

High-Affinity Oligonucleotide Ligands to Human IgE Inhibit Binding to Fcε Receptor I

Torsten W. Wiegand,^{1*} P. Brock Williams,^{2†} Stephen C. Dreskin,[†] Marie-Helen Jouvin,[‡] Jean-Pierre Kinet,[‡] and Diane Tasset*

Using the systematic evolution of ligands by exponential enrichment (SELEX) method, we have identified oligonucleotides that bind to human IgE with high affinities and high specificity. These ligands were isolated from three pools of oligonucleotides, each representing 10^{15} molecules: two pools contained 2'-NH₂ pyrimidine-modified RNA with either 40 or 60 randomized sequence positions, and the third pool contained ssDNA with 40 randomized sequence positions. Based on sequence and structure similarities, these oligonucleotide IgE ligands were grouped into three families: 2'-NH₂ RNA group A ligands are represented by the 35-nucleotide truncate IGEL1.2 ($K_d = 30$ nM); 2'-NH₂ RNA group B ligands by the 25-nucleotide truncate IGEL2.2 ($K_d = 35$ nM); and the ssDNA group ligands by the 37-nucleotide truncate D17.4 ($K_d = 10$ nM). Secondary structure analysis suggests G quartets for the 2'-NH₂ RNA ligands, whereas the ssDNA ligands appear to form stem-loop structures. Using rat basophilic leukemia cells transfected with the human high-affinity IgE receptor FcεRI, we demonstrate that ligands IGEL1.2 and D17.4 competitively inhibit the interaction of human IgE with FcεRI. Furthermore, this inhibition is sufficient to dose-dependently block IgE-mediated serotonin release from cells triggered with IgE-specific Ag or anti-IgE Abs. Therefore, these oligonucleotide ligands represent a novel class of IgE inhibitors that may prove useful in the fight against allergic diseases. *The Journal of Immunology*, 1996, 157: 221–230.

The SELEX³ (systematic evolution of ligands by exponential enrichment) process is a methodology of rapid drug discovery, utilizing the power of combinatorial chemistry to create a large pool of potential therapeutic candidates in conjunction with in vitro selection to isolate the molecules possessing the desired characteristics (1–3). It has become apparent that nucleic acid molecules are capable of adopting a diverse set of three-dimensional structures (4, 5). A large pool of single-stranded oligonucleotides, differing in nucleotide sequence, will, therefore, represent a vast array of differently shaped molecules, some fraction of which has a high affinity for binding to a given target protein. These rare ligands can be isolated from the pool of undesired molecules by the SELEX process using iterative rounds of affinity-based enrichment alternating with oligonucleotide amplification. This approach resembles the immune system, in which recombination and mutation are used to create a library of different Abs. Igs that are useful in the protection of the host from the environment by binding tightly to Ags are selected and rapidly amplified (6, 7). The SELEX methodology has been used to isolate ligands with high affinities for diverse targets, including both nu-

cleic acid-binding proteins (1, 8–11) and non-nucleic acid-binding proteins (12–15), as well as simpler organic molecules like antibiotics (16), theophylline (17), and organic dyes (18). More recently, in vitro selection schemes have yielded oligonucleotides capable of catalyzing organic reactions, spurring the interest to recreate the “RNA world” (19–21).

A particularly interesting immunologic target for the SELEX process is human IgE. IgE Abs have been demonstrated to play a role in the mammalian defense system against parasites (22, 23) by eliciting physiologic responses including local inflammation, itching, mucus production, and bronchial constriction to purge the parasites from the organism. In atopic individuals, however, exposure to environmental Ags can cause the pathologic overproduction of allergen-specific IgE, causing severe IgE-mediated diseases including allergies, atopic dermatitis, and allergic asthma (24, and references therein). A crucial step in eliciting these responses is the binding of IgE to the high affinity receptor FcεRI (25, 26) with a K_d of approximately 10^{-10} M (24). The contact between these proteins has been mapped and includes the N-terminal Cε3 domain of IgE (27, 28) and the second domain of the FcεRI α-polypeptide (29, 30). Cross-linking of these IgE/FcεRI receptor complexes, located on the surface of mast cells and basophils, by multivalent IgE-specific Ags triggers a complex series of intracellular events leading to the activation of these cells. Within minutes, activated mast cells and basophils release preformed mediators, stored within their granules, and newly formed lipid mediators. In addition, over several hours these cells synthesize and secrete pro-inflammatory cytokines. Taken together, these inflammatory mediators elicit many of the symptoms of acute and chronic inflammation (31). A promising approach to intervene in IgE-mediated diseases is to block the interaction of IgE with the FcεRI receptor (32). Attempts to develop therapeutic IgE antagonists have been limited to the use of recombinant peptides composed of structural elements from IgE or FcεRI (33, 34) and the engineering of humanized anti-IgE mAbs (35). In this work, we describe and

*NeXstar Pharmaceuticals, Boulder, CO 80301; [†]Division of Allergy and Clinical Immunology, Department of Medicine, University of Colorado Health Sciences Center, Denver, CO 80262; and [‡]Department of Pathology, Beth Israel Hospital, Boston, MA

Received for publication December 13, 1995. Accepted for publication April 17, 1996.

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¹ Address correspondence and reprint requests to Dr. Torsten Wiegand, NeXstar Pharmaceuticals, 2860 Wilderness Place, Suite 200, Boulder, CO 80301.

² Current address: IBT Reference Laboratory, Lenexa, KS 66214.

³ Abbreviations used in this paper: SELEX, systematic evolution of ligands by exponential enrichment; RBL, rat basophilic leukemia.

characterize oligonucleotide ligands, isolated by the SELEX process, that bind human IgE with high affinities. Moreover, we demonstrate that two of the three ligand groups inhibit the binding of IgE to the high affinity receptor FcεRI.

Materials and Methods

Reagents and cell culture

Human IgE used in this SELEX procedure was purchased from Athens Research Technology (Athens, GA). Myeloma IgE_{PS}, used in the serotonin release assay, was the gift of Dr. Henry Metzger (National Institutes of Health, Bethesda, MD). Serum JP, obtained with informed consent (kind gift of Dr. John C. Selner, Allergy Respiratory Institute, Denver, CO) from a patient with seasonal allergic rhinitis, contained 1.28 µg/ml of IgE; 12.2% of this IgE was specific for grass Ags (CAP System; Pharmacia, Columbus, OH). Goat anti-human IgE was raised by immunization of a goat with polyclonal IgE obtained from a patient with the hyperIgE (Job's) syndrome (36), and was affinity purified on a myeloma IgE_{PS} agarose column. Timothy grass extract (100,000 allergenic U/ml; Pharmacia) was used without further purification. Cells from the rat basophilic leukemia cell line RBL-2H3 were electroporated with the human FcεRI α- and β-chain cDNAs subcloned into vector pCDL-Sra 296 (37) and the γ-chain cDNA subcloned into vector pBJ1neo (38). Cell line RBL SX-38 was isolated by testing neomycin-resistant clones for surface expression of human FcεRI using flow cytometry with an FITC-labeled anti-human α Ab. For the oligonucleotide ligand assays described herein, RBL SX-38 cells were carried in continuous culture as published for RBL-2H3 cells (39), except that 1.2 mg/ml of geneticin (Life Technologies, Gaithersburg, MD) was added every other time the cells were split. The TAN IgE (40) was purified from cell culture supernatant by affinity chromatography on a 3-nitro-4-hydroxy-5-iodophenylacetic acid BSA Sepharose 4B column, and biotinylated using biotin-X-N-hydroxy-succinamide (Calbiochem, San Diego, CA). The 2'-NH₂-modified CTP and UTP nucleotides were prepared according to a method previously described (41). DNA oligonucleotides were either synthesized on an Applied Biosystems (Foster City, CA) 394 DNA synthesizer using Millipore Corp. (Bedford, MA) phosphoramidites, according to manufacturers' specifications, or obtained from Operon Technologies (Alameda, CA). All other reagents and chemicals were of the highest purity available and were purchased from standard commercial sources.

The SELEX process

The SELEX process has been described in detail elsewhere (1, 8, 42). For the 2'-NH₂ SELEX experiments, the DNA templates were designed to contain 40 or 60 random nucleotides, flanked by 5' and 3' regions of defined sequence. The constant regions include DNA primer-annealing sites for PCR and cDNA synthesis, as well as the consensus T7 promoter region to allow in vitro transcription. Two nanomoles (approximately 10¹⁵ molecules) of these ssDNAs were converted into double-stranded, transcribable templates by PCR amplification. The PCR reactions were performed in buffer containing 50 mM KCl; 10 mM Tris-Cl, pH 9; 0.1% Triton X-100; 3 mM MgCl₂; and 0.5 mM each of dATP, dCTP, dGTP, and dTTP, using 0.1 U/µl of Taq DNA polymerase. Transcription reactions contained 5 µM DNA template; 5 U/µl T7 RNA polymerase; 40 mM Tris-Cl (pH 8.0); 12 mM MgCl₂; 5 mM DTT; 1 mM spermidine; 0.002% Triton X-100; 4% polyethylene glycol 8000; 2 mM each of 2'-OH ATP, 2'-OH GTP, 2'-NH₂ CTP, and 2'-NH₂ UTP; and 0.25 µM [α-³²P]2'-OH ATP. The resulting RNA molecules were incubated with IgE in PBS, modified to contain 1 mM of Mg²⁺ ions (138 mM NaCl, 2.7 mM KCl, 8.1 mM Na₂HPO₄, 1.1 mM KH₂PO₄, and 1 mM MgCl₂, pH 7.4) for 10 min to allow binding to occur. Protein-RNA complexes were separated from unbound RNA by nitrocellulose filter partitioning, as described below. Bound RNA was recovered from filters by phenol/urea extraction. The RNA was reverse-transcribed into cDNA by avian myeloblastosis virus reverse transcriptase at 48°C for 60 min in buffer containing 50 mM Tris-HCl (pH 8.3), 60 mM NaCl, 6 mM Mg(OAc)₂, 10 mM DTT, 50 pmol DNA primer, 0.4 mM each of dNTPs, and 1 U/µl avian myeloblastosis virus reverse transcriptase. PCR amplification of this cDNA resulted in approximately 500 pmol of dsDNA that was used to initiate the next round of SELEX. The ssDNA SELEX experiments were initiated with pools of 10¹⁵ molecules (2 nmol) containing a stretch of 40 random nucleotides, flanked by fixed sequences necessary for PCR amplification. Two biotin moieties were incorporated into the 3' PCR primer to allow the isolation of the nonbiotinylated strand of the PCR products on denaturing polyacrylamide gels. This material was enriched for IgE ligands, as described below, and amplified by PCR to complete the cycle.

Nitrocellulose filter partitioning

Nitrocellulose filter partitioning was used to enrich for oligonucleotides that bind tightly to IgE. Filter disks (nitrocellulose/cellulose acetate-mixed matrix, 0.45-µm pore size; Millipore Corp.) were placed into a vacuum manifold (Millipore Corp.) and wetted by aspiration of 5 ml of modified PBS buffer (see above). ³²P-labeled oligonucleotide pools were incubated in 2- to 10-fold molar excess of IgE in modified PBS for 10 min at 37°C and aspirated through the filter disks, followed immediately by a 5-ml modified PBS wash. The concentration of oligonucleotides and proteins was decreased during successive rounds from 10⁻⁶ M and 5 × 10⁻⁶ M to about 10⁻⁹ M and 2 × 10⁻⁹ M, respectively, to keep the fraction of protein-bound ligands below 5% of input material. The background of oligonucleotides binding nonspecifically to the filter membrane was reduced by an additional wash under mildly denaturing conditions (5 ml of 0.5 M urea) and by prefiltration of the oligonucleotides through nitrocellulose filters in later rounds of the SELEX experiment.

K_d determination

Nitrocellulose filter partitioning, as described above, was used to determine the equilibrium dissociation constants (K_ds) of the enriched ligand pools, the full-length clones, and the truncated ligands. The concentration of unlabeled ATP in transcription reactions for the RNA ligands was reduced to 0.2 mM to obtain higher specific activities. Ligands at concentrations of less than 5 × 10⁻¹¹ M were incubated with IgE dilution series ranging from 10⁻¹⁰ M to 10⁻⁶ M. Oligonucleotide concentrations were calculated from absorbance readings at 260 nm (43). The filter disks were air dried and quantitated in liquid scintillation counters (Beckmann Instruments, Palo Alto, CA). Equilibrium dissociation constants were determined using equations described elsewhere (15). The data sets were fitted by the least square method with the Kaleidagraph software package (Synergy Software, Reading, PA).

Cloning and sequencing

During the last round of the SELEX process, the PCR-amplification step was performed with primers that contain recognition sites for the restriction endonucleases *Hind*III (5' primer) and *Bam*HI (3' primer). Using these restriction sites, the DNA sequences were inserted directionally into pUC19 (2'-NH₂ RNA SELEX) or pUC9 (ssDNA SELEX), and the recombinant plasmids were transformed into *Escherichia coli* strain SURE (Stratagene, La Jolla, CA). Plasmid DNA was prepared with the ClearCut Miniprep kit (Stratagene, La Jolla, CA), and was sequenced with the Sequenase sequencing kit (United States Biochemical Corp., Cleveland, OH).

Ligand truncation

Truncation experiments (15) were conducted to determine the minimal sequence necessary for high-affinity binding of the 2'-NH₂ RNA ligands to IgE. For 3'-boundary determination, RNA ligands were 5' end labeled with [γ-³²P]ATP using T4 polynucleotide kinase. 5' boundaries were established with ligands 3' end labeled using [α-³²P]pCp and T4 RNA ligase. After partial alkaline hydrolysis, radiolabeled RNA ligands were incubated with IgE at concentrations ranging from 1 nM to 150 nM, and protein-bound RNA was separated by nitrocellulose partitioning. RNA truncates were analyzed on a high resolution denaturing polyacrylamide gel. A ladder of radioactively labeled ligands terminating with G residues was generated by partial RNase T1 digestion, and was used as marker.

IgE receptor-binding assay

A cell-based ELISA was used to measure the ability of the high-affinity oligonucleotide ligands to inhibit binding of IgE to the FcεRI receptor. RBL cell line SX-38 that expresses the α, β, and γ subunits of the human FcεRI IgE receptor was plated at a concentration of 10⁵ cells per well in 96-well flat-bottom microtiter plates. After overnight growth, the cells were washed three times in modified PBS and incubated for 1 h at 37°C in 30 µl of 1.5 µg/ml of biotinylated TAN IgE (40). The cells were washed three more times and incubated in 30 µl of 1/100 dilution of horseradish peroxidase conjugated to avidin (Molecular Probes, Eugene, OR) for 1 h at 37°C. After three final washes, 30 µl of 3,3',5,5'-tetramethylbenzidine (Kirkegaard and Perry, Gaithersburg, MD) was added as a chromogenic substrate for the horseradish peroxidase, and the plate was transferred immediately to the microtiter plate reader (Bio-Tek Instruments, Winooski, VT) for analysis. The absorbance at 630 nm was measured every 60 s over a time span of 15 min. The rate of color development is proportional to the extent of IgE binding to the FcεRI receptor, and was determined from the linear region of graphs, in which absorbance was plotted vs time. To test the effectiveness of oligonucleotide ligands to inhibit IgE binding to the receptor, biotinylated TAN IgE at a concentration of 8 × 10⁻¹² M was

preincubated with oligonucleotides at concentrations ranging from 10^{-5} M to 3.3×10^{-9} M for 30 min at 37°C. As control reactions, addition of oligonucleotides or IgE was omitted (maximum rate and background rate, respectively). The inhibition activities of the oligonucleotide ligands were determined using the following formula:

$$I_B = 100 \times (R_R - B_R) / (M_R - B_R)$$

I_B = relative percentage of IgE bound
 R_R = reaction rate
 B_R = background rate
 M_R = maximum rate

Inhibition data were fit by a standard competition equation and fitting procedure (44), assuming a 1:1 stoichiometry for the IgE/ligand interaction. Values for the inhibitory dissociation constants (K_i) were calculated based on the following estimates: 10^5 cells per well and 10^5 receptors per cell (unpublished data) in 30- μ l volume, resulting in a receptor concentration of 5×10^{-10} M. The IgE concentration in this assay was 8×10^{-12} M, and the dissociation constant for the IgE/receptor complex is approximately 10^{-10} M (24). Substituting transfected RBL SX-38 cells with the untransfected parental cell line resulted in reaction rates similar to the background rate (data not shown).

Serotonin release assay

Eighteen hours before an experiment, cells (2.5×10^5) were resuspended in 500 μ l of culture medium without geneticin, but with 1 μ Ci/ml of [^{3} H]serotonin (DuPont NEN, Boston, MA), and plated into 24-well tissue culture dishes. On the day of the experiment, 2'-NH₂ RNA or DNA ligands were diluted with water to 300 μ g/ml, and then with sodium PIPES buffer (20 mM Na₂ PIPES, 100 mM NaCl, 5 mM KCl, 0.8 mM MgCl₂, and 0.1% BSA, pH 7.0) to achieve one-sixth of the desired final concentration. Finally, 10 μ l of the diluted IgE ligands were added to 50 μ l of sera, resulting in final concentrations of 2.8×10^{-5} M to 3.3×10^{-8} M. The sera plus buffer, 2'-NH₂ RNA ligands, or DNA ligands were left at room temperature for 30 min. At this time, 375 μ l of assay buffer was added to each treated serum sample, and this mixture was added to duplicate samples of cells that had been washed twice with assay buffer. The cells were then incubated for 2 h at 37°C in 5% CO₂, 95% humidity to allow sensitization. The cells were washed twice with sodium PIPES buffer and finally resuspended in Na₂ PIPES buffer containing 100 μ M of 5'-N-ethylcarboxamide adenosine (Sigma Chemical Co., St. Louis, MO), an analogue of adenosine that is known to synergize with Fc ϵ RI (45, 46). The duplicate wells were then incubated for 30 min at 37°C with either buffer, goat anti-human IgE (3 μ g/ml; the optimal dose), or grass extract (8 allergenic U/ml; the optimal dose). The values for serotonin release were calculated by division of the [^{3} H]serotonin in the supernatant by the sum of [^{3} H]serotonin left in the cells (determined by solubilization with 0.1% Triton X-100 in PBS), plus the [^{3} H]serotonin in the supernatant. The resulting fractions were converted into percentages by multiplication with 100. The background release of [^{3} H]serotonin during the 30-min incubation without stimulus varied between 4 and 10% in the experiments shown, and has been subtracted.

Competition assays

^{32} P-labeled oligonucleotide IgE ligands at a final concentration of 50 nM were added to a dilution series of unlabeled competitor ligands spanning the concentration range of 3×10^{-10} M to 10^{-5} M. After addition of IgE to a final concentration of 50 nM, the reactions were incubated and partitioned under conditions described for the filter-binding assay. Self-competitions were performed as controls. In all competition experiments, the labeled and unlabeled ligands were mixed before the addition of IgE to eliminate potential effects of different ligand/IgE off-rates.

Results

RNA sequences and affinities

Separate SELEX experiments targeting human IgE were performed using 2'-NH₂ RNA or ssDNA random sequence libraries. To address the question as to whether a longer random region results in isolation of ligands with higher affinities to the target protein, the RNA SELEX experiments were initiated with two separate pools of 2'-NH₂ RNA containing either 40 or 60 random nucleotides in the central portion of the molecules (40N7 and 60N7, respectively). These random regions are flanked by 5'- and 3'-fixed regions that are required as primer binding sites for reverse transcription and PCR amplification (Fig. 1A). All of the RNA molecules in these experiments were transcribed with 2'-

A) 2'-NH₂ RNA SELEX

Starting RNA: 40N7 or 60N7

5' - GGGAGGACGAUGCGG - 40N/60N - CAGACGACUGCCCGA - 3'

5' PCR Primer: 5P7

5' - **TAATACGACTCACTATAGG**GAGGACGATGCGG - 3'

3' RT / PCR Primer: 3P7

5' - TCGGGCGAGTCGTCTG - 3'

B) ssDNA SELEX

Starting DNA: 40NBHI

5' - CTACCTACGATCTGACTAGC - 40N - GCTTACTCTCATGTAGTTCC - 3'

5' PCR Primer: 5BHI

5' - CTACCTACGATCTGACTAGC - 3'

3' PCR Primer: 3BBHI

5' - **AJAJAGGA**ACTACATGAGAGTAAGC - 3'

FIGURE 1. Sequences of starting RNAs, DNAs, and PCR primers used in the SELEX experiments. The T7 promoter is shown in boldface and biotin moieties are indicated by the letter J.

NH₂-modified CTP and UTP to increase the in vivo stability of the potential IgE ligands (13). The starting pools of 3×10^{14} RNA molecules (500 pmol) bind IgE with estimated K_d s of greater than 50 μ M. IgE-bound ligands were separated from unbound RNA molecules on nitrocellulose filters and reamplified, as described in *Materials and Methods*. After nine rounds of ligand partitioning and reamplification, the affinities of the evolving pools for IgE had improved about three orders of magnitude and did not shift further in subsequent rounds (data not shown). RNA pools from round 9 RNA were bulk sequenced and found to be nonrandom. The cDNAs from this round were cloned into pUC19, and 35 clones each from the 40N7 and the 60N7 SELEX experiment were sequenced.

Twenty-five clones from the 40N7 SELEX experiment and 30 clones from the 60N7 SELEX experiment were found to be unique. Based on sequence similarities, high-affinity IgE ligands from both the 40N7 and the 60N7 libraries can be divided into two groups, A and B, as depicted in Figure 2A. In addition, a group of nitrocellulose filter-binding clones was identified (data not shown) that were not further analyzed. Ligands in groups A and B contain domains of highly conserved sequence comprising 10 or 20 nucleotides, respectively, that are located immediately adjacent to the 5'-fixed region (Fig. 2A). The remaining bases of the V regions 3' to these conserved stretches show no observable sequence conservation and are, therefore, unlikely to contribute to the specific binding of IgE. As groups A and B contain members from both the enriched 40N7 and 60N7 SELEX pools, independent SELEX experiments with different length variable regions resulted in the isolation of essentially identical ligands. In addition to these two groups of IgE ligands, nine orphan clones were identified that show no identifiable homology to these families or to each other.

Binding affinities of representative members of groups A and B, as well as orphan sequences to human IgE, were determined by

A		5' Fixed Region	Random Region	3' Fixed Region
Group A				
Clone	gggaggacgaugcgg -			- cagacgacucgcccga
IGEL 1.1			GUGUGAAUGGUGUGUGAGG	UUACUGUACUUCGGUGGCUG
IGEL21.1			GUGUGAACGGUGUGUGAGG	UUACUGUACUUCGGUGGCUG
IGEL22.1			GUGCGAAUGGUGUGUGAGG	AGCCUAAAUACGGAUUGGU
IGEL23.1			GUGUGAAUGGUGUGUGAGG	ACUCCGAAAUUCCCCAGGGC
IGEL24.1			GUGUGAAUGGUGUGUGCGAGG	CAUGCAGGAGGCGCUGUGU
IGEL25.1			GUGUGAAUGGUGUGUGAGG	ACUUUACAGGCUCGUGUG
IGEL26.1			GUGUGAAUGGUGUGUGAGG	UUACUGCACUUCGGCGCU
IGEL27.1			GUGUGCAUGGUGUGUGAGG	CUGAGUAUAGGGCCUGCGU
IGEL28.1			GUGUGAAUGGUGUGUGAGG	AUGGAUUCGACAUAGCGAU
IGEL29.1			GUGUGCAUGGUGUGUGCGAGG	CAAAAUAACAGCGCAUUAUUCUGGCCAUGUUGCGUGCAUA
IGEL30.1			GUGCGAAUGGUGUGUGAGG	AGUGAAUAUAGGUGGAUACCCUUAACAACUGCGUGGGU
IGEL31.1			GUGUGAAUGGUGUGUGAGG	UUUCUGACUGUUUGUGUCUAGCCGUACUUUAGCCUCCG
Consensus			GUGUGAAUGGUGUGUGAGG	
Minimal Sequence	aggacgaugcgg		GUGUGAAUGGUGUGUGAGG	
IGEL1.2	gggaggacgaugcgg		GUGUGAAUGGUGUGUGAGG	
Group B				
Clone	gggaggacgaugcgg -			- cagacgacucgcccga
IGEL 2.1			GUGUGGGGCGAAUGGAGAAACGUUACCAGGAAAUGCGACUG	
IGEL41.1			GUGAGGGGCGAAUGGAGAAACAUAGAGACAGGAGAAUGCGG	
IGEL42.1			GAGUGGGGCGAAGGUAAUGUUGAGACGAUGUAAGACUGGU	
IGEL43.1			GCUGGGGCGCAUUCAUUAUACUGCUUAAAGGUCACGGGUC	
IGEL44.1			GUCCGGGCGAGUAUAUGAAACUUGGCUUGGUAAUGAUCAGAAUGAGAGAACUGGGUG	
IGEL45.1			GAGUGGGGCGUAGGAUUUUGCCACUUGGAUUUGGACAGUGAGCAUCAGAGUCAUACCCGC	
IGEL46.1			GAGUGGGGCGGAUAUAACUAUGUGUGCGUAAUUGUCCUGUCGGUGUCACGAACCUUGUG	
IGEL47.1			GUGUGGGGCGGAUAUAUGAGUGAAACAGAGUGAAAUCCAGCGUACGCAGACUGUGCUGCG	
IGEL48.1			GAGUGGGGCGAUGAGAGAGAUACAGAGAACUAGAAUGAUACAAAUCUGAGGUUGUGCG	
IGEL49.1			GUGUGGGGCGGAUAUAUGAGUGAAACAGAGUGAAAUCCAGUGUAGCCAGACUGUGCUGUGC	
Consensus			GUGUGGGGCG	
Minimal Sequence	aggacgaugcgg		GUGUGGGGCG	
IGEL2.2	gggaggacgaugcgg		GUGUGGGGCG	
B		5' Fixed Region	Random Region	3' Fixed Region
Clone	ctacctacgatctgactagc -			- gcttactctcatgtagttcc
D 58.0			agcCCCTTCATCCGTTA	CTCTTAAGTGGGGGCTTGCGATTTCGAGT
D 169.0			gcGGCTTTATCCGTAACTCT	TTAGTGGGCCGCTCGCTTCACA
D 162.0			TGGCATTCATCCGCTCT	CTCCTAAGTGGTGGCTTGTCGCCCA
D 152.0			AGGGATCTTCATCCGTTT	CTCTCAAGTGGCATCCCGTGGCT
D 61.0			CTACGCGCTTTATCCGTTT	CTCCCAAGTGGCGGGCGGCTTC
D 53.0			CGAAGTTAAATTTATCCGTCC	CTCCTAAGTGGTCTTAACAGCG
D 31.0			ACGGGTGAGTTGATCCGTTA	CTCTTAAGTGGTGAACCTTGT
D 172.0			CTTGCTCCATTTATCCGTTT	CTCCCAAGTGGTGGTTGTCATG
D 80.0			GTAGCGCGCTTTATCCGTTT	CTCCCAAGTGGCGGGCGGCTTC
D 2.0			GGCCGGAGCTTTATCCGTTA	CTCTCAAGTGGGTGACTGTC
D 153.0			CAAAGTTAAATTTATCCGTCC	CTCTCAAGTGGTTAACAGCG
D 59.0			GATGGGAGCTTTATCCGTTT	CTCTCAAGTGGCTCTCTCAG
D 12.0			CTACAGCCCATTTATCCGTTT	CTCCTAAGTGGTGGGCTGCT
D 11.0			ACCGGAGTACTTTATCCGTCC	CTTCTAAGTGGGTACCCGTA
D 56.0			TGCTGGACAAATTTATCCGTTA	CTCTTAAGTGGTTGTGTGCT
D 173.0			AATGGTCCAGCTTTATCCGCT	CTTCTAAGTGGCGTCATT
D 51.0			AAGGCGACTACTTTATCCGTTT	CTCTTAAGTGGGTATCCG
D 165.0			GTGMCGGGATCTTTATCCGTTA	CTCTTAAGTGGGTCTCG
D 69.0			GGCCGTAAAGCACTTTATCCGTAACTCT	CTCAAGTGGGTA
D 17.0			CAATGAGTGATCACCTTTATCCGTCC	CTCCTAAGTGGGCTG
D 52.0			CGCGGTACGAGCACCTTCATCCGTCC	CTCCTAAGTGGGCTGct
D 54.0			AGGTCATGCGAGTATGCTTTATCCGTAACTCT	CTCAAGTGGgc
D 160.0			CAATTGCTGAAGGAAGCAATTTATCCGTTC	CTCTTAAGTGGTgctt
D 9.0			ATGGGAGAACACTTAGCCCTTCATCCGTTC	CTCCTAAGTGGgctt
D 66.0			ACAGCATGAGAGATATAGCTTTATCCGTGA	CTCTCAAGTGGgct
D 151.0			TACCCGCGATGAGAGTAAGTTTATCCGTGTACTCT	TTAGTGGcttactctcat
Consensus			(N ₁ -11) - TTTATCCGTTT CTCTTAAGTGG - (N ₁ -11)	
D17.1			CACGTTTATCCGTCC	CTCCTAAGTGGGCTG
D17.4			GGGGCACGTTTATCCGTCC	CTCCTAAGTGGCGTGCCCC

	Ligand	SELEX Pool	Dissociation Constant (K_d)
2'-NH ₂ RNA			
Group A			
	IGEL1.1	40N	138 nM
	IGEL25.1	40N	186 nM
	IGEL29.1	60N	141 nM
	IGEL31.1	60N	134 nM
	IGEL1.2	Truncate	30 nM
Group B			
	IGEL2.1	40N	41 nM
	IGEL44.1	60N	34 nM
	IGEL48.1	60N	30 nM
	IGEL49.1	60N	39 nM
	IGEL2.2	Truncate	35 nM
Orphans			
	IGEL3.1	40N	56 nM
	IGEL4.1	40N	87 nM
	IGEL5.1	40N	145 nM
	IGEL7.1	40N	142 nM
	IGEL8.1	40N	225 nM
	IGEL9.1	60N	105 nM
	IGEL10.1	60N	69 nM
	IGEL11.1	60N	129 nM
	IGEL13.1	60N	50 nM
ssDNA			
	D2.0	40N	6 nM
	D11.0	40N	8 nM
	D31.0	40N	22 nM
	D17.0	40N	9 nM
	D17.1	Truncate	82 nM
	D17.4	Truncate	10 nM

To determine the minimal sequence information necessary for high-affinity binding to IgE, truncation analysis was performed on representative members of group A (IGEL1.1 and IGEL31.1) and group B (IGEL2.1 and IGEL48.1). For these experiments, ligands were 5' or 3' radiolabeled, subjected to limited alkaline hydrolysis, and bound to IgE. After partitioning of the protein-bound RNA, the RNA fragments that retain high affinity for IgE were analyzed on high resolution denaturing polyacrylamide gels (1). The 3' boundaries of these ligands are located precisely at the end of the conserved sequence motifs in the variable region. This verifies the prediction that these conserved sequence motifs are necessary for binding to IgE, whereas the nonconserved remaining nucleotides

IGEL1.2

[illegible]

of the variable regions and the 3'-fixed regions are dispensable. The 5'-boundary determinations revealed that the entire 5'-fixed regions, with the possible exception of the first three guanines, are necessary for binding to IgE. Since efficient initiation of transcription from the T7 promoter requires G residues at the start of the transcript (47), the truncates were designed to contain the entire 5'-fixed region. The minimal consensus sequence IGEL1.2, representing group A ligands, is 35 nucleotides long; IGEL2.2, representing group B ligands, is 25 nucleotides in length. Using a thermodynamically based folding algorithm to predict secondary RNA structures (48), no convincing stem-loop motifs could be identified. The absence of stable stem-loop structures and the high G content of the isolated ligands makes it likely that the 2'-NH₂ RNA ligands form G quartets (49), as suggested for ligand IGEL1.2 in Figure 3A. Representative binding curves of these truncated RNA ligands in comparison with the starting RNA pools are shown in Figure 4A, and the K_d s of the oligonucleotide ligands for binding to human IgE are summarized in Table I. While the K_d

FIGURE 2. Aligned sequences of IgE ligands. The 5'- and 3'-fixed regions are shown in lower case letters; nucleotides derived from the variable region are shown in upper case letters. The consensus sequence motifs are indicated in bold type. A, 2'-NH₂ RNA group A and group B ligands. B, ssDNA ligands. Underlined nucleotides flanking the consensus region are believed to be base paired.

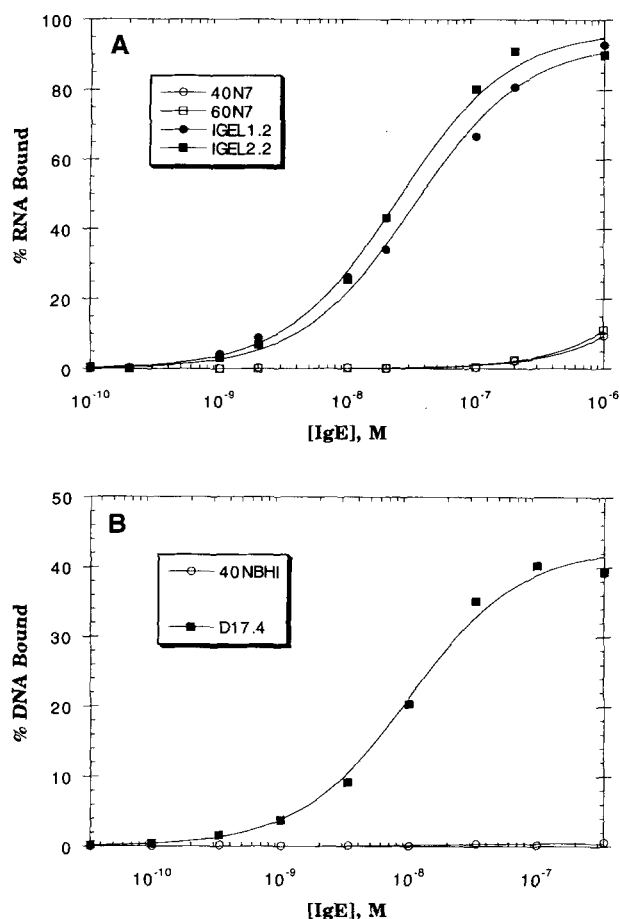


FIGURE 4. Representative binding curves of oligonucleotide ligands to IgE. The fraction of ^{32} P-labeled oligonucleotides bound to nitrocellulose filters is plotted as a function of total protein concentration, and the data points were fitted as described (15). Oligonucleotide concentrations were kept below 5×10^{-11} M. A, 2'-NH₂ RNA ligands. B, ssDNA ligands.

of IGEL2.2 is essentially unaffected by the truncation, IGEL1.2 actually binds several-fold better as a truncate compared with the full-length IGEL1.1 ligand. This result demonstrates that the removal of nucleotides that are not contributing to the binding of a protein can increase the affinity of this interaction, possibly due to the relief of steric hindrance or electrochemical repulsion caused by these unnecessary nucleotides, thereby allowing a tighter fit of the RNA to the surface of the protein.

DNA sequences and affinities

The ssDNA SELEX experiment was performed with a pool of molecules containing 40 random nucleotides. The starting DNA sequences and corresponding PCR primers are shown in Figure 1b. Approximately 10^{15} molecules (2 nmol) of 40NBHI DNA were used to initiate this SELEX experiment, and after 15 rounds of ligand partitioning and reamplification, the K_d of the DNA pool for human IgE had decreased from greater than $50 \mu\text{M}$ to about 10 nM. DNA molecules from round 15 were cloned into pUC9, and sequence information was obtained for 87 clones, 26 of which were found to be unique. Sequence analysis showed that all of these clones contain a 21-nucleotide conserved sequence motif that is located within the 40-nucleotide random region (Fig. 2B). In contrast to the 2'-NH₂ RNA ligands for which the minimal binding sequences include the 5'-fixed region, the binding motif of the

Table II. Dissociation constants of human IgE oligonucleotide ligands for rodent IgE and human IgG

Ligand	K_d for Mouse IgE	K_d for Rat IgE	K_d for Human IgG
IGEL1.2	$\geq 10 \mu\text{M}$	$\geq 10 \mu\text{M}$	$\geq 20 \mu\text{M}$
IGEL2.2	$\geq 10 \mu\text{M}$	$\geq 10 \mu\text{M}$	$\geq 20 \mu\text{M}$
D17.4	$\geq 10 \mu\text{M}$	$\geq 10 \mu\text{M}$	$\geq 20 \mu\text{M}$

DNA ligands does not extend into either of the fixed regions. This consensus sequence can be folded into a stem-loop structure. Nucleotides flanking this consensus sequence can base pair with each other, thereby extending the conserved stem by an additional 2 to 11 bp (Fig. 3A). Extensive covariation of this stem extension indicates that no specific primary sequence is required for this part of the IgE-binding motif.

To determine the minimal sequence requirements for high-affinity binding to human IgE, three ssDNA species were synthesized: D17.0, the full-length ligand of clone 17 (80 nucleotides); D17.1, containing the 21-base conserved sequence flanked by eight nucleotides forming a 4-bp stem extension (29 nucleotides); and 17.4 (Fig. 3B), which extends D17.1 with an additional four 5' G residues and four 3' C residues (37 nucleotides), resulting in a longer stem. Affinities of these and other representative DNA ligands for human IgE were determined by nitrocellulose filter-binding experiments, and the resulting K_d s are listed in Table I. The affinities of ligands tested from this pool are approximately 10 nM and show little variation from each other, which affirms the homogeneity of the enriched DNA pool. Compared with the full-length ligand D17.0 ($K_d = 9$ nM), the 29-base truncate D17.1, containing the conserved sequence motif and the 4-bp extended stem, binds with a K_d of 82 nM, indicating a ninefold weaker affinity to IgE. This affinity, however, could be restored to the level of the full-length ligand by the addition of a 4-bp stem extension resulting in the 37-nucleotide truncate D17.4 ($K_d = 9$ nM). It is possible that the shorter stem in D17.1 is not stable enough to prevent the formation of an alternative secondary structure and that the additional base pairs in D17.4 stabilize the conformation necessary for IgE binding. A representative binding curve of ligand D17.4 compared with the starting pool 40NBHI is shown in Figure 4B.

Ligand specificity

To test the specificity of the high-affinity interaction between the oligonucleotide ligands and human IgE, nitrocellulose filter-binding experiments were performed with rodent IgE (Table II). The K_d s of ligands IGEL1.2, IGEL2.2, and D17.4 for mouse IgE and rat IgE are greater than $10 \mu\text{M}$, which is 300- to 1000-fold higher than the corresponding dissociation constants to human IgE. Furthermore, the IgE ligands were tested for binding to human IgG, and those K_d s were determined to be greater than $20 \mu\text{M}$. These results demonstrate that the oligonucleotide ligands not only bind human IgE tightly, but are also very specific and do not bind significantly to related molecules.

Inhibition of the IgE/FcεRI interaction

To test the ability of the oligonucleotide ligands to inhibit the binding of IgE to the FcεRI receptor, a tissue culture cell-based assay was performed, as described in *Materials and Methods*. RBL SX-38 cells, expressing the human FcεRI receptor on the surface, were incubated with biotinylated IgE. Using streptavidin-conjugated horseradish peroxidase, the relative amount of IgE bound to the receptor is determined by measuring the rate of conversion of

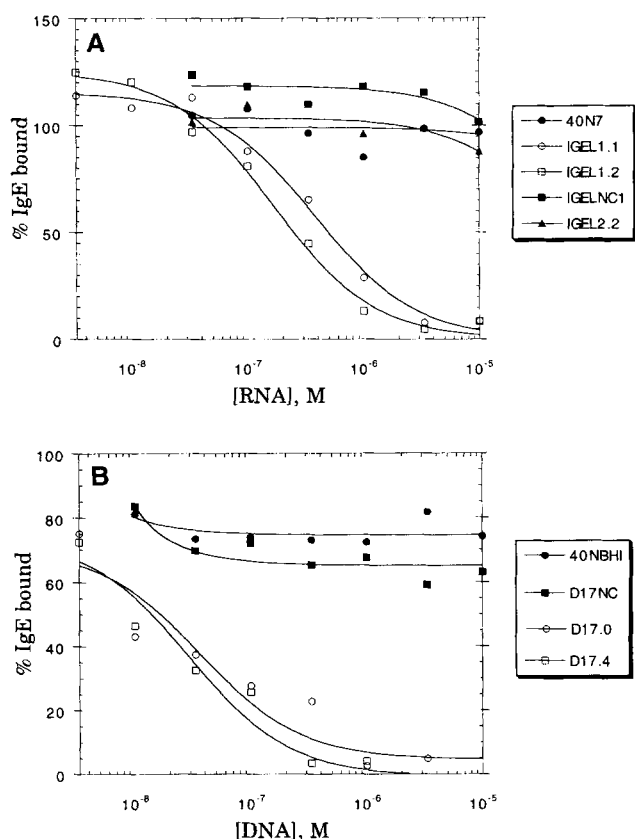


FIGURE 5. Inhibition of the IgE/FcεRI interaction by oligonucleotide ligands. Biotinylated IgE was incubated with increasing amounts of oligonucleotide ligands, and then allowed to bind to human FcεRI receptors expressed on the surface of RBL SX-38 cells. A chromogenic assay based on horseradish peroxidase-conjugated avidin was used to determine the fraction of IgE bound to FcεRI. *A*, 2'-NH₂ RNA ligands: IGEL1.1 (open circles) $K_i = 44$ nM; IGEL1.2 (open rectangles) $K_i = 21$ nM. *B*, ssDNA ligands: D17.0 (open circles) $K_i = 11$ nM; D17.4 (open rectangles) $K_i = 6$ nM.

a chromogenic substrate. The oligonucleotides to be tested for inhibition were incubated with the IgE before the mixture was added to the RBL cells. The results for the RNA ligands are shown in Figure 5A. Random 40N7 RNA does not inhibit IgE binding to the FcεRI receptor. Group A ligands IGEL1.1 and IGEL1.2, however, inhibit this interaction with inhibitory dissociation constants (K_i) of 44 nM and 21 nM, respectively. To further demonstrate that inhibition is dependent on the specific sequence and, therefore, on the structure of these ligands, a scrambled version of IGEL1.2 was designed. IGELNC1, containing the nucleotides of IGEL1.2 in randomized order, does not bind IgE better than the random starting pool (data not shown), and also does not inhibit the interaction of IgE with the FcεRI receptor (Fig. 5A). The orphan ligands tested in this assay show varying degrees of inhibition, but all of them are weaker inhibitors than IGEL1.1 and IGEL1.2 (data not shown). Although group B ligands show significant sequence similarity to group A ligands and bind IgE with similar affinities, IGEL2.2, representing group B ligands, does not show significant inhibition of the IgE/FcεRI interaction in this assay. This observation suggests that ligands IGEL1.2 and IGEL2.2 either bind IgE at different areas of the molecule, or that the binding orients the IGEL2.2 ligand differently and does not allow inhibition of the IgE/receptor interaction. Figure 5B shows the results of the inhibition assay for the DNA ligands. Random 40NBH1 DNA and D17NC, a scram-

bled sequence version of D17.4 with no enhanced affinity to IgE over the random DNA pool (data not shown), do not inhibit this assay. In contrast, DNA ligands D17.0 and D17.4 inhibit the interaction with K_i s of approximately 10 nM. In summary, these results demonstrate that binding of group A 2'-NH₂ RNA ligands and the ssDNA ligands to IgE causes specific and competitive inhibition of the interaction with the FcεRI receptor.

Inhibition of IgE-mediated serotonin release

To determine whether the level of inhibition by the IgE ligands is sufficient to prevent IgE-dependent cellular degranulation, a second assay based on the RBL SX-38 cells was used. RBL SX-38 cells, containing [³H]serotonin in their granules, were sensitized by incubation in serum from a grass-allergic patient. The cells were triggered by addition of either anti-IgE Abs or grass extract, and the release of [³H]serotonin was quantitated. To measure the ability of the IgE ligands to inhibit this release, the IgE-containing serum was preincubated with various concentrations of oligonucleotides. Figure 6, *A* and *B*, shows the results of this experiment for the RNA ligands, while Figure 6, *C* and *D*, shows results obtained for the DNA ligands. The RNA ligand IGEL1.2 and the DNA ligand D17.4, which showed inhibition of the IgE/FcεRI interaction, also block the IgE-mediated release of serotonin. The IC₅₀ values in this assay are approximately 2 to 6 × 10⁻⁶ M when the RBL cells are triggered with anti-IgE Abs (Figure 6, *A* and *C*) and 2 to 3 × 10⁻⁷ M for the Ag triggering. A possible explanation for the observation that Ag-triggered serotonin release is easier to block than Ab-triggered release, is that the serum collected from the grass-allergic patient contains only 12% grass-specific IgE. The Ag in the grass extract can only cross-link grass-specific IgE, whereas the anti-IgE Abs will cross-link all IgE regardless of specificity. This is corroborated by the observation that optimal anti-IgE Abs give greater net degranulation (55–65%, Figure 6, *A* and *C*) than does optimal grass Ag (40–45%, Figure 6, *B* and *D*). As expected, the RNA ligand IGEL2.2, which does not inhibit the IgE/FcεRI interaction, as well as the negative control ligands IGELNC1 and D17NC, all fail to inhibit the IgE-mediated serotonin release in this assay.

IgE ligand competition

Since ligands IGEL1.2 and D17.4 both inhibit the interaction of IgE with the FcεRI receptor, it seems likely that they both bind to the same site on IgE that hinders binding to FcεRI. To test this hypothesis, competition experiments, using the nitrocellulose filter-binding assay, were performed, in which ligands competed either with themselves or with each other for binding to IgE. Self-competition of ligands IGEL1.2, IGEL2.2, and D17.4 resulted in K_C values similar to previously established K_d values (data not shown). In cross-competitions, ligands IGEL1.2 and D17.4 compete efficiently for binding to IgE (Fig. 7). This result indicates that IGEL1.2 and D17.4 bind either to the same site on IgE or to sites in close proximity, ruling out the simultaneous binding of both ligands. In contrast, competition experiments of IGEL2.2 with IGEL1.2 or with D17.4 exhibit only partial competition, indicating that ligand IGEL2.2 binds at a site on IgE not identical with the contact point of ligands IGEL1.2 and D17.4. The most likely reason that IGEL2.2 does not block the IgE/FcεRI interaction is, therefore, that this ligand binds IgE at a site in which it does not cause steric interference with the contact of IgE and FcεRI.

Discussion

Using the SELEX methodology, three different classes of high-affinity oligonucleotide ligands to IgE were isolated: 2'-NH₂ RNA ligands group A and group B, and ssDNA ligands. Two of these

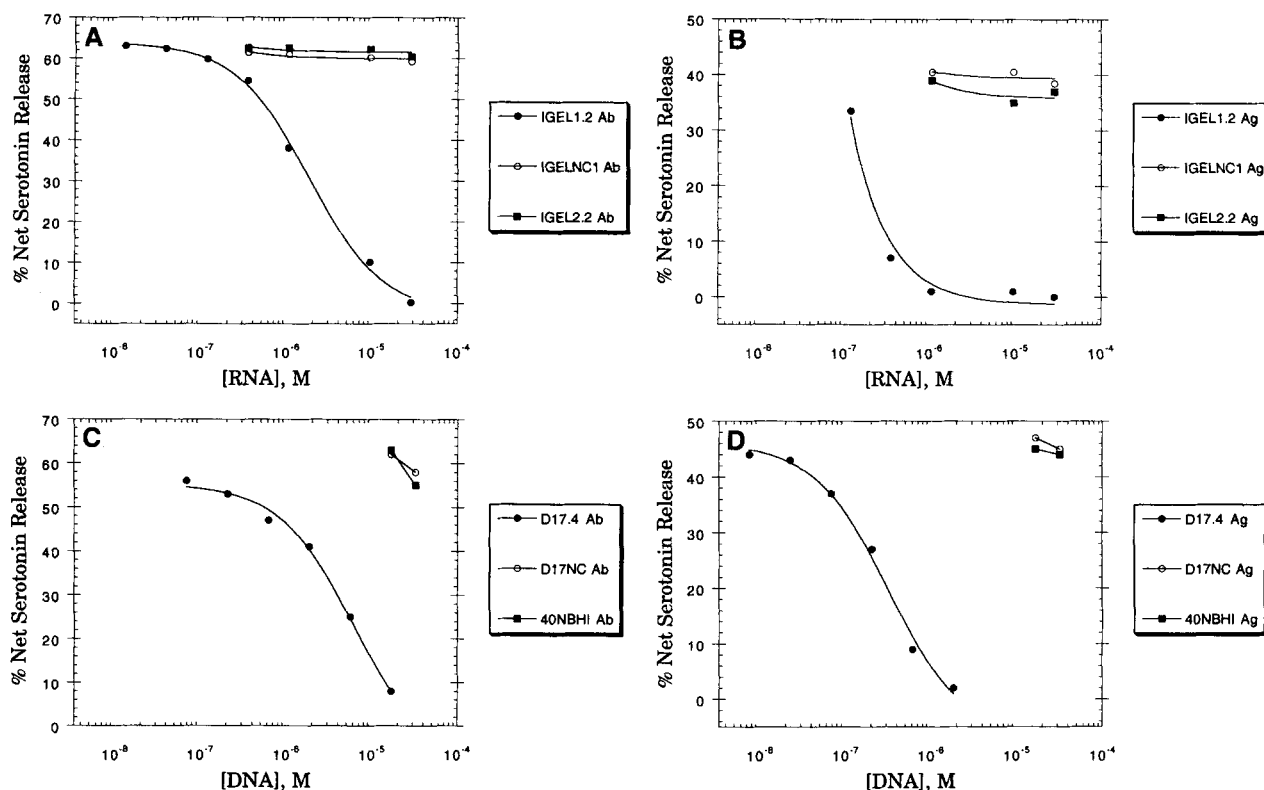


FIGURE 6. Inhibition of IgE-mediated serotonin release by oligonucleotide ligands. Whole serum containing grass-specific IgE was incubated without or with increasing amounts of oligonucleotide ligands, and the mixture was used to sensitize RBL cells expressing human FcεRI receptors. The RBL cells were triggered by the addition of affinity-purified anti-IgE Abs (3 μg/ml; A and C) or grass extract (8 allergenic U/ml; B and D). Net serotonin release was determined and plotted against the concentration of oligonucleotide IgE ligands. The range of duplicate samples was generally less than 5% of the mean values. Net serotonin release in the absence of IgE ligands was 66% (A), 44% (B), 53% (C), and 48% (D). A, 2'-NH₂ RNA ligands, cells triggered with Abs; IC₅₀ = 1.8×10^{-6} M. B, 2'-NH₂ RNA ligands, cells triggered with Ag; IC₅₀ = 2×10^{-7} M. C, ssDNA ligands, cells triggered with Abs; IC₅₀ = 6.1×10^{-6} M. D, ssDNA ligands, cells triggered with Ag; IC₅₀ = 3.2×10^{-7} M.

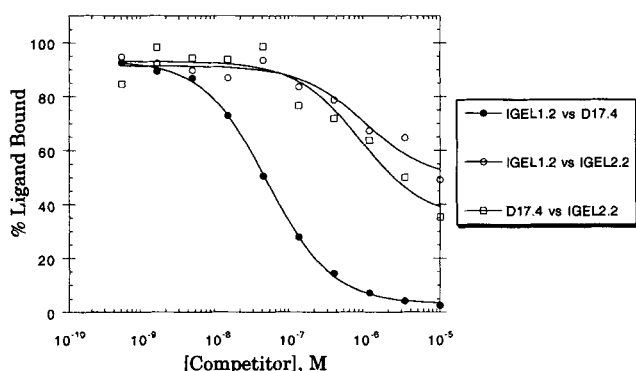


FIGURE 7. Competition-binding analysis. ³²P-labeled oligonucleotide ligands at a concentration of 50 nM were mixed with increasing amounts of unlabeled competitor ligands. The mixture was bound to IgE and partitioned by nitrocellulose filter binding. The labeled ligands are listed first in the legend; the unlabeled competitors are listed second. IGEL1.2 vs D17.4 (closed circles); IGEL1.2 vs IGEL2.2 (open circles); D17.4 vs IGEL2.2 (open squares).

three classes inhibit the interaction of IgE with its high-affinity receptor FcεRI. IGEL1.2, a 35-nucleotide 2'-NH₂ RNA oligonucleotide, representing the RNA group A ligands, and D17.4, a 37-nucleotide ssDNA oligonucleotide, representing the ssDNA ligands, have K_d and K_i values of about 30 nM and 10 nM, respectively. The third class, 2'-NH₂ RNA group B, represented by the

25-nucleotide ligand IGEL2.2, binds IgE with about the same affinity, but does not block its interaction with FcεRI. Together with the observation that ligands IGEL1.2 and D17.4 compete efficiently with each other but only partially with IGEL2.2 for binding to IgE, these results indicate that we have identified two distinct but probably overlapping oligonucleotide binding sites on IgE. One site, recognized by IGEL1.2 and D17.4, is identical with or at least in close proximity to the contact point of IgE with FcεRI, located in the Cε3 region of the protein. A surface map of electric charge distribution (Jeff Davis, personal communication) based on the molecular model of the IgE Fc domain (50, 51) shows a positively charged patch in this area that might favor interaction with polyanionic oligonucleotides. It has previously been suggested that proteins contain immunodominant domains for nucleic acid binding (15). This notion is supported by the finding that two different kinds of oligonucleotides, 2'-NH₂ RNA and ssDNA, bind IgE at the same site. However, we also identified a second binding site on IgE, defined by the interaction with ligand IGEL2.2. This second site is likely to be in the same vicinity and may overlap with the first binding site because of the partial competition of IGEL2.2 with IGEL1.2 and D17.4. It appears to be a distinct site, however, since occupation of this ligand binding site does not prevent the interaction of IgE with the FcεRI receptor.

When comparing potential structures of the 2'-NH₂ RNA and ssDNA ligands it becomes apparent that the same task, high-affinity binding to IgE, can be fulfilled with different molecular shapes. Based on a thermodynamic folding algorithm (48), the ssDNA family forms an easily identifiable stem-loop structure.

The existence of the double-stranded stem is confirmed by extensive covariation of nucleotides flanking the highly conserved central consensus sequence. The 2'-NH₂ RNA ligands, however, cannot be folded easily into stable secondary structures using Watson-Crick base pairing. It is known that 2'-NH₂-substituted nucleotides destabilize RNA and DNA duplexes (52), and the formation of alternative structures for these oligonucleotides has been reported (13). We suspect that these RNA molecules form G-quartet structures in which four guanine bases aligned in a plane interact through hydrogen bonds, resulting in a cyclical array (49, 53, 54). Two or more of these G quartets can be stacked to create a stable and compact three-dimensional structure. This G-quartet structure could also help to explain a second major difference between the classes of IgE ligands: the ssDNA ligands do not involve the fixed regions in binding to IgE, whereas both groups of 2'-NH₂ RNA ligands use the entire 5'-fixed region. Clearly this is not due to the fact that the random region of the 2'-NH₂ RNA ligands is too short, since the ligands isolated from the RNA pool with 40 random nucleotides utilize only one-half of the random region for binding to IgE, and an increase of this random region to 60 nucleotides does not prevent the utilization of the 5'-fixed region. It seems plausible that the 5'-fixed region forms an integral part of the binding domain due to its G richness and, therefore, its ability to participate readily in G-quartet structures. To determine whether we had isolated any sequences that do not involve the fixed regions in binding to IgE, truncation experiments were performed on the nine orphan sequences identified in the 2'-NH₂ RNA SELEX experiments. Eight of these ligands involve parts of the 5'-fixed region, the 3'-fixed region, or both, but one ligand, IGEL11.1, could be truncated to contain only nucleotides from the random region without significant loss of IgE binding affinity (data not shown). Ligand IGEL11.1 does not bind IgE tighter than ligands IGEL1.2 and IGEL2.2 (Table I). This result, however, does not rule out the existence of IgE ligands with higher affinities to IgE.

Since IgE-dependent cellular degranulation is a critical step involved in serious and widespread diseases including allergic rhinitis, atopic dermatitis, allergic asthma, and IgE-mediated anaphylaxis, the ability of ligands IGEL1.2 and D17.4 to inhibit the IgE/FcεRI interaction and, thereby, block IgE-dependent mediator release is clearly of therapeutic interest. The nature of the oligonucleotide pools, 2'-NH₂ RNA and ssDNA, was chosen to make them less susceptible against degradation in serum or other body fluids, and the isolated ligands can be further modified if necessary. Since the potency of these inhibitors depends on their affinities to IgE, a more extensive interface between ligand and target protein, resulting in tighter binding, would be desirable. A SELEX "walking" experiment (Craig Tuerk, manuscript in preparation) could be performed, in which the consensus ligand sequences are placed adjacent to a region of random nucleotides to form new starting pools for the selection of higher affinity, extended molecules. Presumably, such ligands would have an increased ability to block the IgE/FcεRI interaction. In conclusion, this work demonstrates that oligonucleotide-based compounds have the potential to become a new class of potent therapeutic agents in the fight against IgE-mediated diseases.

Acknowledgments

We thank Katie Lynott and Carol Bell for assistance with the IgE receptor-binding assay, and Tim Fitzwater and Ying Chang for help with sequencing and ligand truncations. We are grateful to Brenda Javornik and Dominic Zichi for advice with the multiple sequence alignments. We thank David Parma for many helpful discussions, and Laurie Wiegand, Drew Smith, David Pribnow, Joe Alper, Barry Polisky, and Larry Gold for critical reading of the manuscript.

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