Isolation of a Nuclease-resistant Decoy RNA That Selectively Blocks Autoantibody Binding to Insulin Receptors on Human Lymphocytes

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Summary

An RNA containing 2′-amino pyrimidines has been isolated using in vitro selection techniques that specifically and avidly (apparent K_d ~30 nM) binds a mouse monoclonal antibody called MA20. This 2′-amino–derivatized RNA is at least 10,000-fold more stable than unmodified RNA in serum, and can act as a decoy and block MA20 binding to its natural antigen, the human insulin receptor, on lymphocytes. Furthermore, this RNA decoy can inhibit MA20-mediated downmodulation of insulin receptor expression on human lymphocytes in culture by up to 90%. Surprisingly, the decoy RNA cross-reacts with autoantibodies from patients with extreme insulin resistance and can inhibit these antiinsulin receptor antibodies from downmodulating insulin receptor expression by up to 80% without impeding insulin binding to its receptor. These results suggest that in vitro–selected decoy RNAs may be able to specifically and selectively block oligoclonal autoimmune responses to self-antigens in patients with autoimmune diseases.

Autoimmune diseases are often caused by abnormal targeting of self-antigens by autoantibodies (1). A small "decoy" ligand could potentially interrupt this process if it specifically and avidly bound the autoantibodies and blocked them from interacting with their target antigen. A small peptide decoy seems the most obvious choice for such an inhibitor because a peptide should be able to structurally mimic the autoantigenic epitope on a self-protein. Unfortunately, isolation of small peptides that bind avidly to target proteins has been difficult, probably because short peptides cannot adopt stably folded structures very readily (2). By contrast, short RNA molecules can form fairly stable structures through basepairing interaction (3), and RNA decoys have been reported that avidly bind and block a variety of proteins (4–22). Expression of TAR and RRE decoy RNAs has been shown to render $CD4^+$ T cells resistant to HIV replication by inhibiting the functions of the RNA-binding proteins tat and rev (8–12). Furthermore, by using in vitro selection techniques, "designer" decoy RNAs have been isolated from large pools of random RNA molecules that bind with high affinity and specificity to several proteins, including a few that do not naturally interact with nucleic acids (13, 15–22).

We previously identified a designer decoy RNA that binds and blocks the mAb, MA20 (18). This antibody naturally recognizes the main immunogenic epitope on the human insulin receptor (23) that elicits autoantibodies in patients with severe insulin resistance (type B) (24–26). Although this RNA was shown to be able to block MA20 binding to purified insulin receptors in solution in a test tube, we realized that the therapeutic utility of such a decoy RNA would be limited by its short half-life in the presence of serum nucleases. In addition, it was unclear if an RNA decoy made against MA20 could block an oligoclonal autoantibody response to the immunogenic epitope on the insulin receptor. Therefore, we sought to isolate a nuclease-resistant decoy RNA specific for MA20 and to determine if this RNA could inhibit patient autoantibodies from binding the insulin receptors on human cells in culture.

To generate nuclease-resistant RNA decoys, in vitro selection was performed using an RNA library that contains 2′-amino–modified pyrimidine nucleotides. Changing the 2′ position on the pyrimidines of an RNA from a hydroxyl to an amino group substantially increases the stability of RNA in serum (27). The modified pyrimidine nucleotides are compatible with in vitro selection procedures because 2′-amino–2′-deoxypyrimidine triphosphates can be incorporated into RNA by T7 RNA polymerase (28), and RNA with 2′-amino pyrimidines can serve as substrates for AMV reverse transcriptase. Using similar methodology, RNase-resistant ligands with 2′-amino pyrimidines that bind human neutrophil elastase (17, 20), basic fibroblast growth factor (21), or vascular permeability factor/vascular endothelial growth factor (22) have been identified.

Here, we report the isolation of a nuclease-resistant decoy RNA that specifically and avidly binds the MA20 anti-

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body and cross-reacts with autoantibodies from patients with severe insulin resistance. Furthermore, the decoy RNA can block MA20 and patient autoantibodies from binding to and downmodulating expression of insulin receptors on human lymphocytes in culture without impeding insulin binding.

Materials and Methods

Antibodies and Cells. An mAb, MA20, was kindly provided by Ira Goldfine and Betty Maddux (University of California, San Francisco, CA). An mAb, 83-7, was a gift from Kenneth Siddle (Addenbrooke's Hospital, University of Cambridge, Cambridge, UK). Serum samples from three patients with severe insulin resistance type B, called B7, B10, and Bd, were provided by Domenico Accili and Simeon Taylor (National Institutes of Health, Bethesda, MD). IM-9 human lymphoblasts (ATCC 159-CCL) express high levels of insulin receptors on their plasma membranes (29) and were used in insulin receptor downregulation experiments.

Selection Procedure. A random pool of RNA oligonucleotides was generated by in vitro transcription of synthetic DNA templates using 2′ amino derivatized CTP and UTP (Amersham, Arlington Heights, IL) and normal GTP and ATP in the reaction. The sequence of the resulting RNA is 5′-GGGAGAGCGGAAGCGUGCUGGGCCN$_{40}$CAUAACCCAGAGGUCGAUGGAUCCCCCC-3′, where N_{40} represents 40 nucleotides (nts)[1] with eqimolar incorporation of A, G, C, and U at each position. First, the RNA library (30 μg) was incubated with 5 μl normal mouse IgGs (1 mg/ml) in 100 μl binding buffer (30 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10 mM $MgCl_2$, 2 mM dithiothreitol, and 1% BSA) for 30 min at room temperature with shaking. Then, 20 μl of goat anti–mouse IgG–coated magnetic beads (Dynal, Oslo, Norway) or 20 μl of protein G–Sepharose beads (Pharmacia Biotech, Piscataway, NJ) were added to the binding reaction and incubated for an additional 30 min. Antibody–RNA complexes were immunoprecipitated and discarded to remove RNAs that bind to the constant region of the antibodies or adhere nonspecifically to the beads. The precleared supernatant was transferred to a new tube and incubated with 5 μl of mouse mAb MA20 (0.5 mg/ml) for 30 min at room temperature. The mAb–RNA complexes were then immunoprecipitated using magnetic beads or protein G–Sepharose beads as mentioned above, and the pellets were washed three times with 0.5 ml of the binding buffer. RNA was eluted from the pellets by a 5-min incubation with 100 μl of 0.1 M EDTA and phenol extraction. The RNA eluate was then applied to a Sephadex G-50 spin column to remove EDTA and salts, and reverse-transcribed for 30 min at room temperature using avian myeloblastosis virus reverse transcriptase (RT; Boehringer Mannheim, Indianapolis, IN) and a 28-nt DNA primer complementary to the fixed 3′ sequence of the RNA pool. The RT was then inactivated by heating the sample to 95°C for 5 min. The resulting cDNAs were amplified by PCR for 20 cycles using Taq DNA polymerase (Perkin-Elmer Cetus, Norwalk, CT) and a second DNA primer which was identical to the fixed 5′-end of the RNA pool and contained the 17-nt promoter sequence for T7 RNA polymerase. The amplified DNA was phenol extracted, ethanol precipitated, and resuspended in 25 μl of TE (10 mM Tris-HCl, pH 8.0, 1 mM EDTA). 40% of this DNA template was transcribed in vitro with T7 RNA polymerase and the 2′-amino–modified mix of NTPs to generate RNA for the next round of selection. Subsequent rounds were performed in the same manner using either 50% (rounds 1–9) or 20% (rounds 10–12) of the transcribed RNA. Goat anti–mouse IgG–coated beads were used on odd-numbered and protein G–Sepharose beads on even numbered rounds of selection. From round 10, MA20 concentration was reduced fivefold, and mAb-RNA pellets were washed five or more times. After 12 rounds of selection, the amplified DNA was cloned and several clones were sequenced.

Analysis of Selected RNAs by Immunoprecipitation. Plasmid DNA encoding 2′-amino RNA No. 1 was transcribed with T7 RNA polymerase and the 2-amino pyrimidine mix of NTPs in the presence of [α-^{32}P]ATP (Amersham). Transcribed RNA was gel isolated and purified RNA incubated with different antibodies in 100 μl of binding buffer for 30 min at room temperature. Antibody–RNA complexes were immunoprecipitated by goat anti–mouse IgG magnetic beads or protein G–Sepharose beads. The immunoprecipitates were washed three to five times with 0.5 ml of binding buffer. Bound RNA was eluted by addition of 15 μl of 0.1 M EDTA and phenol extraction, and was analyzed on a 6% polyacrylamide gel with urea.

Gel Shift Analysis of RNA–Antibody Complexes. Radiolabeled 2′-amino RNA No. 1 was incubated with MA20 in binding buffer at room temperature for 30 min. The binding reaction was then mixed with one sixth of its volume of loading buffer containing 30% glycerol. Then the sample was loaded onto a 4% nondenaturing polyacrylamide gel containing 1× TBE buffer, 10 mM $MgCl_2$, and 2% glycerol. Electrophoresis was performed with 0.5× TBE buffer at 4°C for 4 h.

RNA Stability Analysis in Serum. 10 nmol of ^{32}P-body-labeled 2′-amino RNA No. 1 was incubated in 200 μl of 10% human serum or 10% fetal bovine serum at 37°C. Aliquots of the mixture were removed at various times, quenched with 0.5 M EDTA and formamide, and analyzed on a 10% polyacrlyamide gel with urea.

Measurement of Antibody Binding to Insulin Receptors on IM-9 Cells. IM-9 cells (5×10^4) were washed on ice in FACS® buffer (PBS, 1% BSA, 0.1% sodium azide) containing 10 mM $MgCl_2$ and then resuspended in 100 μl FACS® buffer containing 10 mM $MgCl_2$ with normal mouse IgG (isotype control, 50 ng), MA20 (50 ng), or 83-7 (3 μl) preincubated with or without pool RNA (10 μM) or selected RNA (10 μM). After a 20-min incubation on ice, cells were washed once with FACS® buffer and stained with either a PE-conjugated F(ab′) goat anti–mouse IgG2b antibody (Southern Biotechnology Associates, Birmingham, AL) for MA20 containing samples or a FITC-conjugated F(ab′) donkey anti–mouse IgG antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) for 83-7–containing samples. Next, cells were washed with FACS® buffer and then fixed with FACS® buffer containing 0.4% paraformaldehyde. Stained cells were analyzed on a FACScan® apparatus (Becton Dickinson & Co., Mountain View, CA). Results were displayed as frequency distribution histograms of log fluorescence.

Monitoring Insulin Receptor Downmodulation on IM-9 Cells. Insulin receptor downregulation was assayed as described previously (23), with a few modifications. 1.0×10^4 IM-9 cells were resuspended in 75 μl of media (DMEM) with 10 mM $MgCl_2$, 1% BSA, and 10% FBS. MA20 (1 nM), autoantibodies or insulin (1 μM) were incubated in 25 μl binding buffer with the original amino RNA pool, 2′-amino RNA No. 1, or no RNA for 30 min at room temperature. These mixtures were then added to IM-9 cells and incubated at 37°C for 15 min. Next, the cells were washed twice with a phosphate buffer, pH 6.0, containing 154

[1] *Abbreviation used in this paper:* nts, nucleotides.

mM NaCl and 0.1% BSA for 30 min at 30°C to remove receptor-bound ligands (23). To quantitate the number of insulin receptors remaining on the cell surface, the cells were resuspended in 100 μl of 100 mM Hepes buffer, pH 7.8, containing 120 mM NaCl, 1.2 mM $MgSO_4$, 1 mM EDTA, 15 mM sodium acetate, 10 mM glucose, and 1% BSA, and incubated with 150 pM ^{125}I-insulin for 70 min at 15°C. The cells were pelleted and washed twice with 10 mM Tris-HCl buffer, pH 7.8, containing 154 mM NaCl at 4°C. The washed cells were pelleted again and the radioactive ^{125}I-insulin bound to them was determined using a γ 5,000 counter (Packard Instruments, Meriden, CT). Incubation of IM-9 cells with 1 nM of MA20 by itself reproducibly reduced insulin receptor expression by ~50% (data not shown). This level of antigenic downregulation was considered 100%, and other values were normalized to this amount. Because 10 mM $MgCl_2$ is optimal for the binding of the selected RNA to MA20 in vitro, cell culture experiments were performed in media containing 10 mM $MgCl_2$. For antigenic downregulation experiments involving patient autoantibodies, patient serum or insulin was added to IM-9 cells at concentrations determined to engender similar levels of antigenic downregulation as 1 nM of MA20: 1 μl of a 1:25 dilution of B7, 1 μl of a 1:12 dilution of B10, 1 μl of undiluted Bd sera, or 1 μM of insulin (data not shown).

Results

Isolation of Nuclease-resistant RNAs That Specifically Bind the MA20 Antibody. An RNA library of ~10^{14} different molecules was generated with every pyrimidine modified at its 2′ position by an amino group. Each molecule in the library contained a 40-nt long region derived from a random sequence flanked by defined sequences. The amino-derivatized RNA library was preincubated with normal mouse IgGs, and RNA–antibody complexes immunoprecipitated and discarded. The precleared amino–RNA pool was then incubated with the mouse mAb MA20, and RNA–antibody complexes immunoprecipitated. Bound RNAs were eluted and reverse transcribed. The resulting cDNAs were amplified by PCR and transcribed to generate RNA for the next cycle of selection. After 12 rounds of selection (see Materials and Methods for details), the amplified cDNAs were cloned, and 18 different clones sequenced.

Three different RNAs were selected that specifically bind the variable region of the MA20 mAb (Fig. 1). As shown in Fig. 1 *A*, all the RNAs were 6 nts shorter than the original 92-nt-long pool RNAs. This change probably resulted from deletion of part of the random region during reverse transcription and PCR amplification. In addition, the three RNAs have very similar sequences and were found in multiple clones. Interestingly, the sequences of these 2′-amino derivatized RNAs (2′-amino RNAs) are very different from the sequences of unmodified RNAs that we previously generated to bind MA20 (18). 2′-amino RNA No. 1 was chosen for further characterization because it appeared most frequently in the sequenced clones. To determine if 2′-amino RNA No. 1 binds specifically to the MA20 antibody, an immunoprecipitation experiment was performed using internally radiolabeled RNA. The selected RNA was shown to bind only MA20 and not other

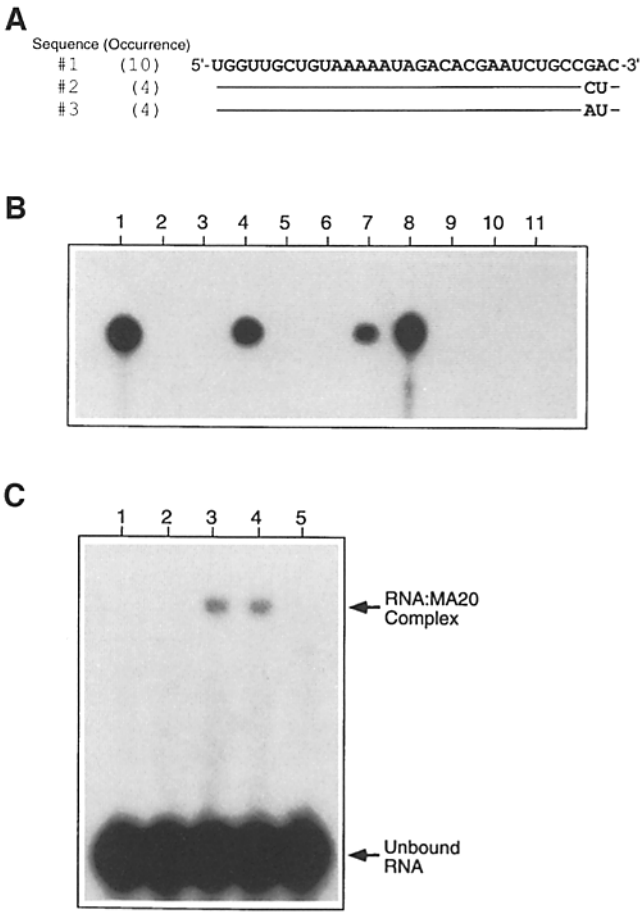

Figure 1. Selected 2′amino RNA sequences and their specific binding to the mAb MA20. (*A*) After 12 rounds of in vitro selection, the sequence of 18 selected RNAs was determined, and all were found to contain 34-nt-long inserts. Only three different but very similar RNA sequences were found in these clones, with each being present multiple times (numbers in parentheses). Only two nucleotides near the 3′-end of the inserts varied. The line drawn for sequences Nos. 2 and 3 indicates that nucleotides found at these positions are identical to those shown for sequence No. 1. C and U in this figure correspond to 2′-amino C and 2′-amino U. (*B*) 2′-amino RNA No. 1 specifically binds MA20. Internally radiolabeled RNA No. 1 (lanes *1–7*, 2.5 nM) or original pool RNA (lanes *8–11*, 2.5 nM) was incubated without any antibodies (lanes *2*, *5*, and *9*) or with either normal mouse IgGs (lanes *3*, *6*, and *10*, 100 nM) or the MA20 antibody (lanes *4*, *7*, and *11*, 100 nM). The antibody–RNA complexes were immunoprecipitated with protein G–Sepharose beads (lanes *2–4* and *9–11*) or goat anti–mouse IgG magnetic beads (lanes *5–7*). Immunoprecipitated RNAs were extracted and electrophoresed in a 6% polyacrylamide gel with urea. Lanes *1* and *8* contain 20% of the input-labeled RNA No. 1 or pool RNA, respectively. (*C*) Formation of 2′ amino RNA No. 1 and MA20 antibody complexes can only be inhibited by specific RNA competitors. Internally radiolabeled 2′-amino RNA No. 1 (100 pM) was incubated without antibody (lane *1*), with normal mouse IgGs (lane *2*, 20 nM), or with the MA20 antibody (lanes *3–5*, 20 nM). Antibody–RNA complexes were separated from unbound RNA in a 4% nondenaturing polyacrylamide gel. The original pool RNA (lane *4*, 1 μM unlabeled) or 2′-amino RNA No. 1 (lane *5*, 1 μM unlabeled) were added in a 10,000-fold excess to the binding reaction to determine if either could inhibit the formation of the 2′-amino RNA No. 1–MA20 complex.

mouse IgGs (Fig. 1 *B*). Furthermore, this binding was independent of the type of beads used to immunoprecipitate the MA20–RNA complexes (Fig. 1 *B*). In addition, the 2′-amino pool RNA does not nonspecifically bind to MA20 (Fig. 1 *B*, lanes *8–11*).

To confirm that 2′-amino RNA No. 1 specifically binds the MA20 antibody, a gel retardation assay was developed. As shown in Fig. 1 *C*, 2′-amino RNA No. 1 efficiently forms a "shifted" nucleoprotein complex with MA20, but not with normal mouse IgGs. Formation of this nucleoprotein complex can be blocked by addition of a 10,000-fold excess of unlabeled 2′-amino RNA No. 1, but not by a nonspecific competitor, such as the original 2′-amino pool RNA (Fig. 1 *C*, lanes *4* and *5*).

As a working model, the most stable secondary structure of 2′-amino RNA No. 1 was predicted using the MULFOLD program (30), and is shown in Fig. 2 *A*. In this folded structure, the 34-nt-long sequence, derived from the randomized region of the RNA library, is predicted to form basepairing interactions with the defined sequences that flank the random region in the 2′-amino RNA pool. To begin to test the validity of this predicted structure, as well as to determine if a shortened version of 2′-amino RNA No. 1 could be made that would still bind MA20, a series of truncated RNAs were generated and tested for antibody binding (Fig. 2 *B*). These shortened RNAs ranged in size from 34 to 83 nts; however, only the 83-nt long version of 2′-amino RNA No. 1 retained the ability to bind MA20. Thus, 2′-amino RNA No. 1 (originally 86 nt long) could not be shortened to any significant extent and still adopt the structure recognized by the antibody. Unfortunately, this result does not strongly support or refute the working model shown in Fig 2 *A*, but it did underscore the need to use the nearly full-length 83-nt long 2′-amino RNA No. 1 in the following experiments.

To investigate the structural significance of the 2′-amino groups on the pyrimidine nucleotides, selected RNA No. 1 was generated with 2′-hydroxyl pyrimidines and tested for binding to the MA20 antibody. As shown in Fig. 2 *C*, alteration of the 2′ position disrupted the interaction between RNA No. 1 and MA20. Similarly, the 2′ position appears to be critical for binding of our selected unmodified RNA (18) to MA20. Changing the 2′ position on the pyrimidines of this RNA from hydroxyls to aminos yielded a modified RNA that did not bind MA20 (Fig. 2 *C*). Thus, the 2′ position on both selected RNAs appears to be important for proper RNA folding and MA20 binding.

To estimate the equilibrium K_d for the interaction between 2′-amino RNA No. 1 and MA20, a gel retardation assay was used (Fig. 3 *A*). Trace amounts of radiolabeled RNA were incubated with increasing amounts of antibody and complexes separated on nondenaturing acrylamide gels. The same apparent K_d of ~30 nM was detected at three different concentrations of 2′-amino RNA No. 1 (Fig. 3 *B*). Thus, selected 2′-amino RNA No. 1 binds fairly avidly to the MA20 antibody.

Even though the 2′-amino RNA No. 1 specifically and avidly binds MA20, this decoy would be of little therapeutic utility if the RNA is quickly degraded by serum nucleases. Therefore, the stability of the 2′-amino RNA No. 1 in serum was measured (Fig. 4). In 10% human serum, RNA with 2′-hydroxyl groups (unmodified RNA) was

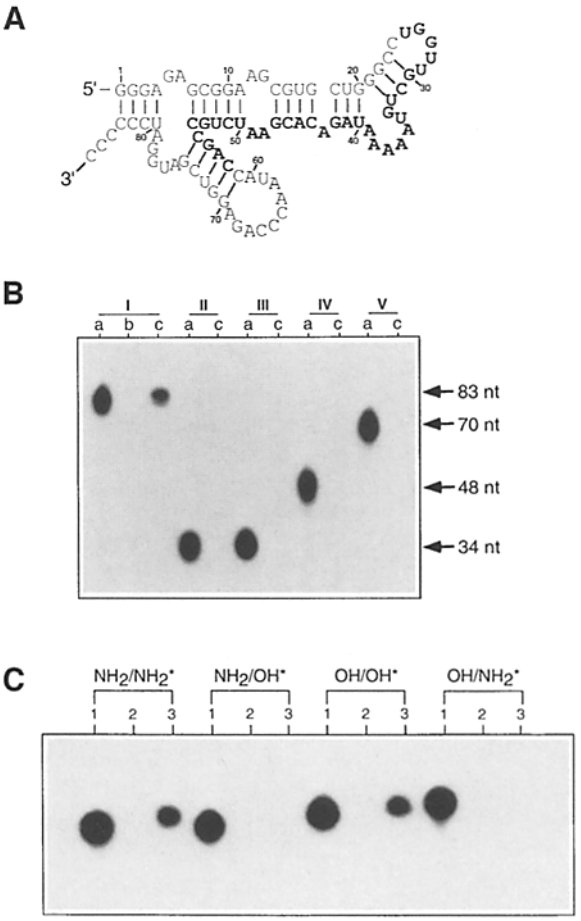

Figure 2. Structural analysis of 2′-amino RNA No. 1. (*A*) Predicted secondary structure of 2′-amino RNA No. 1. Nucleotides shown in plain letters originate from the fixed flanking sequences found in the RNA library. The 34 nts shown in bold indicate the sequence selected from the randomized region of the RNA library that is present in 2′-amino RNA No. 1. U and C represent 2′-amino U and 2′-amino C. Every 10th nt of the RNA is numbered. (*B*) Most of 2′-amino RNA No. 1 is required for binding to MA20. Truncated versions of 2′-amino RNA No. 1 were transcribed and tested for binding to MA20. Tested derivatives contain 83 nts of 2′-amino RNA No. 1 (nts 1–83; sample I), the 34 nts selected RNA sequence (nts 25–58; sample II), 34 nts of the RNA (nts 20–53; sample III), 48 nts of the RNA (nts 7–54; sample IV), or 70 nts of the RNA (nts 5–74; sample V). The RNAs (2.5 nM) were incubated with normal mouse IgGs (lane *b*, 100 nM) or MA20 (lane *c*, 100 nM). The antibody–RNA complexes were immunoprecipitated and analyzed on an 8% polyacrylamide gel with urea. Lane *a* contains one-fifth of the amount of radiolabeled RNA that was incubated with the antibody. (*C*) The 2′ position on the pyrimidine of the selected RNAs is important for antibody binding. Internally radiolabeled RNAs (2.5 nM) were incubated with normal mouse IgGs (lane *2*, 125 nM) or MA20 (lane *3*, 125 nM). RNAs immunoprecipitated by these antibodies were electrophoresed on a 6% polyacrylamide gel with urea. Lane *1* contains one fifth of the amount of input RNA that was used in the immunoprecipitation reaction. The 2′-amino RNA No. 1 was made with either 2′-amino pyrimidines (NH_2/NH_2*) or 2′-hydroxyl pyrimidines (NH_2/OH*). The 2′-hydroxyl RNA No. 9, which we previously described (18), was made with either 2′-hydroxyl pyrimidines (OH/OH*) or 2′-amino pyrimidines (OH/NH_2*).

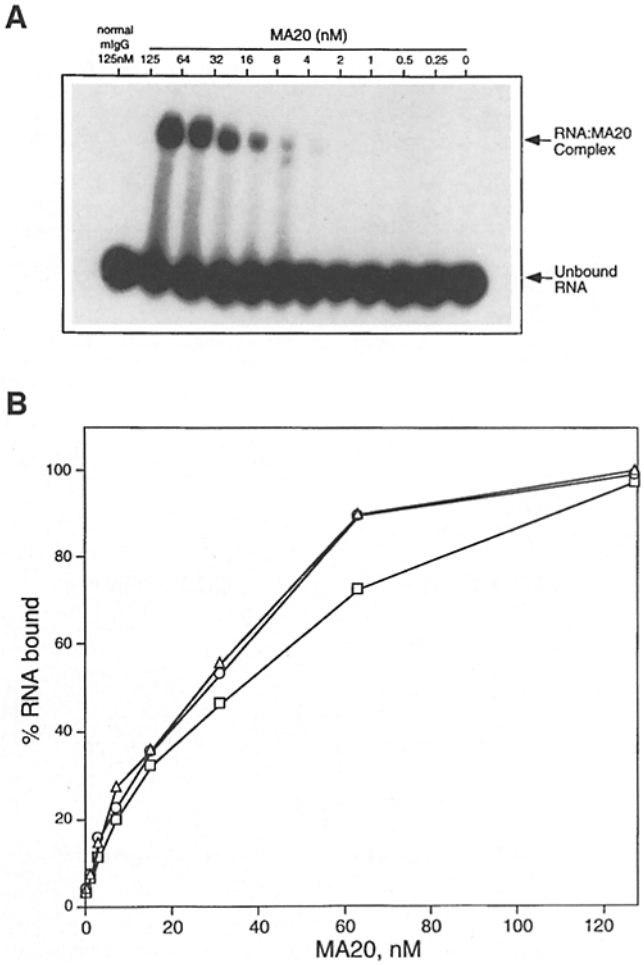

Figure 3. Determining the binding affinity between the selected 2′-amino RNA No. 1 and MA20. (*A*) Internally radiolabeled 2′-amino RNA No. 1 (200 pM) was incubated with normal mouse IgGs (lane *1*, 125 nM) or decreasing amounts of MA20 (lanes *2–12*, 125–0 nM). The MA20–RNA complexes were separated from unbound RNAs by a gel mobility shift assay performed on a 4% nondenaturing polyacrylamide gel. (*B*) Three different concentrations of internally radiolabeled 2′-amino RNA No. 1 were incubated with the increasing amounts of the MA20 antibody, and the MA20–RNA complexes were analyzed as described in *A*. The percentage of RNA bound to MA20 was calculated by determining the fraction of radioactivity present in the MA20–RNA complexes. A maximum of 40–50% of RNA formed MA20–RNA complexes, even in the presence of extremely high concentration of MA20. Thus, plotted numbers have been normalized to that amount. □, 50 pM RNA; ○, 100 pM RNA; △, 200 pM RNA.

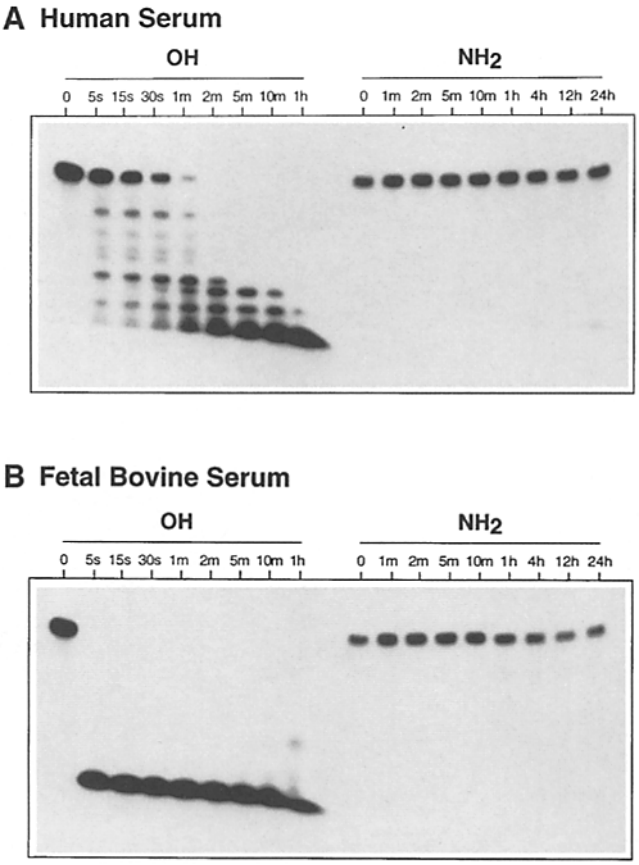

Figure 4. Stability of selected 2′-amino RNA in human serum (*A*) or fetal bovine serum (*B*). Internally radiolabeled selected RNA No. 9 (18) containing 2′-hydroxyl pyrimidines (OH, 10 nM) or RNA No. 1 containing 2′-amino pyrimidines (NH_2, 10 nM) were incubated at 37°C in 200 μl of PBS containing 10% serum. Aliquots (20 μl) of the reaction were removed at the times indicated, quenched with 5 μl of 0.5 M EDTA, phenol extracted, and fractionated on a 10% polyacrylamide gel with urea (*s*, seconds; *m*, minutes; *h*, hours).

rapidly degraded by serum nucleases ($t_{1/2}$ ~15 s). In contrast, 2′-amino RNA No. 1 was resistant to nuclease degradation with most of the RNA remaining full-length, even after 24 h of incubation. In the presence of 10% fetal bovine serum, this difference in stability was even greater. Once again, 2′-amino RNA No. 1 was refractory to serum nucleases with most of the RNA remaining intact, even after 24 h of incubation, while most of the unmodified RNA was totally degraded by 5 s. Similar results were obtained when RNAs were incubated in 100% serum (data not shown). Therefore, 2′-amino RNA No. 1 is more than 10,000-fold more stable in serum than unmodified RNA, confirming previously published results describing the nuclease resistance of 2′-amino–substituted RNA (17, 20–22, 27).

2′-Amino RNA No. 1 Blocks MA20 Binding to and Downmodulation of Insulin Receptors on Human Lymphocytes. To determine if selected 2′-amino RNA No. 1 can specifically inhibit MA20 binding to insulin receptors on the surface of human lymphocytes, we incubated IM-9 cells with the MA20 antibody in the presence of 2′-amino RNA No. 1 or a nonspecific RNA competitor. MA20 binding was then quantitated by the addition of a secondary goat anti–mouse PE-conjugated antibody and subsequent analysis of stained cells via FACS®. As shown in Fig. 5 *A*, addition of the original RNA pool as a competitor did not affect the binding of MA20 to IM-9 cells. Addition of selected 2′-amino RNA No. 1, however, greatly inhibited MA20 binding to these cells. Fluorescent staining of IM-9 cells was reduced by almost 95% when 2′-amino RNA No. 1 was used as the competitor. In contrast, selected

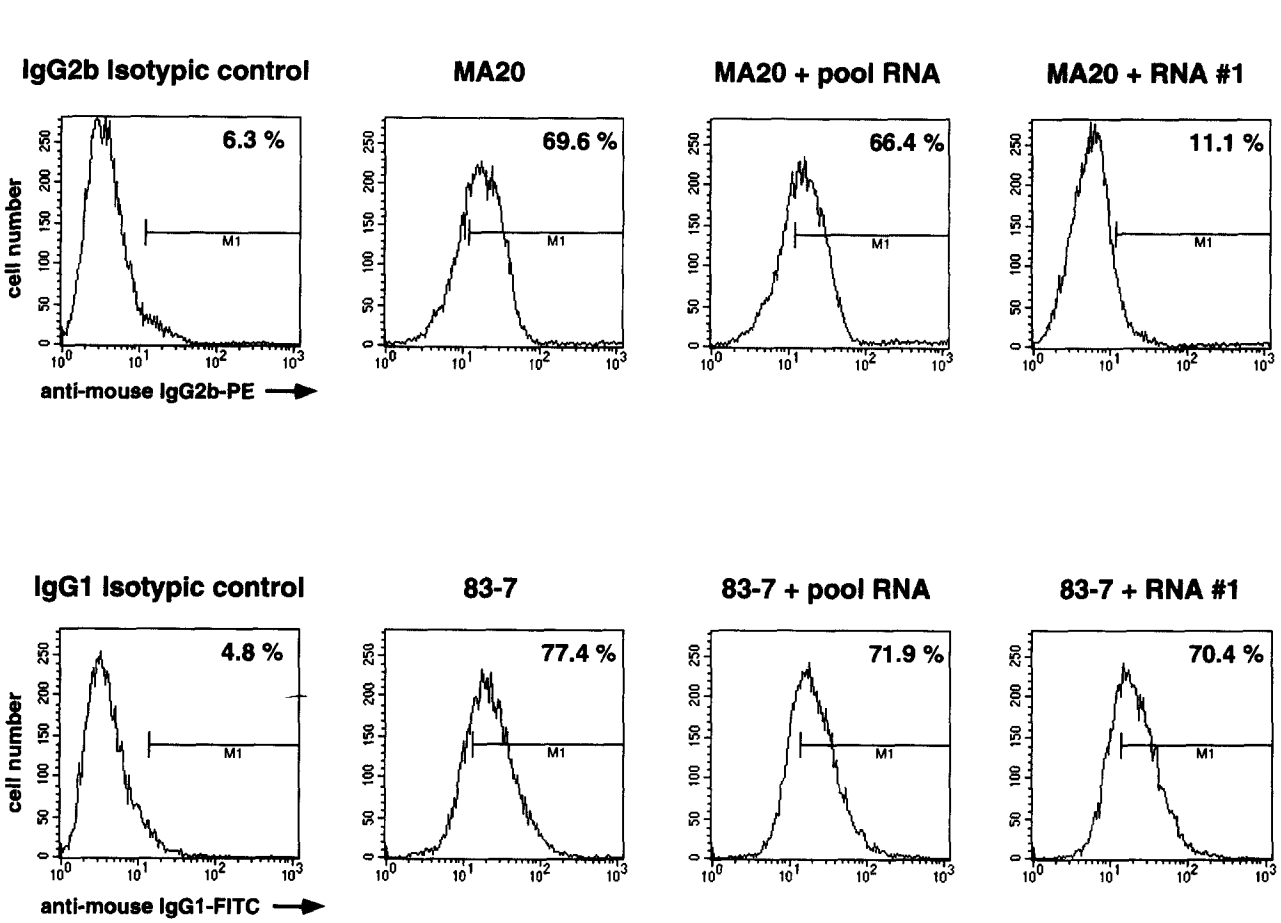

Figure 5. 2′-amino RNA No. 1 inhibits MA20 binding to insulin receptors on IM-9 cells. The mAbs MA20 (50 ng) (*A*) or 83-7 (*B*) were preincubated in buffer alone or with unselected pool RNA (10 μM), or selected 2′-amino RNA No. 1 (10 μM) as competitors. Then, these antibody mixtures were incubated with 5×10^4 IM-9 cells and antibody binding was monitored by FACS® analysis, as described in the Materials and Methods. IM-9 cells were stained with the IgG2b or IgG1 isotypic control antibodies to set the counting gates for staining with the MA20 or the 83-7 antibodies, respectively. The percentage of cells found in gate M1 is shown at the top of each histogram.

2′-amino RNA No. 1 did not interfere with the staining of IM-9 cells by a different mAb, called 83-7, which recognizes a different epitope on the human insulin receptor (26, 31, 32, and Fig. 5 *B*). Therefore, 2′-amino RNA No. 1 can specifically inhibit MA20 binding to its normal antigen (the insulin receptor), suggesting that this RNA decoy binds near the combining site on the antibody.

Once we had ascertained that 2′-amino RNA No. 1 was fairly stable in serum and that it could block MA20 binding to the insulin receptors expressed on IM-9 cells in serum-free conditions, we wanted to determine if this decoy RNA could protect cells from MA20 in culture media containing serum. Incubation of IM-9 cells with the MA20 antibody has been shown to cause a dose- and time-dependent decrease in the number of insulin receptors present on the surface of these cells (33). This process, termed antigenic downregulation, can be easily monitored by determining the ability of the cells to bind ^{125}I-insulin (33). To determine if 2′-amino RNA No. 1 could protect cells from the effects of MA20 and inhibit antibody-mediated downregulation of insulin receptor expression, IM-9 cells were incubated with MA20 in the presence or absence of 2′-amino RNA No. 1. As shown in Fig. 6 *A*, a nonspecific RNA competitor, such as the original 2′-amino RNA pool, could not inhibit MA20-mediated downregulation of insulin receptor expression. However, decoy 2′-amino RNA No. 1 protected IM-9 cells from MA20 and inhibited antigenic downregulation by 75%. Furthermore, 2′-amino RNA No. 1–mediated inhibition of antigenic downregulation was dose dependent with an ED_{50} of ~0.6 μM (Fig. 6 *B*). A maximum inhibition of 80–90% was obtained at the highest doses tested. Thus, this nuclease-resistant decoy RNA can protect cells from the MA20 antibody in the presence of serum nucleases.

2′-Amino RNA No. 1 Inhibits Autoantibody-mediated, but not Insulin-mediated, Downregulation of Insulin Receptor Expression. Because MA20 naturally recognizes the main immunogenic epitope on the insulin receptor (23) that elicits autoantibodies in patients with severe insulin resistance type B (24–26), we wanted to determine if 2′-amino

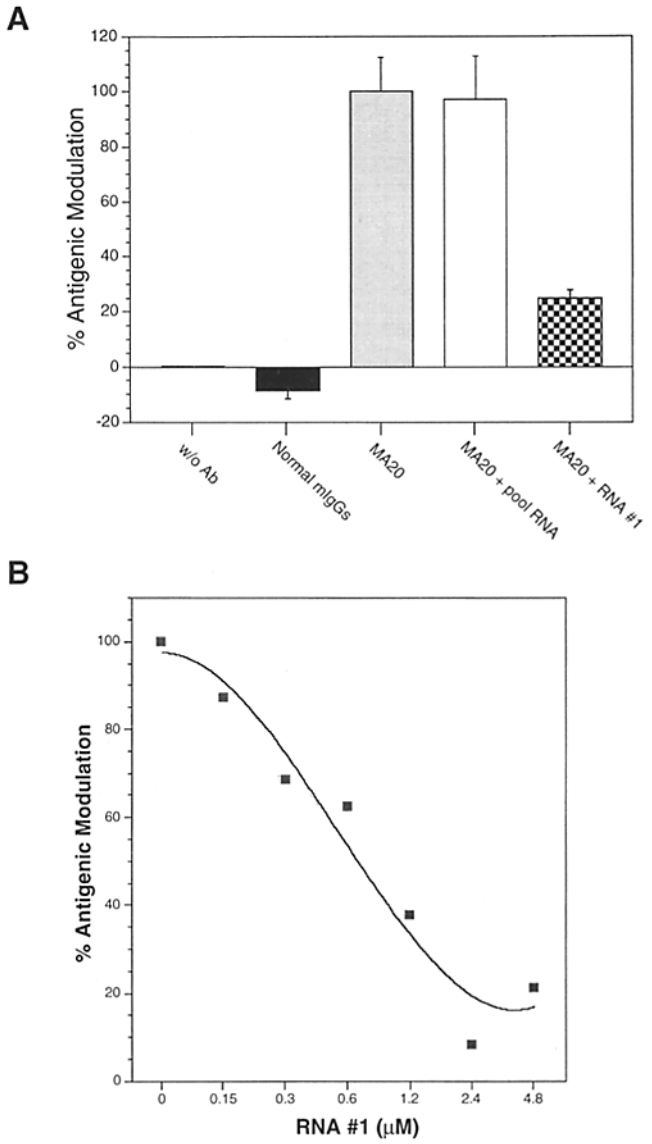

Figure 6. 2′-amino RNA No. 1 inhibits MA20-mediated downmodulation of insulin receptor expression on human lymphocytes in culture. (*A*) IM-9 cells were incubated in complete cell culture media containing no antibody (*w/o Ab*), normal mouse IgGs (mIgs, 1 nM), MA20 (1 nM), MA20 (1 nM) plus 2′-amino pool RNA (3 μM), or MA20 (1 nM) plus 2′-amino RNA #1 (3 μM). Downmodulation of insulin receptor expression was assayed by ^{125}I-insulin binding, as described in the Materials and Methods. Values are expressed as a percentage of the downmodulation engendered by the MA20 antibody (1 nM) alone (percent of antigenic modulation). Values shown are averages ± SD of measurements performed in triplicate. (*B*) IM-9 cells were incubated in culture media containing MA20 (1 nM) and increasing amounts of 2′-amino RNA No. 1, and downmodulation of insulin receptor expression was assayed as above.

RNA No. 1 would also bind any of the antiinsulin receptor autoantibodies from these patients. Therefore, an immunoprecipitation experiment was performed using radiolabeled 2′-amino RNA No. 1 and antibodies from sera of three different patients called B7, B10, and Bd. As shown in Fig. 7 *A*, all three of the autoimmune sera were found to contain antibodies that can bind and immunoprecipitate 2′-amino RNA No. 1. In contrast, the selected RNA was not precipitated by normal mouse IgGs, the 83-7 mAb (26, 31, 32), or antibodies from normal human serum. Thus, 2′-amino RNA No. 1 appears to specifically cross-react with some fraction of the patients' autoantibodies.

This result raised the intriguing possibility that selected 2′-amino RNA No. 1 may resemble or mimic the structure of the main antigenic epitope on the human insulin receptor. If this were indeed the case, then one would predict that such a mimotope should be able to block a significant fraction of the patient autoantibodies from binding insulin receptors on the surface of human cells. To test this hypothesis, we evaluated the ability of 2′-amino RNA No. 1 to protect cells from the effects of the three autoimmune sera, B7, B10, and Bd. Incubation of IM-9 cells with the patient sera was shown to cause a dose- and time-dependent decrease in insulin receptor expression, as monitored by the subsequent inability of these cells to bind ^{125}I-insulin (data not shown). Addition of selected 2′-amino RNA No. 1 protected the cells from the autoantibodies in the patient sera and inhibited downmodulation of insulin receptor expression by ~54% with B7, 75% with B10, and 40% with Bd (Fig. 7 *B*). Furthermore, 2′-amino RNA No. 1 protected cells from the autoantibodies in B10 serum as effectively as it protected cells from the MA20 mAb. By contrast, addition of the original 2′-amino pool RNA as a competitor did not inhibit the antigenic downmodulation engendered by the patient sera, indicating that 2′-amino RNA No. 1 specifically blocks the antiinsulin receptor autoantibodies in these sera. In addition, 2′-amino RNA No. 1 did not inhibit insulin-induced loss (34) of insulin receptors on IM-9 cells (Fig. 7 *B*). Thus, 2′-amino RNA No. 1 can block an autoimmune response to the insulin receptor without impeding insulin binding to its receptor. Finally, to determine how effectively 2′-amino RNA No. 1 could protect IM-9 cells from the autoantibodies present in the serum of patient B10, a dose-escalation experiment was performed. As shown in Fig. 7 *C*, at high concentrations, 2′-amino RNA No. 1 can block ~70–80% of the B10 autoantibodies responsible for insulin receptor loss. Such protection is dose dependent with an ED_{50} of ~1.2 μM (Fig. 7 *C*). Therefore, 2′-amino RNA No. 1 can protect IM-9 cells from a significant fraction of the antiinsulin receptor autoantibodies present in a patient with severe insulin resistance.

Discussion

Previously, we and others have described the isolation of small RNA molecules that avidly bind specific antibodies and block them from binding their natural antigens in vitro (13, 14, 18). These observations have led to the speculation that such RNA decoys may be useful as therapeutics to combat autoimmune responses in patients. While these initial studies allowed for much conjecture, they were unable to critically test many aspects of this decoy hypothesis. Specifically, the short half-life of RNAs in the presence of se-

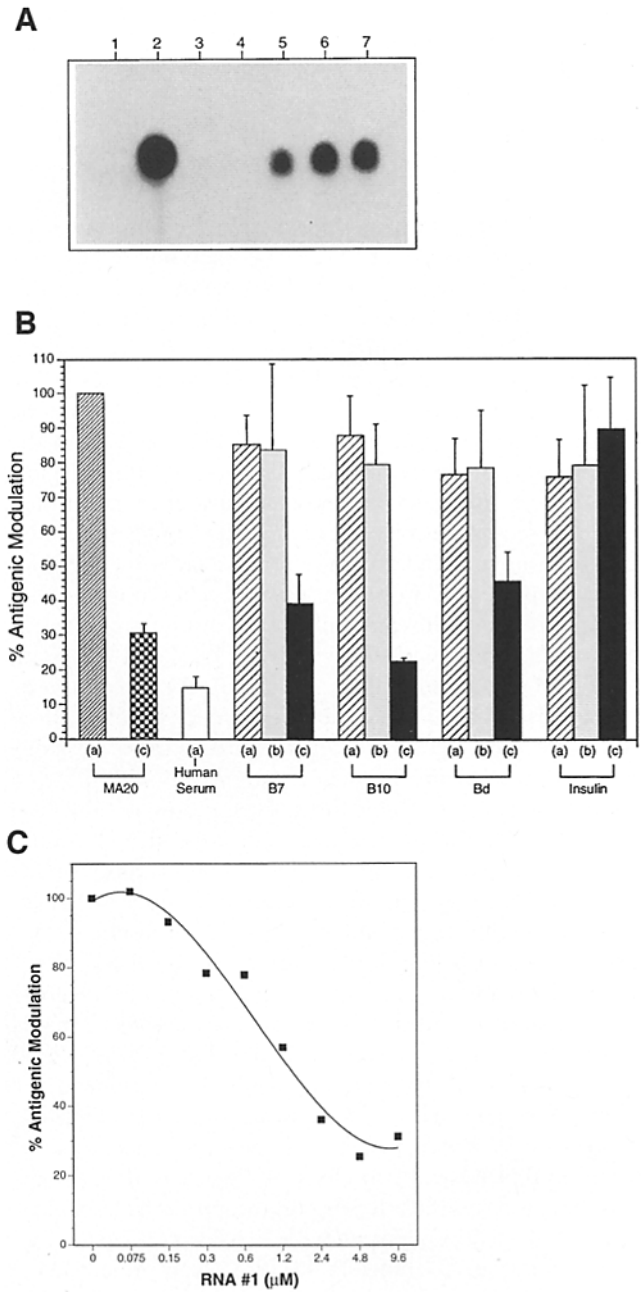

Figure 7. 2′-amino RNA No. 1 can act as a decoy for autoantibodies against the human insulin receptor. (*A*) 2′-amino RNA No. 1 is recognized by human autoantibodies in sera from patients with severe insulin resistance type B. Radiolabeled 2′-amino RNA No. 1 (2.5 nM) was immunoprecipitated with normal mouse IgGs (lane *1*, 100 nM), MA20 (lane *2*, 100 nM), the 83-7 mAb (lane *3*, 3 μl), normal human sera (lane *4*, 50 μl), or three autoimmune patients' sera (lane *5*, serum B7; lane *6*, serum B10; lane 7, serum Bd, 50 μl each). Bound RNA was eluted and electrophoresed on a 6% polyacrylamide gel with urea. (*B*) 2′-amino RNA No. 1 inhibits autoantibody-mediated downmodulation of insulin receptor expression. IM-9 cells were incubated in complete cell culture media containing MA20 (1 nM), normal human sera (1 μl), B7 patient sera (1 μl of 1:25 dilution), B10 patient sera (1 μl of 1:12 dilution), Bd patient sera (1 μl), or insulin (1 μM) that had been preincubated in the absence of RNA competitors (*a*) or with either 3 μM pool RNA (*b*), or 3 μM selected RNA No. 1 (*c*; see Materials and Methods for details). Downmodulation of insulin receptor expression was assayed by ^{125}I-insulin binding, as previously described. Values are expressed as a percentage of the downmodulation engendered by the MA20 antibody (1 nM; percent of antigenic modulation), and the dilutions of patient sera used were chosen because they engender similar levels of downmodulation as 1 nM MA20 (see Materials and Methods for details). Values shown are means ±SD of three measurements obtained from three separate experiments. (*C*) IM-9 cells were incubated in culture media containing B10 patient sera (1 μl of 1:12 dilution) and increasing amounts of 2′-amino RNA No. 1, and downmodulation of insulin receptor expression was assayed as above. Values are expressed as the percentage of the downmodulation engendered by B10 patient sera alone.

rum nucleases made it impossible to determine if a decoy RNA could protect human cells from a patient's autoantibodies. To address this shortcoming, we performed the experiments described herein.

In vitro selection techniques (4, 35, 36) were used to identify a small, nuclease-resistant RNA molecule that binds to a mouse mAb, called MA20, which naturally recognizes the main autoantigenic epitope on the human insulin receptor. To yield nuclease-resistant RNA, selection was performed using a modified RNA pool containing pyrimidine nucleotides with 2′-amino groups (27). The selected RNA binds to the MA20 antibody specifically (Fig. 1) and with high affinity (Fig. 3). The presence of such 2′-amino groups increased the stability of the RNA in serum by more than 10,000-fold, as compared to unmodified RNA (Fig. 4). This selected RNA can serve as a decoy and block MA20 binding to insulin receptors on the surface of human lymphocytes (Fig. 5). This RNA can also protect cells from the MA20 antibody and prevent MA20-mediated downmodulation of insulin receptor expression (Fig. 6). Furthermore, this selected RNA cross-reacts with autoantibodies from three patients with extreme insulin resistance (type B) and can inhibit autoantibody-mediated downmodulation of insulin receptor expression without impeding insulin binding to its receptor (Fig. 7). Thus, a nuclease-resistant decoy RNA can protect human cells from patient autoantibodies.

The three sets of human autoimmune sera contain antibodies that naturally recognize the same region of the human insulin receptor as MA20. This immunogenic region is contained within amino acids 450–601 of the α subunit of insulin receptor and appears to elicit the majority of autoantibodies in patients with extreme insulin resistance (26). Since small RNAs, selected to bind MA20, cross-react with autoantibodies that recognize the same epitope on insulin receptor as MA20 (18 and Fig. 7 *A*), we hypothesized that such selected RNAs may mimic the structure of the receptor that is bound by the antibodies. Such RNA mimotopes would be expected to be able to block a significant fraction of a patient's autoantibodies even though the patient's immune response to the antigenic epitope would probably be oligoclonal. As shown in Fig. 7 *B*, selected 2′-amino RNA No. 1 can significantly block the action of autoantibodies from three different patients, but to varying degrees. These different levels of inhibition may be caused by differences in the patient's immune response to the in-

sulin receptor, with certain patients making a greater percentage of their autoantibodies against the main immunogenic region than others. In the best case, the RNA decoy appears to be able to inhibit the downmodulation of insulin receptor expression that is engendered by the autoantibodies from patient B10, as effectively as it inhibits the MA20 mAb (Fig. 7, *B* and *C*). Thus, remarkably a single RNA decoy can apparently impede a significant fraction of a patient's autoantibodies from binding their targeted self-antigen. This result suggests that one or a few selected decoy RNAs may be able to block autoimmune responses, even though such responses are probably oligoclonal in nature.

The decoy RNA does not appear to inhibit insulin binding to and down-regulation of its receptor on the surface of human lymphocytes (Fig. 7 *B*). This observation that the decoy RNA selectively blocks antibodies is consistent with the hypothesis that the RNA mimics the structure of the immunogenic epitope on the insulin receptor. MA20 has been shown to bind the receptor near but not at the site of insulin binding (23). Thus, a decoy RNA that structurally resembles the antigenic region of the insulin receptor would not be expected to interact with insulin.

Our results suggest that designer decoy RNAs may become useful reagents to inhibit autoimmune responses. To be of any therapeutic value however, more effective RNA decoys will probably be required. To isolate an RNA decoy that can block a greater fraction of autoantibodies from a variety of patients, in vitro selections can be performed using a pool of human autoantibodies. Furthermore, it remains to be determined if nuclease-resistant decoy RNAs can block immune responses in animals or if they will elicit immune responses rather than block them.

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