining active groups were blocked with carbonate aminobutyrate buffer, pH 8 (20 ml/g Sepharose; 2 h under agitation). After washings in acetate buffer (pH 4) and borate NaCl (pH 9), the immunoabsorbent was equilibrated in Eagle's minimum essential medium.

Sera were incubated once with anti-IgE coupled Sepharose for 2 h at 20°C in a sterile tube, further incubated overnight at 4°C and

centrifuged (2,000 g). The IgE-depleted sera were further tested in the platelet cytotoxicity assay. Total IgE as evaluated before and after immunoabsorption by a PRIST assay and specific IgE antibodies against *D. pt* by a RAST assay.

Results

Stimulation of Platelets by Peptides Derived from *Der p I* Allergen

When platelets from patients were incubated in the presence of autologous serum and with various concentrations of peptides (or with different dilutions of a *D. pt*-antigenic preparation), a dose-dependent stimulation of platelets was observed. As evidenced by the anti-parasite platelet-dependent cytotoxicity, a final concentration of 5 μ g/ml for the peptides A1 and A2 led to a significant degree of stimulation (80.5 ± 3.2 and $42.4 \pm 2.8\%$ of cytotoxicity, respectively) but usually lower than that obtained with the *Der p I*-containing antigen preparation (90% of cytotoxicity at the dilution of 10^{-1} ; fig. 1).

The same phenomenon was observed when platelets

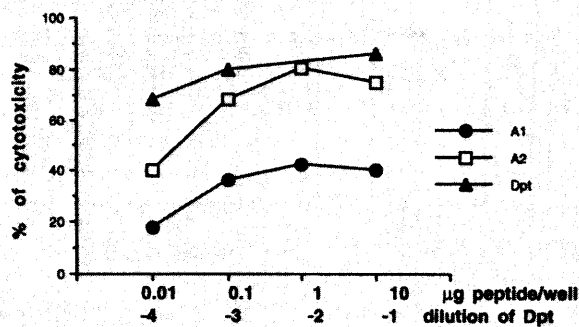


Fig. 1. *Der p* I peptide-dependent stimulation of platelets from *D. pt*-sensitive patients. Platelets from a patient sensitive to *D. pt* were incubated with peptide A1 or peptide A2 at various concentrations. After 24 h, the percentage of cytotoxicity was calculated. The positive control consisted of *Der p* I-containing *D. pt* extract. In the absence of peptides, the background cytotoxicity did not exceed 10%.

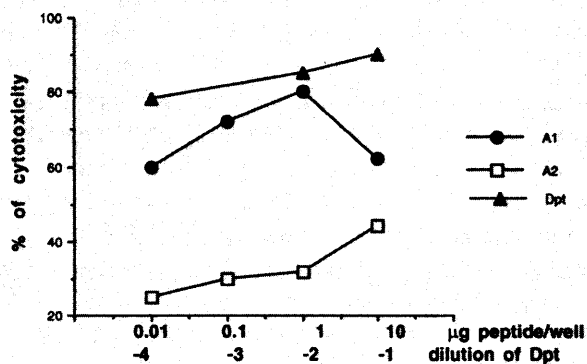


Fig. 2. *Der p* I peptide-dependent stimulation of platelets from non *D. pt*-sensitive patients. Platelets from a healthy donor were preincubated with the serum from a patient sensitive to *D. pt* (different from the patient of fig. 1) and further stimulated with peptides (A1 or A2) or with *D. pt* at various doses. After 24 h, the percentage of cytotoxicity was calculated. In the absence of peptides, the background cytotoxicity did not exceed 10%.

from healthy donors (without allergic manifestations) were passively sensitized with the serum of another patient sensitive to *D. pt* and incubated with the two peptides (fig. 2). It is noteworthy to indicate that in both cases platelets incubated with autologous serum or platelets sensitized with a heterologous serum containing anti-*D. pt* IgE

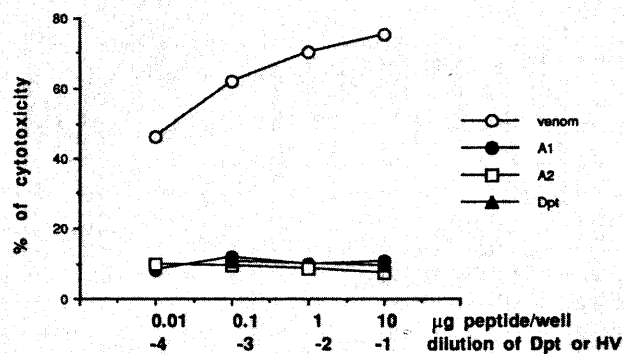


Fig. 3. Specificity of platelet reactivity. Platelets from a patient sensitive to *Hymenoptera* venom (HV) were incubated with the corresponding autologous serum (10%) and further incubated with *Der p* I-derived peptides or the relevant *Hymenoptera* antigen. In the absence of the peptides, the background cytotoxicity did not exceed 10%. Data correspond to the results of one representative experiment ($n = 4$).

antibodies could be also activated following addition of anti-IgE antiserum (data not shown).

Allergen Specificity of the Platelet Reactivity

The reactivity of platelets to the peptides appeared to be allergen specific. Indeed, when platelets from patients sensitive to *Hymenoptera* venom were incubated with the corresponding autologous serum and with A1 or A2, no cytotoxic effect was observed (fig. 3). In contrast, the incubation with the corresponding *Hymenoptera* venom antigen did induce a platelet stimulation. In addition, the incubation of platelets from non allergic healthy donors ($n = 7$) with A1 or A2 did not lead to a platelet reactivity (fig. 4), excluding a direct nonspecific effect of the two synthetic peptides.

Implication of IgE in the Peptide-Dependent Platelet Stimulation

As platelet cytotoxicity could be induced by the passive sensitization of platelets with a serum containing specific IgE antibodies and consecutive incubation with the related allergen, the role of IgE in the peptide-dependent platelet reactivity was investigated in two series of experiments.

As indicated in figure 5a, when the serum from a *D. pt*-sensitive patient exhibiting higher platelet reactivity with A1 than with A2 (78.2 ± 4.2 and $25.4 \pm 2.8\%$, respectively) had been heated, the platelet-dependent cytotoxicity was dramatically reduced (16.5 ± 2.1 and $8.5 \pm 1.4\%$, respec-

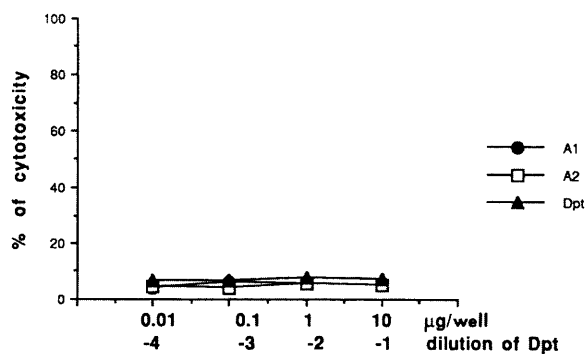


Fig. 4. Failure of *Der p* I peptides to stimulate nonsensitized platelets. Platelets from healthy donors ($n = 7$) were incubated with serum of a nonallergic patient (10%) and further incubated with peptides or *Der p* I-containing antigen extract. In the absence of peptides, the background cytotoxicity did not exceed 10%.

tively). Heating a serum 3 h at 56 °C is known to affect the Fc portion of the IgE molecule.

Similar results were obtained after specific immunoadsorption of the IgE molecules. As shown in figure 5b by using the serum of another *D. pt*-sensitive patient exhibiting a higher reactivity to A2 ($70.4 \pm 3.5\%$) than to A1 ($47 \pm 2.8\%$), no cytotoxic effect was elicited after specific IgE depletion in platelets incubated with the same doses of peptides ($6.6 \pm 2.1\%$ with A2; $8 \pm 1.4\%$ with A1). After immunoadsorption of IgG, no significant decrease was observed in the percentage of cytotoxicity (data not shown), suggesting the absence of direct implication of specific IgG antibodies.

The results, confirmed with three other sera tested before and after IgE depletion (data not shown), indicated that IgE molecules could play a pivotal role in the platelet stimulation triggering induced by *Der p* I-derived peptides.

Pattern of the Response of *D. pt*-Sensitive Patients

In fact, in a series of 19 dust mite-allergic patients, we observed that platelets could be efficiently stimulated by selected peptides. Taking into account the highest value of cytotoxicity obtained for each patient (fig. 6) it appeared that the majority of patients responding to *D. pt* (from 56.5 ± 3.5 to $94.5 \pm 2.1\%$ of cytotoxicity; mean $77.8 \pm 9.45\%$) responded to the same extent to A1 (from 28.5 ± 8.6 to $92.5 \pm 3.5\%$ of cytotoxicity; mean $62.6\% \pm 18.5\%$) and/or to A2

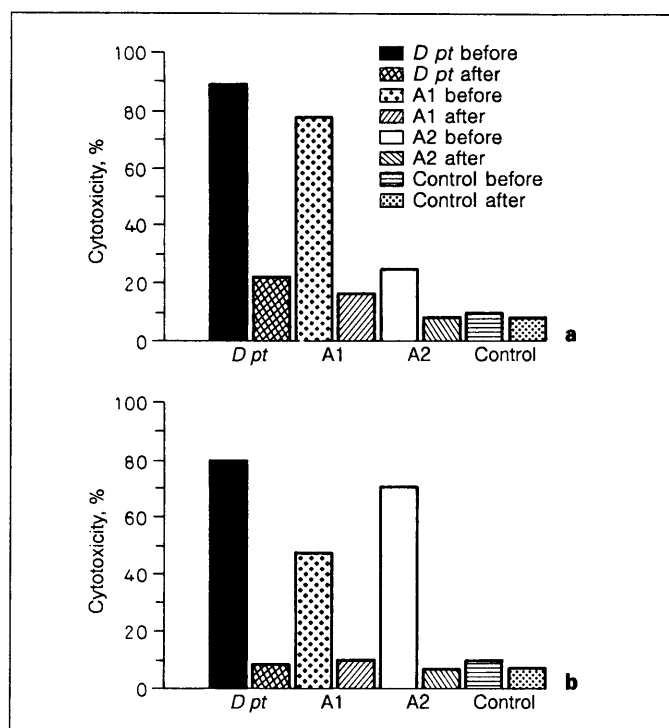


Fig. 5. Role of the IgE in the peptide-dependent platelet stimulation. Platelets from a healthy donor were incubated either with serum A (total IgE: 960 kIU/ml; specific anti *D. pt* IgE: 52 U RAST) or with serum B (total IgE: 790 kIU/ml; specific anti-*D. pt* IgE: 45 U RAST) from patients allergic to *D. pt* and further stimulated by addition of *D. pt* extract (at the final dilution of 10^{-3}) or peptides (at the final concentration of 10 $\mu\text{g/ml}$). The percentage of cytotoxicity was determined before and after heating serum A (a) or before and after specific IgE depletion of serum B by using immunosorbent (b). In both cases, a synthetic peptide unrelated to *Der p* I was used as a control.

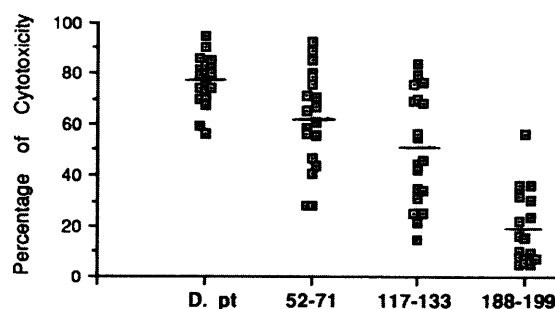


Fig. 6. Pattern of reactivity of *D. pt*-sensitive patients to *Der p* I-derived peptides. Platelets from patients sensitive to *D. pt* ($n = 19$) were incubated with peptides A1, A2 or A3 at various concentrations. Only the highest percentage of cytotoxicity was taken into account. The positive control consisted of *Der p* I containing *D. pt* extract. In the absence of peptides, the background cytotoxicity did not exceed 10%.

(from 15.0 ± 2.8 to $84.7 \pm 6.5\%$ of cytotoxicity, mean $50.1 \pm 21.4\%$). In contrast, the same patients were demonstrated to respond to A3 less intensively and less frequently (from 5.0 ± 1.4 to 56.5 ± 2.8 ; mean $19.4 \pm 14.1\%$ of cytotoxicity). For 8 patients, values superior to 20% of cytotoxicity were obtained, and only 1 patient exhibited a cytotoxicity higher than 40%.

Discussion

The participation of platelets in the allergic reactions has been suggested by several authors in various models [30–35]. For example, in rabbit, it was demonstrated that following specific depletion of platelets, the allergen-dependent anaphylactic shock could not be induced any longer [36]. In asthmatic patients, the plasmatic levels of the platelet-derived PF4 and β -thromboglobulin were increased after bronchial challenge with allergen [37, 38]. Moreover, recent studies indicate that the number of the Fc ϵ RII-bearing platelets increased in allergic patients [24, 25, 34] and that in *Hymenoptera* venom hypersensitivity the IgE-mediated stimulation of platelets was reduced after rush desensitization [26, 39, 40].

In the present study, we demonstrate that, following incubation with the synthetic *Der p* I peptides, platelets from *D. pt*-sensitive patients could exhibit a significant cytotoxic activity. This platelet stimulation observed in *D. pt*-sensitive patients was allergen specific and could be mediated by IgE. In fact, an efficient platelet triggering could be observed in two situations: either by incubating platelets from a sensitive patient with the peptides in the presence of the corresponding autologous serum or by sensitizing platelets from a healthy (nonallergic) donor with the serum of a *D. pt*-allergic patient and a further incubation with the *Der p* I peptides.

Similar results were obtained in both experimental procedures; however, the second method enabled us to show the participation of the IgE molecules in the platelet activation process. Indeed, platelets either sensitized in vivo or in vitro were equally stimulated by anti-IgE antibodies (between 64.5 and 91.2%), indicating the presence of IgE molecules on Fc ϵ RII of platelets. These data also suggest that patients sensitive to *D. pt* have IgE antibodies directed against the peptides A1, A2 and/or A3, antibodies which were able to bind to the platelet low-affinity Fc ϵ RII [28, 29, 41, 42]. Two recent observations seem to confirm this hypothesis.

First, all the *D. pt*-sensitive patients who were able to respond in vitro to at least one peptide have specific anti-

Der p I IgE (as determined by a specific immunoassay). In contrast, as estimated by our platelet stimulation test, patients sensitive to *Hymenoptera* venom or to pollen antigens (data not shown, with high IgE serum level, but without specific anti-*Der p* I antibodies) fail to respond to *Der p* I peptides. Second, the higher the level of serum anti-*Der p* I IgE the higher is the capacity of *D. pt*-sensitive patients to respond to several *Der p* I-derived peptides [42]. However, though with a variable intensity, all patients tested for platelet reactivity responded more frequently to peptides A1 and A2 than to peptide A3.

In previous interesting experiments using murine monoclonal IgG antibodies, species-specific and cross-reacting epitopes were defined [15, 16, 43]. In cross-inhibition studies, some monoclonal antibodies were demonstrated to reduce significantly the binding of human IgE antibodies to cross-reacting epitopes [43]. However, the sequence of amino acid residues recognized by these antibodies were not really known.

Recently, starting from a series of 215 *Der p* I-derived overlapping peptides bound to polyethylene rods, an IgE Elisa profile was obtained with a pool of sera from patients sensitive to *Der p* I [27]. In this pioneering approach, several sequential IgE (and IgG) epitopes were revealed and IgE appeared to recognize particularly areas around residues 68, 121 and 151. Thus, though our study investigated a limited number of peptides and the peptide 52–71 could stimulate more frequently platelets from *D. pt*-sensitive patients, the results that we obtained by analyzing the potential IgE-dependent reactivity of *Der p* I synthetic peptides seem to comfort the idea that the amino acid sequence 117–133 could contain IgE epitopes. It must be emphasized that this sequence corresponds to a region of high hydrophilicity and accessibility (table 1) and has been revealed to be located on the surface on a modeled *Der p* I molecule [42].

Nonetheless, though at variable degree, the three peptides tested could specifically induce the IgE-dependent activation of platelets from *D. pt*-sensitive patients, and we cannot consider one single peptide as an immunodominant as already mentioned [27, 43]. In fact, recent experiments carried out with four synthetic peptides (52–71, 117–133, 176–187, 188–199) in order to determine their capacity of inducing specific histamine released led to similar conclusions. However, the histamine release pattern from basophils was different from that observed for the platelet stimulation. In particular, *D. pt*-sensitive patients responded more frequently to the 117–133 than to 52–71.

It is known that, in *D. pt*-allergic patients, addition of crude extracts of *D. pt* or purified *Der p* I allergen can in-

duce activation of FcεRI-bearing mast cells or basophils, generating release of histamine [44]. In the same manner, platelets from patients sensitive to *D. pt* are susceptible to be activated by the specific allergen, through IgE receptors of low affinity present on their surfaces [24, 25, 34]. Though the participation of platelets in allergic diseases is still debated [45], the model of platelet activation could be useful for the evaluation of the IgE-dependent biological activities of other *Der p* I-derived peptides.

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