Inhibition of Antigen-Induced Eosinophilia and Late Phase Airway Hyperresponsiveness by an IL-5 Antisense Oligonucleotide in Mouse Models of Asthma

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Inhibition of Antigen-Induced Eosinophilia and Late Phase Airway Hyperresponsiveness by an IL-5 Antisense Oligonucleotide in Mouse Models of Asthma

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Chronic airway eosinophilia is associated with allergic asthma and is mediated in part by secretion of IL-5 from allergen-specific Th2 lymphocytes. IL-5 is a known maturation and antiapoptotic factor for eosinophils and stimulates release of nascent eosinophils from bone marrow into the peripheral circulation. An antisense oligonucleotide found to specifically inhibit IL-5 expression in vitro was observed to significantly reduce experimentally induced eosinophilia in vivo, in both the murine OVA lung challenge and allergic peritonitis models. Intravenous administration resulted in sequence-dependent inhibition of eosinophilia coincident with reduction of IL-5 protein levels, supporting an antisense mechanism of action. Potent suppression of lung eosinophilia was observed up to 17 days after cessation of oligonucleotide dosing, indicating achievement of prolonged protection with this strategy. Furthermore, sequence-specific, antisense oligonucleotide-mediated inhibition of Ag-mediated late phase airway hyperresponsiveness was also observed. These data underscore the potential utility of an antisense approach targeting IL-5 for the treatment of asthma and eosinophilic diseases. *The Journal of Immunology,* 2000, 164: 5409–5415.

Eosinophils are granular cells that secrete a number of proinflammatory proteins and lipid mediators and are believed to play a prime role in the pathophysiology of asthma (1). The accumulation of activated eosinophils within the airways of the lung is a characteristic of asthma of various etiologies. On engagement of their Ig receptors or exposure to soluble mediators such as leukotrienes or platelet-activating factor, eosinophils release basic proteins, eicosanoids, and cytokines into the airway (2–4), likely resulting in damage to the epithelium and contributing to a state of chronic airway hyperresponsiveness. Numerous clinical studies have documented the presence of elevated levels of eosinophil-derived proteins in the bronchoalveolar lavage fluid of asthmatic patients (5–7).

Although several cytokines and chemokines have been demonstrated to affect eosinophil maturation, tissue infiltration, degranulation, and survival, IL-5 appears to be critical for producing a specific tissue eosinophilia. It has been demonstrated that mRNA for IL-5 is up-regulated within the airways of both atopic and nonatopic asthmatic patients and that IL-5 expression inversely correlates with pulmonary function (8, 9). In addition, IL-5 expression directly correlates with the number of eosinophils detected in asthmatic airways (10) and is decreased after corticosteroid treatment (11). IL-5 has been shown to be selectively elevated in helminth-infected patients with eosinophilia (12) and a role for IL-5 in human eosinophilia has also been suggested in both idiopathic hypereosinophilic syndrome (13) and eosinophilia-myalgia syndrome (14). Several lines of evidence also implicate IL-5 as the central cytokine for producing eosinophilia in mice: 1) inhibition of IL-5 responses with neutralizing Ab prevents blood and tissue eosinophilia in response to helminth infection or exposure to Ag (15, 16); 2) transgenic mice overexpressing IL-5 develop a selective eosinophilia (17); 3) IL-5-deficient mice do not respond with increased numbers of eosinophils on Ag challenge (18, 19); and 4) mice deficient in the IL-5 receptor α subunit required for binding IL-5 are unable to respond to parasitic infections with increased numbers of eosinophils (20). Collectively, these observations suggest that inhibition of IL-5 expression would result in impaired eosinophil infiltration into the asthmatic lung, thus effectively reducing the damage produced by eosinophilic components and thereby improving the clinical symptoms.

As an alternative to searching for small molecule inhibitors that prevent the interaction of IL-5 with its receptor, we used an antisense oligonucleotide approach to inhibit IL-5 gene expression. Antisense oligonucleotides that support RNase H-mediated degradation of murine IL-5 mRNA were developed using an in vitro model system of inducible IL-5 expression. After characterization in vitro, a lead oligonucleotide was tested in the OVA lung challenge and OVA-induced peritonitis mouse asthma models as well as in mouse airway hyperresponsiveness studies. Here we report the development and characterization of a murine IL-5 antisense oligonucleotide that reduces IL-5 protein expression and effectively inhibits Ag-induced eosinophil infiltration and airway responses in vivo in a manner that is consistent with an antisense mechanism of action.

**Materials and Methods**

**Cell culture**

The murine thymoma cell line EL-4 was obtained from the American Type Culture Collection (Manassas, VA) and was maintained and stimulated to express IL-5 as previously described (21).

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Table I. Oligonucleotides used in this study

<table>
<thead>
<tr>
<th>ISIS No.</th>
<th>Sequence</th>
<th>Descriptor</th>
</tr>
</thead>
<tbody>
<tr>
<td>20391</td>
<td>GGTTATCCTGGCTACATTA</td>
<td>IL-5 antisense (5 Me-C)</td>
</tr>
<tr>
<td>16999</td>
<td>GGTTATCCTGGCTACATTA</td>
<td>IL-5 antisense</td>
</tr>
<tr>
<td>17983</td>
<td>GGTTATCCTAGGCTACATTA</td>
<td>1-base mismatch for 16999</td>
</tr>
<tr>
<td>17984</td>
<td>GGTTATCCTGAGGCTACATTA</td>
<td>3-base mismatch for 16999</td>
</tr>
<tr>
<td>17985</td>
<td>GGTTAACGTAGCCAACATTA</td>
<td>5-base mismatch for 16999</td>
</tr>
<tr>
<td>20393</td>
<td>GGTTAACGTAGCCAACATTA</td>
<td>5-base mismatch for 20391 (5 Me-C)</td>
</tr>
<tr>
<td>21969</td>
<td>CACCTGATTGTGTCTTGTCA</td>
<td>Near-scrambled control (5 Me-C)</td>
</tr>
</tbody>
</table>

* All oligonucleotides shown are phosphorothioates. Underlined regions indicate residues that are 2'-O-MOE modified. The cytosine residues in these sequences are 5-methylated (5 Me-C) for ISIS 20391, 20393, and 21969.

Results

In vitro characterization of a murine IL-5 antisense oligonucleotide

To identify an antisense oligonucleotide against murine IL-5, we utilized the EL-4 thymoma, which has been shown to express IL-5 mRNA and protein after treatment with phorbol ester and cyclic AMP-elevating agents (21) as an in vitro screening model. An active 20-base oligonucleotide complementary to sequence within the 3’-untranslated region of the murine IL-5 mRNA was identified. This oligonucleotide is a chimeric molecule containing a uniform phosphorothioate backbone and a stretch of ten 2'-deoxy residues in the center of the molecule which supports RNase H-mediated cleavage. The 2'-deoxy core is flanked by five bases at each of the 5' and 3' ends that are MOE modified and thus convey greater resistance to exonuclease activities and higher affinity for hybridization to RNA (22–24). The cytosines within the sequence were methylated at the 5'-position to reduce the immunostimulatory potential sometimes observed with oligonucleotides in rodent studies.

The IL-5 antisense oligonucleotide decreased PMA plus dibutyryl-cAMP-stimulated IL-5 mRNA expression after electroporation into EL-4 cells in a dose-dependent manner, whereas the same sequence carrying five base mismatches was without effect (Fig. 1A), as determined by Northern blot. To further confirm an antisense mechanism of IL-5 mRNA reduction, the active sequence was tested for potency in reducing IL-5 mRNA while containing one, three, or five base mismatches. Activity was compromised after incorporation of a single base mismatch (Fig. 1B) and was completed abrogated by mismatching three or five bases in the sequence, indicating the importance of RNA hybridization for this effect.

PMA plus dibutylryl-cAMP-stimulated IL-5 protein production in EL-4 cells was also dose-dependently inhibited by the IL-5 antisense (Fig. 2A), whereas the five-base mismatch control had no effect. Antisense-mediated reduction of IL-5 protein expression was long-lived, with potent suppression of the response apparent up to 72 h after a single treatment (Fig. 2B).

Reduction of Ag-induced lung and tissue eosinophilia after IL-5 antisense oligonucleotide treatment

Administration of the IL-5 antisense oligonucleotide i.v. in BALB/c mice beginning 8 days after Ag sensitization and continuing daily through day 21, with i.p. Ag boost occurring on day 14 and repeated aerosol lung challenges on day 20, 21, and 23, resulted in potent inhibition of lung eosinophilia (Table II), similar to that produced by dexamethasone. A control oligonucleotide that contained five base mismatches did not show significant activity in
this model, indicating that this effect is sequence specific and consistent with an antisense mode of action. This effect was maintained up to 17 days after the last dose of oligonucleotide, with lung eosinophils reduced from 41 ± 4.5% of the total BAL cells in OVA-sensitized and -challenged mice to 7.5% (± 1.6) eosinophils, 2.0% in OVA near-scrambled control oligonucleotide-treated mice, whereas OVA IL-5 antisense oligonucleotide sequence results in graded loss of potency. Data are representative of at least two separate experiments. The non-5-methylated version of ISIS 20391 (ISIS 16999) was used in B.

### Table II. Effect of IL-5 antisense oligonucleotide on BAL eosinophilia in OVA-induced murine lung asthma model

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>% Eosinophils</th>
</tr>
</thead>
<tbody>
<tr>
<td>OVA</td>
<td>37.6 ± 1.6 (N = 10)</td>
</tr>
<tr>
<td>No sensitization</td>
<td>1.6 ± 0.4 (N = 5)</td>
</tr>
<tr>
<td>IL-5 antisense</td>
<td></td>
</tr>
<tr>
<td>5 mg/kg</td>
<td>11.8* ± 1.3 (N = 10)</td>
</tr>
<tr>
<td>10 mg/kg</td>
<td>5.5* ± 0.6 (N = 10)</td>
</tr>
<tr>
<td>20 mg/kg</td>
<td>3.8* ± 0.7 (N = 10)</td>
</tr>
<tr>
<td>5-Base mismatch control</td>
<td></td>
</tr>
<tr>
<td>10 mg/kg</td>
<td>33.6 ± 1.7 (N = 10)</td>
</tr>
<tr>
<td>20 mg/kg</td>
<td>28.4 ± 1.4 (N = 8)</td>
</tr>
<tr>
<td>Dex</td>
<td>5.8* ± 0.9 (N = 5)</td>
</tr>
</tbody>
</table>

* BALB/c-ByJ mice were injected i.v. with the indicated doses of IL-5 antisense or mismatch control oligonucleotide daily from day 8 through day 21 and immunized with OVA (20 μg i.p. in alum) or injected with saline on days 0 and 14 followed by challenge with aerosolized OVA (2% in PBS for 1 min) on days 20, 21, and 23. A control group received only the vehicle during sensitization but was also challenged with OVA aerosol (no sensitization). Dexamethasone (Dex, 2.5 mg/kg) was administered i.p. 1 day before sensitization, 2 h before and again 18 h after challenge. Cells were collected from bronchoalveolar lavage samples; cytospin slides were prepared and stained with Diff-Quick. The differential percentage of cells was determined based on light microscopic evaluation of >500 cells/slide. Values are means ± SEM. N = number of animals. Two animals died in the five-base mismatch control 20 mg/kg group after OVA challenge, during the late phase reaction. *p ≤ 0.01; Dex and IL-5 antisense at 5 mg/kg vs OVA, IL-5 antisense vs 8-base mismatch control at 10 mg/kg, respectively.

### Lack of immunostimulation in IL-5 antisense oligonucleotide-treated mice

The IL-5 antisense oligonucleotide does not contain any known immunostimulatory sequence motifs. One potential explanation for oligonucleotide-mediated reduction of eosinophilia is the induction of a Th1 immune response that may counteract an ongoing Th2 cytokine profile characteristic of allergic inflammatory states (26). To test for potential immunostimulatory activities of this oligonucleotide in the experimental models used in this study, BALB/c mice were dosed i.v. with either a CpG-containing oligonucleotide, the IL-5 oligonucleotide, or the mismatch control oligonucleotide and cytokine gene expression and spleen weight subsequently examined. After four consecutive daily doses of the IL-5 oligonucleotide at 10 mg/kg, no change in Th1 or Th2 cytokine gene expression was observed using a RNase protection assay (RPA), whereas the CpG-containing oligonucleotide induced a predominantly Th1 cytokine gene response; dosing throughout the immunization schedule (daily, day 2 to day 14) with the IL-5 oligonucleotide did not result in splenomegaly, whereas the CpG oligonucleotide almost tripled spleen weight (data not shown). In other experiments, the IL-5 antisense oligonucleotide was further shown not to affect mRNA levels of IL-10 or IL-3 (data not shown), suggesting that targeting was highly specific.

### IL-5 protein knock-down in vivo

We next examined whether IL-5 mRNA and protein levels were decreased in vivo in antisense-treated animals. IL-5 mRNA levels were evaluated either by RPA of whole spleen RNA or by quantitative RT-PCR analysis of poly(A)-purified mRNA from splenic T cells isolated using Thy1.2 Ab-conjugated magnetic beads. In both instances, although IL-5 mRNA levels were decreased in animals dosed with the IL-5 antisense relative to OVA or to either mismatch or control oligonucleotide-treated animals (data not shown), the low level of IL-5 mRNA expression complicated analyses. Reductions on the order of 50–60% compared with the five-base mismatch control were observed by quantitative RT-PCR
FIGURE 2. IL-5 antisense oligonucleotide-mediated inhibition of IL-5 protein levels in EL-4 cell culture supernatants. A, EL-4 cells were transfected as in Fig. 1, after which supernatants were collected from centrifuged cultures 48 h later and IL-5 protein was analyzed by sandwich ELISA. ■, no oligonucleotide control; □, IL-5 antisense; △, mismatch control. * Statistical significance compared with mismatch control, p ≤ 0.001. B, Kinetic analysis of IL-5 protein levels in untreated, antisense, or five-base mismatch control oligonucleotide-treated EL-4 cell supernatants collected from centrifuged cells. IL-5 analysis was performed by ELISA. Error bars indicate SEM. Analyses shown are representative of at least two separate experiments performed with quadruplicate cultures per treatment group. * Statistical significance compared with mismatch control, p ≤ 0.001. ISIS 16999 was used in B.

FIGURE 3. Reduction of eosinophilia mediated by an IL-5 antisense oligonucleotide in an OVA-induced allergic peritonitis model. BALB/c-ByJ mice were injected i.v. with the indicated doses of IL-5 antisense or mismatch control oligonucleotides daily from day −2 through day 14 and immunized with OVA (20 μg s.c. in alum) or injected with saline on day 0 and again on day 7 followed by challenge with OVA (10 μg i.p.) on day 15. Peritoneal lavage was performed 24 h after challenge, and cells were collected by centrifugation and total and differential counts determined. ■, OVA-sensitized and challenged control; □, IL-5 antisense; △, mismatch control. Values are mean ± SEM; n = 6/group. * Statistically significant from mismatch control; p ≤ 0.05. Data are representative of two separate experiments.

FIGURE 4. Antisense oligonucleotide-mediated inhibition of IL-5 protein in vivo. BALB/c-ByJ mice were injected i.v. with 20 mg/kg IL-5 or near-scrambled sequence control oligonucleotide daily on either day −2 through day 5 or day 0 through day 7 (0–7) and immunized with OVA (20 μg s.c. in alum) or injected with saline on day 0 and again on day 7 followed by challenge with OVA (10 μg i.p.) on day 15. Peritoneal lavage fluid was collected 10 h after challenge and analyzed for IL-5 protein content by sandwich ELISA. ■, OVA-sensitized and -challenged control; □, IL-5 antisense; △, near-scrambled sequence control. The day −2 through day 5 dosing schedule was chosen based on results indicating that inhibition of eosinophilia occurred to a similar degree as that observed using the longer dosing schedule (data not shown). * Statistically significant from mismatch control; p ≤ 0.05. The day −2–5 data are representative of two separate experiments, n = 8.
evaluated after OVA rechallenge. $P_{eh}$ was recorded during both the early phase (up to 30 min after Ag challenge) and the late phase (from 2 until 9 h after OVA challenge) airway responses (Fig. 5A).

Although there were no significant effects of IL-5 oligonucleotide treatment on the early phase response, as expected, a dose-related decrease in the late phase allergic airway response was observed (Fig. 5B). Significant decreases in late phase hyperreactivity of the airways were noted in IL-5 antisense oligonucleotide-treated mice at both 10- and 20-mg/kg doses compared with the mismatch control-treated and the saline-treated groups (Fig. 5, C and D), suggesting that IL-5 is a contributing factor to the late phase allergic airway response in this model.

Discussion

Previous studies have documented disparate roles for the contribution of IL-5 to the induction of allergic airway hyperresponsiveness in mouse models of asthma, depending on the mouse strain used and/or the Ag sensitization route and intensity (31–33). Targeting IL-5 with mAbs has resulted in prevention of lung eosinophilia and airway hyperresponsiveness in mouse, guinea pig, and monkey models of pulmonary inflammation (34–37). IL-5-deficient mice also do not exhibit airway hyperresponsiveness to Ag but can be "reconstituted" to express this phenotype with recombinant vaccinia virus expressing an IL-5 cDNA (19). A humanized IL-5 mAb currently in clinical trials has been shown to inhibit lung eosinophilia in allergic monkeys while not producing the general immunosuppression typical of corticosteroids in guinea pigs (37).

Thus, evidence suggests that blocking the function of IL-5 in vivo may be an important strategy for developing a therapeutic to treat asthma and other eosinophilic diseases. Conversely, in BALB/c mice, other reports have identified non-IL-5-mediated CD4$^+$ T cell-dependent mechanisms of allergic airway hyperreactivity that either do (31) or do not (32) involve IL-4. The data reported in this study correspond well with the IL-5 Ab studies that implicate a role for IL-5 in allergic airway responses and suggest that an antisense approach may represent another option for therapeutic intervention in IL-5-mediated diseases. In support of this concept, an antisense oligonucleotide targeted against the adenosine A1 receptor has been shown to reduce receptor levels and bronchial hyperreactiveness in a dust mite-conditioned allergic rabbit model of asthma (38).

Although IL-5 antisense did inhibit the late phase airway response to OVA rechallenge in a dose-related manner, complete abrogation of this response was not observed (Fig. 5). The initiation of an IL-5-dependent or -independent response in BALB/c mice has been suggested to depend on the sensitization protocol utilized (32). Specifically, s.c. sensitization as performed by Corry et al. (31) resulted in IL-5-independent airway responses while intraperitoneal or airway sensitization yielded hyperreactivity responses with an IL-5-dependent component (32, 35, 39). In addition, a recent study evaluating respiratory delivery of anti-IL-5 in BALB/c mice also showed an IL-5-dependent effect on airway hyperreactivity after i.p. sensitization and Aeroallergen challenge (40). Thus, available data indicate that an IL-5-dependent allergic
airway response can be measured in BALB/c mice under conditions of sensitization similar to those used in this study and suggest that multiple pathways of airway hyperreactivity can occur in BALB/c mice, some of which do not involve IL-5. Earlier studies have found similar induction of airway hyperreactivity in methacholine-treated anesthetized and conscious mice, utilizing either airway constriction measurements in cannulated mice or whole body plethysmography, respectively (32). However, the relative importance of various mechanisms of airway hyperreactivity may also differ in these two experimental systems and therefore contribute to discordant observations on the role of IL-5 in murine models of asthma. The contribution of non-IL-5-mediated airway hyperreactivity in our model may account for the lack of complete suppression of airway hyperresponsiveness, despite dramatic suppression of lung eosinophilia. In light of current knowledge on the differential manifestation of airway hyperresponsiveness in BALB/c mice, the lack of a correlation between eosinophilia and airway reactivity in some asthma patients is perhaps not surprising and suggests that additional pathways distinct from IL-5 may also control airway responsiveness in some instances in humans.

Our in vivo target reduction experiments suggest that oligonucleotide treatment is most efficacious when administered early in the immunization period (at or before Ag sensitization) and for more than two consecutive daily doses (at 20 mg/kg; Fig. 4). In vivo distribution studies have shown that appreciable bone marrow uptake occurs after i.v. administration of oligonucleotide in rats (41, 42) and, combined with the improved nuclelease resistance of the 2′-MOE chemistry used in the 5′- and 3′-ends of the oligonucleotide (23, 24), these data suggest that one site of action is likely to be the bone marrow. Recent evaluation of oligonucleotide uptake in various lymphoid tissues of mice by capillary gel electrophoresis analysis of i.v. injected oligonucleotide has shown that phosphorothioate and 2′-MOE-modified oligonucleotides accumulate chiefly in lymph node and bone marrow and that the 2′-MOE chemistry used in the experiments presented herein was recovered from these tissues largely intact (K. J. Myers and M. J. Graham, unpublished observations). Intranasal administration of the IL-5 oligonucleotide in mice at 24 and 2 h before challenge failed to reduce eosinophil infiltration in the OVA-induced lung model (data not shown). The failure of intranasal administration of the oligonucleotide to reduce OVA-induced lung eosinophilia may be due to poor oligonucleotide uptake by mature T cells in the lung, given that studies have shown poor staining of mature peripheral T cells with fluoroscence-labeled oligonucleotides (43, 44).

In vivo application of antisense technology for inhibition of genes linked to asthma pathology has been limited. In addition to adenosine A1 receptor targeting in a rabbit model of asthma (38), Molet et al. have described the lack of activity of an IL-5 antisense oligonucleotide on the late phase allergic response in adoptive T cell transfer experiments (45). The disagreement between the observations made in this study and ours, we believe, most likely represent differences in the site(s) of action of the antisense in vivo vs T cell transfection ex vivo. In particular, the lack of effect following intranasal administration of the IL-5 oligonucleotide in mice in our study suggests that it may be important to target IL-5-producing cells in vivo that are perhaps at a more immature stage than those recovered from lymph node after Ag sensitization. Thus, an IL-5 antisense strategy that targets the bone marrow tissue directly may be more effective than an approach aimed only at peripheral T cells. Further, the antisense treatment schedule may be important for in vivo activity as well, because we observed less potent activity when administering the antisense compound after the Ag challenge in our immunization model (unpublished observation).

In humans, IL-5 is thought to be specific for promoting eosinophil and basophilic maturation (46) and eosinophil survival and chemotaxis (47). Although discovered as a B cell growth and differentiation factor in mice, its actions on human B cells have been controversial but appear to be considerably more limited. Human IL-5 has no activity in standard human B cell growth factor assays (48) and appears to be costimulatory only with restricted mitogens or with the addition of IL-2 or IL-4 (49–51). These data suggest that selectively blocking the function of human IL-5 in vivo would predominantly affect eosinophils and avoid the side effects that are currently encountered with the broad acting antiinflammatory steroids.

In summary, we have shown that treatment of mice with an IL-5 antisense oligonucleotide resulted in decreased eosinophil infiltration into the lung and peritoneal cavity and improved airway function in mouse models of asthma. These pharmacological effects correlated with a significant reduction of IL-5 protein levels in vivo, supporting an antisense mechanism of action. Importantly, IL-5 blockade with an antisense oligonucleotide imparted significant protection from late phase airway hyperresponsiveness as measured in a whole body plethysmography model, suggesting a connection between IL-5-mediated eosinophilia and reduced airway performance in this setting. Moreover, few therapeutics currently available for asthma characteristically ameliorate the late phase reaction; thus, it is important to further characterize the dosing schedule required for this effect and the efficacy of other routes of administration and to determine the duration of action of this oligonucleotide-mediated effect.

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References


