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Immunology

β-Adrenergic agonist activity of a monoclonal anti-idiotypic antibody

(anti-idiotypic network/β-adrenergic receptor/A431 cells)

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ABSTRACT **Hybridoma cells bearing monoclonal antibody against the β-adrenergic ligand alprenolol were used as an immunogen to raise monoclonal anti-idiotypic antibodies. Of six anti-idiotypic antibodies, which inhibit ligand binding, three were able to recognize β-adrenergic receptors. One of them, mAb2B4, an IgM that could be amplified into ascites, binds to the β-adrenergic catecholamine receptors of intact epidermoid A431 cells and precipitates receptors solubilized from plasma membranes by digitonin. This antibody identifies the β_2-adrenergic receptor of A431 cells as a single 55-kDa protein and stimulates adenylate cyclase activity. This stimulation is inhibited by the β-adrenergic antagonist propranolol.**

Rabbit polyclonal anti-idiotypic antibodies raised against anti-catecholamine antibodies bind to β-adrenergic catecholamine receptors and modulate the hormone-sensitive adenylate cyclase (1, 2). The anti-idiotype thus acts as the "internal image" of the natural ligand as defined in the immune network theory of Jerne (3). The rapid neutralization and disappearance of the anti-receptor anti-idiotypic antibodies due to induction of anti-anti-idiotypic antibodies has provided evidence for the physiological significance of such an immune network (4). To gain further insight into the interaction between anti-idiotypes and receptors, the unlimited availability of purified components would be of considerable advantage. A monoclonal antibody (mAb37A4) directed against alprenolol was chosen for induction of anti-idiotypes because it showed stereospecificity and binding characteristics similar to those of the β-adrenergic receptor (5). Monoclonal anti-idiotypic antibodies were obtained from BALB/c mice immunized intravenously with glutaraldehyde-fixed hybridoma cells secreting mAb37A4 antibody. Of the 24 anti-idiotypic monoclonal antibodies able to recognize mAb37A4, six inhibited alprenolol binding and three of these specifically recognized cells carrying β-adrenergic receptors (6). One of the latter, the IgM mAb2B4, was chosen for further investigation.

MATERIALS AND METHODS

Monoclonal Antibodies. Large amounts of ascitic fluid containing the anti-idiotypic antibody Ab2B4 or the anti-muscarinic receptor antibody mAb35 (7), used as control, were obtained by weekly intraperitoneal transfer in pristane-sensitized mice. Both IgM antibodies were purified on an Ultrogel ACA-22 molecular sieve (IBF, Paris, France) in 0.5 M NaCl/0.05 M Tris-HCl, pH 7.8, for the study of their physiological effects on the receptor.

Anti-Idiotypic Immunoassay. Ascitic fluids at decreasing dilutions in 0.01 M phosphate buffer/0.14 M NaCl, pH 7.2 (P_i/NaCl) were adsorbed overnight at room temperature on Millititer HA 96 nitrocellulose plates (Millipore). After four washes and blocking for 6 hr at room temperature in P_i/NaCl/0.1% Tween 20, the wells were washed and filled with 100 μl of a biosynthetically labeled [^{3}H]leucine 37A4 anti-alprenolol monoclonal antibody diluted 1:5 in P_i/NaCl/0.1% Tween 20. After overnight incubation at room temperature and washing with P_i/NaCl/0.1% Tween 20, the bottom of each well was cut out, transferred to a scintillation vial, treated with 0.2 ml of 1 M HCl and 3 ml of Biofluor (New England Nuclear), and counted in a liquid scintillation counter (Intertechnique, Paris).

Anti-Receptor Immunoassay. Human epidermoid A431 cells were grown to confluence in 96-well Linbro microtiter culture plates (Flow Laboratories). After washing, the cells were fixed with 0.25% glutaraldehyde in P_i/NaCl, free aldehyde groups were blocked with 0.2 M glycine, and the wells were treated with P_i/NaCl/0.1% Tween 20 containing 0.25% gelatine (Sigma). After washing in P_i/NaCl/0.1% Tween 20, the wells were incubated overnight at room temperature with dilutions of the ascitic fluids in P_i/NaCl/0.1% Tween 20. After washing, 100 μl of a 1:1000 dilution of rabbit anti-mouse IgM (Miles) was added, the wells were incubated for 1 hr at 37°C, washed in P_i/NaCl/0.1% Tween 20 and further incubated for 1 hr at 37°C with a goat anti-rabbit peroxidase-conjugated antibody (Miles) at a 1:1000 dilution in P_i/NaCl/0.1% Tween 20. After washing, peroxidase activity was assessed using 2,2′-azino-di(3-ethylbenzthiazoline sulfonic acid) (Sigma) as indicator. The enzymatic reaction was stopped with 50 μl of 10% $NaDodSO_4$ and the optical density was determined at 405 nm in an ARTEK apparatus (Dynatech, New York).

Immunoblot. After $NaDodSO_4$/polyacrylamide gel electrophoresis of an A431 membrane preparation (8), electrophoretic transfer of the proteins was performed in methanol/glycine/Tris buffer as described (9). After blocking of the nitrocellulose filter with P_i/NaCl/0.1% Tween 20/0.25% gelatin, strips were incubated overnight at 4°C with 1:50 dilutions of ascitic fluids or serum. After washing the strips in P_i/NaCl/0.1% Tween 20, they were incubated with rabbit anti-mouse IgM or anti-mouse IgG (dilution 1:500) 4 hr at room temperature. After washing, goat anti-rabbit peroxidase-labeled antibody was added at a 1:1000 dilution and peroxidase activity was finally revealed with chloronaphthol (Sigma).

Receptor Immunoprecipitation. After washing in cold P_i/NaCl, 6×10^7 A431 cells were solubilized in 15 ml of lysis buffer (10 mM Tris·HCl, pH 7.4/90 mM NaCl/2 mM EDTA/1% digitonin/0.5 mM phenylmethylsulfonyl fluoride/25 mM benzamidine/5 μg of leupeptin per ml/7 μg of pepstatin per ml/10,000 units of Trasylol per ml/100 μg of bacitracin per ml/15 μg of soybean trypsin inhibitor per ml). After centrifugation for 30 min at 50,000 × *g*, 300 μl of the lysate was incubated overnight at 4°C with 1:50 dilutions of anti-A431 membrane mouse antiserum or ascitic fluids. Rabbit anti-mouse IgG or rabbit anti-mouse IgM was added to the reaction mixture and after centrifugation, supernatants were tested for residual [^{3}H]dihydroalprenolol binding activity as described (10).

Indirect Immunofluorescence on A431 Cells. A431 cells, grown on microscope slides and incubated for 45 min at 37°C

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in the presence of specific β-adrenergic ligands were washed in cold P_i/NaCl and fixed in 1% paraformaldehyde. Unreacted aldehyde was blocked with 0.2 M glycine in P_i/NaCl and the slides were incubated with P_i/NaCl/0.1% Tween 20/0.25% gelatin before immunostaining with Ab2B4 (dilution 1:100). Binding of anti-idiotype was revealed by incubation with rabbit anti-mouse IgM and fluorescein-labeled goat anti-rabbit antibodies.

Adenylate Cyclase Activity of A431 Cells. Cells (2×10^6 per ml) were incubated for 120 min at 37°C in Hanks' balanced salt solution buffered with Hepes (pH 7.4) and supplemented with 1 mM ascorbic acid/8 mM theophylline in the presence of antibodies purified from ascitic fluids (50 μg/ml) or 1 nM *l*-isoproterenol with or without 10 nM *l*-propranolol. Cells were then removed by scraping, boiled for 5 min, and centrifuged. cAMP was determined on an aliquot of the supernatants by a radiochemical assay using the cAMP kit of Amersham (11).

RESULTS

The anti-idiotypic antibody mAb2B4 specifically binds biosynthetically labeled anti-alprenolol antibody mAb37A4 and inhibits the binding of alprenolol (6). This antibody also binds to A431 cells known to possess a large number of β_2-adrenergic receptors (12) (Fig. 1). This binding was shown to be specific for the β-receptors, because other cells bearing the receptor (turkey erythrocytes or murine mastocytoma cells) were also labeled while rabbit lymphoma cells, which do not carry the receptor, showed no binding (6). After solubilization of the β-receptors of the A431 cells in digitonin, mAb2B4 was able to immunoprecipitate 80% alprenolol binding activity from the supernatant, in contrast to an anti-A431 mouse antiserum, which had no detectable anti-receptor activity (Fig. 2*B*). Immunoblotting of A431 membrane proteins with the mAb2B4 antibody revealed a major polypeptide chain of 55 ± 3 kDa and a minor component of >100 kDa. The anti-A431 mouse antiserum, which did not immunoprecipitate β-receptor, did not reveal this 55-kDa protein either (Fig. 2*A*). Photoaffinity labeling with the β-adrenergic specific antagonist iodo-*p*-azidobenzylcarazolol (13) of A431 membranes showed two polypeptides with apparent molecular sizes of 57 ± 5 and 65 ± 3 kDa, respectively (data not shown). Iodination of affinity-purified A431 β-receptors (14), yielded a protein with apparent molecular mass of 52 ± 3 kDa (unpublished results). This confirms that the polypeptide recognized by mAb2B4 is the β-receptor of the A431 cells.

The specific recognition of the β-adrenergic receptors of A431 cells by mAb2B4 was further assessed by indirect immunofluorescence studies (Fig. 3). Untreated cells or cells treated with the antagonist alprenolol at 100 μM were specifically labeled by indirect immunofluorescence using mAb2B4 and a fluoresceinated rabbit anti-mouse IgM (Fig. 3 *A* and *B*). Pretreatment of A431 cells with the agonist *l*-isoproterenol at 400 μM resulted in disappearance of at least 50% of the binding sites for the radiolabeled hydrophilic β-adrenergic antagonist CGP-12177 (15) and also considerably reduced the surface immunofluorescence revealed by mAb2B4 (Fig. 3*C*). Simultaneous treatment with both agonist and antagonist led to a partial loss of the specific immunofluorescence (Fig. 3*D*).

Since both stimulatory (1) and inhibitory (2) effects of polyclonal anti β-adrenergic anti-idiotypic antibodies have been reported, the effect of mAb2B4 on the adenylate cyclase activity of intact A431 cells was assessed. From the data presented in Fig. 4, it is clear that mAb2B4 has stimulatory properties. In contrast to the rabbit polyclonal anti-idiotypes, the increase in cAMP accumulation induced by the antibody could be totally reversed in 10 μM β-adrenergic antagonist *l*-propranolol. The potency of mAb2B4 was compa-

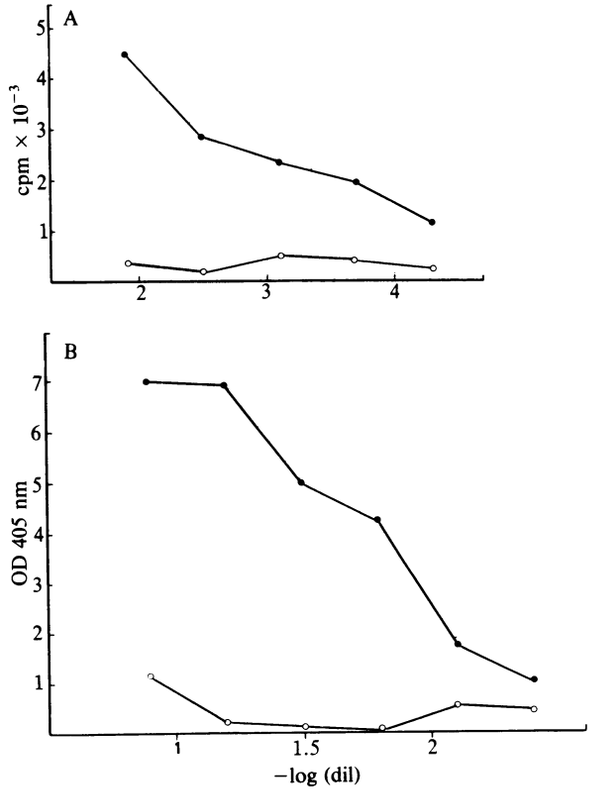

FIG. 1. Titration curves of monoclonal anti-idiotypic antibody to monoclonal anti-alprenolol antibody (mAb37A4) (*A*) and to A431 epidermoid cells (*B*). (*A*) Binding of biosynthetically labeled mAb37A4 on mAb2B4 (●) and mAb35 (○) ascitic fluids. (*B*) Binding of mAb2B4 (●) and mAb35 (○) on fixed A431 cells. Binding was monitored by a rabbit anti-mouse IgM coupled to peroxidase and revealed by 2,2'-azino-di(3-ethylbenzthiazoline sulfonic acid) at OD 405 nm.

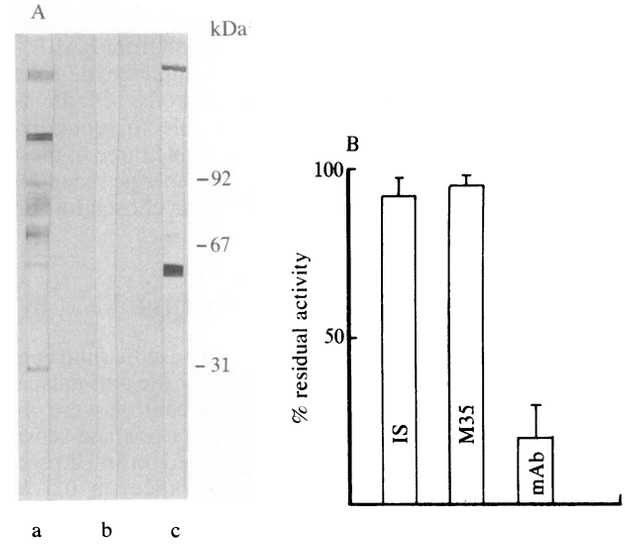

FIG. 2. Reactions of Ab2B4 antibodies with β-adrenergic receptors solubilized from A431 cells assessed by immunoblotting (*A*) and by removal of alprenolol binding activity (*B*). (*A*) Immunoblots of A431 membrane proteins are shown for an anti-A431 mouse antiserum (lane a), for a control monoclonal IgM (lane b), and for mAb2B4 (lane c). (*B*) Percentage of residual alprenolol binding on A431 solubilized membranes after immunoprecipitation with an anti-A431 mouse antiserum (IS), with a control monoclonal IgM (M35), and with mAb2B4 (mAb).

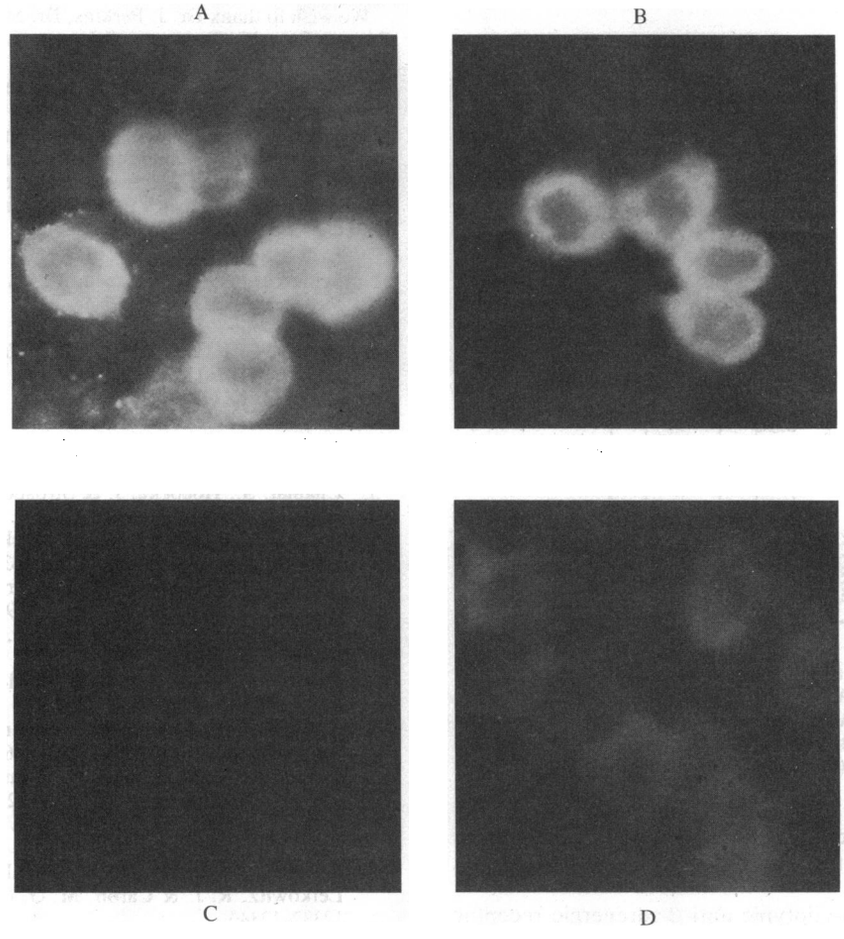

FIG. 3. Visualization of β-adrenergic receptors on A431 cells by the anti-idiotypic monoclonal antibody mAb2B4. Untreated A431 cells (*A*) or cells pretreated for 30 min at 37°C with the antagonist alprenolol (*B*), the agonist isoproterenol (*C*), or a mixture of alprenolol and isoproterenol (*D*) were fixed and β-receptors were revealed by indirect immunofluorescence using mAb2B4.

rable to that of the agonist *l*-isoproterenol at 1 nM. On a molar basis, mAb2B4 is therefore 1/25th as active as the agonist in inducing cAMP accumulation.

DISCUSSION

Since the observation by Sege and Peterson that anti-idiotypic antibodies against anti-insulin antibodies induce a physiological response (16), several reports have suggested that anti-idiotypic antibodies against anti-hormone antibodies can induce physiological effects similar to those of the corresponding hormone (reviewed in ref. 17). To dissect these effects at the structural level, the use of homogeneous reagents such as monoclonal anti-idiotypic antibodies would be of considerable advantage. We report here the characterization of a monoclonal anti-idiotypic antibody that has a physiological effect on the β_2-adrenergic receptors of A431 cells.

This antibody, mAb2B4, of the IgM class has all the properties of an anti-receptor antibody. It recognizes not only the β-adrenergic receptor on intact cells but also immunoprecipitates active receptor after solubilization of plasma membranes by digitonin. The immunoblotting by mAb2B4 of a 55-kDa membrane protein confirms the recognition of the β-adrenergic receptor since this molecular size was also obtained by photoaffinity labeling and radioiodination after affinity chromatography of the receptor. Moreover, this molecular size is very close to that found for β_2-adrenergic receptors in other systems (18).

The modulation of the surface immunofluorescence of mAb2B4 by pretreatment of the A431 cells with β-adrenergic agonists or antagonists confirms the receptor specificity of the anti-idiotypic antibody. The nearly complete disappearance of the immunofluorescence after agonist treatment suggests that the remaining receptors, although detectable by antagonist binding, may be modified and, therefore, not recognized by the anti-idiotypic antibody. Furthermore, the receptor could still be accessible to the smaller antagonist, but not to the much larger IgM anti-idiotype as a result, for instance, of vesicular reorganization of the membrane (19). Further visualization experiments at the optical and the electron microscopic level can verify these hypotheses.

Various physiological effects of the anti β-adrenergic anti-idiotypic antibodies have been reported. While workers in this laboratory found that rabbit polyclonal anti-idiotypic antibodies stimulated cAMP production (1), Homcy *et al.* (2), using similar reagents, reported the inhibition of adenylate cyclase. These divergent results may reflect different balances between stimulatory and inhibitory antibodies present in the anti-idiotypic antisera. The data presented here clearly show that mAb2B4 has stimulatory properties. In contrast to the rabbit polyclonal anti-idiotypes, the increase in cAMP accumulation induced by the antibody could be totally reversed by the β-adrenergic antagonist propranolol.

The anti-idiotypic antibody, mAb2B4, induced against an antibody that itself was raised against the antagonist, would have been expected to act as the internal image of an antagonist, not of an agonist. However, the anti-alprenolol antibody also recognizes agonists and, therefore, the anti-idiotype probably mimics what is common to agonists and antag-

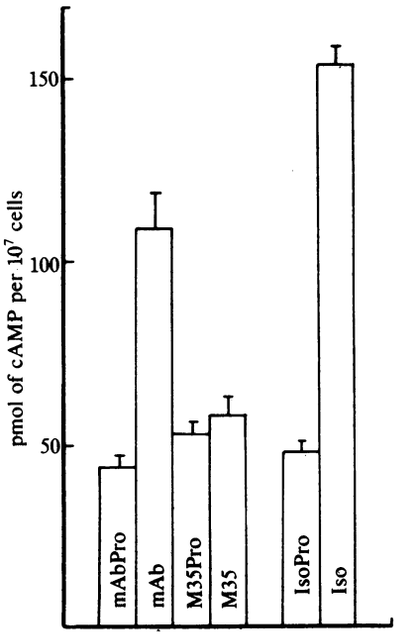

FIG. 4. Stimulation of adenylate cyclase activity of A431 cells by anti-idiotype mAb2B4. cAMP accumulation was measured in cells treated with mAb2B4 (mAb), a control monoclonal IgM (M35), or 1 nM isoproterenol (Iso) in the absence or the presence of the antagonist propranolol (10 μM) (Pro).

onists: the β-adrenergic ligand binding activity. In addition, the polymeric nature of mAb2B4 may also contribute to its agonist character.

The monoclonal anti-idiotypic anti-β-adrenergic receptor antibody described in this report appears to be a useful tool for the visualization of the β-adrenergic receptor and for its structural characterization. Its intrinsic physiological activity as a β-adrenergic agonist could be helpful in unraveling the mechanisms of hormonal signal transduction. Moreover, the availability of monoclonal antibodies that recognize β-adrenergic ligands and of a monoclonal anti-idiotypic antibody recognizing a monoclonal idiotypic antibody and the β-adrenergic receptor will allow the investigation of the structural determinants underlying the concept of the "internal image."

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