

Targeted Fc_{2'-3}-PE₄₀ chimeric protein abolishes passive cutaneous anaphylaxis in mice

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SUMMARY

The alarming increase in the incidence of allergic diseases in the past decade has led to a clear call for more effective treatment. Recently, we reported on the construction of a chimeric protein for targeted elimination of cells expressing FcεRI receptors. This chimeric protein, designated Fc_{2'-3}-PE₄₀, is composed of a Fc fragment of mouse IgE attached to a truncated form of Pseudomonas exotoxin. The Fc_{2'-3}-PE₄₀ chimeric protein was found to be highly cytotoxic to mouse mast cell lines and primary mouse mast cells. We now demonstrate that Fc_{2'-3}-PE₄₀ successfully prevents the development of passive cutaneous anaphylaxis reaction (PCA) in mice. Treatment with Fc_{2'-3}-PE₄₀ for 7 days prevented the PCA reaction in mice by 80% compared with that in control mice given only PBS. Fc_{2'-3}-PE_{40M}, the mutated, enzymatically inactive analogue of Fc_{2'-3}-PE₄₀, did not display this activity. Fc_{2'-3}-PE₄₀ was also effective when given as a single dose 16 h before antigen exposure, resulting in complete inhibition of the PCA reaction. Moreover, treatment with Fc_{2'-3}-PE₄₀ did not cause mast cell degranulation, as the serum histamine values of mice treated with Fc_{2'-3}-PE₄₀ were within the range obtained for control, untreated mice. Thus, the Fc_{2'-3}-PE₄₀ chimeric protein offers a novel approach to the treatment of allergic disorders.

Keywords mast cells basophils allergy cytotoxicity Fc receptors *in vivo* animal models

INTRODUCTION

The worrisome increase in the prevalence of allergic diseases such as asthma, allergic rhinitis, food allergies and atopic dermatitis in most industrialized countries in recent decades has led to a clear need for novel, more effective therapeutic strategies.

Mast cells and basophils play a pivotal role in allergic responses. Degranulation of these cells, triggered by allergen cross-linking of the FcεRI receptor, results in the release of mediators provoking various physiological changes associated with allergy [1].

The fact that the distribution of the FcεRI receptor is restricted to cells participating in the allergic response makes it an attractive candidate for targeted therapy by chimeric proteins. Chimeric proteins, a class of targeted molecules constructed by gene fusion techniques, are composed of cell-targeting and cell-killing moieties. The specific binding of the targeting moiety to a particular structure (e.g. receptor) expressed on the membrane of the targeted cell enables the elimination of the particular cell population, sparing other cell types.

Pseudomonas exotoxin A (PE), widely used in chimeric protein constructs, inhibits protein synthesis by ADP-ribosylation

of elongation factor 2, causing cell death [2]. Chimeric proteins constructed by fusion of cDNAs encoding various growth factors or single-chain antibodies with PE derivatives lacking intrinsic cell binding capacity were shown to be effective in various models of autoimmune disorders, graft rejection and cancer [3–5].

We recently reported the construction of a new chimeric toxin for targeted elimination of cells expressing FcεRI receptors, as a new approach to the treatment of allergic disorders [6]. A sequence encoding amino acids 301–437 of the Fc region of the mouse IgE molecule was genetically fused to PE₄₀, a truncated form of PE lacking the cell-binding domain. The *in vitro* activity of the chimeric protein, designated Fc_{2'-3}-PE₄₀, was evaluated in tissue culture models. Fc_{2'-3}-PE₄₀ specifically and efficiently killed mouse mast cell lines expressing the FcεRI receptor, as well as primary mast cells derived from bone marrow. The cytotoxicity is specific, as the mutated, enzymatically inactive analogue of Fc_{2'-3}-PE₄₀ failed to exhibit the cytotoxic effect, and the activity of Fc_{2'-3}-PE₄₀ toward target cells could be blocked by the addition of whole IgE. The chimeric protein had no effect on other cell lines of haematopoietic origin or on cells expressing the low-affinity IgE binding structures FcγRII/III, FcεRII and εBP. Therefore, it appears that the cytotoxic activity of Fc_{2'-3}-PE₄₀ is restricted solely against cells expressing FcεRI. Moreover, the interaction of Fc_{2'-3}-PE₄₀ with its target cells *in vitro* does not trigger cellular degranulation.

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Here we demonstrate that Fc_{2'-3}-PE₄₀ can target murine mast cells *in vivo*. Treatment with Fc_{2'-3}-PE₄₀, given for a period of 7 days or as a single dose before antigen challenge, abolishes the passive cutaneous anaphylaxis reaction (PCA) in mice. Moreover, Fc_{2'-3}-PE₄₀ treatment does not result in an increase in the plasma histamine levels of the treated animals. Our results indicate that the Fc_{2'-3}-PE₄₀ chimeric protein holds promise as a novel agent for the treatment of allergic disorders.

MATERIALS AND METHODS

Reagents

Monoclonal mouse IgE anti-DNP, mouse IgG, DNP-HSA and Evans blue were purchased from Sigma (Rehovot, Israel), avertine was purchased from Aldrich (Rehovot, Israel), and a histamine detection kit was purchased from Immunotech (Marseilles, France).

Protein expression and partial purification

Cloning of Fc_{2'-3}-PE₄₀ and Fc_{2'-3}-PE_{40M} was previously reported [6].

The chimeric proteins were expressed in *Escherichia coli* strain BL21(ADE3). Induction was performed at optical density (OD)₆₀₀ 0.5 for 180 min in the presence of isopropyl β-D-thiogalactoside (IPTG; 1 mM final concentration). A pellet of expressing cells was suspended in TE buffer (50 mM Tris pH 8.0, 1 mM EDTA) containing 0.2 mg/ml lysozyme, sonicated (three 30-s bursts) and centrifuged at 30 000 g for 30 min. The supernatant (soluble fraction) was removed and kept for analysis. The pellet was denatured in extraction buffer (6 M guanidine-hydrochloride, 0.1 M Tris pH 8.6, 1 mM EDTA, 0.05 M NaCl and 10 mM DTT) and stirred for 30 min at 4°C. The suspension was cleared by centrifugation at 30 000 g for 15 min. The supernatant was then diluted 1:100 in refolding buffer (0.1 M Tris pH 8.0, 1 mM EDTA, 0.25 mM NaCl, 0.25 μM L-arginine and 5 mM dithiothreitol) and left to refold for 48 h at 4°C. The refolded protein solution was dialysed against PBS at 4°C, centrifuged at 30 000 g for 15 min and aliquots of the resulting supernatant were kept at -20°C until use.

Animals

Female 8–10-week-old BALB/c mice were purchased from the Harlen Breeding Farm (Jerusalem, Israel). Food and water were provided *ad libitum*.

PCA assay

Mice lightly anaesthetized with avertin were injected intradermally in their shaved backs with 100 ng of IgE in 20 μl PBS at two sites and with 100 ng of IgG or PBS alone at two control sites. The PCA reaction was induced 2 h later by the i.v. injection of 0.3 mg HSA-DNP in 0.3 ml of 1% Evans blue in PBS. After 30 min the mice were killed, the skins were removed and the developed colour on the inside of the skin was scored from 0 to 4, according to size and colour intensity. The scoring was performed in a blind manner. The sum total obtained for all the IgE-injected sites in the PBS-treated group was considered a 100% positive reaction.

Treatment protocol

Mice were injected with Fc_{2'-3}-PE₄₀ intraperitoneally, in a volume of 0.5 ml PBS. Control animals were given Fc_{2'-3}-PE_{40M} (the mutated, enzymatically inactive analogue of Fc_{2'-3}-PE₄₀) or PBS

alone under identical conditions. In the prolonged treatment assay mice received 20 μg of Fc_{2'-3}-PE₄₀ every 12 h for 7 days and the PCA reaction was performed on day 8. In the short treatment assay, mice were injected with a single dose of 50 μg and the PCA assay was performed 16 h later.

Histamine release

Mice were injected with a single dose of 50 μg Fc_{2'-3}-PE₄₀. At different time points the mice were bled from the tails and the plasma was separated by centrifugation and stored in aliquots at -20°C until use. Plasma histamine levels were determined with a histamine kit, according to the manufacturer's instructions.

β-Hexosaminidase activity

The degree of *in vivo* degranulation following Fc_{2'-3}-PE₄₀ injection was also determined by measuring the release of active β-hexosaminidase. Samples were incubated with the substrate *p*-nitrophenyl-N-acetyl-β-D-glucosamine in 0.1 M of sodium citrate pH 4.5 for 90 min at 37°C. The reaction was terminated by the addition of 1 ml of 1 M Tris-HCl pH 9.0. The release of the product 4-*p*-nitrophenol was followed by absorbance at 405 nm.

RESULTS

To test the ability of the Fc_{2'-3}-PE₄₀ chimeric protein to target mast cells *in vivo*, we used the PCA reaction in mice. This is a common method of assessing immediate cutaneous hypersensitivity in rodents. We assumed that if Fc_{2'-3}-PE₄₀ is cytotoxic to mast cells, it would be able to prevent or diminish the PCA reaction. The influence of Fc_{2'-3}-PE₄₀ on the PCA reaction was tested after both prolonged and single-dose treatments with the chimeric protein.

Prolonged Fc_{2'-3}-PE₄₀ treatment

Mice were treated with 20 μg of Fc_{2'-3}-PE₄₀ every 12 h for 7 consecutive days. Two other groups were treated in the same manner with Fc_{2'-3}-PE_{40M}, which is an enzymatically inactive analogue of Fc_{2'-3}-PE₄₀, or with the same volume of PBS, to assess the specificity of the Fc_{2'-3}-PE₄₀ activity. The PCA reaction was induced on day 8. The results of the three separate experiments are summarized in Table 1 and a representative experiment (no. 3) is shown in Fig. 1.

Allergen challenge of control mice treated for 7 days with PBS resulted in development of the PCA reaction at most of the sites preinjected with IgE. No reaction was visualized at sites preinjected with IgG or PBS (Fig. 2). In contrast, mice treated for 7 days with Fc_{2'-3}-PE₄₀ exhibited a greatly reduced ability to develop cutaneous anaphylaxis at the IgE preinjected sites (Table 1 and Fig. 1). On average, Fc_{2'-3}-PE₄₀-treated mice showed only 22% of the reaction developed by the PBS group. The Fc_{2'-3}-PE_{40M} mutated protein did not induce this effect. Mice treated with this enzymatically inactive analogue of Fc_{2'-3}-PE₄₀ exhibited 90% of the PCA reaction.

Single-dose treatment

Mice were treated with a single dose of 50 μg Fc_{2'-3}-PE₄₀ 16 h before assessment of the PCA reaction. Control animals were treated with Fc_{2'-3}-PE_{40M} or PBS.

Single-dose treatment with Fc_{2'-3}-PE₄₀ completely inhibited the ability of the mice to respond to the antigenic challenge. The results of the three separate experiments are summarized in

Table 1. Inhibition of the passive cutaneous anaphylaxis (PCA) reaction in mice treated with Fc_{2'-3}-PE₄₀ for 7 days

Treatment	Exp. 1		Exp. 2		Exp. 3		Average, %
	No. of mice	%	No. of mice	%	No. of mice	%	
Fc _{2'-3} -PE ₄₀	3	11	6	33	5	16.5	22.4
Fc _{2'-3} -PE _{40M}	ND		7	92	5	88.5	90.5
PBS	4	100	6	100	5	100	100

%, Percent of positive reaction; the total score obtained for all the IgE-injected sites in the PBS-treated group = 100%.

Each mouse in each subgroup was injected intradermally in its shaved back with 100 ng of IgE at two sites and with 100 ng of IgG or PBS alone one site each.

Table 2, and a representative experiment (no. 2) is shown in Fig. 3.

The effect of Fc_{2'-3}-PE₄₀ on mast cell degranulation *in vivo*

Mast cell degranulation following antigen challenge results in clinical manifestations of allergy, which can be as severe as anaphylaxis and even culminate in death. As Fc_{2'-3}-PE₄₀ is being developed for human treatment, it was important to check whether it is capable of causing mast cell degranulation. We previously demonstrated, using an *in vitro* mast cell culture model, that Fc_{2'-3}-PE₄₀ does not trigger mast cell degranulation at any stage of its interaction with the cell [6]. Since cells in their native environment may react differently from cultured ones, we reassessed this property *in vivo*.

Mice were treated with a single dose of 50 µg Fc_{2'-3}-PE₄₀. Control animals were treated with Fc_{2'-3}-PE_{40M} or PBS. Each treatment group was divided into three subgroups of three mice each. Blood was collected at 1, 4 and 24 h following treatment, from three mice at each time point and from a different subgroup

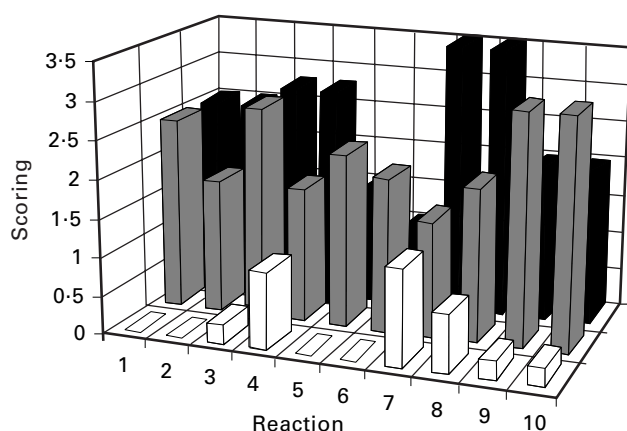


Fig. 1. Passive cutaneous anaphylaxis (PCA) reaction in mice treated for 7 days with Fc_{2'-3}-PE₄₀. Mice were treated intraperitoneally with 20 µg Fc_{2'-3}-PE₄₀ (□) every 12 h for 7 days. Control animals were given Fc_{2'-3}-PE_{40M} (hatched) or PBS alone (■) under identical conditions. On day 8 PCA was induced and evaluated as described in Materials and Methods. A total score for all IgE-injected sites in the PBS-treated group was taken as a 100% positive reaction.

so as to prevent a possible bias in histamine values due to changes in circulation volume caused by recurrent blood collection. Serum histamine levels reflected the degree of degranulation.

Since there were no available data regarding normal murine histamine values, as measured by ELISA, we first estimated the normal plasma histamine values. Plasma values ranging from 38 to 292 nM were found in six untreated mice of the same sex and age. The following histamine values were obtained for treated mice: 34–510 nM for the Fc_{2'-3}-PE₄₀-treated group, 25–377 nM for the Fc_{2'-3}-PE_{40M}-treated group and 33–338 nM for the PBS-treated group. The results of the experiment are presented in Fig. 4. With the exception of one measurement of 510 nM, all the values obtained were similar to those in the untreated mice. No significant differences were found between the Fc_{2'-3}-PE₄₀-treated group and the two control groups.

In addition, we measured the *in vivo* release of active β-hexosaminidase following treatment of mice with Fc_{2'-3}-PE₄₀, as another marker for degranulation. Levels of active β-hexosaminidase were similar for both control and Fc_{2'-3}-PE₄₀-treated mice (results not shown).

DISCUSSION

The alarming increase in the prevalence of allergic diseases over the past decade has led to a clear need for more effective treatment. The Fc_{2'-3}-PE₄₀ chimeric protein represents a new approach to the therapy of allergic responses, based on targeted elimination of cells expressing FcεRI. We previously showed that Fc_{2'-3}-PE₄₀ is highly cytotoxic *in vitro* to mouse mast cell lines and primary mouse mast cells. Using the PCA reaction in mice, we now demonstrate that the Fc_{2'-3}-PE₄₀ chimeric protein can effectively prevent an allergic reaction *in vivo*.

Treatment with Fc_{2'-3}-PE₄₀ for 7 days resulted in inhibition of the PCA reaction by 80%, relative to that in control mice treated with PBS. The same dose of the mutated, enzymatically inactive analogue Fc_{2'-3}-PE_{40M} did not cause any significant inhibition (Table 1, Fig. 1).

Fc_{2'-3}-PE₄₀ was also effective in blocking the PCA reaction when given as a single dose. Treatment with the chimeric protein 16 h before antigen exposure resulted in complete inhibition of the PCA reaction, indicating that the effect is induced within a relatively short period of time. Therefore, if administered after the appearance of clinical symptoms, the chimeric protein is likely rapidly to arrest the allergic process, making it useful also for treatment in allergic emergencies.

The possibility that treatment with the Fc_{2'-3}-PE₄₀ may cause anaphylaxis is a major concern in relation with this new treatment. Therefore, due to the possible clinical application of Fc_{2'-3}-PE₄₀, it was important to test whether treatment of mast cells with the chimeric protein results in the release of allergic mediators. As Fc_{2'-3}-PE₄₀ is cytotoxic to mast cells, it may be expected that degranulation will occur upon treatment with the chimeric protein. Lately, however, there has been growing evidence that the toxic moieties of chimeric proteins or recombinant immunotoxins, including PE, are capable of inducing apoptosis and that these molecules kill cells by certain apoptotic pathways [7–9]. Although not tested directly for Fc_{2'-3}-PE₄₀, it may be suggested that the target mast cells are killed via an apoptotic pathway. As well accepted, apoptotic cells die without any leakage of their content, therefore killing of the mast cells without degranulation of their granules may occur. Nevertheless, the issue of cell

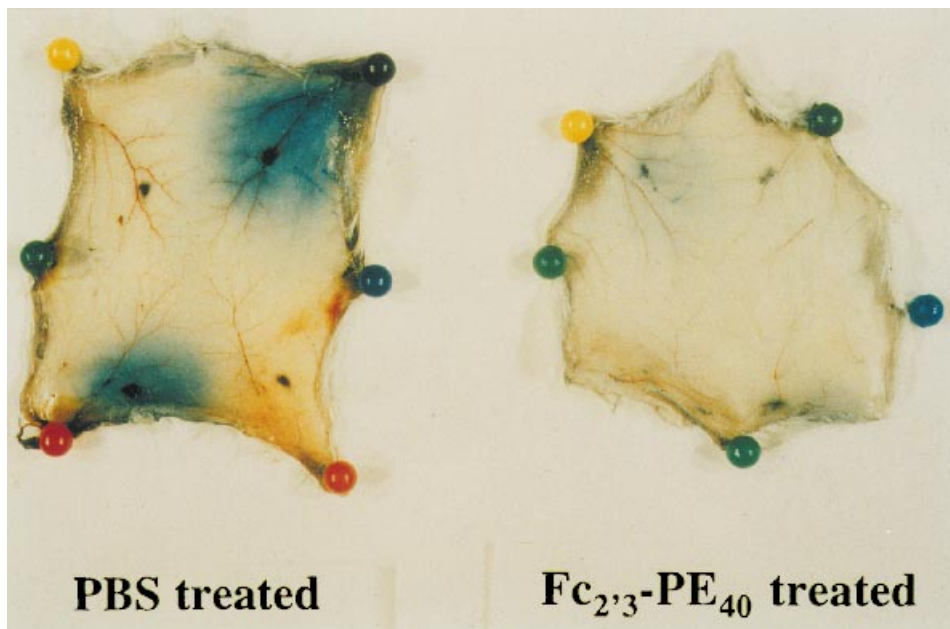


Fig. 2. Inhibition of the passive cutaneous anaphylaxis (PCA) reaction in mice treated with $Fc_{2',3}\text{-PE}_{40}$. The PCA reaction is visualized as a blue spot on the inner side of the skin. The experimental conditions are as described in Fig. 1. The black dots mark the sites of injection. The order of injections, from the upper left clockwise, is as follows: the PBS-treated mice—PBS, IgE, IgG, IgE; the $Fc_{2',3}\text{-PE}_{40}$ -treated mice—IgE, IgG, IgE, PBS.

degranulation as a result of treating cells with the chimeric protein was challenged experimentally.

We previously showed, using an *in vitro* model, that $Fc_{2',3}\text{-PE}_{40}$ does not trigger degranulation at any stage during its interaction with the cell [6]. We now demonstrate that the chimeric protein also does not trigger mast cell degranulation *in vivo*. The serum histamine values of mice treated with $Fc_{2',3}\text{-PE}_{40}$ were within the range obtained for untreated mice. Moreover, these values were not significantly different from those found for control mice treated with PBS or with the $Fc_{2',3}\text{-PE}_{40M}$ mutant protein. Therefore, $Fc_{2',3}\text{-PE}_{40}$ does not cause histamine degranulation of mast cells in tissue culture or in the intact animal. Moreover, we examined another marker for degranulation, such as the *in vivo* release of active β -hexosaminidase, and found no evidence of mast cell degranulation *in vivo*. In addition, the mice did not show any signs of an anaphylactic reaction. Moreover, following the mice for prolonged periods post-treatment did not reveal any signs of delayed symptoms.

At present the major groups of drugs used in the treatment of asthma and allergic disorders are β_2 -agonists, methylxanthines, glucocorticoids, antihistamines and chromolyn sodium [10]. Yet these drugs have notable disadvantages. As these drugs are not mast cell-specific, they do not prevent the allergic reaction, but only reverse or block its effects. Moreover, due to their non-specificity, they act on various organs, causing serious side-effects. The $Fc_{2',3}\text{-PE}_{40}$ chimeric protein may have a number of advantages over the existing drugs. As it affects mast cells and can prevent the allergic reaction, it could be of great value as a prophylactic treatment. Moreover, early expression of the $Fc\epsilon RI$ receptor in the maturation of mast cells [11,12] should allow the elimination of immature target cells before they are capable of mediator release. As the $Fc\epsilon RI$ receptor is of restricted distribution, $Fc_{2',3}\text{-PE}_{40}$ is expected to have few side-effects. Since the receptor is not expressed on stem cells, no damage to

bone marrow should be incurred and natural, normal renewal of mast cells should occur upon withdrawal of the treatment.

Aside from their role in allergic diseases, mast cells can develop hyperplasias and malignancies, such as systemic mastocytosis, and are implicated in various pathologic conditions such as arteriosclerosis and chronic inflammatory diseases, including scleroderma and rheumatoid arthritis [13–15]. Therefore, targeted elimination of mast cells by the chimeric protein should have broad clinical applications.

A number of attempts to develop novel agents for anti-allergy therapy have been reported in recent years. These include recombinant peptides comprising structural elements from IgE [16] or $Fc\epsilon RI$ [17,18], and non-degranulating MoAbs against IgE [19,20] or $Fc\epsilon RI$ [21], all based on interference with the IgE– $Fc\epsilon RI$ interaction. However, once bound to the $Fc\epsilon RI$, the IgE molecule remains attached to the cell membrane for several weeks ($K_M = 10^{-10}$ M) and mast cells can be triggered to degranulate at receptor occupancy as low as 5% [22]. This makes the IgE– $Fc\epsilon RI$

Table 2. Inhibition of the passive cutaneous anaphylaxis (PCA) reaction in mice treated with a single dose of $Fc_{2',3}\text{-PE}_{40}$

Treatment	Exp. 1		Exp. 2		Exp. 3		Average, %
	No. of mice	%	No. of mice	%	No. of mice	%	
$Fc_{2',3}\text{-PE}_{40}$	3	0	3	0	3	4	1
$Fc_{2',3}\text{-PE}_{40M}$	3	66	3	73	3	43	61
PBS	2	100	3	100	3	100	100

%, Percent of positive reaction; the total score obtained for all the IgE-injected sites in the PBS-treated group = 100%.

Details are as in Table 1.

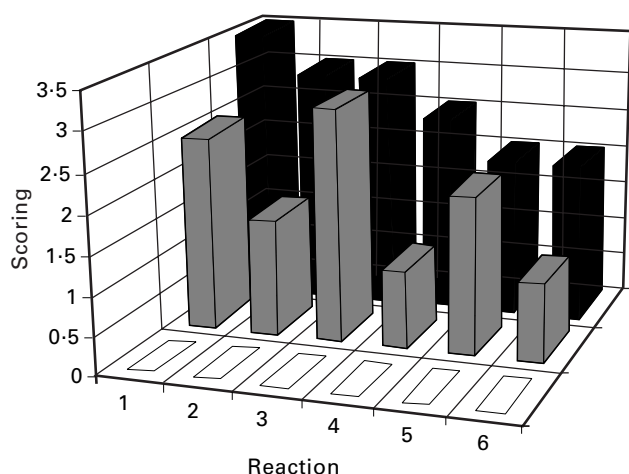


Fig. 3. Passive cutaneous anaphylaxis (PCA) in mice treated with a single dose of Fc_{2'-3}-PE₄₀. Mice were treated with a single i.p. dose of 50 µg Fc_{2'-3}-PE₄₀ (□). Control animals were given Fc_{2'-3}-PE_{40M} (hatched) or PBS alone (■) under identical conditions. The PCA reaction was performed 16 h. later. All other experimental conditions are as described in Fig. 1.

interaction hardly competitive, limiting the clinical value of these agents. In contrast, our anti-allergy molecule does not have to vie with IgE, since once having entered the target cell through a non-occupied receptor, it will affect the cell.

As the chimeric protein is not of human origin, its prolonged administration can result in the generation of antibodies. However, using other chimeric proteins, based on diphtheria toxin, in clinical trials it was reported that responses could be obtained despite the presence of antibody [23].

If Fc_{2'-3}-PE₄₀ is to be adopted for human treatment,

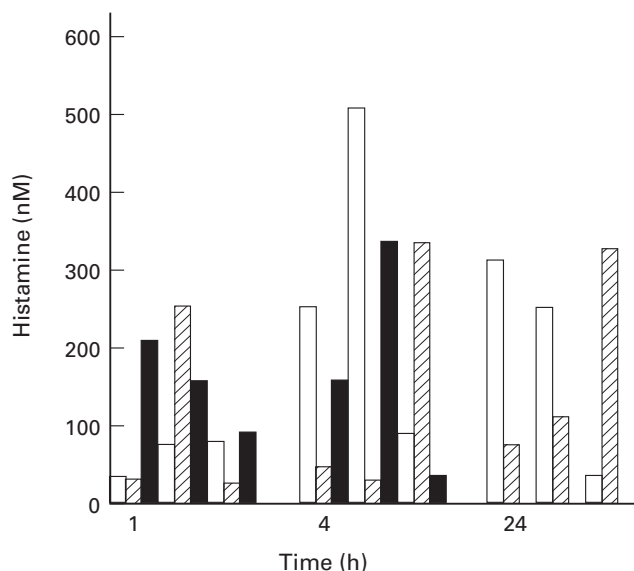


Fig. 4. Plasma histamine levels after administration of Fc_{2'-3}-PE₄₀. Mice were injected intraperitoneally with a single dose of 50 µg Fc_{2'-3}-PE₄₀ (□). Control animals were given Fc_{2'-3}-PE_{40M} (hatched) or PBS alone (■) under identical conditions. At different time points after injection the mice were bled from the tail, three mice from each subgroup, and the plasma separated. Histamine levels were determined as described in Materials and Methods.

consequences of the elimination, even temporary, of the mast cell population from the organism must be considered. Mast cell degranulation is a culmination of Th2-type response to invading pathogens. Recent evidence suggests that Th2 responses are probably not critical to human life. Subjects homozygous for a deletion involving the ϵ gene appear perfectly normal [24]. Moreover, IL-4-deficient mice that do not develop Th2 responses are better protected than wild-type animals against the great majority of infections, with the exception of gastrointestinal nematode infection [25].

More recently it was suggested that mast cells are important in the host defence against bacterial infections [26,27]. However observing the treated mice for long periods did not reveal any increase in sensitivity of those mice to viral or bacterial infections.

As demonstrated in this study, Fc_{2'-3}-PE₄₀ is a highly selective and potent molecule for eliminating mast cells. Thus, Fc_{2'-3}-PE₄₀ can be regarded as a prototype for a new generation of targeting anti-allergy reagents.

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