

Molecular mechanisms in allergy and clinical immunology

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Oligonucleotide therapy of allergic asthma

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The recent increase in the prevalence of and mortality from asthma has inspired several new molecular techniques to improve treatment. Because asthma is a disease of gene polymorphism, gene therapy is unlikely to be effective. Alternative methods use oligonucleotides (ODNs) in the form of (1) DNA vaccination expressing CpG motifs that mimic bacterial DNA or (2) antisense ODNs inhaled and locally deposited into pulmonary airways to specifically modulate receptors for inflammatory mediators. DNA vaccination, a form of "molecular immune surveillance," attenuates a TH2 predominance. Antisense directed against the adenosine A₁ receptor abrogates A₁ sensitivity, improves allergen-induced immediate airway obstruction, and inhibits the expected increase in histamine responsiveness in allergic rabbits. Adenosine receptor inhibition lasts for an average of 7 days and the majority of the antisense remains in the lung. ODN therapy for asthma seem unlimited, but confirmation awaits the extension from animal models to human studies. (*J Allergy Clin Immunol* 1999;104:260-6.)

Key words: Asthma, oligonucleotide therapy, antisense, DNA vaccination, CpG DNA

The increasing prevalence, morbidity, and mortality from asthma and other allergic diseases¹ has prompted the development of a number of new molecular tools and possible treatment modalities. These include such things as humanized anti-IgE monoclonal antibody,² small molecule receptor antagonists,³ or inhibitors of cellular adhesion.⁴ Anti-IgE monoclonal antibody has been very effective for both asthma^{5,6} and rhinitis⁷ with few side effects. Anti-VLA4 antibodies and intercellular adhesion molecule antibodies as well as CS1 peptide have been shown to be effective in animal models, and some are now undergoing clinical investigation in human asthma.⁴ This review will deal with other ways to modify recep-

Abbreviations used

ASON:	Antisense oligonucleotide
BHR:	Bronchial hyperresponsiveness
ISS:	Immune stimulatory sequences
mRNA:	Messenger RNA
NK:	Natural killer
ODN:	Oligonucleotides
PC ₅₀ :	Provocative concentration at 50%

tors,⁸ enzymes, cytokines, or transcription factors⁹ at a molecular level using oligonucleotides to alter molecular immune surveillance or to cause epigenetic attenuation of inflammatory receptors in allergic asthma.

GENE OR EPIGENE THERAPY

Many attempts at *gene* therapy have been made to correct mutations by inserting normal genetic material where mutated genetic material is functionally defective, such as in cystic fibrosis.¹⁰ *Epigenetic* therapy refers to modulation of gene expression rather than manipulation of the gene itself to "arrest" translation of the gene into its product(s). Antisense therapy, an example of epigenetic therapy, has been described in the context of inflammatory models for asthma.¹¹ Although gene therapy may replace the defective gene and DNA CpG motifs enhance innate immunity nonspecifically, the primary benefit of epigenetic therapy is specificity¹² in eliminating gene products, usually proteins such as cytokines, transcription factors, or receptors (Fig 1).

Concern over the increasing prevalence and morbidity of asthma has increased during the past 10 to 15 years. Asthma is a disease that affects at least 15 million Americans (up to 15% of the population) and is highly correlated with atopy (>60%). These rising rates of prevalence and mortality have been attributed most recently to the possibility that, as society becomes more privileged, greater immunization and protection from early bacterial infections may negate the usual development of potent innate TH1 immunity, allowing predominance of TH2-driven allergic responses.¹³ Consequently, sales of asthma therapeutics have reached more than \$10 billion, an increase of 10% in some previous years. Most patients

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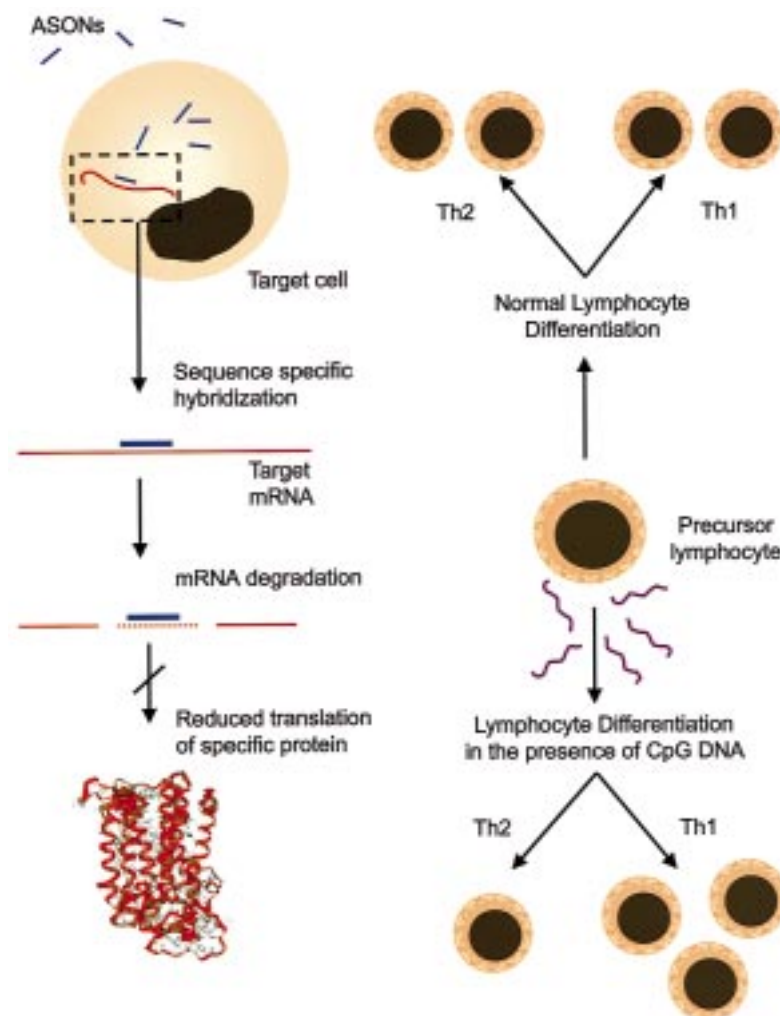


FIG 1. Left, Antisense oligonucleotides (ASONS) incorporated intracellularly hybridize with messenger RNA (mRNA) and through degradation by ribonuclease H cause translational arrest and subsequent attenuation of receptor protein. Right, DNA vaccination with CpG motifs results in modified immune surveillance and induces enhanced TH1 immune response. (Drawn by S. Leonard.)

take 2 or more drugs simultaneously, and multiple medications may together cost as much as \$10,000 per year. Mortality in eastern North Carolina, for example, has reached as high as 3 to 4 per 100,000. The risk of near-fatal asthma has also increased in lower socioeconomic areas because of lack of education, resources, and access to a physician or caregiver.¹⁴ Several other hypotheses include the overuse of highly potent β -agonists,^{15,16} recently addressed by selectively producing isomers of the β -agonists with reduced side effects, exposure to cockroach antigen in the inner city,¹⁷ or obesity¹⁸ and lack of exercise.¹⁹

The current view of asthma is that it is mostly stimulated by allergen, enhanced by late-phase airway inflammation, and possibly results in "airway remodeling."²⁰ Thus anti-inflammatory medications seem essential for the treatment of asthma. Prevention of asthma, however, is as important as treatment. Therefore (1) avoidance, (2) adequate anti-

inflammatory medications, (3) allergy immunotherapy, (4) adherence, and (5) accessibility represent the five As of treating allergic diseases. However, because not all asthma or rhinitis is allergic, antiviral or other treatments may also be important. Cockcroft and Swystun²¹ have proposed that "the most common reason for suboptimal control of asthma is failure to adhere to asthma treatment guidelines." This implicates not only inappropriate treatment but also inadequate adherence to regimens that educators and physicians have prescribed to caregivers or patients.²² Allergy immunotherapy is the only method known to prolong modification of the immune response (a TH2 to TH1 switch), but there is concern for safety, and its efficacy for asthma has been difficult to prove. *Oligonucleotide therapy* is one of the newer ways to deliver immunomodulating DNA (called DNA vaccination) or to prevent the production, with antisense DNA, of critical mediators of allergic inflammation or their receptors.

TARGETS FOR OLIGONUCLEOTIDE THERAPY IN ASTHMA

Modern concepts of allergic rhinitis and asthma originate from the use of the fiberoptic bronchoscope to analyze bronchoalveolar lavage fluid^{23,24} and biopsy specimen tissue²⁵ after late-phase allergic reactions. These are inflammatory and lead to previously observed increases in persistent bronchial hyperresponsiveness (BHR). A switch from the normal balance between TH1- and TH2-biased immunity leads to predominance of TH2 cytokines in the face of allergen exposure. Even in mild or asymptomatic asthma, inflammatory cells are present,²⁶ and the epithelium is modified or sloughed. In poorly treated asthmatic patients with persistent disease subbasement membrane thickening can develop over a period of time as a result of deposition of fibrin and collagen III and V,^{27,28} a process called "remodeling." It has severe implications resulting in so-called "irreversible" changes in airway function. Asthmatics, in fact, generally lose airway function at a greater rate than healthy nonsmoking individuals do.²⁹ In rhinosinusitis, nasal polyps may represent comparable remodeling of inflamed epithelium. These observations have led to an understanding of the connection between the upper and lower airway in these disease states.³⁰

A role for adenosine in asthma

The influx of inflammatory cells into the nose and lung creates a complex milieu of mediators, cytokines, chemokines, and newly differentiated progenitor cells. Activation of adhesion molecules initiates rolling, adhesion, and finally migration of cells from circulation into tissue. The activated eosinophil characterizes both the allergic and nonallergic disease. Neutrophils may participate in severe acute-onset asthma, and macrophages and natural killer (NK) cells serve as a reservoir for cytokines such as IL-12 and INF- γ .²⁶ Persistent inflammation leads to smooth muscle hypertrophy and up-regulation of receptors. One of these mediators of inflammation is adenosine. The adenosine A₁ receptor is up-regulated in the allergic rabbit asthma model for early- and late-phase airway inflammation³¹ and in humans.³² Recently, with use of in vitro cell culture Hakonarson et al³³ have shown that incubation of smooth muscle with high-titer serum IgE up-regulates A₁ receptors, a process mediated through the low-affinity Fc ϵ _{II} (CD23) receptor for IgE. Adenosine is perhaps one of the most underrecognized mediators in allergic disease.⁸ Not only does the adenosine A₁ receptor appear to be up-regulated on airway smooth muscle through an IgE mechanism, but 3 additional receptors, A_{2A}, A_{2B}, and A₃, found on mast cells³⁴ and eosinophils³⁵ may influence mast cell degranulation and eosinophil chemotaxis. Hypoxic stress generates adenosine from many cell types. Theophylline (an adenosine antagonist), has for some time been a reliable bronchodilator with some anti-inflammatory properties for asthma therapy that may suppress nuclear factor- κ B.³⁶ An adenosine pulmonary challenge is highly spe-

cific for allergic asthma,³⁷ suggesting a hypothesis for allergic asthma where adenosine is a central mediator. This information led to our use of the allergic rabbit model to demonstrate the effectiveness of an A₁ antisense to reduce A₁ receptor number and improve airway physiologic features and BHR. Thus prevention of these processes by inhibiting inflammatory mediators, adhesion molecules, transcription factors, or receptors is considered a useful goal by targeting them with antisense oligonucleotides (ODNs) or modifying immunity with CpG immunoadjuvants.

METHODS USING OLIGONUCLEOTIDE TECHNOLOGY

DNA vaccination

Krieg³⁸ and others³⁹ have reviewed the use of CpG DNA as a vaccine adjuvant to promote TH1 cells by increasing secretion of INF- γ from B cells or NK cells. It has been known for some time that bacterial DNA contains numerous unmethylated CpG motifs (70%-90%, unmethylated), whereas vertebrate DNA has fewer CpG motifs and is mostly methylated (5% unmethylated). These differences in human DNA, methylation, and lack of CpG motifs permit modification of human DNA to mimic bacterial DNA. When the immune system recognizes this modified formulation as aberrant, it suppresses IgE synthesis, which could be called "molecular immune surveillance." It was discovered that the CpG sequence needed a certain order of ODNs, containing 8 to 30 or more oligonucleotides bases, to cause immune stimulation. If there was only a single CpG dinucleotide, it could not be preceded by C or followed by G. Furthermore, it had to be unmethylated, and if it was replaced by 5-methylcytosine, the oligonucleotide lost its immune stimulatory activity.¹⁵ Most CpG motifs in vertebrate DNA are actually preceded by G or followed by G, which typically makes them nonimmunostimulatory. Krieg³⁸ has outlined a hypothesis for the mechanism by which CpG motifs stimulate antigen-processing cells and B cells to induce NK cell INF- γ and drive the system toward a TH1 population. Kline et al⁴⁰ initially showed this could transform an immunized murine model to down-regulate IgE. Broide et al⁴¹ showed that DNA could reduce eosinophilia, BHR, and IL-5. Sur et al⁴² have recently confirmed the ability of immunostimulatory CpG motifs to prevent allergic lung inflammation. Their observations extended over a period of 2 months with repeated doses, and after cessation of drug administration they found a prolonged beneficial effect for at least 6 weeks.

Spiegelberg et al⁴³ originally reported the inhibition of IgE synthesis with immune stimulatory sequences (ISS) that code for specific antigens. They used a gene construct coded for β -galactosidase that reduces specific IgE in Balb/c mice by approximately 75% in 6 weeks. Huang³⁹ proposed that through the use of newer approaches identifying gene expression, including complementary DNA and oligonucleotide microarrays, serial

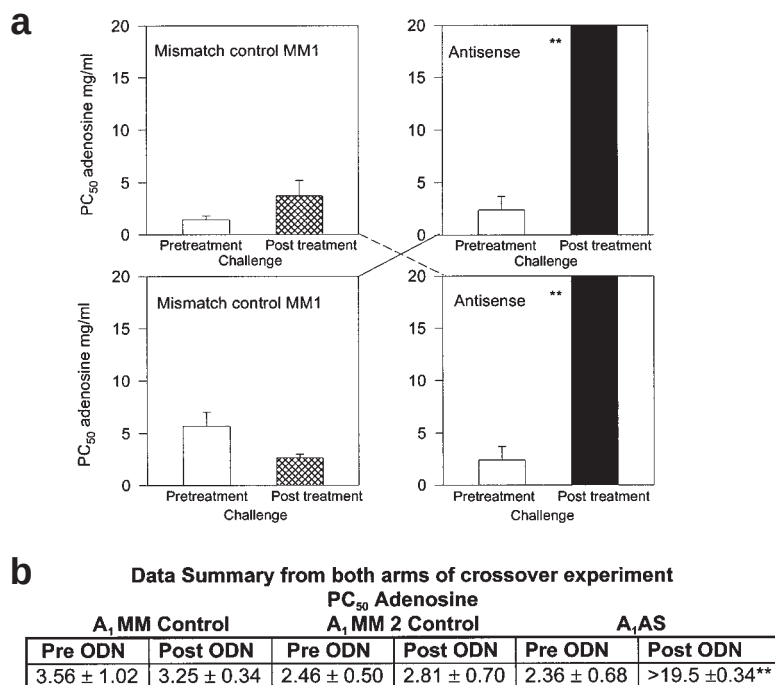


FIG 2. **a**, Effects of adenosine A₁ receptor antisense ODN on provocative concentration at 50% (PC₅₀) values in asthmatic rabbits. PC₅₀ adenosine values were determined before and after administration of aerosolized A₁AS or A₁MM intratracheally to allergic rabbits as described in Methods. After 2-week rest period between arms of experiment, animals were then crossed over, with those that had received A₁AS in first arm of experiment now receiving A₁MM and those that had received A₁MM in first arm of experiment now receiving A₁AS. A₁MM2-treated animals comprised separate group. **b**, Results are presented as the mean ± SEM. Significance was determined by repeated-measures analysis of variance and Tukey's protected *t* test. *Two asterisks*, Significantly different from all other groups, *P* < .01. (From Nyce JW, Metzger WJ. DNA antisense therapy for asthma in an animal model. *Nature* 1997;385:721-5. Reprinted by permission Macmillan Magazines Ltd.)

analysis of gene expression and differential display, new genes may be identified as targets for DNA vaccination or for antisense.⁴⁴

Modified allergen DNA with or without CpG motifs

Many modifications of allergen immunotherapy have been attempted to improve its safety and effectiveness.⁴⁵ None of these have been overwhelmingly successful for one reason or another. However, allergen has been modified in several ways by molecular techniques.

Allergen variants. First, the allergen itself has been modified by site-directed mutagenesis to dictated IgE binding regions while retaining T-cell reactivity.^{46,47} Allergen variants may become useful immunogens with excellent safety profiles, reducing the possibility of anaphylaxis.

Allergen DNA

Several methods use plasmid (naked) DNA constructs of the allergen gene that can be selected to induce the responses.³⁹ Antigens such as hepatitis B can be given with CpG motifs and are better adjuvants than cholera toxin.⁴⁸ Antigen alone produces no immune response.

Finally, DNA may be modified to contain CpG motifs and stimulate primarily TH1 responses,^{49,50} induce tolerance,⁵¹ and inhibit IL-5 eosinophilic inflammation and BHR.⁴¹ These may be administered alone or as ISS or be modified from allergen-derived sequences with CpG to have an adjuvant effect.³⁹

Mechanism of action and effects of immune CpG DNA

Theoretically, DNA-binding proteins on the cell surface help DNA move into the cytosol compartment where they are acidified and degraded.³⁸ DNA internalization may also be mediated by absorptive endocytosis. Although all cells seem to be capable of internalizing DNA, monocyte-derived B cells may be induced to take up DNA rapidly.

CpG DNA not only promotes nonspecific innate immunity but also generates antigen-specific immune responses. CpG motifs synergize with the antigen receptor⁴⁸ on B cells to prevent apoptosis and stimulate a sustained immune response. They may synergize with costimulatory molecules such as class II MHC and other costimulatory genes⁵² to enhance the immune response, or they may stimulate antitumor immune responses to

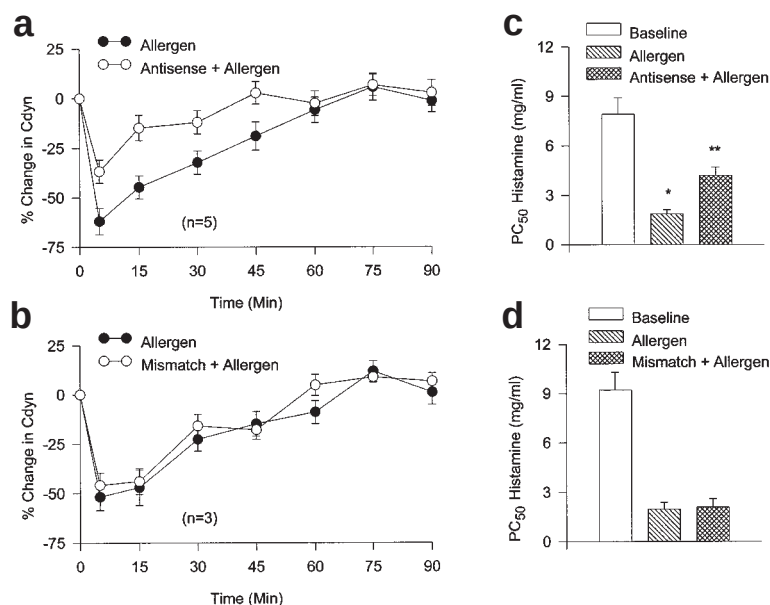


FIG 3. Effect of antisense and mismatch ODNs on allergen-induced airway obstruction and BHR in allergic rabbits. **a**, Effect of A₁AS antisense ODN in allergen-induced airway obstruction. As calculated from area under curve, A₁AS significantly inhibited allergen-induced airway obstruction (55%, $P < .05$; repeated-measures analysis of variance and Tukey's test). **b**, Lack of effect of mismatch control A₁MM on allergen-induced airway obstruction. **c**, Effect of A₁AS antisense ODN on allergen-induced BHR. As calculated from PC₅₀ histamine, A₁AS significantly inhibited allergen-induced BHR in allergic rabbits (61%, $P < .05$; repeated measures analysis of variance, Tukey's t test). **d**, Lack of effect of A₁MM mismatch control on allergen-induced BHR. (From Nyce JW, Metzger WJ. DNA antisense therapy for asthma in an animal model. *Nature* 1997;385:721-5. Reprinted by permission Macmillan Magazines Ltd.)

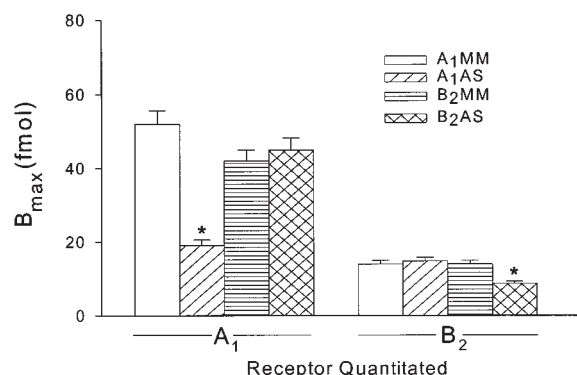


FIG 4. Maximum receptor binding of rabbit lung tissue after treatment with adenosine A₁ receptor antisense (A₁AS) or bradykinin B₂ receptor antisense (B₂AS). A₁ antisense or mismatch did not affect B₂ receptor binding; B₂ antisense or mismatch did not decrease A₁ receptor binding. (Adapted from Metzger JW, Nyce JA. DNA antisense therapy for asthma. In: Marone G, Austin KF, Holgate ST, Kay BA, Lichtenstein LM, editors. *Asthma and allergic diseases, physiology, immunology, and treatment*: 5th international symposium. London: Academic Press; 1998. p 382.)

tumor antigens. They have been described to be actually more potent than Freund's adjuvant in producing TH1-like vaccine responses.⁵³ These vaccines are relatively inexpensive (\$200 per gram or less),³⁸ provide nuclease resistance through phosphorothioate oligonucleotide syn-

thesis, and have a relative lack of toxicity at low doses.⁵⁴ An effective dose of human DNA vaccine has been estimated to be approximately 500 μ g.

Potential toxicity of DNA

Inappropriate or excessive immune response to CpG DNA is a theoretic possibility. In certain models increased production of INF- γ and TNF- α made animals susceptible to mortality and sepsis and to the toxic effects of TNF- α . Naturally occurring DNA with CpG motifs has been found in cystic fibrosis and may participate in an inflammatory role in chronic infections. Krieg and Steinberg⁵⁵ have proposed that it may also act as a trigger for lupus and may stimulate B cells.

ANTISENSE TECHNOLOGY FOR ASTHMA

Antisense oligonucleotides

Antisense oligonucleotides (ASOs) are single-stranded nucleic acids, generally 15 to 24 bases long, chemically modified to prevent rapid nuclease digestion, which can impede the template properties of messenger RNA (mRNA). The end result is the loss of the ability to synthesize the target protein. Cellular quantities of the target protein then diminish over time with kinetics that depend on the half-life of the protein and DNA strand, the half-life and total amount of the mRNA, and other factors.

Respirable ASONs in an animal model for asthma

Recently we have extended ODN technology^{8,11,12} to administer antisense DNA by aerosol directly into the lung of allergic rabbits to modify the production of receptors for adenosine. This local administration of antisense has been called respirable ASON. This came about as a result of a serendipitous homology between the human A₁ receptor and the rabbit A₁ receptor.¹² In these initial studies antisense against A₁ was aerosolized into allergic rabbits on 2 successive days (10 mg each day in divided doses). Treated rabbits were found to have undetectable responses to adenosine challenge and modified immediate airway responses and reduced histamine sensitivity (Figs 2 and 3).¹² Several controls with a bradykinin B2 antisense and mismatch or A1 antisense and mismatch documented specificity of the antisense for the A1 receptor (Fig 4). Later experiments demonstrated that the duration of effect for the antisense, measured by a reduction in sensitivity to adenosine, averaged 6.83 days compared with 1.7 days for rabbits given sodium chloride solution (Fig 5). In these studies occasional rabbits were found to have effective antisense reduction in adenosine sensitivity for as much as 10 days. Preliminary studies have shown inhibition of late-phase airway responses, in addition to the early phase of airway obstruction.

Advantages of antisense over traditional small-molecule drugs

The advantages of antisense therapy over traditional drugs are that (1) it targets only mRNA, (2) it is many times more efficient, (3) it has remarkable specificity, (4) there is design simplicity in that both target and receptor share some common chemistry, (5) small doses can be given to enhance safety, and (6) in our studies accessibility seems to be possible with low toxicity and dispersion throughout the airways.⁵⁶

Future of antisense technology

Antisense applications have been used for HIV, cancer, and organ transplantation.^{54,57} For respiratory diseases the potential is great, including lung cancer, chronic obstructive pulmonary disease, BHR and asthma, and other respiratory diseases. Antisense technology can target virtually any mediator or receptor, cytokine, chemokine, adhesion molecule, enzyme, transcription factor, or second messenger molecule. Antisense for GATA-3 or CCR3, for example, may be uniquely suited for antisense therapy in asthma, and antisense for IgE has already been proved effective in an animal model.⁵⁸

Future of ODN therapy

Therefore ODNs represent enormous potential for the treatment of allergic diseases with enhanced efficacy, specificity, and safety. ODNs with CpG motifs may induce immune surveillance at the molecular level; combined with antigen, there could be an improved form of allergy immunotherapy. Antisense technology provides

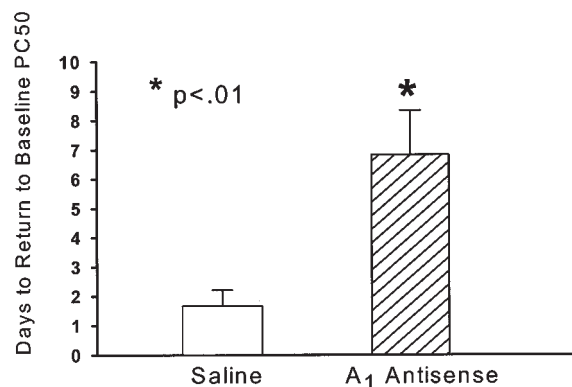


FIG 5. Average duration of effect of A₁ antisense (6.83 ± 1.4 days) compared with sodium chloride (1.7 ± 0.5 days) in rabbit model, as measured by response to adenosine.

unique specificity and probably safety when given locally and could possibly be combined to incorporate several targets. Potentially, the beneficial effectiveness of DNA vaccination and antisense DNA could be imagined to work together in a new more effective form of allergy immunotherapy with safety. The proof of this, of course, awaits human studies, which are in progress.

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