Enhanced Antigen–Antibody Binding Affinity Mediated by an Anti-idiotypic Antibody†

David G. Sawutz, Richard Koury, and Charles J. Homcy*
Harvard Medical School and Cardiac Unit, Massachusetts General Hospital, Boston, Massachusetts 02114
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ABSTRACT: We previously described the production of four monoclonal antibodies to the β-adrenergic receptor antagonist alprenolol. One of these antibodies, 5B7 (IgG_{2a}, κ), was used to raise anti-idiotypic antisera in rabbits. In contrast to the expected results, one of the anti-idiotypic antisera (R9) promotes [^{125}I]iodocyanopindolol (ICYP) binding to antibody 5B7. In the presence of R9, the dissociation constant decreases 100-fold from 20 to 0.3 nM. This increase in binding affinity of antibody 5B7 for ICYP is not observed in the presence of preimmune, rabbit anti-mouse or anti-idiotypic antisera generated to a monoclonal antibody of a different specificity. Furthermore, R9 in the absence of 5B7 does not bind ICYP. The F(ab) fragments of 5B7 and R9 behaved in a similar manner, and the soluble complex responsible for the high-affinity interaction with ICYP can be identified by gel filtration chromatography. The elution position of the complex is consistent with a 5B7 F(ab)–R9 F(ab) dimer, indicating that polyvalency is not responsible for the enhanced ligand binding. Kinetic analysis of ICYP–5B7 binding revealed that the rate of ICYP dissociation from 5B7 in the presence of R9 is approximately 100 times slower than in the absence of R9 [k_{-1}(+R9) = 0.025 min^{-1} vs. k_{-1}(−R9) = 2.04 min^{-1}], consistent with the 100-fold change in binding affinity of 5B7 for ICYP. The available data best fit a model in which an anti-idiotypic antibody binds at or near the binding site of the idiotype participating in the formation of a hybrid ligand binding site. This would allow increased contact of the ligand with the idiotype–anti-idiotype complex and result in an enhanced affinity of the ligand interaction.

The early work of Oudin and Michel (1963) and Kunkel et al. (1963) demonstrated that, paradoxically, immunoglobulin molecules possess "non-self" antigenic determinants that can promote immune responses within the same organism. On the basis of this work, Jerne (1974, 1975) proposed that the immune system is regulated through a network of idiotypic and anti-idiotypic antibodies. Evidence now exists to support this concept (Rajewsky & Takemori, 1984; Jerne, 1984).

An idiotype has been defined as a set of antigenic determinants (idiotypes) unique to a single molecule (or small set of molecules) that is expressed on the V region of a particular antibody (Rajewsky & Takemori, 1984). Antibodies directed against these determinants are called anti-idiotypic antibodies. Of particular interest are anti-idiotypic antibodies with binding sites that mimic the antigenic structure of the original antigen to which the idiotype was raised. These antibodies are said to contain "internal images" of the antigen (Urbain et al., 1984). Anti-idiotypic antibodies of this type have recently been used in the study of cell surface receptors (Strosberg et al., 1981; Homcy et al., 1982; Venter et al., 1984). For example, Sege and Peterson (1984) demonstrated that anti-idiotypic antibodies generated against anti-insulin antibodies were able to inhibit ^{125}I-insulin binding to isolated rat epididymal fat cells. Recently, anti-idiotypic antibodies have also been used to identify a receptor for suppressor T cells containing the I-J determinant (Zupko et al., 1985).

Several groups have reported the production of polyclonal (Schreiber et al., 1980; Homcy et al., 1982) and monoclonal anti-idiotypic antibodies (Guillet et al., 1984) to anti-alprenolol antibodies that recognize the β-adrenergic receptor. These antibodies inhibited binding of β-adrenergic ligands to the receptor and also modulated agonist-stimulated adenylate cyclase activity. Monoclonal antibodies to the β-adrenergic receptor antagonist alprenolol have been raised by Strosberg and co-workers (Strosberg, 1984). We also reported the production of four anti-alprenolol monoclonal antibodies (Sawutz et al., 1985). In an attempt to obtain anti-idiotypic antibodies that recognize the β-adrenergic receptor and which are specifically directed to the hormone binding site, we used one of these anti-alprenolol monoclonal antibodies, 5B7, to generate polyclonal anti-idiotypic antibodies in rabbits. One anti-idiotypic antiserum (R9) produced an unexpected 100-fold increase in ligand–antibody binding affinity. Studies into the molecular mechanism underlying this effect form the basis for this report.

Materials and Methods

Materials. Anti-digoxin monoclonal antibodies were kindly donated by Dr. Meredith Mudgett-Hunter (Mudgett-Hunter et al., 1985), Cardiac Unit, Massachusetts General Hospital, Boston, MA. Mouse immunoglobulins (IgG, IgM, and IgA) were purchased from Cappel, Inc. Rabbit anti-mouse (RAM) antisera were obtained from Antibodies Incorp. or generated by immunizing New Zealand white rabbits with 200 μg of mouse IgG's in adjuvant on a monthly basis and obtaining weekly bleeds. AcA-34, Sephadex G-25, tris(hydroxymethyl)aminomethane (Tris) base, 1-alprenolol tartrate, bovine serum albumin (fraction V), hemoglobin, IgG (bovine), mercury papain, phenylmethanesulfonyl fluoride (PMSF), and sodium azide were all purchased from Sigma, Inc. DEAE-cellulose and (carboxymethyl)cellulose were obtained from Whatman, Inc. Propanol (reagent grade) was purchased from Fisher. Agarose–protein A was purchased from Bethesda Research Labs, Inc. [^{125}I]Iodocyanopindolol (2000 Ci/mmol) and Na^{125}I were purchased from Amersham, Inc. Cyanogen bromide activated Sepharose 4B-Cl was purchased from

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* Correspondence should be addressed to this author.

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Pharmacia. All tissue culture reagents were obtained from Biofluids Inc. and Freund's adjuvants from Gibco.

Production of Antibodies. The production and characterization of anti-alprenolol monoclonal antibody 5B7 (IgG_{2a},κ) have already been described (Sawutz et al., 1985). Antibody 5B7 was isolated from ascites fluid by DEAE-cellulose chromatography, and 1 mg of antibody emulsified in complete Freund's adjuvant was injected subcutaneously into New Zealand white rabbits. The second and all subsequent immunizations were given at monthly intervals using 1 mg of 5B7 in Freund's incomplete adjuvant. Anti-5B7 antiserum was collected on a weekly basis following each monthly boost and stored at −20 °C until needed.

Purification of 5B7 IgG and F(ab) Fragments. The IgG fraction of 5B7 ascites (20 mL) was precipitated with 40% ammonium sulfate at 4 °C, resuspended in phosphate-buffered saline containing 0.02% sodium azide (PBSA, pH 7.4), and then extensively dialyzed against PBSA. The antibody was incubated with 15 mL of a Sepharose–nadolol (analogue of alprenolol) resin (Homcy et al., 1983) overnight at 4 °C. The resin was washed with ice-cold PBSA, and 5B7 was eluted in three steps by incubating the resin first with 15 mL of PBSA plus alprenolol (100 μM), then with 15 mL of 1 M NaCl plus alprenolol for 1 h, and finally with 15 mL of 5 M guanidine hydrochloride (pH 7.4) for 15 min. The elutions were immediately dialyzed against PBSA (3 × 6 L). A total of 55 mg of 5B7 IgG was recovered from all three elutions. The alprenolol and high-salt elutions (40 mg) were combined, and the F(ab) fragments were prepared with papain essentially as described by Oi and Herzenberg (1979). The papain reaction mixture was dialyzed overnight against 50 mM phosphate buffer (pH 8.0) and then against 5 mM phosphate buffer (pH 8.0). The 5B7 F(ab) fraction was applied to a DEAE-cellulose column equilibrated in 5 mM sodium phosphate buffer (pH 8.0) and eluted with 5 mM phosphate buffer (pH 8.0), yielding 18 mg of 5B7 F(ab). All 5B7 F(ab) and R9 F(ab) preparations were passed over a 1-mL protein A–agarose column to ensure removal of any Fc′ fragments and undigested IgG.

Purification of R9 Anti-idiotypic IgG and F(ab) Fragments. R9 anti-idiotypic antiserum was precipitated with 40% ammonium sulfate, and the IgG fraction was isolated by DEAE-cellulose chromatography with 10 mM sodium phosphate buffer, pH 7.0 (Good et al., 1980). The IgG fraction was dialyzed against PBSA and then passed twice over a Sepharose 4B-CL normal mouse IgG column (17 mL of packed resin; 50 mg of mouse IgG) to absorb nonspecific rabbit anti-mouse antibodies. The "fall through" from the column was used for subsequent solid phase assays and F(ab) preparations.

R9 F(ab) fragments were made by incubating R9 IgG (100 mg) with 1 mg of papain in 15 mL of 100 mM sodium phosphate buffer, pH 7.0, containing 2 mM ethylenediaminetetraacetic acid (EDTA) and 10 mM L-cysteine for 90 min at 37 °C. The papain digestion was terminated by the addition of iodoacetamide (20 mM). The digestion mixture was dialyzed against 10 mM sodium acetate buffer (pH 5.8), and the R9 F(ab) fragments were purified by (carboxymethyl)cellulose chromatography in 10 mM acetate buffer, pH 5.8. No IgG was detected following sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (Laemmli, 1970) in any F(ab) preparation.

Affinity Purification of R9 IgG. R9 IgG was affinity purified by 5B7–Sepharose column chromatography. Antibody 5B7 (IgG) was incubated overnight at 4 °C with cyanogen bromide activated Sepharose 4B-CL (Pharmacia) and then with 100 mM ethanolamine for 1 h to block any unreacted coupling sites. The resin was washed extensively with PBSA, and aliquots were tested for binding activity. ICYP binding to 5B7–agarose aliquots, which was inhibited by alprenolol, indicated the presence of intact 5B7 on the resin. The 5B7–Sepharose resin (6 mL) was packed in a column and equilibrated with PBSA that included 10 μM alprenolol. DEAE-cellulose-purified R9 antibody in PBSA containing alprenolol was repeatedly passed over the resin. The resin was then washed with 3 column volumes of 1 M NaCl containing alprenolol (10 μM). The R9 IgG was eluted with guanidine (5 M) in PBSA, and the eluate was immediately dialyzed against PBSA (3 × 4 L). The dialyzed IgG was then used in subsequent binding experiments.

Solid Phase Assay for Determining Anti-idiotypic Antibody Activity. Solid phase assay plates were prepared by coating 96-well flexible microtiter plates with 50 μL of a 0.1 mg/mL R9 (anti-5B7) IgG solution overnight at 4 °C. The plates were washed with PBSA, and nonspecific binding sites were blocked with 10% normal horse serum in PBSA for 1 h at 23 °C. Antibody 5B7 (10 μg) was iodinated with $Na^{125}I$ using chloramine-T (Greenwood et al., 1963), and ^{125}I-5B7 (600 Ci/mmol) was separated from free ^{125}I by Sephadex G-25 column chromatography in PBSA. Radiolabeled 5B7 (50000 cpm/25 μL) was incubated for 2 h at 23 °C with 25 μL of culture medium diluted 1:5 in PBSA or with 25 μL of increasing concentrations of 5B7 IgG or F(ab) fragments. After the incubation period, each well was washed extensively with PBSA, then excised, and counted for 1 min in a Micromedic γ counter (efficiency = 80%).

5B7 F(ab)–R9 Antibody Binding Experiments. Five micrograms of affinity-purified 5B7 F(ab) was iodinated as described above to achieve a final specific activity of 44.2 μCi/μg (1990 Ci/mmol). R9 IgG (3.4 mg; 17 mL) was incubated with 500 μL of agarose–protein A resin (Bethesda Research Labs, Inc.) for 90 min at 23 °C. The suspension was centrifuged at 7500 rpm for 10 min, and the resin was resuspended in 15 mL of PBSA containing 6.0 mg of Fc′ isolated from anti-digoxin monoclonal antibody 44-10 to block any remaining protein A binding sites. The resin was incubated for 1 h at 23 °C, the supernatant was removed by centrifugation, and the resin was washed in 50 mL of buffer consisting of 10 mM Tris (pH 7.4), 150 mM NaCl, 1 mM PMSF, 0.02% NaN_3, and 0.1% bovine serum albumin (BSA). The washed resin was resuspended in 10 mL of the same buffer and used for the binding assays.

In the 5B7 F(ab) competitive inhibition assays, 20 μL of R9–agarose solution was incubated with ^{125}I-5B7 F(ab) [(0.5–1.0) × 10^5 cpm/50 μL] in the absence or presence of increasing concentrations of unlabeled 5B7 F(ab) for 1 h at 23 °C. At the end of the incubation period, 4 mL of ice-cold buffer was added to each tube, and the contents were filtered under vacuum through S&S glass fiber filters. The filters were then washed with 3 × 4 mL of ice-cold buffer and counted for 1 min in a Micromedic γ counter (efficiency = 80%).

Iodocyanopindolol Binding Assays. The specific details of the individual [^{125}I]iodocyanopindolol (ICYP) equilibrium binding experiments are presented in the figure legends. Generally, 5B7 [F(ab) or IgG] was incubated with ICYP (0.1 nM) in the presence of R9 IgG or F(ab) for 1 h at 37 °C. Rabbit anti-mouse (RAM) antiserum served as a nonspecific control for R9, and all assays were done in the absence or presence of alprenolol (10 μM), which was used to determine nonspecific binding. A buffer consisting of 10 mM Tris-HCl (pH 7.4), 150 mM NaCl, and 0.1% BSA was used in all

binding assays at a final assay volume of 300 μL. The ICYP binding assays were terminated by one of three methods, each of which gave comparable results. Precipitation of the binding complexes with 1-propanol (Couraud et al., 1985) was accomplished by adding 1 mL of ice-cold propanol to each tube, mixing by vortex, and immediately centrifuging the tubes at 7500 rpm for 20 min at 4 °C. The supernatants were removed by aspiration, and the pellets were counted for radioactivity.

A propanol filtration assay was used in addition to the precipitation assay. Following the addition of ice-cold 1-propanol (1 mL) to the assay tubes, the contents were immediately filtered under vacuum through S&S glass fiber filters, and each filter was washed with 2 × 4 mL of cold propanol. The filters were then counted for trapped radioactivity.

The third assay employed was a double antibody precipitation assay. After the incubation period, the assay tubes were placed in an ice–water bath, and 100 μL of rabbit anti-mouse antibody (1:100 dilution in assay buffer) was added to each tube. This was followed by 50 μL of goat anti-rabbit IgG antiserum, and the assay tubes were incubated at 4 °C for 30 min during which a precipitate formed. To each tube was added 4 mL of cold assay buffer, and the contents were filtered under vacuum through S&S glass fiber filters. Each filter was washed with 3 × 5 mL of buffer and then counted for trapped radioactivity. For each binding protocol, nonspecific binding was always less than 5% of the total radioactivity added in the assay.

Data Analysis. Dissociation binding constants (K_D) determined from equilibrium binding studies were calculated from the untransformed radioligand binding data using LIGAND (Munson & Rodbard, 1980), a mass action based, weighted, nonlinear curve-fitting program. IC_{50} values from competitive inhibition curves were calculated by using the computer program ALLFIT (De Lean et al., 1978). Analysis of mean and standard deviation data was done by using the Student's *t* test.

RESULTS

Characterization of Anti-5B7 Anti-idiotypic Antibody R9. Competitive inhibition studies, using affinity-purified ^{125}I-5B7 in a solid phase assay system, were conducted to determine whether anti-5B7 anti-idiotypic antibodies were present in the rabbit antiserum. Anti-alprenolol monoclonal antibody 5B7 inhibits the binding of ^{125}I-5B7 to wells coated with R9 immunoglobulins, as shown in the top panel of Figure 1. Antibody 5D9 (IgG_1,κ), which has a lower affinity for alprenolol (Sawutz et al., 1985), also significantly inhibits 5B7 binding to R9. Neither a third monoclonal anti-alprenolol antibody, 1B7 (IgG_{2b},κ), nor four anti-digoxin monoclonal antibodies possessing various specificities for digoxin (Mudgett-Hunter et al., 1985) inhibit the binding. Inhibition of ^{125}I-5B7 binding to R9 by unlabeled 5B7 IgG and 5B7 F(ab) fragments is demonstrated in the bottom panel of Figure 1. The inhibition of binding is dose dependent, and the IC_{50} values for the inhibition curves shown in the bottom panel of Figure 1 are calculated to be 12 and 15 ng for intact 5B7 IgG and 5B7 F(ab), respectively.

The affinity of 5B7 F(ab) to R9 can be estimated by measuring ^{125}I-5B7 F(ab) binding to immunoaffinity-purified R9 IgG coupled to agarose–protein A beads. Figure 2 demonstrates that 5B7 F(ab) inhibits the binding of ^{125}I-5B7 F(ab) to R9 IgG in a dose-dependent manner. An approximation of the affinity of 5B7 F(ab) for R9 IgG from three experiments is calculated to be 1.8 ± 0.27 nM on the basis of IC_{50} values obtained from ALLFIT analysis of the competitive inhibition curves.

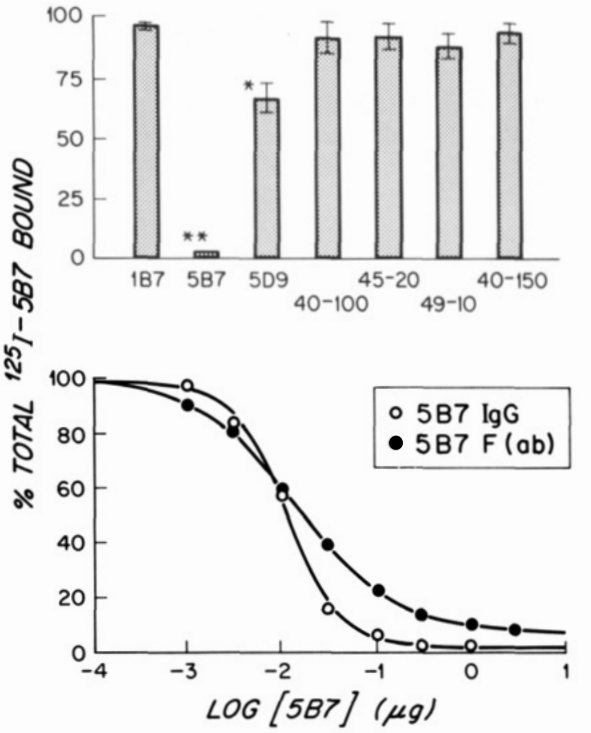

FIGURE 1: Demonstration of R9 anti-idiotypic activity in a solid phase assay system. R9 anti-idiotypic activity was determined by using a solid phase assay as described under Materials and Methods. The monoclonal antibodies tested included the anti-alprenolol monoclonal antibodies 1B7 (γ_{2b},k), 5B7 (γ_{2a},k), and 5D9 (γ_1,k) plus the anti-digoxin monoclonal antibodies 40–100 (γ_1,k), 45–20 (γ_{2a},λ_1), 49–20 (γ_1,k), and 40–150 (γ_1,k). The data in the top panel represent means and standard deviations from two experiments and were analyzed by using the student's *t* test. Significance is defined as $p < 0.05$ for one asterisk and $p < 0.001$ for two asterisks. The dose–response curves in the bottom panel were analyzed with ALLFIT (De Lean et al., 1978). IC_{50} values were 12 and 15 ng for intact 5B7 IgG and 5B7 F(ab), respectively.

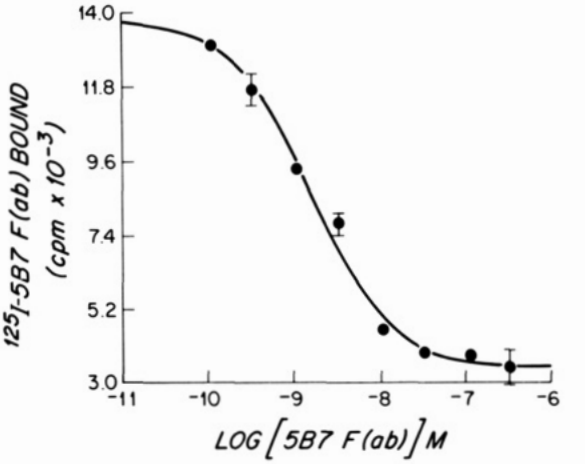

FIGURE 2: Inhibition of ^{125}I-5B7 F(ab) binding to R9 attached to agarose beads. R9 IgG was coupled to protein A–agarose, and 5B7 F(ab) competitive inhibition curves with ^{125}I-5B7 F(ab) were generated as described under Materials and Methods. The binding of ^{125}I-5B7 F(ab) to the R9 agarose beads is specific for 5B7 because unrelated IgG and F(ab) fragments of antibodies with different specificities do not inhibit the antibody binding. The data presented are mean and standard errors of triplicate determinations from one of three similar experiments. The mean IC_{50} value from all three experiments was calculated to be 1.8 ± 0.3 nM. Nonspecific binding (3000 cpm) was approximately 5% of the total ^{125}I-5B7 F(ab) added to each assay tube.

Anti-5B7 Promotion of Ligand Binding to Antibody 5B7. Screening of the anti-idiotypic antiserum for inhibition of ligand binding to 5B7 demonstrated that R9 produces a

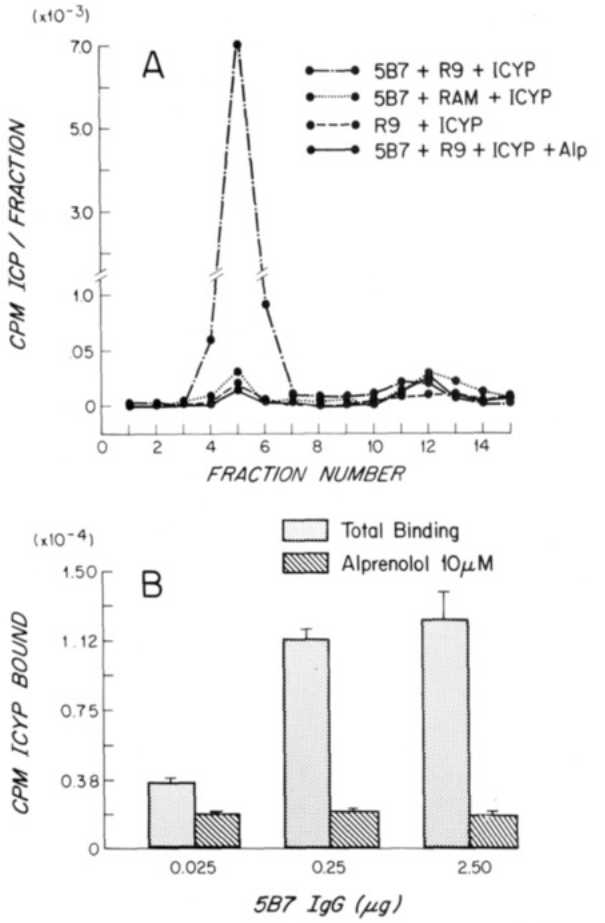

FIGURE 3: Promotion of ICYP binding to 5B7 by R9 anti-idiotypic antiserum. (A) A 10-mL Sephadex G-25 column was equilibrated in assay buffer consisting of 150 mM NaCl, 10 mM Tris-HCl, and 0.1% BSA, pH 7.4. Antibody 5B7 culture medium (100 μL of a 1:30 dilution) was incubated with ICYP (0.15 nM final concentration) and R9 antiserum (1:10^3 dilution), with R9 antiserum plus 10 μM alprenolol, or with rabbit anti-mouse antiserum (1:10^3 dilution) for 1 h at 23 °C in a final assay volume of 300 μL. The contents of each assay tube were then transferred to the G-25 column, and 1-mL fractions were collected and assessed for radioactivity. The data are presented as total cpm of ICYP per fraction. No binding of ICYP by R9 antiserum was detected in a separate run under identical conditions. (B) The effect of affinity-purified R9 IgG on the ability of affinity-purified 5B7 to bind [^{125}I]iodocyanopindolol (ICYP) was determined with a propanol precipitation assay as described under Materials and Methods. R9 (2.5 μg) was incubated with ICYP (0.1 nM) plus increasing concentrations of purified 5B7 in the absence and presence of alprenolol (10 μM) which was used to determine nonspecific binding. The assay tubes were incubated for 1 h at 37 °C to reach equilibrium. Nonspecific radioactivity trapped in the pellet in the absence of antibody was less than 5% of the total radioactivity added to each tube. The data presented in (B) are means and standard deviations of duplicate determinations from a typical experiment.

marked enhancement of ^{125}I-iodocyanopindolol (ICYP) binding to 5B7. The following experiment suggests that this effect is due to an enhancement in the binding affinity of 5B7 for ligand in the presence of R9. Figure 3A demonstrates the results of an experiment wherein the binding of ICYP to 5B7 is measured by separating bound ligand on a Sephadex G-25 column. By holding the concentration of ICYP to 0.1 nM (significantly less than the K_D of 5B7 for ICYP), no ICYP binding to 5B7 is observed. However, in the presence of R9, ICYP binding to antibody 5B7 is readily apparent (Figure 3A). ICYP binding to 5B7 is not observed if the R9 antisera are replaced with rabbit anti-mouse (RAM) antiserum. ICYP binding to R9 does not occur in the absence of 5B7, and the binding of ICYP to the presumed 5B7–R9 complex is completely inhibited by alprenolol (10 μM). Neither R9 preimmune serum nor rabbit anti-idiotypic antiserum generated against a monoclonal antibody of the same isotype as 5B7 (but specific for the cardiac glycoside digoxin) produces the enhanced ligand binding. The effect of R9 antiserum is dose dependent. Half-maximal enhancement of ligand binding is observed at a serum dilution of 1:10^4, and promotion of ligand binding reached its peak at a serum dilution of 1:10^3 (data not shown).

The increase in ICYP binding to 5B7 can also be demonstrated in a precipitation assay with affinity-purified immunoglobulin fractions of 5B7 and R9. In the presence of a fixed concentration of R9 (2.5 μg), ICYP binding was determined at three different concentrations of 5B7 that correspond to R9:5B7 molar ratios of 100:1, 10:1, and 1:1 (Figure 3B). Maximal enhancement of ICYP binding is observed at a 10:1 R9:5B7 molar ratio. Higher concentrations of 5B7 (2.5 μg; molar ratio = 1:1) produce no further increase in binding. Alprenolol (10 μM) completely inhibits ICYP binding in the presence of purified R9, again indicating the specificity of the interaction. We presume that a 10:1 ratio of R9 to 5B7 is required since the anti-idiotypic antibody is a polyclonal reagent and the IgG species producing the enhanced binding may therefore be a small percentage of the total R9 IgG pool.

Characterization of 5B7 F(ab)–R9 F(ab) Binding Complexes. F(ab) fragments were prepared from IgG fractions isolated from both 5B7 ascites and R9 antiserum to determine whether the increased binding observed with the anti-idiotype requires a polyvalent interaction. R9 F(ab) promoted ICYP binding to both whole 5B7 IgG and 5B7 F(ab) with equal potency (when compared to the effect of R9 IgG, data not shown). To further characterize the nature of the 5B7 F(ab)–R9 F(ab) interaction, the binding complexes were identified by size-exclusion gel filtration chromatography. Figure 4 (top) demonstrates that ICYP binding to the 5B7 F(ab)–R9 F(ab) complex elutes from an AcA-34 column earlier (i.e., as a higher molecular weight species) than either F(ab) species alone and before the molecular weight standard, hemoglobin. The specificity of binding is demonstrated by its inhibition in the presence of 10 μM alprenolol. These results are consistent with a complex consisting of R9 F(ab), 5B7 F(ab), and ICYP. The molecular weight of this proposed ternary complex is characterized more accurately with a Spherogel TSK-3000 high-performance liquid chromatography (HPLC) column (Figure 4, bottom). The open triangles indicate the elution position of ^{125}I-5B7 F(ab) alone. In the presence of R9 F(ab), the peak of radioactivity (closed triangles) shifts to an earlier elution position [elution time (et) = 10 min] between that of the molecular weight standards IgG (150 000, et = 8.7 min) and hemoglobin (67 000, et = 11.5 min). This is consistent with an R9 F(ab)–5B7 dimer. To determine if the peak of ligand binding activity coincides with the elution position of the dimeric complex, we repeated the same protocol after incubating unlabeled F(ab) fragments of 5B7 and R9 in the presence of ICYP. As can be seen in the figure, the two peaks (□, ▲) are in fact superimposable, indicating that a species composed of 5B7 F(ab) and R9 F(ab) in a 1:1 stoichiometry is responsible for the enhancement of ligand binding. Specificity is again demonstrated by the ability of alprenolol to completely inhibit ICYP binding to the complex.

Effect of R9 on 5B7–ICYP Binding Affinity. Both propanol and double antibody precipitation assays were used to quantitate the increase in ICYP binding to 5B7 in the presence of

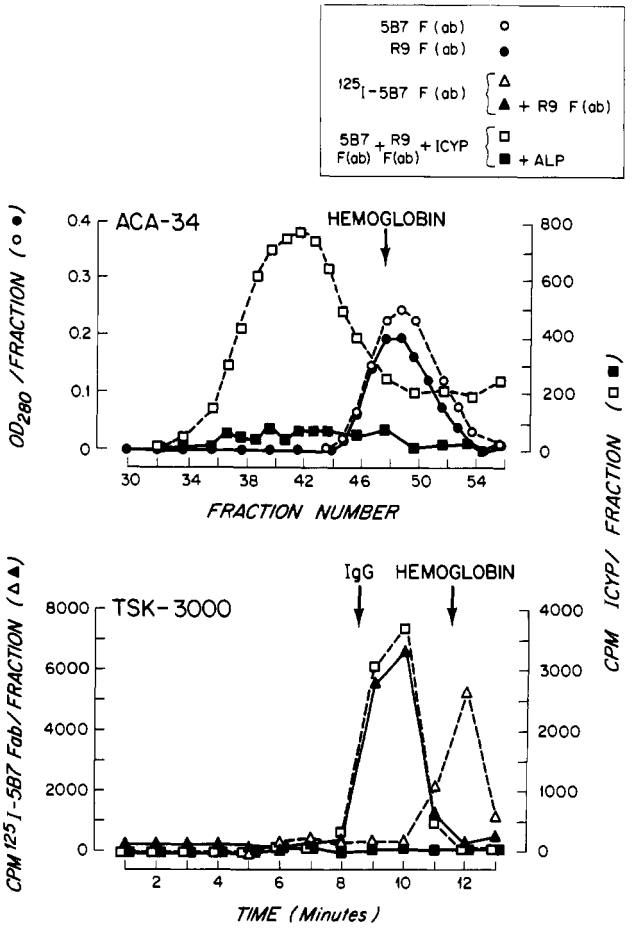

FIGURE 4: Separation of 5B7–R9–ICYP F(ab) complexes by size-exclusion chromatography. (Top) An AcA-34 column (100-mL bed volume) was equilibrated in PBSA, pH 7.4. 5B7 (○) and R9 (●) F(ab) fragments were chromatographed separately on the AcA-34 column, and the absorbance at 280 nm was measured for each of the 1-mL fractions. Dextran blue and hemoglobin were found to elute at fractions 25 and 48, respectively, in separate runs. 5B7 and R9 F(ab) fragments, 10 and 100 μg, respectively, were then incubated with ICYP (80 pM final concentration) in the absence (□) or presence (■) of 10 μM alprenolol for 1 h at 37 °C. At the end of the incubation period, the contents of each assay tube were transferred to the AcA-34 column and eluted with PBSA. Fractions containing 1 mL of eluate were collected and assessed for radioactivity by counting for 1 min in a Micromedic γ counter (efficiency = 80%). (Bottom) 5B7–R9 F(ab) complexes and ICYP binding activity were separated by size-exclusion chromatography on a TSK-3000 Spherogel HPLC column. The column, attached to a Beckman (Model 332) HPLC, was equilibrated in prefiltered PBSA at a pressure of 250 psi and a flow rate of 1 mL/min. The column was calibrated by making separate 15-min runs with bovine γ-globulins (et = 8.7 min) and hemoglobin (et = 11.5 min). Samples of 200 μL were applied to the column for all HPLC runs. ^{125}I-5B7 F(ab) was incubated for 20 min at 37 °C in the absence (△) or presence (▲) of 1.0 μg of R9 F(ab) before injection onto the TSK-3000 column. Fractions were collected every minute and assessed for radioactivity. In separate binding experiments, 1 μg of 5B7 F(ab) and 10 μg of R9 F(ab) were incubated with ICYP (80 pM final concentration) in the absence (□) and presence (■) of 10 μM alprenolol for 1 h at 37 °C. The assay mixtures were injected onto the HPLC column, and 1-min fractions were collected. The data are presented as total cpm of ^{125}I-5B7 or ICYP per fraction vs. time (minutes).

R9. Saturable binding of ICYP to 5B7 can be demonstrated in the presence of R9 (Figure 5), and these binding data are transformed into linear Scatchard plots by using the mass action based computer program LIGAND (Munson & Rodbard, 1980). In 14 experiments, ICYP (in the presence of excess R9) bound to 5B7 with a dissociation constant (K_D) of 0.30 ± 0.04 nM, and the data fit to a single class of binding sites. This K_D is approximately 100-fold higher than the K_D of 5B7

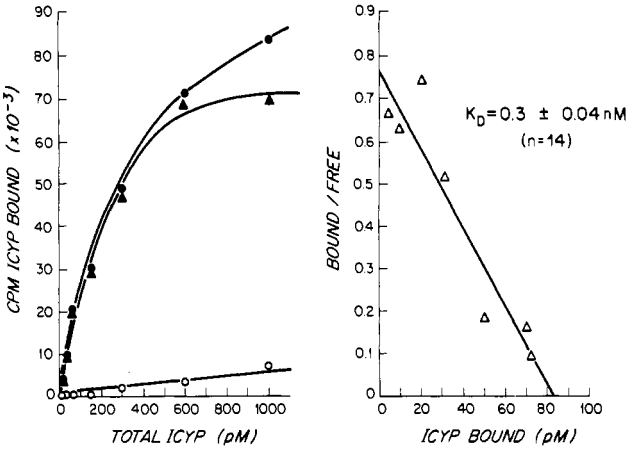

FIGURE 5: Binding of ICYP to 5B7 in the presence of anti-idiotypic antibody R9. 5B7 culture medium (1:5 dilution) was incubated for 1 h at 37 °C with R9 anti-idiotypic antiserum (1:300 dilution) and increasing concentrations of ICYP from 10 pM to 1 nM. Alprenolol (10 μM) was used to determine nonspecific binding. Termination of the binding assay was accomplished with either the propanol precipitation assay or the double antibody precipitation assay as described under Materials and Methods. The data represent a typical binding experiment with a calculated K_D of 0.3 ± 0.04 nM (n = 14) based on LIGAND computer analysis of the saturation binding data. Similar results were also obtained with affinity-purified 5B7 and R9 antibodies. Total (●), nonspecific (○), and specific (▲) binding.

for alprenolol, which was previously reported to be 20 nM (Sawutz et al., 1985). Specific ICYP binding to 5B7 was also obtained in a double antibody precipitation assay with RAM; however, saturation binding curves could not be completed because of the low affinity of 5B7 for ICYP in the absence of R9. An approximation of the K_D for ICYP–5B7 binding in the presence of RAM was obtained from competitive inhibition assays. Increasing concentrations of alprenolol inhibit ICYP binding to 5B7 in a dose-dependent manner, with a calculated K_D of 25 nM (which agrees with our previous results). Similar results can be demonstrated with the β-adrenergic receptor antagonist pindolol. The effect of R9 on the binding affinity of 5B7 for ICYP is depicted graphically in Figure 6. When the percent occupancy of 5B7 is plotted against the log of the ligand concentration used in the saturation and competitive inhibition binding experiments, the presence of anti-idiotypic antibody causes the binding curve to shift 100-fold to the left.

Kinetics of Ligand–5B7 Binding. Kinetic studies of ICYP–5B7 binding revealed that the dissociation rate constant (k_{-1}) of ICYP from 5B7 was significantly reduced in the presence of R9 (Figure 7). The dissociation rate constant was determined after elimination of the forward reaction by the addition of a high concentration of alprenolol (100 μM). A dissociation rate constant of 0.025 ± 0.005 min^{-1} (n = 3) was determined in the presence of R9 compared to 2.04 ± 0.16 min^{-1} (n = 3) calculated in the absence of R9. The 100-fold difference in k_{-1} values closely approximates the difference in K_D values (0.3 vs. 25 nM) obtained from the saturation binding experiments and competitive inhibition curves.

In another series of experiments, the association rate constant for ICYP binding to 5B7 was measured either at the same time as the formation of the 5B7–R9–ICYP complex or after formation of an R9–5B7 complex. When R9 and 5B7 are coincubated at 37 °C for 30 min before the addition of ICYP, a monophasic binding isotherm is observed. If R9 is coincubated with ICYP for 30 min at 37 °C and the binding assay is initiated by the addition of 5B7, then a biphasic curve is observed (data not shown), even though binding reaches

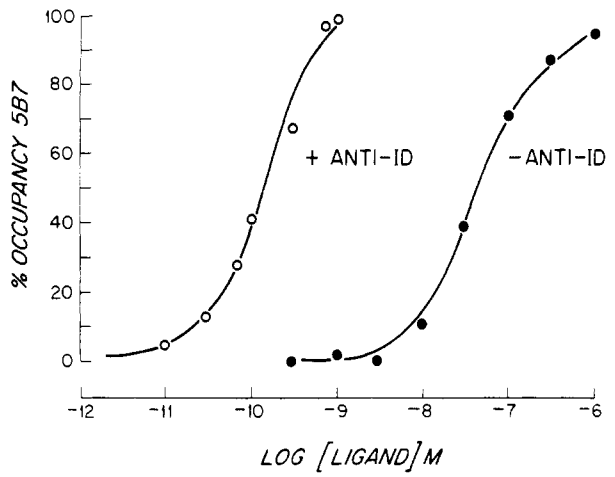

FIGURE 6: Effect of R9 anti-idiotypic antiserum on the binding of ligand to 5B7. The affinity of 5B7 for ICYP in the presence of R9 (○) was determined by ICYP saturation binding experiments (see Figure 5). To determine the affinity of ligand for 5B7 in the presence of rabbit anti-mouse (RAM) antiserum (●), a competitive inhibition assay was used because a complete saturation binding isotherm for ICYP to 5B7 could not be generated due to its intrinsically low affinity. 5B7 culture medium (1:5 dilution) and RAM (1:300 dilution) were incubated with ICYP (0.5 nM final concentration) and increasing concentrations of (−)-pindolol for 1 h at 37 °C. The competitive inhibition assays were terminated with the double antibody precipitation protocol described under Materials and Methods. LIGAND analysis of the binding data from two similar experiments revealed a K_D of 42 nM for (−)-pindolol binding to 5B7 in the presence of RAM. The data in this figure, demonstrating the 100-fold shift in binding affinity, are from individual representative experiments.

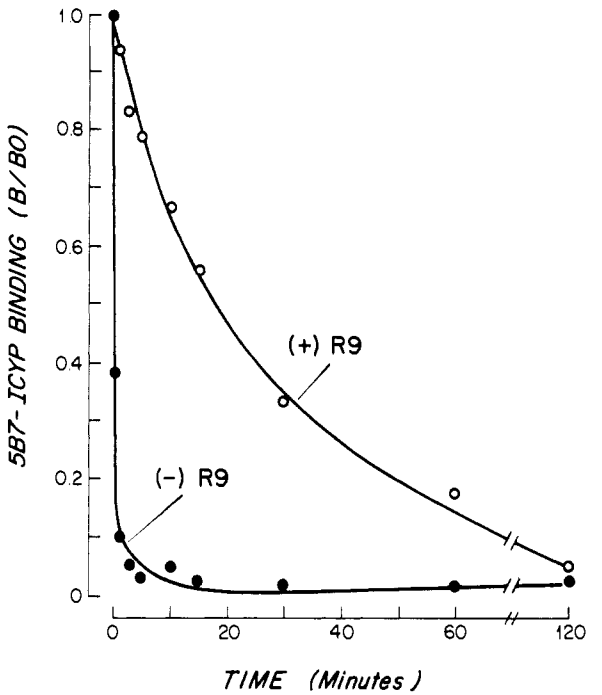

FIGURE 7: Effect of R9 on the rate of ICYP dissociation from 5B7. ICYP binding assays were constructed to give similar levels of total binding. In the absence of R9, 5B7 (50–100 μg) and ICYP (0.5 nM) were coincubated for 30 min at 37 °C. In the presence of R9 (5–10 μg), 5B7 (0.5–1.0 μg) and ICYP (0.1 nM) were incubated under the same conditions. At the end of the incubation period, alprenolol (0.1 mM) was added, and the binding assays were terminated at various time points from 0 to 120 min using the propanol filtration method. Three dissociation experiments were done for each condition. ICYP dissociation rate constants (k_{-1}) of 2.04 ± 0.16 and 0.025 ± 0.005 min^{-1} in the absence and presence of R9, respectively, were calculated by plotting ln (B/B_0) against time (minutes), the slope of which is equal to $-k_{-1}$.

equilibrium at the same time. These data indicate that several processes may occur during the first few minutes of the interaction resulting from R9 binding to 5B7, ICYP binding to 5B7, and ICYP binding to a 5B7–R9 complex.

Effect of Temperature on ICYP–5B7 Binding Affinity. Thermodynamic analyses of ICYP binding to 5B7 in the absence and presence of R9 were done in order to gain further insight into the molecular basis of the 5B7–ICYP–R9 interaction. Changes in the entropic and enthalpic components of ICYP–5B7 binding in the presence of R9 might reflect conformational changes in the antibody binding site. The effect of increasing temperatures (4–50 °C) on the inhibition of ICYP binding to 5B7 by alprenolol revealed that there are no differences in the binding affinity of 5B7 for alprenolol at any temperature (Table I). Similar results were obtained from analysis of saturation binding experiments for ICYP binding to 5B7 in the presence of R9.

The standard Gibbs free energy change ($\Delta G°$) of binding was calculated from the equation:

$$\Delta G° = -RT \ln K_A \quad (1)$$

where K_A is the equilibrium association constant ($1/K_D$), T is the temperature in degrees kelvin, and R is the gas constant (1.99 cal mol^{-1} deg^{-1}). Changes in the standard Gibbs free energy are determined by changes in entropy ($\Delta S°$) and enthalpy ($\Delta H°$) of the binding at various temperatures as shown in eq 2:

$$\Delta G° = \Delta H° - T\Delta S° \quad (2)$$

By using the van't Hoff equation:

$$\ln K_A = -\Delta H°/RT + \Delta S°/R \quad (3)$$

a plot of ln K_A against $1/T$ provides an approximation of the enthalpic change ($\Delta H°$) because the slope of the line is $-\Delta H°/R$. From the $\Delta H°$ and $\Delta G°$ values, the relative change in entropy ($\Delta S°$) can then be calculated.

Table I: Effect of Temperature on the Thermodynamics of 5B7–Ligand Binding with and without R9 Anti-idiotypic Antibody[a]

	−R9			+R9		
temp (°C)	K_I (nM)	$\Delta G°$ (kcal/mol)	$\Delta S°$ (eu)	K_D (nM)	$\Delta G°$ (kcal/mol)	$\Delta S°$ (eu)
6	22.7	−9.77	+39.5	0.36	−12.17	+50.2
16	19.9	−10.19	+39.6	0.40	−12.31	+49.1
24	25.6	−10.33	+38.9	0.34	−12.92	+48.7
37	26.9	−10.75	+38.7	0.39	−13.40	+49.1
50	25.2	−11.24	+38.7	0.54	−13.73	+48.2

[a] K_D values for ICYP binding to 5B7 in the presence of R9 were calculated from saturation binding experiments. Antibody 5B7 (0.1–1.0 μg) antibody was incubated with R9 (1–10 μg) and increasing concentrations of ICYP (10 pM to 2 nM) in the absence and presence of 10 μM alprenolol to determine nonspecific binding. The assays were terminated by the propanol precipitation method described under Materials and Methods. In the absence of R9, K_I values were calculated from alprenolol competitive inhibition curves of ICYP binding to 5B7. ICYP (0.3 nM) was incubated with 1.0 μg of 5B7 and increasing concentrations of alprenolol (1 nM to 1 μM). The competitive inhibition assays were terminated by the double antibody precipitation protocol as described under Materials and Methods. The incubation times for each temperature were 10 min (50 °C), 60 min (37 °C), 120 min (24 °C), 15 h (16 °C), and 24 h (6 °C) in every experiment. The binding constant data presented are mean values of two alprenolol competitive inhibition experiments and three ICYP saturation binding experiments at each temperature.

A van't Hoff plot of ligand binding to 5B7 at several temperatures (Table I) with or without R9 is presented in Figure 8. The plots are linear over the entire temperature range, suggesting that the change in enthalpy is independent of

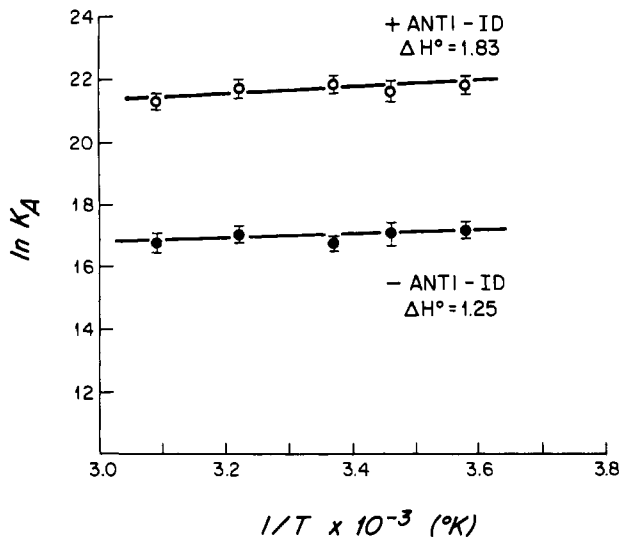

FIGURE 8: van't Hoff plots of the dependence of K_A on temperature. The data presented are for ligand binding in the absence (●) or presence (○) of R9 anti-idiotypic antibody. K_A values (means and SD) were calculated from the binding constants obtained from two to three binding assays for each condition as described in Table I. The slope of the line obtained from plotting ln K_A against $1/T$ ($\times 10^{-3}$) is equal to $-\Delta H^\circ/R$. Linear regression analysis of the binding data revealed ΔH° values of 1.83 and 1.25 kcal/mol in the presence and absence of R9, respectively.

temperature. ΔH° values calculated by regression analysis are essentially the same for the binding of ligand to 5B7 in the absence (ΔH° = 1.25 kcal/mol) and presence (ΔH° = 1.83 kcal/mol) of R9.

Discussion

The data presented here demonstrate that an anti-idiotypic antibody can be raised that will enhance the binding of antigen to the original idiotype. A rabbit anti-idiotypic antiserum, R9, was raised against the anti-alprenolol monoclonal antibody 5B7. Promotion of ICYP binding to 5B7 in the presence of R9 was observed, and this enhancement appears to be the result of a 100-fold increase in the binding affinity of 5B7 for the ligand. An increase in the binding of ^{125}I-pindolol and [^{3}H]dihydroalprenolol to 5B7 in the presence of R9 was also observed. The affinity of R9 antiserum for ligand was of such low affinity that ICYP binding could not be measured to R9 alone. The increase in ICYP binding could not be duplicated with rabbit antisera to murine antibodies of specificities other than that of 5B7. R9 was selective for 5B7, with regard to the increase in ligand binding, although it did produce a small increase in ICYP binding to anti-alprenolol antibody 5D9 (data not shown). This correlates with the 20–30% inhibition of ^{125}I-5B7 binding to R9 by 5D9 (see Figure 2) and is consistent with idiotypy shared by the two monoclonal antibodies.

A dimer composed of a 5B7 molecule and an R9 molecule appears to be responsible for the increase in ligand binding. This conclusion is based on the size-exclusion gel filtration data presented in Figure 4. These studies also clearly indicate that polyvalency was not required for this interaction to occur. The increase in binding affinity can be directly related to the decrease in the rate of dissociation of the ligand from the 5B7–R9 complex. A 100-fold decrease in the dissociation rate constant was observed in the presence of anti-idiotypic antibody. Measurement of the association rate of ligand binding was performed in one of two ways. Either the ligand was allowed to bind to the preferred idiotype–anti-idiotype complex or the three reactants were added simultaneously. Under the first set of conditions, a linear monophasic rate of association was observed in comparison with the biphasic pattern observed in the latter case. An initial rapid rate of association preceded a somewhat slower on rate, which was identical with that observed when the 5B7–R9 complex had been preassociated. It is likely that this latter rate represents the association rate of the ligand with the 5B7–R9 complex that had formed within the initial minutes of the incubation. Nevertheless, once the ligand is appropriately oriented, its rate of dissociation from the 5B7–R9 complex is markedly slower (as shown in Figure 7).

Previous studies demonstrate that select antibodies, by virtue of possessing an internal image of the ligand, can induce functional (conformational) changes in a protein after ligand binding. It has been previously reported that rabbit anti-alprenolol anti-idiotypic antibodies can either promote (Schreiber et al., 1980) or inhibit (Homcy et al., 1982) β-adrenergic receptor agonist-stimulated adenylate cyclase activity in turkey erythrocyte membranes. Sege and Peterson (1978) demonstrated that anti-insulin anti-idiotypic antibodies promote uptake of aminoisobutyric acid in rat hepatocytes to the same extent as does insulin. In addition, it is known that antibodies may enhance the activity of certain enzymes. For example, a defective mutant of the enzyme β-galactosidase of *Escherichia coli* has conformation-dependent determinants to which specific antibodies bind, resulting in a 1000-fold increase in activity of the mutant enzyme (Celada et al., 1971). Subsequent studies by deMacario et al. (1978) suggested that this enzyme activation was the result of antibody promoting the formation of active 16S tetramers of the enzyme mutant (based on sedimentation analysis) from inactive 10S dimers of the enzyme. All of these prior reports, however, are different from the findings we report here, which characterize the increase in binding affinity of an antibody for its antigen by an anti-idiotypic antibody.

Recently, Chamat et al. (1986) also reported that a rabbit anti-idiotypic antibody to anti-alprenolol monoclonal antibody 37A4 (Chamat et al., 1984) promoted the binding of [^{3}H]-dihydroalprenolol to 37A4 in a dose-dependent manner. The effect they observed was small (2-fold increase in binding), and they did not characterize it further. They speculated that the increase in ligand binding was due to an enhancement in the affinity of the interaction.

In this report, we demonstrate that the binding of a monovalent anti-idiotype F(ab) fragment to a monovalent idiotype F(ab) fragment enhances ligand binding affinity. How the anti-idiotype produces this effect is unclear. There are at least two potential mechanisms: the anti-idiotypic antibody could bind to a site distant from the ligand binding site and yet effect a conformational change in the binding site of the idiotypic antibody. Alternatively, the anti-idiotypic antibody may bind in the near vicinity of the ligand binding site itself and thus provide additional contact residues for that portion of the ligand which is not in direct contact with the idiotype. If a major conformational change in the binding site of the idiotype by the anti-idiotypic antibody is the mechanism for this enhanced binding affinity, then changing temperature might be predicted to alter the magnitude of this effect, but no effect on ligand binding was detected at any temperature (Table I). The energy contribution to this effect, in terms of kilocalories per mole of free energy, could be quite small, however, since a 100-fold increase in affinity would result from a net favorable energy gain of only 2–3 kcal/mol.

The kinetics of the interaction among ligand, idiotype, and anti-idiotype are also of interest in terms of delineating the mechanism underlying the promotion of ligand binding affinity.

A biphasic ligand association rate is observed when ligand, idiotype, and anti-idiotypic antibodies are allowed to interact simultaneously. However, only a monophasic rate of association is observed when ligand binds to the preformed antibody complex. We believe that the observed kinetics under these conditions favor the second model for the following reason. Since binding of the anti-idiotype at or near the binding site of the idiotype might be expected to hinder association of the ligand with the resulting antibody complex, a biphasic rate of association would be predicted as the formation of the idiotype–anti-idiotype complex reaches steady state. However, when all of the idiotype exists as a complex with the anti-idiotypic antibody (under conditions of anti-idiotypic antibody excess as shown in Figure 3B), a slower monophasic association rate would be observed.

Delineation of the exact mechanism underlying the enhancement of antigen affinity described in these studies will benefit from the production of a monoclonal anti-idiotypic antibody that produces a similar effect. It might then be possible to pinpoint the exact site on 5B7 to which it binds. A monoclonal anti-idiotypic antibody producing this effect would also allow for biophysical approaches to be employed to identify the specific residues which may be participating in ligand contact in the idiotype–anti-idiotype complex.

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