An Antagonistic IL-4 Mutant Prevents Type I Allergy in the Mouse: Inhibition of the IL-4/IL-13 Receptor System Completely Abrogates Humoral Immune Response to Allergen and Development of Allergic Symptoms In Vivo

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An Antagonistic IL-4 Mutant Prevents Type I Allergy in the Mouse: Inhibition of the IL-4/IL-13 Receptor System Completely Abrogates Humoral Immune Response to Allergen and Development of Allergic Symptoms In Vivo

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We have analyzed in vivo effects of the murine IL-4 mutant Q116D/Y119D (QY), which forms unproductive complexes with IL-4Rα and is an antagonist for IL-4 and IL-13 in vitro. Treatment of BALB/c mice with QY during immunization with OVA completely inhibited synthesis of OVA-specific IgE and IgG1. BALB/c-derived knockout mice lacking either IL-4 or IL-4Rα also did not develop specific IgE or IgG1, but mounted a much stronger IgG2a and IgG2b response than wild-type mice. In contrast, QY treatment of normal BALB/c mice suppressed specific IgG2a, IgG2b, and IgG3 synthesis, which may indicate the development of tolerance toward the allergen. Associated with the lack of IgE synthesis in QY-treated wild-type mice and in IL-4−/− mice used as a control was the failure to develop immediate cutaneous hypersensitivity or anaphylactic shock upon rechallenge. Interestingly, QY treatment also inhibited humoral immune responses and allergic reactivity in SJL/J mice, a strain that did not produce IgE, but displayed IgE-independent mast cell degranulation mediated by specific IgG1. We conclude that QY inhibits Ag-specific humoral immune responses and allergic symptoms mediated either by IgE or IgG1. It needs to be clarified how QY abrogates synthesis of IgG2a, IgG2b, and IgG3, but the induction of tolerance toward nonhazardous protein Ags should be advantageous for therapy of atopic disorders and other Th2-dominated diseases.

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IL-4 has a central role in Th2 dominated diseases like type I allergies, helminthic infections, and some autoimmune diseases, like systemic sclerosis and systemic lupus erythematosus (1–3). Inhibition of IL-4 may be particularly helpful in type I allergic reactions, where IL-4 is responsible for the differentiation of allergen-specific Th2 cells, and, together with the closely related cytokine IL-13, for the class switching of activated B cells to IgE. IL-13 has very similar effects to IL-4 on B cells, monocytes, and other cell types, but T cells appear to lack an IL-13 binding receptor component and do not respond to IL-13 (4). The structural basis for the overlapping functions of IL-4 and IL-13 is a shared receptor subunit, IL-4Rα, which organizes intracellular signals in response to both cytokines (5, 6). Signal transduction is induced by heterodimerization of the IL-4Rα with a second subunit, which may vary in different cell types.

Specific inhibition of IL-4 can be achieved with antagonistic IL-4 mutants. Variants of human IL-4 that bind to the first receptor subunit (IL-4Rα) but not to the second (either γc or IL-13Rα1) are competitive antagonists for IL-4 (7, 8). IL-13 is inhibited by the same variants, which form unproductive complexes with IL-4Rα (5, 9). The single site human IL-4 mutant Y124D has been used as IL-4/IL-13 inhibitor in various studies (7–17), but this variant retains some residual agonistic activity, which could be relevant for in vivo applications (7, 8). The double mutant R121D/Y124D lacks detectable activity and appears to be a perfect antagonist for human IL-4 and IL-13 (5, 18). A major obstacle for testing the effects of antagonistic IL-4 mutants in vivo is the species specificity of IL-4. Human IL-4 does not bind to the mouse receptor and vice versa, so human IL-4 antagonists cannot be tested in mice.

We have recently developed a highly efficient murine IL-4 antagonist (QY), where the amino acids glutamine 116 and tyrosine 119 were mutated to aspartic acid (19). This murine mutant is analogous to the R121D/Y124D double mutant of human IL-4. QY binds with high affinity to the murine IL-4Rα without inducing signal transduction, has no detectable activity upon proliferation or differentiation of murine cells, and an excess of QY completely inhibits responses toward wild-type IL-4 (19). Like its human analogue, the QY mutant is an antagonist for IL-13 (B. Schnarr et al., unpublished observations). Recent experiments with monocytic cells from mice lacking a functional γc gene showed, that QY is a complete inhibitor for IL-4 in the absence of γc as well (20).

In this study we have examined the effects of QY in vivo, using a mouse model of allergy against the protein antigen OVA. The effects of the inhibition of IL-4 and IL-13 by QY on the humoral immune response were compared with knockout mice lacking either IL-4 (21) or IL-4Rα (M. Mohrs et al., manuscript in preparation). We show here that QY treatment prevented humoral immune responses toward the Ag, as well as clinical manifestations of type I allergic symptoms.
Materials and Methods

Mice

Female BALB/c and SJL/J mice between 8 and 12 wk of age were purchased from Charles River (Sulzfeld, Germany). The animals were kept under specific pathogen-free conditions and maintained on OVA-free diets. BALB/c IL-4−/− mice and BALB/c IL-4Rα−/− mice were obtained from the Max-Planck-Institut für Immunobiologie (Freiburg, Germany).

Allergic sensitization procedure

Mice were immunized by i.p. injection with 10 μg OVA (Sigma, Deisenhofen, Germany; Grade V) in a solution containing 200 μl PBS and 70 μl Inject Alum (Pierce/KMF, St. Augustin, Germany) as an adjuvant.

IL-4 antagonist (QY)

The QY mutant was synthesized and purified as described (19). Briefly, the protein was expressed in Sf9 insect cells after infection with recombinant baculoviruses. QY was secreted as active protein into the cell culture supernatant and purified by ion exchange chromatography and reversed phase HPLC to a final purity between 80 and 90%. Protein was lyophilized and stored at −20°C until use. For in vivo therapy, QY was first dissolved in water. Mice were injected i.p. twice a day with QY diluted in 200 μl PBS. At day 0, QY was applied 2 h pre- and post-OVA immunization as a 50-μg dose each. QY treatment was continued from day 1 to 8 with 30 μg QY per injection twice a day.

Serum Ab titers

Anti-OVA IgE and IgG subclasses were measured by ELISA. Plates were coated for 6 h at 37°C with 100 μl 0.1 M NaHCO3 containing 100 μg OVA per milliliter. The plates were blocked 2 h at 37°C with 200 μl 3% BSA in PBS. Plates were washed, and 100 μl of 1:40 serum dilutions with PBS containing 1% BSA were applied overnight at 4°C. The amount of bound Ab was analyzed using horseradish peroxidase-conjugated Abs against mouse heavy chain classes. Rat mAbs against mouse IgE and IgG1 were from Pharmingen (Hamburg, Germany), goat antiserum against mouse IgG2a, IgG2b, and IgG3 were from SBA (Biozol, Eching, Germany). Plates were read in a microplate autoreader (Dynatech MR5000, Denkendorf, Germany) at 405 nm. Serum titers for all Abs were expressed as relative ELISA units (RU),3 referring to a laboratory standard sera pool, which was collected 20 days after sensitization with OVA (22). Standard sera were applied on each plate in a 1:10 dilution. To obtain optimal signal/noise ratios for each subtype, plates were read when the standard samples reached a previously defined optical density (OD 0.130 or 130 units for IgE and OD 2–2.5 or 2000–2500 units for the IgG subtypes). All samples from one experiment were developed simultaneously on a single plate.

Immediate cutaneous hypersensitivity

Active cutaneous anaphylaxis was tested by skin test after i.v. injection of 200 μl of 0.5% Evans Blue dye in PBS. The skin of the belly was shaved with an electric clipper and four injection sites were marked with a felt tip pen on the skin. Two of the marked sites were injected intradermally with 50 μl PBS containing 1 mg/ml OVA, and the other two sites with protein-free PBS. After 15 min, the mice were killed by cervical dislocation and the skin was stripped off for inspection of the injected sites. The intensity of bluing was scored on the dorsal side of the skin. Data are summarized in Table I.

Anaphylactic shock

Mice were injected i.v. with 200 μl 0.5% Evans Blue solution containing 500 μg OVA. After 15 min, symptoms of an anaphylactic shock were assessed by two independent observers who were unaware of the sensitization status of each animal.

Results

QY inhibits specific humoral immune response to OVA

Groups of wild-type BALB/c, BALB/c IL-4−/−, BALB/c IL-4Rα−/−, and SJL/J mice were immunized i.p. with 10 μg OVA plus Alum as adjuvant. Negative control animals were injected only with the adjuvant. In one group of mice, QY was applied 2 h pre- and post-OVA immunization as a 50-μg dose each and was continued twice a day from day 1 to 8 after sensitization with 30 μg QY per injection. A second group was injected with PBS instead of QY. Mice were bled at day 0 before immunization with OVA, and 10 days later. OVA-specific serum Abs were measured by ELISA. Data for BALB/c and IL-4−/− mice were collected in three independent in vivo tests. SJL/J and IL-4Rα−/− animals were tested in one in vivo test. Figure 1 shows data from one representative in vivo test for each mouse strain.

As seen in Figure 1A, there was a massive induction of IgE in OVA-sensitized, PBS-treated animals (10 μg OVA BALB/c IL-4−/−). In contrast, QY therapy (QY + 10 μg OVA BALB/c IL-4−/−) completely inhibited induction of OVA-specific IgE in wild-type BALB/c mice. A total of 19 mice were treated with QY during sensitization in three separate experiments. None of these mice showed an Ab titer above the baseline level found in non-sensitized animals (0 μg OVA BALB/c IL-4−/−). Furthermore, there was no detectable induction of OVA-specific IgG1 in OVA-sensitized IL-4 knockout mice (10 μg OVA BALB/c IL-4−/−), IL-4R knockout mice (10 μg OVA BALB/c IL-4−/−), or immunized SJL/J mice (10 μg OVA SJL/J) in comparison with untreated mice (0 μg OVA SJL/J).

There was no induction of Ag-specific IgG1 in immunized but QY-treated BALB/c mice, or in either of the knockout strains, while BALB/c mice treated with PBS had a high titer of specific IgG1 (Fig. 1B). In contrast to the results from IgE, SJL/J mice showed a pronounced production of OVA-specific IgG1, which could be inhibited by QY.

There was no induction of specific IgG2a or IgG2b over background levels in QY-treated BALB/c or SJL/J mice, whereas in IL-4 knockout mice and in IL-4R knockout mice these subclasses were dramatically up-regulated compared with immunized wild-type BALB/c mice (Fig. 1, C and D).

QY inhibited OVA-specific IgG3 synthesis in SJL/J and BALB/c mice, but no significant differences in this Ab subclass were found between the two knockout strains and BALB/c wild-type mice (Fig. 1E). Taken together, these data demonstrate that QY inhibits the specific humoral immune response to OVA in vivo in two different mouse strains.

Prevention of immediate cutaneous hypersensitivity in IL-4−/− mice and QY-treated BALB/c or SJL/J wild-type mice

We have further assessed immediate hypersensitivity in skin responses upon rechallenge of sensitized mice. The mice had been analyzed for OVA-specific Ab synthesis, 10 days after i.p. immunization with 10 μg OVA. Following i.v. injection of 0.5% Evans Blue dye solution, mice received two intradermal injections with OVA in PBS, and two injections with PBS alone. Positive reactions upon intradermal injection of Ag resulted in mast cell degranulation and fluid extravasation, which led to the formation of a blue patch on the injection site. The intensity of bluing was scored on the dorsal side of the skin. Data are summarized in Table I.

Strong fluid extravasation was observed in immunized, PBS-treated mice (10 μg OVA BALB/c IL-4−/−). Local OVA challenge did not lead to any detectable skin bluing in QY-treated mice (QY + 10 μg OVA BALB/c IL-4−/−), and in mice lacking either IL-4 (10 μg OVA BALB/c IL-4−/−) or IL-4Rα (10 μg OVA BALB/c IL-4Rα−/−). Non-sensitized wild-type animals (0 μg OVA BALB/c IL-4−/−) also failed to respond to the OVA injection. Immunized SJL/J mice (10 μg OVA SJL/J) developed positive skin reactions, despite the fact that these mice did not have a detectable level of OVA-specific IgE (Fig. 1A). In QY-treated SJL/J mice, OVA sensitization did not lead to active cutaneous anaphylaxis (QY + 10 μg OVA SJL/J). These results indicate that

3 Abbreviations used in this paper: RU, relative ELISA units.
FIGURE 1. Serum levels of OVA-specific Abs in mice, 10 days after immunization with OVA. Ab subclasses tested were IgE (A), IgG1 (B), IgG2a (C), IgG2b (D), and IgG3 (E). Mice were either SJL/J or BALB/c wild-type, or BALB/c-derived knockout mice lacking IL-4 (IL-4^{-/-}) or the IL-4 receptor (IL-4Rα^{-/-}) as indicated. Serum titers for OVA-specific Ab subtypes were measured by ELISA. Data are given in relative ELISA units (RU), referring to laboratory standards of OVA-specific antisera pools. Error bars indicate the SD, and the number of mice used under each condition is indicated as (n = x).
application of QY during the sensitization phase prevents development of active cutaneous anaphylaxis in response to IgE-mediated as well as to IgE-independent mast cell degranulation.

Prevention of anaphylactic shock in IL-4 knockout mice and QY-treated mice

We have assessed development of anaphylactic shock in wild-type BALB/c and in IL-4 knockout mice, which had been analyzed previously for OVA-specific Ab synthesis, 10 days after i.p. immunization with 10 μg OVA, mice were injected i.v. with 500 μg OVA in an Evans Blue solution. Reactions were scored according to four criteria: Fluid extravasation, piloerection, spontaneous activity, and responsiveness to external stimuli.

OVA sensitized wild-type mice (10 μg OVA BALB/c IL-4+/+) developed anaphylactic symptoms, which lead to a severe shock 15 min after injection. The severity of the symptoms was scored as shown in Table II. Nonimmunized animals (0 μg OVA BALB/c IL-4+/+; 0 μg OVA BALB/c IL-4−/−) did not show any reactions. Shock symptoms were also absent in sensitized but QY treated mice (QY + 10 μg OVA BALB/c IL-4+/+) and in IL-4−/− mice (10 μg OVA BALB/c IL-4−/−).

Discussion

We have shown that the antagonistic IL-4 double mutant QY inhibits allergen-specific IgE and IgG1 synthesis in vivo, and that this inhibition prevents the development of allergic symptoms upon local or systemic challenge. Under some pathophysiologic conditions, as in murine retrovirus-induced immunodeficiency syndrome (MAIDS), significant IL-4-independent IgE synthesis has been observed (23). Such a mechanism appears not to be involved in allergy, since IgE synthesis was completely inhibited by QY and was also absent in OVA-immunized IL-4−/− mice.

It has been reported that the antagonistic human IL-4 mutant Y124D leads to decreased ongoing IgE synthesis, which occurs independently of cytokine treatment in a humanized SCID mouse model (13, 14). Soluble versions of IL-4R (sIL-4R) or inhibitory anti-IL-4 Abs inhibit IgE synthesis in mice in response to helminthic infections, treatment with anti-IgD (24–26), and inhaled allergens (27, 28). Both sIL-4R and inhibitory anti-IL-4 Abs have to be used cautiously in vivo, because binding to these proteins prolongs the serum lifetime of IL-4 and can actually increase its efficiency (26, 29). Class switching to murine IgG1 and its human analogue IgG4 is regulated by IL-4 (30), but the inhibition of IL-4 by sIL-4R or inhibitory Abs in vivo results in most cases only in a moderate decline of IgG1 levels (25, 27, 28) or has no effect at all (24). In contrast, specific IgG1 synthesis was completely inhibited by QY treatment. Mast cell degranulation can be stimulated independently of IgE, presumably mediated through FcγRII receptors (31). We found that IgE-independent mast cell degranulation occurred in SJL/J mice, which produce very low levels of IL-4 and

Table I. Prevention of immediate type cutaneous hypersensitivity

<table>
<thead>
<tr>
<th>Mouse Strain</th>
<th>Sensitization/Therapy</th>
<th>50 μl PBS i.d.*</th>
<th>50 μl OVA (1 mg/ml) i.d.</th>
</tr>
</thead>
<tbody>
<tr>
<td>SJL/J</td>
<td>0 μg OVA</td>
<td>0, 01</td>
<td>0, 0</td>
</tr>
<tr>
<td></td>
<td>10 μg OVA</td>
<td>0, 0, 0, 0, 0</td>
<td>3, 2, 1, 1</td>
</tr>
<tr>
<td></td>
<td>10 μg OVA + QY</td>
<td>0, 0</td>
<td>0, 0</td>
</tr>
<tr>
<td>BALB/c IL-4+/+</td>
<td>0 μg OVA</td>
<td>0, 0, 0, 0, 0, 0</td>
<td>0, 0, 0, 0, 0</td>
</tr>
<tr>
<td></td>
<td>10 μg OVA</td>
<td>0, 0, 0, 0, 0, 0</td>
<td>3, 2, 1, 2, 3, 2, 1, 2</td>
</tr>
<tr>
<td></td>
<td>10 μg OVA + QY</td>
<td>0, 0, 0, 0, 0, 0</td>
<td>0, 0, 0, 0, 0, 0</td>
</tr>
<tr>
<td>BALB/c IL-4−/−</td>
<td>0 μg OVA</td>
<td>0, 0</td>
<td>0, 0</td>
</tr>
<tr>
<td></td>
<td>10 μg OVA</td>
<td>0, 0</td>
<td>0, 0</td>
</tr>
</tbody>
</table>

* Mice were locally challenged with OVA, 10 days after immunization with 10 μg OVA or mock immunization with PBS. After i.v. injection of 200 μl Evans Blue, mice were injected intradermally with 50 μl PBS with or without OVA. Both solutions were applied to two of four premarked injection sites on the skin of the belly. Data were scored by two independent observers and are given as mean scores between both injection sites of each solution for each individual. Reactions were rated as positive when the diameter of the blue patch exceeded 5 mm, which was premarked on the mouse skin. The intensity of bluing was rated as follows: 0, no blue patch formation (negative reaction); 1, slight bluing; 2, marked bluing; 3, strong bluing.

† Individual mice scored.

Table II. Prevention of anaphylactic shock

<table>
<thead>
<tr>
<th></th>
<th>BALB/c IL-4+/+</th>
<th>BALB/c IL-4−/−</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bluing (no = 0; slight = 1; strong = 2)</td>
<td>0, 0, 0, 0*</td>
<td>0, 0, 0, 0</td>
</tr>
<tr>
<td>Piloerection (no = 0; slight = 1; strong = 2)</td>
<td>0, 0, 0, 0</td>
<td>0, 0, 0, 0</td>
</tr>
<tr>
<td>Spontaneous activity (running around = 0; sitting passively = 1; lying = 2)</td>
<td>0, 0, 0, 0</td>
<td>0, 0, 0, 0</td>
</tr>
<tr>
<td>Responsiveness to external stimuli (running away upon touching = 0; slight reaction = 1; no reaction = 2)</td>
<td>0, 0, 0, 0</td>
<td>0, 0, 0, 0</td>
</tr>
</tbody>
</table>

* Mice were immunized with OVA and 10 days later injected i.v. with Evans Blue containing 500 μg OVA. After 15 min, anaphylactic symptoms were assessed by two independent observers. The animals were considered to be in a state of shock if at least three of the four indicated symptoms were observed.

Individual mice scored.
are strongly biased toward Th1 immune reactions (32). In accordance, SII/L/J mice failed to develop specific IgE upon sensitization with OVA. However, they developed specific IgG1 and other IgG subtypes and reacted with mast cell degranulation upon challenge. This response appears to be mediated by IgG1, because IL-4−/− mice, which produced specific IgGs of all subclasses except IgG1, did not display mast cell degranulation.

While IL-4 can at least in human cells contribute to the induction of various IgG subtypes (33), other cytokines are considered to be more involved, like IFN-γ for IgG2a (30) or IL-10 for IgG3 (34). Therefore it was surprising that QY inhibited not only IgE and IgG1 synthesis, but also the development of allergen-specific IgG2a, IgG2b, and IgG3. The QY-induced tolerance to protein Ag was not limited to BALB/c mice, which are prone to develop IL-4 regulated Th2 responses (35), but also occurred in Th1-prone SJL/J mice (32), suggesting a strain-independent mechanism. The QY-induced unresponsiveness cannot be due simply to lack of IL-4 effects, since treatment with inhibitory Abs or sIL-4R does not prevent induction of IgG2a, but rather up-regulates synthesis of this subclass, under conditions where IgE and IgG1 are suppressed (24, 28). In these experiments, the immune response seems to be redirected rather then abrogated by treatment with these agents. This is particularly dramatic in IL-4−/− mice, which show strong superinduction of specific IgG2a, IgG2b, and IgG3 in response to Nippostrongylus brasiliensis infection or upon immunization with (4-hydroxy-3-nitrophenoxy) acetyl chicken γ-globulin (21, 36). In agreement, we found much higher levels of IgG2a and IgG2b upon OVA sensitization in IL-4−/−, and even more so in IL-4+/− mice, compared with wild-type mice. This property of IL-4−/− mice can be interpreted as symptom of a strong Th1 response, which may occur in the absence of counterregulating Th2 cytokines.

Because Th2 development and IgE class switching are both dependent on IL-4 in the mouse, QY may exert its effects on T or B cells. Complete inhibition of Th2 development should prevent the stimulation of B cells to synthesize IgE, but, since B cells respond already to very low IL-4 concentrations (8), the direct inhibition of B cells may also contribute to QY effects in vivo. Since nearly all cell types express IL-4Rα, inhibitory effects of QY on other cells may also be relevant. Comparative studies with IL-4−/− and IL-4+/− mice should help to understand the specific effects of QY in the future. It is presently unclear which receptor and which cells are responsible for the inhibition of IgG subtypes by QY. The agent may suppress class switching or inhibit Ab production in general.

The IL-4−/− knockout, sIL-4R, and inhibitory anti-IL-4 Abs all target IL-4. In contrast, the target of QY is the receptor IL-4Rα. Since this receptor is required for responses toward IL-13 (5, 6), it could be envisioned that inhibition of IL-13 by QY may be responsible for suppression of the humoral immune response. This is not the case, since IL-4Rα−/− mice, which lack a functional IL-4Rα gene (M. Mohrs et al., manuscript in preparation) showed a similar phenotype as IL-4−/−, with significant induction of specific IgG2a, IgG2b, and IgG3 upon sensitization, while no specific IgE or IgG1 could be detected. However, the levels of all three subclasses appeared to be lower than for the IL-4−/− mice, which may indicate that IL-13 contributes to the overall Ig response. Therefore, the mechanisms by which QY inhibits the humoral immune response is not obvious. QY may interact with another binding partner besides IL-4Rα, but it is also possible that lack of an IL-4−/−IL-13 stimulus throughout development in the two knockout strains leads to a different response than short term inhibition by QY.

The induction of tolerance toward an allergen would be advantageous for therapeutic applications. Since allergens are not pathogenic, anergy toward them is the preferred outcome of any treatment.

Acknowledgments

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References


