CYCLICAL ANTIIDIOTYPIC RESPONSE TO ANTI-HORMONE ANTIBODIES DUE TO NEUTRALIZATION BY AUTOLOGOUS ANTI-ANTIIDIOTYPE ANTIBODIES THAT BIND HORMONE*

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When antibodies against a well-defined antigen are used to immunize animals of the same genetic background, the antibodies raised are referred to as antiidiotypic (1–8). It has recently been recognized that four types of antiidiotypic antibodies may actually be induced (5, 6). The first type is directed against idiotopes associated with the combining site of the immunizing antibodies and is generally hapten inhibitable. The second type is composed of antibodies directed against idiotopes associated with the framework of the V region. A third kind of antibodies may interact with epitope- or idiotope-related structures of antibodies. The fourth type of Ig that may result from the injection of antibodies apparently mimics the original antigen. These antibodies are said to constitute the "internal image" of the initial immunogen (7) and have been termed "homobodies" by Lindenmann (8).

Most studies concerning idiotype recognition have involved systems in which the primary antigens are molecules devoid of known biological function, thus making identification of internal image difficult. However, recent reports have described systems in which allotypic (9) or biological properties of antigens are indeed "mimicked" by immunological "internal images." In particular, antibodies raised against anti-hormone antibodies have been observed to bind hormone receptors and to trigger hormone-regulated physiological responses (10–17).

This anti-receptor antiidiotypic response is generally transient. In our experience, anti-catecholamine hormone antiidiotypic antibodies that specifically bound β-adrenergic receptors appeared soon after immunization, but were hardly detectable 6–10 wk later (12, 13). In some instances, additional injections of anti-hormone antibodies caused the reappearance of anti-receptor antibodies. Similar observations have been reported by Homcy et al. (14) for the catecholamine system and by Farid et al. (15) for the thyrotropin-sensitive adenylate cyclase. Wasserman et al. (16) showed that experimental myasthenia gravis induced in rabbits by the injection of antibodies directed against an acetylcholine antagonist was also transient, apparently in direct relationship to the rapid clearance from the sera of the anti-acetylcholine receptor antiidiotypic antibodies.

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This report analyzes the transient character of the anti-receptor antiidiotypic response to antibody against catecholamines. The present results suggest that the synthesis of anti-hormone antibodies subsequent to the immunization with anti-hormone antibody may play a major role in the regulatory network of idiotypic antiidiotypic interactions.

Materials and Methods

Animal Immunization and Preparation of Antibodies. Antiidiotypic antibodies were raised in rabbits by intradermal injection of anti-alprenolol IgG from allotype-matched rabbits. The isolation and purification of IgG was performed by 33% ammonium sulfate precipitation followed by dialysis and ion exchange chromatography on DEAE-cellulose as previously described (12). The IgG fraction was concentrated by a further ammonium sulfate precipitation and stored in aliquots at −20°C.

Affinity Chromatography. Anti-alprenolol IgG was coupled to Sepharose 4B by cyanogen bromide (CNBr)[1] activation as described by Mach et al. (18) using 4 g of Sepharose 4B (net weight), 0.5 g CNBr, and 40 mg of antibody dissolved in phosphate-buffered saline (PBS). The resulting gel was suspended in PBS containing azide and stored at 4°C.

The immunoabsorbent gel was packed in a column (0.5 × 6 cm). IgG fractions were passed through the column and the gel was washed with PBS until no significant absorbance (<0.01) was detected. Elution was then performed with 3% acetic acid; the eluted fractions were immediately neutralized with Tris-HCl (1 M, pH 8.2). Effluents and eluates were extensively dialyzed against PBS at 4°C, concentrated by vacuum dialysis, and stored in aliquots at −20°C.

Preparation of Turkey Erythrocyte Membranes. Turkey blood was mixed with citrate buffer and diluted with 145 mM NaCl. After centrifugation, the plasma and buffy coat were removed. The washed cells were lysed in 5 mM Tris-HCl (pH 7.4), 2 mM $MgCl_2$ buffer and homogenized. After centrifugation, the supernatant was removed and the upper pink layer of the pellet (erythrocyte ghosts) was collected and suspended in 10 mM Tris-HCl (pH 7.4), 145 mM NaCl, 2 mM $MgCl_2$ (buffer A). After washing, the resulting ghosts were suspended in buffer A containing glycerol and stored in liquid nitrogen. The procedure has been described in detail elsewhere (19).

Immunofluorescence Studies. Turkey erythrocytes (10^6 cells) were incubated with IgG fractions for 1 h at 30°C. After washing with PBS, cells were reincubated with fluorescein isothiocyanate-labeled goat anti-rabbit IgG for 1 h at 30°C. The cells were observed in a Leitz fluorescence microscope (E. Leitz).

Binding Assays. Binding of (−)-[^{3}H]dihydroalprenolol (−)-[^{3}H]DHA to each fraction was assayed by means of an ammonium sulfate precipitation assay (Farr assay). Briefly, IgG samples were incubated overnight at 4°C with (−)-[^{3}H]DHA, then precipitated with 50% ammonium sulfate, and finally filtered on glass fiber filters. Antiidiotypic IgG was detected by inhibition of (−)-[^{3}H]DHA to β-adrenergic receptor from turkey erythrocyte membranes. Turkey erythrocyte membranes were preincubated with dilutions of IgG fractions from each bleeding or with dilutions of corresponding passthrough and eluted fractions from the Ab1-Sepharose gel for 1 h at 30°C in 75 mM Tris-HCl buffer, pH 7.4/25 mM $MgCl_2$. Membranes were then tested for their ability to bind (−)-[^{3}H]DHA after 8 min incubation at 30°C and finally filtered on glass-fiber filters (12).

Results

Preparation and Characterization of Anti-alprenolol Antibodies. Antibodies against alprenolol, a potent catecholamine antagonist, were raised in a rabbit by injection of alprenolol linked to bovine serum albumin (20). The antibodies specifically bound

[1] *Abbreviations used in this paper:* Ab1, anti-epitope-antibody; Ab2, antiidiotype antibody; Ab3, anti-antiidiotype antibody; CNBr, cyanogen bromide; (−)-[^{3}H]DHA, dihydroalprenolol; PBS, phosphate-buffered saline.

(−)-[^{3}H]DHA with an apparent dissociation constant of 1.1×10^{-7} M and represented ~1.5% of the total IgG fraction, further designated as Ab1 throughout this report. The Ab1 binds other catecholamine antagonists such as propranolol and agonists such as isoproterenol (12). The antibody-containing IgG fraction obtained from pooled bleedings from a single animal was injected into four allotypically matched rabbits: 31, 35, 57, and 58 to induce the synthesis of antiidiotypic antibodies (Ab2).

Transient Character of the Antiidiotypic Response to Ab1. Antiidiotypic activity was monitored in the serum of the immunized rabbits by following the capacity to inhibit the binding of catecholamine ligands to the anti-alprenolol antibodies and to the β-adrenergic receptors (12). In our initial study (12), this activity was detected only in the serum of rabbit 31. It appeared the 6th wk after initial immunization, and disappeared 10 wk later. It could not be detected in the serum of the other three immunized rabbits. The transient character of the antiidiotypic response to anti-alprenolol antibodies was also described in another study in which only 1 animal out of 11 appeared to respond (14). We were intrigued by this low responsiveness and decided to investigate the presence of antibodies that might neutralize the antiidiotypic antibodies.

Alprenolol-binding Capacity in Sera of Rabbits Immunized with Anti-alprenolol Antibodies. When testing the IgG fraction from each bleeding from rabbit 31, it appeared that the decrease of adenylate cyclase stimulating antiidiotypic antibodies was accompanied by an increase of alprenolol-binding activity. We therefore decided to investigate this activity in the serum of the apparently nonresponding rabbits. In the IgG fractions of rabbits 57 and 58 we detected several periods of production of anti-alprenolol antibodies (Fig. 1). To differentiate this Ab1-like activity from that due to residual Ab1 injected during the preceding weeks, we determined the ligand-binding affinity of the "new" antibodies: except for the first peak, in both rabbits it appeared to be higher than that of the immunogen, and decreased progressively from one peak to the next (Fig. 2). Because we suspected that the Ab1-like response was due in fact to antiidiotypic antibodies against Ab2 molecules themselves raised against the injected Ab1, we prepared an Ab1-agarose affinity gel to isolate the various antibody activities.

Isolation and Characterization of Ab2 and Ab1-like Antibodies. Ig fractions from each bleeding from rabbits 57 and 58 were absorbed on the Ab1 agarose column and both the effluent ("pass-through") and the eluate were analyzed for the capacity to bind (−)-[^{3}H]DHA (Ab1-like activity) (Fig. 1) or to inhibit this binding to the β-adrenergic receptor or to anti-alprenolol antibodies (Ab2 activity).

Alternate peaks of both types of activities could be observed (Fig. 3 B) in either the effluent or the eluate. Ab2 antibodies, not detected in the serum appeared to be able to inhibit almost all the binding of (−)-[^{3}H]DHA to the receptor in a dose-dependent, saturable manner (Fig. 4). The small amount of Ab2 activity detected in the effluent (Fig. 3 A) might be due to saturation of the Ab1 column or to competition with the unretained Ab1-like antibodies.

The presence of antiidiotypic Ab2 antibodies able to interact with the alprenolol binding β-adrenergic receptor (12) was confirmed by immunofluorescence experiments in which turkey erythrocyte membranes known to possess β-adrenergic receptors were incubated with the various fractions obtained during affinity chromatography on the Ab1 agarose gel. When FITC-labeled goat anti-rabbit antibodies were added, only the fractions that inhibited the binding of (−)-[^{3}H]DHA to the β-adrenergic

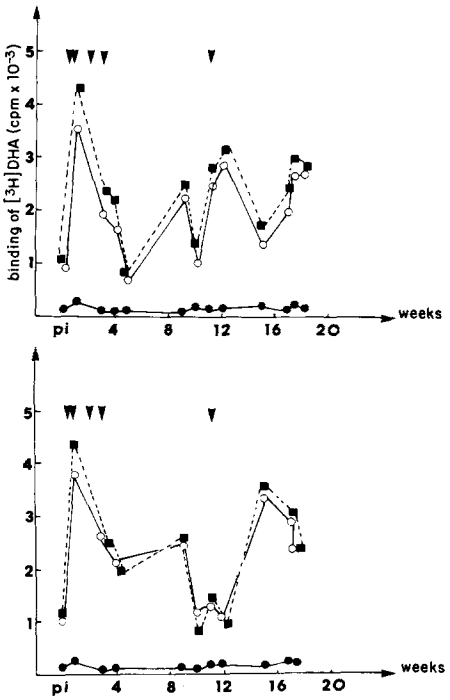

FIG. 1. (−)-[^{3}H]DHA Binding capacity of the first bleedings of rabbit 58 (upper) and rabbit 57 (lower) to (−)-[^{3}H]DHA using unfractionated IgG (■), effluents (○), and eluates (●) fractionated on an anti-alprenolol antibody-Sepharose column. 100 μl of IgG (1 mg/ml in PBS) were incubated with 50 μl of (−)-[^{3}H]DHA. Bound radioactivity was measured by means of an ammonium sulfate precipitation assay (Farr assay). Nonspecific binding was determined in the presence of 1,000-fold propranolol and did not exceed 10% of the specific binding. Means of two experiments are shown. Arrows indicate times of injection of alprenolol antibodies (Abl).

receptor stained the receptor containing membranes.

As for the direct (−)-[^{3}H]DHA-binding capacity, superimposable results were obtained either with entire IgG serum fractions or with the effluent from the Ab1-agarose gel (Fig. 1). The comparison of the Ab2 and Ab1-like (or Ab3) time courses revealed that the peaks of these two kinds of activities appeared exactly in alternance all along the 5-mo-long study, independent of the booster injections (Fig. 3 B).

Interactions of the Ab2 with Both Ab1 and Ab3. The cyclical appearance of the Ab2 and Ab1-like Ab3 antibodies could be explained by reciprocal expansion of idiotypic and antiidiotypic clones (21) and neutralization of antibody activity by antiantibodies. To verify this hypothesis, the Ab2 fractions were preincubated with increasing concentrations of Ab1 or Ab3, then incubated with membranes and tested for their ability to inhibit the binding of (−)-[^{3}H]DHA to the β-adrenergic receptor. Both Ab1 and Ab3 prevented the interaction of Ab2 with the β-adrenergic receptor. A typical result is shown in Fig. 5 for bleeding 58-4.

From this experiment, it appeared that Ab3, for example from bleeding 58-5, which was seen to exhibit a higher affinity for (−)-[^{3}H]DHA than Ab1 (Fig. 3 B) also demonstrated a higher affinity for Ab2 than did Ab1 (Fig. 5). Under the same conditions, the effluent of bleeding 58-4, which was negative in the (−)-[^{3}H]DHA binding test when incubated with Ab2 (eluate from the same bleeding), failed to

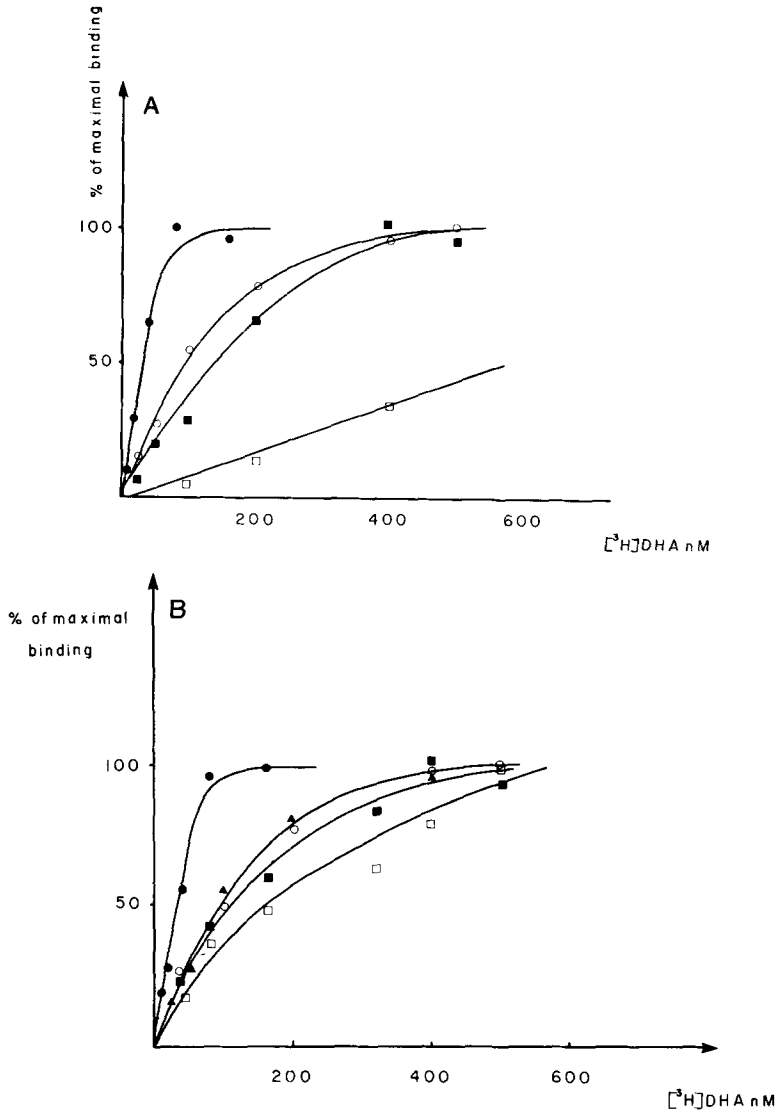

FIG. 2. (*A*) Evolution of the affinity of Ab1-like (−)-[^{3}H]DHA binding capacity. Effluents from bleedings of rabbit 31 corresponding to the peaks of (−)-[^{3}H]DHA binding capacity were incubated overnight at 4°C with increasing concentrations of (−)-[^{3}H]DHA; nonspecific binding was determined in the presence of a 200-fold excess of (d,l)-alprenolol. For each effluent, specific binding was plotted as percent of maximum binding. (○), Ab1; (●), Ab3-peak 1; (■), Ab3-peak 2; (□), Ab3-peak 3. (*B*) Evolution of the affinity of Ab1-like (−)-[^{3}H]DHA binding capacity for rabbit 58, using the experimental conditions described for A. (○), Ab1; (▲), IgG from bleeding 58-1; (●), Ab3 from bleeding 58-5; (■), Ab3 from bleeding 58-8; (□), Ab3 from bleeding 58-11.

inhibit Ab2. These results were in agreement with our interpretation that anti-alprenolol antibodies, appearing in the course of the Ab2 immune response, were antiidiotypic with respect to Ab2. Moreover, the binding affinity of Ab2 for Ab1 and for Ab3 were well conserved during the course of the immune response (Fig. 5),

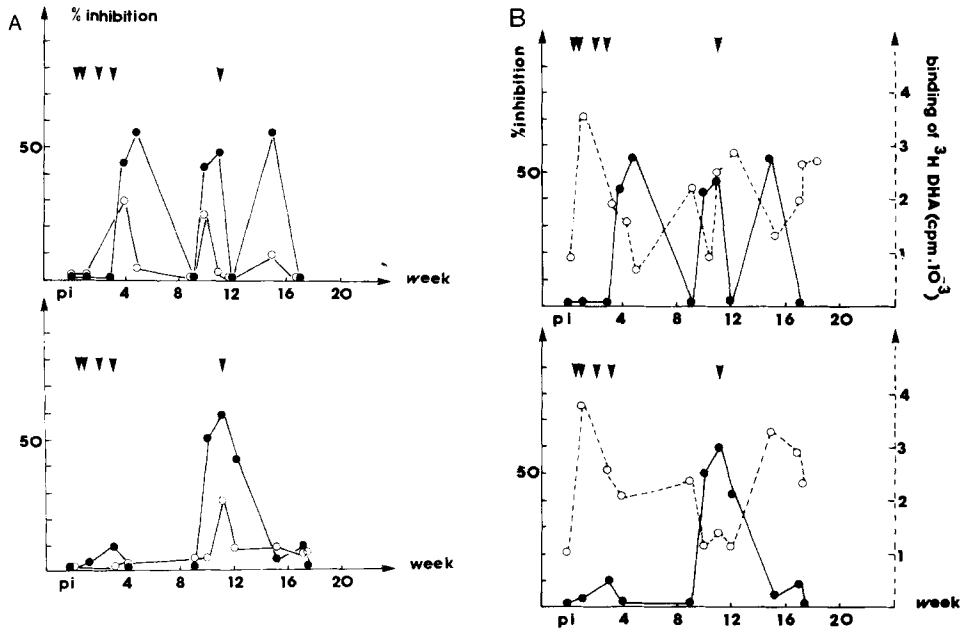

FIG. 3. (A) Inhibition of binding of (−)-[^{3}H]DHA to β-adrenergic receptor of turkey erythrocyte membranes by eluates and effluents separated on the anti-alprenolol antibody-Sepharose column. Turkey erythrocyte membranes were incubated with eluates (●) and effluents (○) in 75 mM Tris-HCl buffer, pH 7.4/2.5 mM $MgCl_2$ at 30°C for 1 h, then tested for their ability to bind (−)-[^{3}H]-DHA after an 8-min incubation at 30°C and filtration on glass fiber filters. Controls were made up with preimmune IgG. Means of two independent experiments are shown. Upper: rabbit 58; lower: rabbit 57. (B) A comparison of the inhibition of binding of (−)-[^{3}H]DHA to β-adrenergic receptor by eluates (●) with the binding capacity of effluents (○) to (−)-[^{3}H]DHA. Upper: rabbit 58; lower: rabbit 57.

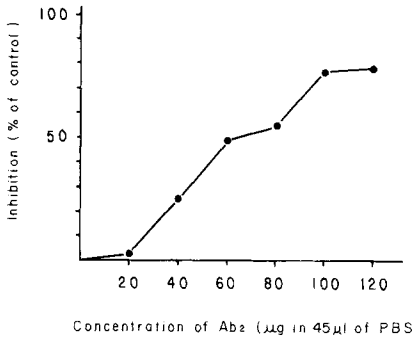

FIG. 4. Inhibition of binding of (−)-[^{3}H]DHA to β-adrenergic receptor by increasing concentrations of Ab2 separated from bleeding 6 of rabbit 57.

whereas as noted above, the affinities of Ab3 for (−)-[^{3}H]DHA or Ab2 decreased from one peak to the other.

Discussion

Jerne's network of idiotypic and antiidiotypic interactions between lymphocytes or their products postulates the existence of a dynamic equilibrium in the immune

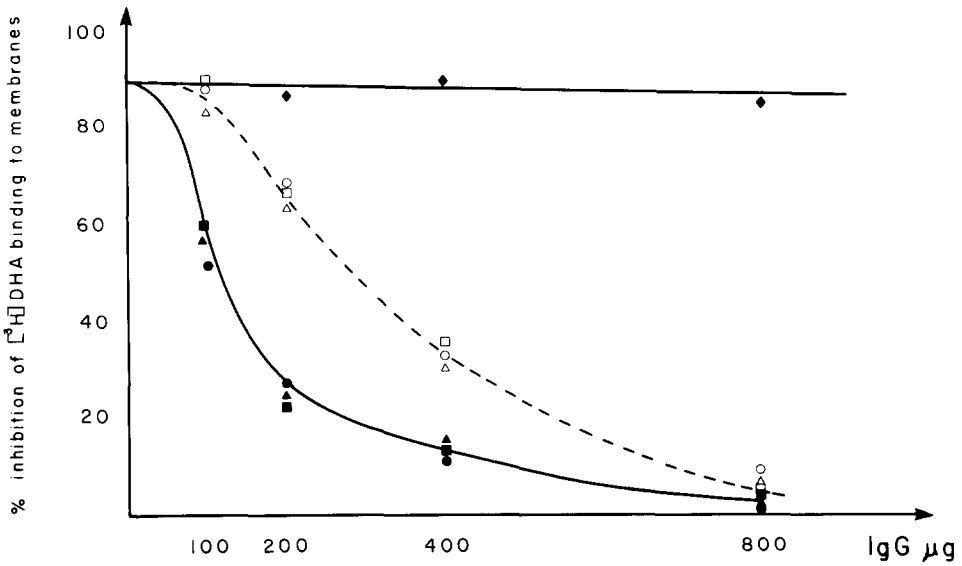

FIG. 5. Inhibition of interaction of Ab2 to β-adrenergic receptor by Ab1 (open symbols) and Ab3 (effluent from bleeding 58-5, closed symbols). 45 µl of Ab2 (1,000 µg/ml) isolated from each of the successive Ab2 peaks (○ and ●, eluate from 58-4; □ and ■, eluate from 58-7; △ and ▲, eluate from 58-9) (Fig. 3) were preincubated with increasing concentrations of Ab1, Ab3, or normal rabbit IgG as control (◆) overnight at 4°C, then incubated with 15 µl of membranes (1 mg/ml) 1 h at 30°C, before (−)-[^{3}H]DHA binding capacity of the membranes was tested in an 8-min incubation at 30°C.

system (7). A foreign antigen would temporarily disturb this state by stimulating the production of Ab1 antibodies. Subsequent stimulation of Ab2 antiidiotypic antibodies would restore the initial steady state. The oscillatory behavior of the system should not, however, remain limited to the Ab1 and Ab2 components; increased Ab2 synthesis should stimulate Ab3 production and so on.

Several (10–17) studies have documented the ability of antiidiotypic antibodies raised against anti-hormone antibodies to bind the corresponding hormone receptors. A detailed analysis of the response to anti-catecholamine antibodies reveals that only a minority of the Ab2 molecules functions as the immunological internal image of the adrenergic hormones. Ab2 does not mimic all the properties of the smaller and specific ligands: thus the syngeneic action between guanyl trinucleotides seen with the hormone is not observed for the antibodies (13). The Ab2 may stimulate both basal- and hormone-sensitive cyclase, suggesting that the site of action is not exactly the same, an expected conclusion considering the large difference in size. Not all anti-receptor Ab2 molecules have similar properties within the heterogeneous population of antibodies. Some may act as agonists, others may behave as antagonists. The analysis of the unfractionated population yields a summation of all effects which appears as agonistic in the animals we have studied. In contrast, in the study of Homcy et al. (14) Ab2 antibodies raised against affinity-purified anti-alprenolol antibodies were predominantly antagonistic.

A time course study of the Ab2 response reveals a cyclic appearance of the anti-idiotypic molecules. Their disappearance could be explained by metabolism. However, such a process is usually slow compared with the rapid decrease in Ab2 concentration seen in our study. We favor an active removal mechanism likely to result from the

synthesis of a third kind of antibody (Ab3) auto-antiidiotypic against Ab2 (21). We isolated Ab3 by affinity chromatography on an antigen-containing affinity column and were able to compare its precise binding characteristics with those of the Ab1 antibodies. Some of the Ab3 molecules thus mimicked the Ab1 original antibodies by binding the catecholamine ligand. It remained to be shown that the Ab3 molecules were not actually Ab1, since both bind the ligands and both inhibit the action of Ab2 in binding to the receptor. However, in each of these experiments, the binding properties of Ab1 and Ab3 were shown to be different.

The results presented here and those described by others (14) on the β-adrenergic system, as well as the results of Shechter et al. (22) on the insulin system, clearly indicate that the auto-antiidiotypic response induced by the injection of anti-hormone antibodies may actually intervene in physiological processes, as had been suggested earlier by Sege and Peterson (11). The synthesis by rabbits of antiidiotypic antibodies that act as anti-receptor antibodies may constitute a potent trigger for the synthesis of autologous anti-antiidiotypic antibodies that could neutralize potentially detrimental effects of the hormone like immunological internal images. A recent study by Wasserman et al. (16) has shown that this neutralization event actually reverses the pathological developments of experimental autoimmune myasthenia gravis induced in rabbits immunized with antibodies against an acetylcholine analog.

In the present study of rabbits immunized with anti-alprenolol antibody Ab1, we have shown a cyclic appearance of antiidiotypic antibodies (Ab2), alternating with the appearance of alprenolol-binding antibodies. Anti-receptor Ab2 response, which is only a fraction of the overall antiidiotypic response to the injection of Ab1, has been documented in several hormonal systems (10–17), wherein most authors have noticed the transient aspect of this response.

Our results confirm that the rapid and cyclic disappearance of anti-receptor Ab2 antibodies is due to a regulation assumed by antiidiotypic antibodies directed against Ab2. This reciprocal expansion of antiidiotypic clones is supported by Jerne's idiotypic network theory (7) and is in agreement with other recent results (21, 23). Moreover, we have shown that at least a fraction of these anti-antiidiotypic antibodies are able to bind the original antigen, i.e., alprenolol, and thus may be considered as Ab1-like antibodies. It is interesting to underline the general decrease in the affinity of these antibodies towards the antigen all along the immune response. This result suggests a degeneracy in the idiotypic response, as if the image would fit less and less its model.

The anti-antiidiotypic antibodies are heterogeneous; some may be directed against the idiotype of Ab2 involved in this interaction with the hormone receptor. These are the Ab1-like antibodies. Others may be directed against different idiotypes of the Ab2 molecule and have no alprenolol-binding capacity. One hypothesis could be that these anti-antiidiotypic antibodies are responsible for neutralizing the anti-receptor Ab2 antibodies, thus masking the Ab2 activity in the serum of immunized animals in varying proportions according to the animal. Fractionation of the serum on an Abl-containing affinity column separates the anti-antiidiotypic antibodies from the retained Ab2 molecules and permits detection of the different binding activities of these complementary Ig.

Summary

Antiidiotype antibodies were raised against anti-catecholamine ligand antibodies. The antiidiotype response was shown to be cyclical and to correspond to the

production of antibodies that could bind to catecholamine β-adrenergic receptors and stimulate adenylate cyclase. Disappearance of these antibodies from the serum could be correlated with the appearance of a catecholamine ligand-binding activity corresponding to the synthesis of autologous anti-antiidiotype antibodies directed against the induced antiidiotypic molecules. Comparison of the injected versus the induced anti-ligand antibodies reveals striking differences in affinities but similarities in the ability to bind to the antiidiotype antibodies and to the ligand-containing affinity gel. The results support the existence of a functional network of idiotype antiidiotype interactions involving external as well as internal antigens, antibodies, and possibly other types of molecules involved in recognition phenomena, such as hormone receptors.

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