

Effective prevention and therapy of experimental allergic asthma using a GATA-3-specific DNzyme

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Background: Allergic bronchial asthma is a chronic inflammatory disease of the airways. The transcription factor GATA-3 was shown to play an important role in T_H2 cell activation, but also in the regulation of other cell types involved in bronchial asthma including mast cells, eosinophils, and epithelial cells. DNzymes represent a new class of antisense molecules that combines the specificity of DNA base pairing with an inherent RNA-cleaving enzymatic activity.

Objective: To develop a GATA-3 mRNA-specific DNzyme and analyze its allergy-preventing activity in murine models of experimental allergic asthma.

Methods: The most active DNzyme (termed *gd21*) was selected by *in vitro* cleavage assays. Allergic airway inflammation was assessed by inflammatory cell and cytokine analysis within bronchoalveolar lavage. Lung histology, including goblet cell hyperplasia and lung function, was analyzed using head-out body-plethysmography.

Results: Intranasal administration of *gd21* prevented airway inflammation and mucus production and inhibited development of airway hyperresponsiveness to methacholine in models of acute allergic airway inflammation. Similar effects were also detected in a model of chronic experimental asthma. Interestingly, *gd21* was at least as effective as other antisense molecules, and off-target effects were not detected. Further experiments indicated that pulmonary surfactant may facilitate the cellular uptake of *gd21* by acting as an endogenous transfectant.

Conclusion: These results indicate that topical application of the GATA-3-specific DNzyme is a promising novel approach for the treatment of allergic bronchial asthma. (J Allergy Clin Immunol ■■■■;■■■:■■■-■■■.)

Key words: Allergic asthma, GATA-3, DNzymes, T_H2 cells, antisense DNA, small interference RNA

Abbreviations used

AHR: Airway hyperresponsiveness
asDNA: Antisense DNA
BAL: Bronchoalveolar lavage
BDNF: Brain-derived neurotrophic factor
FACS: Fluorescence-activated cell sorting
HE: Hematoxylin and eosin
MCh: β -Methacholine
MCP: Macrophage chemoattractant peptide
NGF: Nerve growth factor
PAS: Periodic acid-Schiff
siRNA: Small interference RNA
TLR: Toll-like receptor

Allergic bronchial asthma is a chronic inflammatory disease with increasing prevalence and incidence worldwide. Several hallmarks characterize the complex pathogenesis of the disease, including recurrent and reversible broncho-obstruction with airway inflammation, mucus hypersecretion, and development of airway hyperresponsiveness (AHR).¹ The chronic nature of the disease results in structural changes of the airway wall termed *airway remodeling*. The disease-underlying dysregulation of the immune system is defined by a T_H1/T_H2 cell imbalance resulting in overproduction of many proallergic and proinflammatory cytokines such as IL-4, IL-5, IL-9, and IL-13.^{2,3} The transcription factor GATA-3 promotes the development of naive T cells into T_H2 cells and directly induces production of T_H2 cytokines by transactivation of the promoters for IL-5 and IL-13.^{4,5} Moreover, GATA-3, even expressed at low levels, inhibits the expression of T-bet, which is required for T_H1 cell development and subsequent production of IFN- γ .⁶ More recent data indicate that the postembryonic expression of GATA-3 is not restricted to T cells but is also present in eosinophils, basophils, mast cells, and epithelial cells.^{7,8} Because all of these cell types play an important role in the pathogenesis of bronchial asthma and other allergic diseases, GATA-3 represents an interesting novel target for anti-allergic therapeutic strategies.⁹

DNzymes represent a novel class of antisense molecules that are not yet established in the treatment of any human disease. In terms of inherent enzymatic activity, stability, specificity, and bioavailability, they have several important properties that make them attractive tools for drug development. Among these 10-23 DNzymes are a group of RNA-cleaving DNA molecules that consist of a catalytic and 2 substrate recognition domains.^{10,11} In the current study, we report the *in vitro* selection of 10-23 DNzymes targeting GATA-3 mRNA and compare the efficacy of the

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most active DNAzyme gd21 with that of antisense DNA (asDNA) and small interference RNA (siRNA) in mouse models of experimental asthma. We demonstrate strong antiasthmatic effects of gd21 in an acute and a secondary allergic response as well as in a chronic allergic asthma model.

METHODS

Design of antisense molecules

All DNAzymes possess a central 10-23 catalytic domain (15 bp) flanked at both sides by GATA-3 mRNA-specific binding arms (9 bp; Fig 1, A). It was demonstrated that purine-uracil junctions are the most preferred cleavage sites of 10-23 DNAzymes.¹² Initially, 70 unmodified DNAzymes (Sigma-Genosys, Cambridge, United Kingdom) were tested *in vitro*. The most active DNAzyme, named gd21, modified by an inverted 3'-3'-thymidine (Operon, Cologne, Germany), was then used in the *in vivo* experiments. We applied a previously described 18-mer oligonucleotide (Metabion GmbH, Martinsreid, Germany) as asDNA.⁹ Four siRNA molecules specific for murine GATA-3 mRNA (Mm_GATA3_1-4_HP; Qiagen, Hilden, Germany) were tested *in vitro* for their activity to downregulate GATA-3 mRNA expression in LA-4 cells. The 2 most active siRNAs (Mm-GATA3_1+2_HP) were then applied in the *in vivo* experiments in an equimolar mixture.

In vitro analysis of DNAzyme activities

Full-length murine GATA-3, T-bet, brain-derived neurotrophic factor (BDNF), and nerve growth factor (NGF) cDNA sequences were cloned into the pGEM-7Zf(+/−) or PCR-TOPO cloning vector. mRNA was synthesized by *in vitro* transcription using the MAXIScript T7 Kit (Ambion, Austin, Tex).

A multiplex cleavage assay for screening of GATA-3 mRNA-specific DNAzymes¹³ was used with few modifications. A pool of 20 DNAzymes (5 nmol/L of each DNAzyme) was tested in 1 multiplex cleavage assay; therefore, they were incubated with GATA-3 mRNA (50 ng/μL) for 30 minutes at 37°C in reaction buffer (50 mmol/L TRIS-HCl, pH 7.4; 10 mmol/L MgCl₂; and 100 mmol/L NaCl). Cleaved RNA fragment lengths were determined by primer extension analysis using ThermoScript Reverse Transcriptase (Invitrogen, Carlsbad, Calif) with a 5'-6-fluorescein phosphoramidite-labeled murine GATA-3-specific primer (5'-GGCTGAGGTTCCAGGGCGAGGCGGTG-3') and analyzed by sequencing gel electrophoresis (Seqlab, Göttingen, Germany). The following DNAzymes were selected by this procedure: gd21 (5'-AG GACGGGAGGCTAGCTACAACGAGTGGTTGCC) and hgd40 (5'-GTGG ATGGAGGCTAGCTACAACGAGTCTTGGAG). A scrambled DNAzyme with unspecific binding arms was used as a control: ODNg (5'-TGTGATGT AGGCTAGCTACAACGACAGATGGAA).

Dose response and kinetic experiments were performed by incubating full-length GATA-3 mRNA (10 ng/μL) with increasing concentrations of DNAzyme for 1 hour or for different time intervals, respectively.

Total RNA was purified from cell lines (J774, MLg, LA-4) and lung tissue using the RNeasy Mini Kit (Qiagen).

Animals

Female BALB/c mice 6 to 8 weeks old (Harlan Winkelmann, Hannover, Germany) were maintained under specific pathogen-free conditions. Ovalbumin-free diet and water were supplied *ad libitum*. All animal studies were performed under the approval of the local animal ethics committee.

Animal treatment protocols

Mice were intraperitoneally sensitized to ovalbumin and challenged with aerosolized ovalbumin as previously described.¹⁴ Four different DNAzyme treatment protocols were used: (1) prevention protocol in an acute asthma model, (2) therapeutic protocol or (3) treatment during the first set of 2 challenges, and (4) chronic asthma model. Gd21, asDNA, and siRNA (each with 200 μg per treatment) targeting GATA-3 mRNA were delivered by intranasal applications. The exact protocols are shown in this article's Fig E1 in the Online Repository at www.jacionline.org.

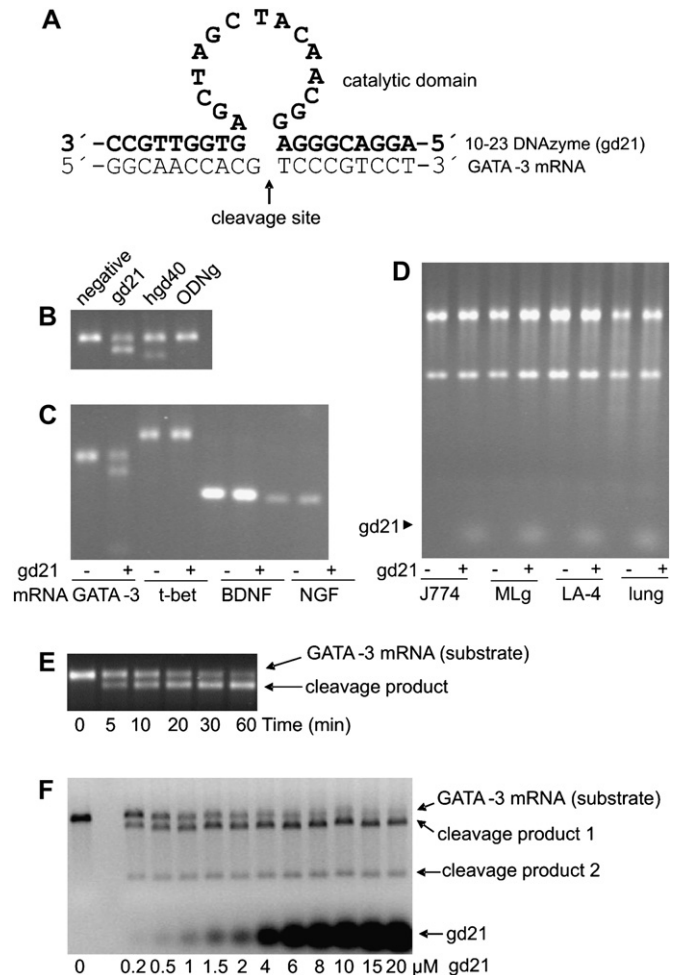


FIG 1. Structure and *in vitro* cleavage activity of the DNAzyme gd21. **A**, DNAzyme gd21 with its target sequence in the GATA-3 mRNA. **B**, Gd21 and hgd40 cleave murine GATA-3 mRNA, but not the control DNAzyme ODNg. **C**, Gd21 cleaves GATA-3 but not T-bet, BDNF, or NGF mRNA. **D**, Gd21 has no unspecific cleavage activity on total RNA. Gd21 activity is time-dependent (**E**) and dose-dependent (**F**).

Differentiation of cells in the bronchoalveolar lavage fluid

Bronchoalveolar lavage (BAL) sample collection and cell differentiation were performed as previously described.¹⁵

Assessment of airway reactivity

Noninvasive measurement of midexpiratory airflow of bronchial responsiveness to β-methacholine (MCh) was performed using head-out body plethysmography as described previously.¹⁴ The MCh concentration that caused a 50% reduction in baseline midexpiratory airflow (mg/mL) is reported.

Measurements of cytokine concentrations

IL-5, IL-13, IFN-γ, and macrophage chemoattractant peptide (MCP)-1 were determined in cell-free BAL fluids by using the BD cytometric bead array system (BD Biosciences, Heidelberg, Germany). IL-6 (BD Biosciences) and TNF-α (BioSource) were quantified by ELISA.

Lung histology

Formaldehyde-fixed and paraffin-embedded lungs sections were stained with hematoxylin and eosin (HE) or periodic acid-Schiff (PAS). Goblet cell hyperplasia was expressed as number of goblet cells/100 μm basement

membrane.¹⁶ Quantitative morphological analysis of mucin was performed by using systematic uniform random tissue samples of lungs fixed by instillation at a constant airway pressure of 20 cm fluid column. The volume of PAS-stained epithelial mucin (V_{mucin}) per surface area of airway epithelial basal membrane (S_{ep}) was determined by means of point and intersection counting using a computer-assisted stereology tool box¹⁷ (CAST-Grid 2.0; Olympus, Albertslund, Denmark) and subsequent calculation according to the following formula: $V_{\text{mucin}}/S_{\text{ep}} = LP \times \sum P_{\text{mucin}} / 2 \times \sum I_{\text{ep}}$ with $\sum P_{\text{mucin}}$ = sum of all points hitting mucin, with $\sum I_{\text{ep}}$ = sum of all intersections of test-lines with epithelial basal membrane, and LP = test-line length at final magnification.

In paraffin sections, collagen was stained by using Fast Green (Rowley Biochemicals, Danvers, Mass)/Sirius Red (Sigma-Aldrich, Hamburg, Germany).

In vitro off-target effects

J774 cells, a murine macrophage cell line, at 1×10^6 cells/mL were incubated with gd21 or mCpG 1668¹⁸ (Operon) as positive control. After 24 hours of incubation, TNF- α and IL-6 concentrations were quantified in cell culture supernatants.

In vivo off-target effects

Mice were treated intraperitoneally with gd21 or murine CpG 1668 (each 200 μg in 50 μL PBS). Peritoneal lavage was performed with 5 mL PBS 24 hours later, and IL-6 levels were measured by ELISA.

DNAzyme transfection

A549 cells, a human lung epithelial cell line, at 6×10^5 cells/mL were incubated in the presence of fluorescein phosphoramidite (FAM)-labeled DNAzyme (3 $\mu\text{g}/\text{mL}$) and the surfactant preparation Alveofact (Boehringer Ingelheim, Biberbach, Germany) for 9 hours. DOTAP transfection reagent (Roche, Mannheim, Germany) was used as positive control (15 $\mu\text{g}/\text{mL}$), and transfection efficiency was determined by fluorescence-activated cell sorting (FACS) analysis. DOTAP was also used to transfect the murine EL-4 cells.

Statistical analysis

Results are presented as mean values $\pm$ SEMs. After passing normality and equal variance tests, data were analyzed for significance by using the Student t test.

RESULTS

In vitro selection of the most active GATA-3 mRNA-specific DNAzyme

Because of secondary and tertiary structures of the target mRNA, not all theoretical target sites are actually accessible for DNAzyme cleavage. Therefore, the cleavage activity of $n = 70$ GATA-3 specific DNAzymes was initially tested by using a multiplex cleavage assay, and active molecules were selected. The DNAzymes gd21 and hgd40 were identified to possess the highest activity (data not shown). Both DNAzymes—but not the control DNAzyme ODNg—cleaved *in vitro* transcribed GATA-3 mRNA (Fig 1, B). The specificity of gd21 was verified by incubating it with *in vitro* transcribed T-bet, BDNF, and NGF mRNA, showing no cleavage activity (Fig 1, C). Gd21 had also no unspecific activity on total RNA purified from cell lines and lung tissue (Fig 1, D). The gd21 cleavage reaction showed a time-dependent and a dose-dependent pattern (Fig 1, E and F). The sequence of gd21 is given in Fig 1, A. Transfection of the T-cell line EL-4 with GATA-3-specific DNAzyme led to a decrease of GATA-3 paralleled by an increase of T-bet mRNA expression (see this article's Fig E2 in the Online Repository at www.jacionline.org).

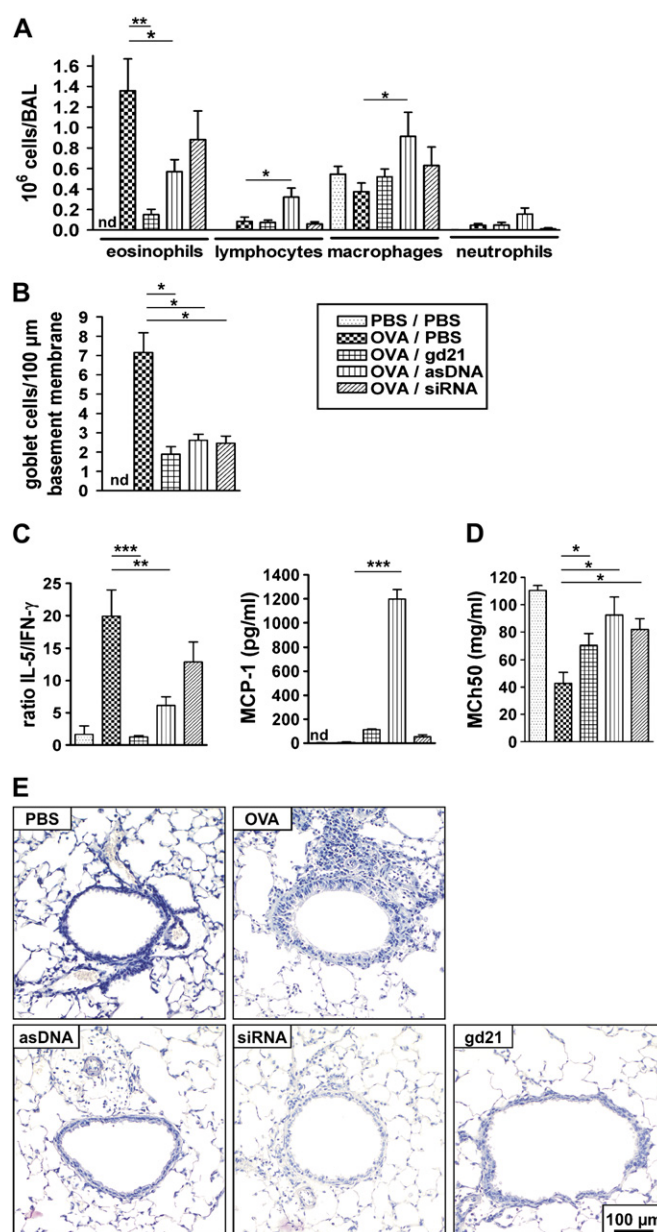


FIG 2. Antisense molecules against GATA-3 inhibit cellular inflammatory response in the airways of allergic mice and improve AHR. **A**, Numbers of white blood cells were quantified in the BAL. **B**, Goblet cells were counted in lungs sections. **C**, IL-5, IFN- γ , and MCP-1 concentrations were analyzed in the BAL. **D**, Lung function was analyzed by head-out body plethysmography. **E**, Representative HE-stained airways are shown. nd, Not detectable; OVA, ovalbumin.

Gd21 inhibits the development of an acute allergic airway response

Initially we analyzed the *in vivo* efficacy of gd21 in an acute model of experimental asthma. This model is characterized by a sensitization phase followed by the elicitation of the allergic inflammatory response in the airways by repeated aerosol allergen challenges. Treatment was started in already sensitized mice by intranasal application of gd21 on 4 subsequent days beginning 1 day before the first allergen challenge (see Fig E1 in the Online Repository). Gd21 significantly reduced the number of BAL eosinophils and mucus-producing goblet cells and decreased the

TABLE I. Production of TNF- α and IL-6 from J774 cells by gd21 or mCpG 1668

	PBS	mCpG 1668		gd21	
		0.05 μ mol/L	0.5 μ mol/L	0.05 μ mol/L	0.5 μ mol/L
TNF- α (ng/mL)	0.12 $\pm$ 0.001	37.9 $\pm$ 12.1	32.61 $\pm$ 3.1	0.1 $\pm$ 0.02	0.45 $\pm$ 0.006
IL-6 (ng/mL)	<DL	17.1 $\pm$ 0.2	19.4 $\pm$ 1.3	<DL	<DL

DL, Detection limit.

Given are means $\pm$ SEMs.

IL-5/IFN- γ ratio in BAL fluids (Fig 2, A-C). The decrease of IL-5/IFN- γ ratio was caused by a decrease in IL-5 paralleled by an increase in IFN- γ production (see this article's Fig E3 in the Online Repository at www.jacionline.org). Furthermore, BAL IL-13 levels were diminished after gd21 treatment. Histologic analysis revealed reduced peribronchial and perivascular infiltration of inflammatory cells (Fig 2, E). In addition to the inflammation-preventive effect, gd21 also significantly inhibited the development of AHR as analyzed by head-out body plethysmography (Fig 2, D). Similar anti-inflammatory activities could be observed after treatment with hgd40, whereas the control DNAzyme ODNg exerted no such effects (see Fig E4 in the Online Repository).

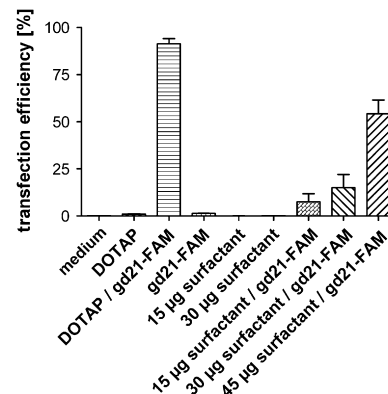
The DNAzyme approach was compared with alternative antisense strategies, asDNA and siRNA, respectively. As asDNA targeting GATA-3, we used an oligonucleotide that has already been established for the treatment for experimental asthma.⁹ siRNAs targeting GATA-3 were selected as described in the Methods section. Compared with gd21, siRNA treatment resulted in a weaker reduction in eosinophil numbers and smaller changes in the IL-5/IFN- γ ratio (Fig 2, A and C). The number of mucus-producing goblet cells and the level of AHR were decreased after treatment with all 3 classes of antisense molecules (Fig 2, B and D). Interestingly, positive effects of asDNA on eosinophils were accompanied by marked increases in numbers of BAL lymphocytes and macrophages and, to lesser extent, in neutrophils (Fig 2, A). This is further underlined by the observation that the asDNA induced high amounts of the proinflammatory chemokine MCP-1 (Fig 2, C), indicating the development of off-target effects.

Gd21 has no off-target effects

Because 10-23 DNAzymes are oligodesoxynucleotides that contain an unmethylated CpG motif within the catalytic domain, further experiments were performed to assess putative Toll-like receptor (TLR)-9-mediated off-target effects of gd21. Therefore, J774 cells were incubated with gd21 for 24 hours. Concentrations of IL-6 and TNF- α were measured in cell culture supernatants. The TLR-9 agonist 1668 mCpG was used as a positive control inducing maximal production of IL-6 and TNF- α . In contrast, no IL-6 and TNF- α production was measured with gd21. Even at an unusually high concentration, only negligible amounts of TNF- α were detected (Table I). To exclude *in vivo* off-target effects by gd21 further, mice received gd21 or 1668 mCpG as a control by intraperitoneal injections. IL-6 was determined in peritoneal lavage fluids 24 hours later. In contrast with 1668 mCpG (25 $\pm$ 10 pg/mL), gd21 did not induce detectable levels of IL-6.

Pulmonary surfactant enhances the uptake of oligonucleotides

Because it was surprising to detect such a high efficacy with the naked gd21 DNAzyme, the hypothesis was tested that pulmonary

**FIG 3.** Surfactant facilitates the uptake of gd21 into lung epithelial cells. The percentage of A549 cells transfected with fluorescence-labeled gd21 using Alveofact or DOTAP was determined by FACS analysis. DOTAP, N-[1-(2,3-dioleoyloxy)]-N, N, N-trimethylammonium propane.

surfactant enhances the cellular uptake of oligonucleotides. A549 cells were incubated in the presence of fluorescence-labeled gd21 with different concentrations of the commercial surfactant preparation Alveofact (Boehringer-Ingelheim, Biberach, Germany) followed by FACS analysis to determine the percentage of successfully transfected cells. This surfactant preparation clearly facilitated the uptake of gd21 by A549 cells in a dose-dependent manner (Fig 3). DOTAP, a cationic lipid widely used for the transfection of DNA, was used as a positive control.

The reduction of experimental asthma by gd21 in a secondary allergic response protocol is dose-dependent

After demonstrating the effectiveness of gd21 in the treatment of acute bronchial asthma, we investigated the *in vivo* efficacy of gd21 in further mouse models featuring more complex pathophysiological aspects of asthma. Gd21 was tested in a model of secondary allergic inflammatory reactions by using different doses of the DNAzyme in this setting. Sensitized animals were exposed to a first round of allergen challenges without any treatment (see Fig E1 in the Online Repository). In this model, treatment was performed in mice with existing airway inflammation and AHR. Gd21 was administered intranasally before and during secondary allergen challenges. The secondary allergic response was largely reduced as indicated by a significantly lower number of BAL eosinophils (Fig 4, A), improved airway reactivity (Fig 4, B) and a marked reduction of inflammatory cell infiltration and goblet cell hyperplasia (Fig 4, C). Dose-response experiments indicated that optimal effects on airway inflammation were obtained at a dose of 200 μ g per treatment,

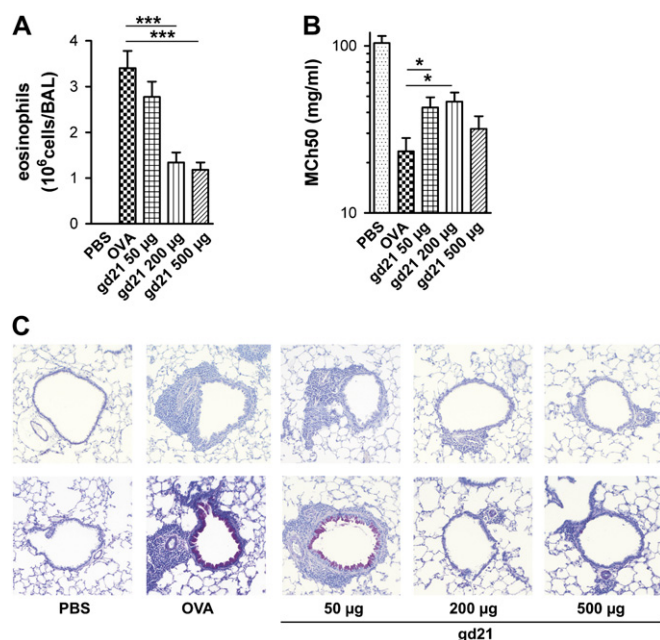


FIG 4. Dose-response relationship of the activity of the GATA-3 DNzyme gd21 on parameters of an already established allergic inflammatory response. The total number of BAL eosinophils (**A**) and airway reactivity to methacholine (**B**) were determined. **C**, Representative HE-staining (upper panel) or PAS staining (lower panel) airways are shown. OVA, Ovalbumin.

whereas significant improvement of AHR was already achieved at lower concentrations.

Gd21 has long-term effects of at least 2 weeks

To evaluate the longevity of gd21 treatment, sensitized mice were treated intranasally with gd21 during the first round of allergen challenges, and 2 weeks later, mice were challenged again (for protocol, see Fig E1 in the Online Repository). The subsequent analysis revealed that even after an interval of 2 weeks, the inflammatory response in airways and lung remained suppressed and airway reactivity remained improved compared with nontreated mice (see this article's Fig E5 in the Online Repository at www.jacionline.org).

Gd21 inhibits allergic airway inflammation and AHR in a mouse model of chronic experimental asthma

Because allergic asthma is a chronic inflammatory disease, gd21 treatment was further investigated in a mouse model of chronic experimental asthma.¹⁴ Sensitized mice were challenged for a total of 14 weeks with 2 allergen challenges each week applied on 2 consecutive days. To mimic the situation of patients with asthma as closely as possible, gd21 was applied only during the last 8 weeks of this protocol when airway inflammation and airway hyperreactivity were fully established (for protocol, see Fig E1 in the Online Repository). Gd21 treatment markedly reduced the number of eosinophils in both BAL fluids (Fig 5, A) and lung tissue (Fig 5, D). Compared with the ovalbumin-positive control group, goblet cell hyperplasia and mucus hypersecretion were significantly suppressed (Fig 5, B) and AHR improved (Fig 5, C) as a consequence of gd21 treatment. Subepithelial collagen deposition as a sign of airway remodeling was significantly reduced in the gd21-treated group.

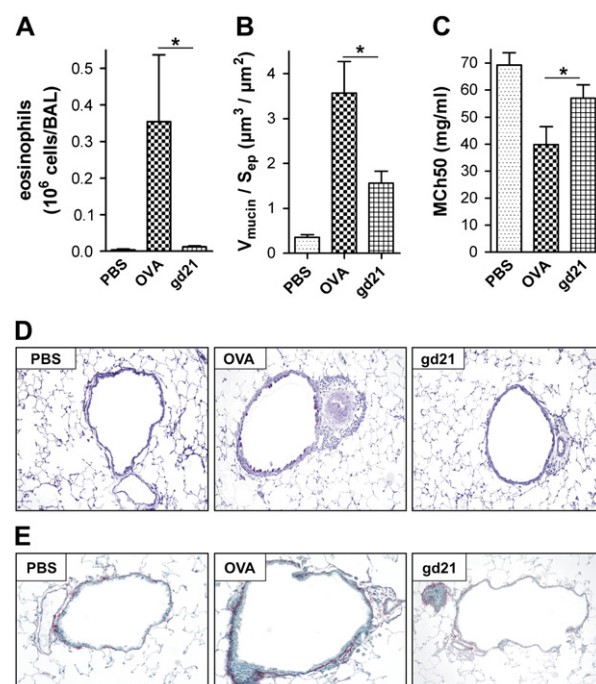


FIG 5. Gd21 inhibits cellular inflammatory response in the airways and improves AHR in a chronic asthma mouse model. **A**, Numbers of eosinophils were quantified in BAL fluid. **B**, Mucin volumes (V_{mucin}) were assessed morphometrically and related to basal membrane area (S_{ep}). **C**, Lung function was analyzed by head-out body plethysmography. **D**, Representative lung histologies of PAS-stained airways are shown. **E**, Collagen fibrils are shown in representative lung sections. OVA, Ovalbumin.

DISCUSSION

Allergic bronchial asthma is a complex inflammatory disorder. Many cellular and molecular mechanisms contribute to the induction and perpetuation of the disease, and several redundant principles are involved. Therefore, strategies of therapeutic intervention that interfere with upstream events in the disease-causing cascade seem to be promising. T_H2 cells are known to play a central role in initiation and maintenance of the allergic inflammatory response,^{1,19} and within these cells, GATA-3 represents a key transcription factor involved in the transcriptional control of T_H2-derived cytokines.^{1,20} In addition, the development of antisense strategies has opened a new field of therapeutic interventions based on the sequence-specific knockdown of gene expression involved in the development of disease at the very upstream transcriptional level. In the current study, we demonstrate that selective silencing of GATA-3 using a 10-23 DNzyme is effective in reducing airway inflammation and mucus production together with significant improvement of AHR in an acute murine model of experimental allergic asthma. Gd21 was more effective than the tested GATA-3 specific asDNA and siRNA molecules. In contrast with these strategies, gd21 showed no off-target effects *in vitro* or *in vivo*. In addition to the positive effects of gd21 in an acute model, the *in vivo* efficacy of gd21 could also be demonstrated in a secondary response, as well as in a chronic model of experimental asthma.

GATA-3 was selected as a promising target for therapeutic intervention of allergic diseases on the basis of its central role in T_H2 cell differentiation and control of T_H2 cytokine transcription. However, several recent studies have shown that in adults,

expression of GATA-3 is not limited to this T-cell effector subset. In addition, many other cell types that play a prominent role in asthma pathology express GATA-3, including mast cells, basophils, eosinophils, and airway epithelial cells.²¹ It has been shown that transcription of IL-4 and IL-5 in eosinophils is controlled by GATA-3 via transactivation of the respective promoters in these cells. Thus, GATA-3 expression in these cells may further contribute to progression and self-perpetuation of the allergic inflammatory response. Furthermore, since Zhang et al²² demonstrated a differential expression of GATA-3 in T_H1 and T_H2 cells, respectively, GATA-3 has been proposed to have great effect on the balance of these 2 opposing T-cell subsets. This assumption was most recently supported by data demonstrating a negative effect of GATA-3 on T_H1 cell differentiation by its ability to suppress signal transducer and activator of transcription 4 and T-bet, both representing transcription factors crucial for T_H1 cell development.^{6,23} *Vice versa*, T-bet acts by downregulating GATA-3, which eventually leads to proper T_H1 development, ultimately leading to induction of IFN- γ gene transcription. Interfering with GATA-3 expression in T cells may not only suppress T_H2 cell activities but also simultaneously induce and promote T_H1 cell development. In concordance with these data, gd21 DNzyme treatment inhibited GATA-3 and indirectly augmented T-bet mRNA expression. Consequently, T_H2 cell dependent cytokines (IL-5 and IL-13) were abolished and the T_H1 cell cytokine IFN- γ was augmented in the BAL fluid.

An important aspect for the *in vivo* application of antisense molecules is the efficacy of the delivery of these molecules to the target tissue and the cells of interest. Several studies using different animal models demonstrated that intranasal application of naked antisense oligonucleotides can elicit beneficial effects in the lung.^{24,25} These observations—which are strongly supported by our results—provide evidence that such molecules are effectively taken up in the lung.²⁶ The underlying mechanism of this process is still poorly understood. One possibility is that pulmonary surfactant may act as an endogenous transfectant supporting the cellular uptake of oligonucleotides.^{27,28} This concept is supported by our experiments using a commercially available surfactant preparation.

A comparison of the 3 classes of GATA-3 antisense molecules was performed. All 3 molecules are able to improve certain key features of the disease significantly. Whereas no differences were observed in the efficacy of the different antisense molecules with regard to their effects on AHR, application of the DNzyme gd21 turned out to be the most efficient molecule for downregulating the airway eosinophilia, followed by GATA-3 specific asDNA and siRNA. This order was properly paralleled by the capability of the different approaches to change the balance of antigen-specific T_H1 and T_H2 cell activities as indicated by the respective changes in IL-5/IFN- γ ratios in the BAL. The dissociation between partial effects on restoration of airway responsiveness and almost complete inhibition of the inflammatory response after DNzyme treatment revealed once again that other mechanisms not directly connected to inflammation are also involved in the regulation of AHR. A similar dissociation between airway inflammation and AHR has been shown in previous studies.^{29,30} The DNzyme approach was not only effective in preventing the primary allergic inflammatory response but also able to influence positively the secondary response, indicating that such a strategy might be promising for the treatment of already established allergic airway inflammation.

A major point of concern with regard to the application of antisense molecules is their potential to induce nonsequence-dependent effects, referred to as *off-target effects*. Off-target effects are partially a result of the interaction of foreign oligonucleotides with pattern recognition receptors, including certain TLRs that are able to recognize single-stranded (TLR-7) or double-stranded (TLR-3) RNA, or CpG-motif-containing DNA molecules (TLR-9).^{31,32} Signaling via these receptors is known to stimulate the host's innate immune system and, thus, support development of inflammatory processes with a strong T_H1 component. Therefore, it is essential to explore carefully such unwanted responses. Off-target effects were absent in GATA-3 DNzyme or siRNA-treated mice. The asDNA molecule was included in the study because it was already shown to exert beneficial effects in a model of experimental asthma.⁹ This approach, however, was accompanied by off-target effects as demonstrated by increased BAL levels of the chemokine MCP-1 and the infiltration of macrophages, lymphocytes, and, to some extent, neutrophils into the airway lumen. Thus, the antiallergic effects of this approach may be explained by both sequence-specific downregulation of GATA-3 mRNA expression and the induction of other immune mechanisms as a result of off-target effects of this asDNA molecule.

In conclusion, the usefulness of a catalytic molecule targeting GATA-3 for the treatment of allergic asthma was investigated. GATA-3 was selected as a promising target on the basis of its central role in both T_H2 cell differentiation and transcriptional control of T_H2 cytokines, as well as in the biology of eosinophils, mast cells, and epithelial cells. This DNzyme prevented acute experimental asthma, reduced secondary allergic responses, and was effective in a chronic model of experimental asthma. Furthermore, off-target effects could be excluded as a relevant mode of action. Altogether, this makes this new class of antisense molecule a promising technology toward therapeutic intervention of allergic bronchial asthma.

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Clinical implications: The DNzyme technology could break new ground for treatment of human allergic asthma.

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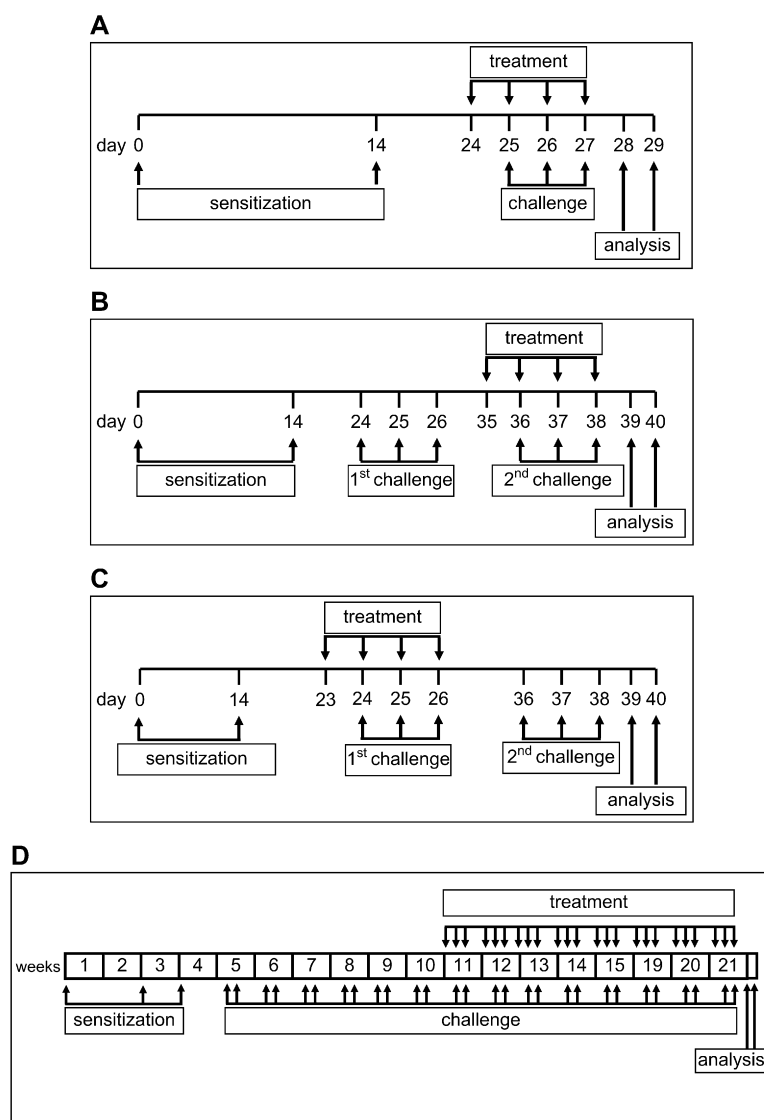


FIG E1. Antisense treatment protocols. BALB/c mice were sensitized to ovalbumin by intraperitoneal injections of ovalbumin adsorbed to alum ($\text{Al}[\text{OH}]_3$) followed by inhalational challenges with an 1% ovalbumin aerosol to induce allergic airway inflammation. Treatment using antisense molecules (200 μg) was performed by i.n. application. Treatment was performed in an acute asthma model (**A**), during a secondary allergen response (**B**), only during a first set of 2 ovalbumin challenges (**C**), and in a chronic asthma model (**D**).

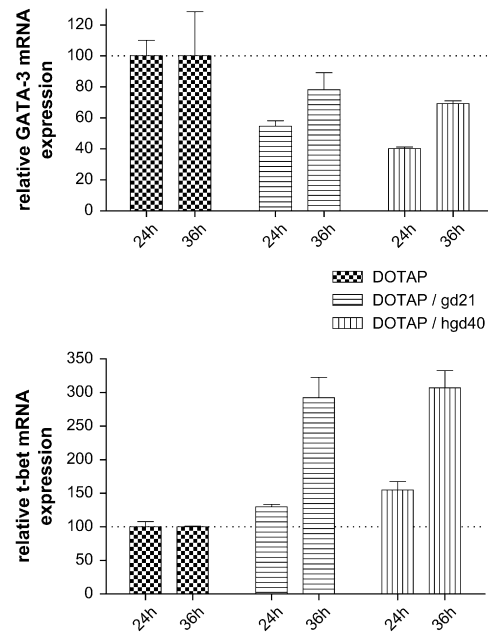


FIG E2. Gd21 and hgd40 lead to a decrease of GATA-3 mRNA and to an increase of T-bet mRNA expression. EL-4, a murine T-cell line, was transfected with gd21 or hgd40 by using DOTAP. The relative expression of GATA-3 and T-bet mRNA was determined by using real-time PCR (LightCycler; Roche, Mannheim, Germany) after sample normalization using the housekeeping gene L32. The GATA-3 and T-bet mRNA expression values from DOTAP control were set at 100%.

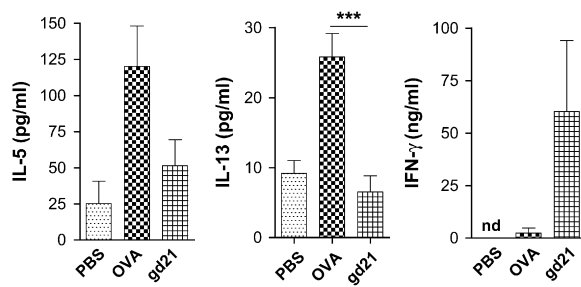


FIG E3. Gd21 reduces IL-5 and IL-13 and increases IFN- γ production in an acute model of allergic asthma. Cytokine levels were determined in cell-free BAL fluid. *nd*, Not detectable; *OVA*, ovalbumin.

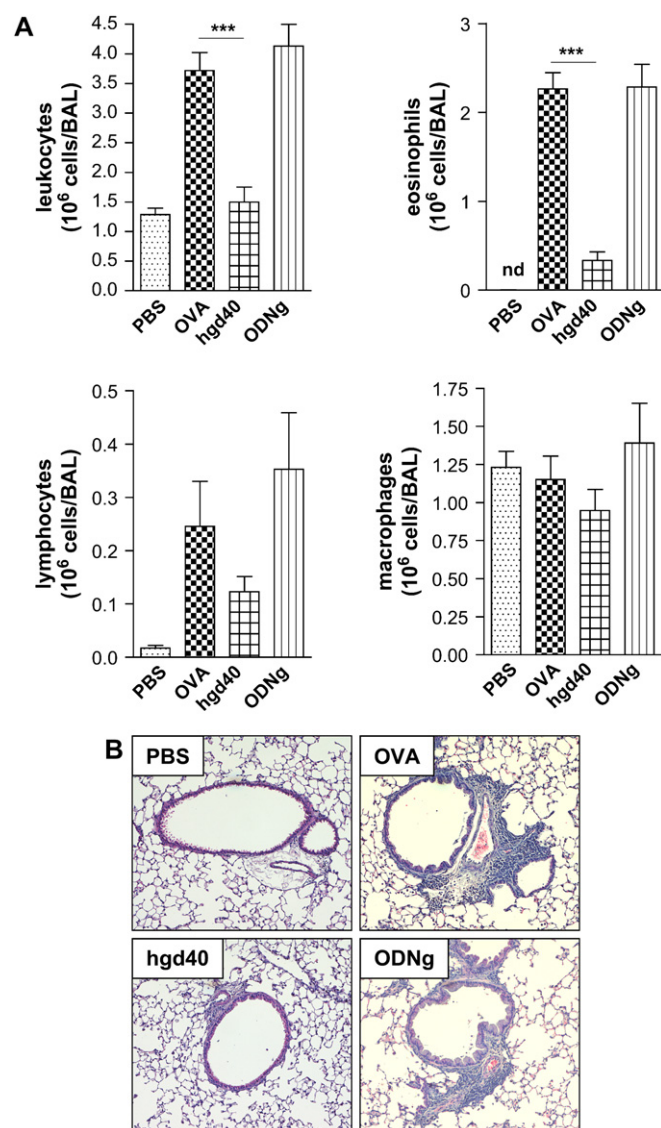


FIG E4. Hgd40—but not the control DNAzyme ODNg—inhibits airway inflammation in a mouse model of acute allergic asthma (Fig E1). **A**, Differential leukocyte counts were quantified in the BAL. **B**, Representative HE-stained airways are shown. *nd*, Not detectable; *OVA*, ovalbumin.

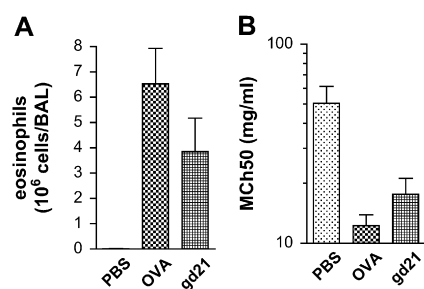


FIG E5. Long-term effect of gd21 in a murine model of experimental asthma. Ovalbumin (OVA)-sensitized mice were intranasally treated with gd21 during the first round of OVA challenges. After 2 weeks, a second set of challenges was performed without treatment. Shown are means $\pm$ SEMs of the total number of BAL eosinophils (**A**) and airway reactivity to methacholine (**B**).