Monoclonal Anti-idiotypic Antibodies to Opioid Receptors*

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Two monoclonal anti-idiotypic antibodies (anti-Id-135 and anti-Id-14, both of the IgM class) which interact with the binding site of opioid receptors were generated. A monoclonal anti-β-endorphin antibody (3-E7) which displays binding characteristics for opioid ligands similar to opioid receptors served as the antigen (Gramsch, C., Meo, T., Riethermüller, G., and Herz, A., (1983) J. Neurochem. 40, 1220–1226; Meo, T., Gramsch, C., Inan, R., Höltt, V., Weber, E., Herz, A., and Riethermüller, G. (1983) Proc. Natl. Acad. Sci. U.S.A. 80, 4048–4052) and the hybridomas obtained were screened for anti-idiotypic antibodies with Fab fragments of 3-E7. The anti-idiotypes were then screened for opioid binding to rat brain membrane receptors, yielding several positive clones two of which were more intensively studied. Both anti-idiotypic antibodies were about equally potent in displacing the μ- and δ-opioid receptor ligands [3H]dihydromorphine, [3H]ethylketazocine, [3H]labelled β-endorphin, [3H]enkephalin and [3H]naloxone from rat brain membrane opioid receptors; no interaction was observed with the ligands [3H]ethyleneketazocine or [3H]bremazocine. The anti-idiotypic antibodies were able to precipitate [3H]diprenorphine binding sites from solubilized opioid receptor preparations. In addition, both antibodies showed opioid antagonistic properties as demonstrated by their abilities to block the inhibitory effect of [odyl-(4,10)-3H]enkephalin on prostaglandin E1-stimulated cAMP accumulation in NG 108-15 hybrid cells.

Our findings demonstrate the successful generation of monoclonal antibodies interacting with membrane-bound and solubilized opioid receptors of the μ- and δ-type.

It has been shown in several systems that the anti-idiotypic antibodies (anti-Id) provide a powerful tool for the characterization and functional modulation of membrane receptors (for review, see Ref. 3). This approach, using polyclonal antibodies, was recently employed to study opioid receptors (4–7). The anti-idiotypic procedure may be advantageous over the classical method of raising anti-receptor antibodies which requires a purified receptor preparation for immunization. Nevertheless, the conventional technique has been successfully applied to the production of anti-opioid receptor antibodies (8).

We first demonstrated that the anti-idiotypic route of raising antibodies is applicable to opioid receptors (5, 6). The antigen used was the monoclonal anti-β-endorphin antibody 3-E7 which binds opioid peptides in a similar manner to opioid receptors (1, 2). The anti-Ids that were generated behaved apparently like an internal image of the 3-E7 antibody, being able to interact with opioid receptors. However, in studying these polyclonal anti-Ids several difficulties are encountered: first, due to a low titer, high concentrations of antiserum are required to inhibit ligand binding to receptors or to initiate a biological response. Thus, additional processing is necessary for the concentration and purification of the limited amounts of antibodies. Second, the cyclic nature of the anti-idiotypic response may result in the disappearance of anti-idiotypic antibodies and appearance of a further set of antibodies, the anti-anti-idiotypes, which neutralize the anti-idiotypic antibodies as well as the ligand for the receptor (9).

Considering our successful anti-idiotypic approach to raising antibodies to opioid receptors and to avoid the drawbacks of polyclonal antibodies, we attempted to generate monoclonal anti-idiotypic anti-opioid receptor antibodies, whose unlimited availability would be of considerable advantage. Several monoclonal anti-idiotypic antibodies were obtained from BALB/c mice immunized with 3-E7; two of these were studied in detail and their characteristics are described below.

EXPERIMENTAL PROCEDURES

Immunization

The mouse monoclonal anti-β-endorphin antibody 3-E7 was raised as described (1). Protein A-purified 3-E7 (Fusion I) or its Fab fragment coupled to bovine thyroglobulin (Fusion II) were used for immunization. BALB/c mice were injected intradermally at multiple sites with 100 μg of antigen emulsified in Freund’s complete adjuvant at 4-week intervals. The immunization efficiency was controlled by periodic testing of mice sera for the development of anti-Fab (3-E7) antibodies by ELISA (details see below). At the end of the immunization period, 100 μg of the immunogen was injected both intraperitoneally and intravenously on 3 consecutive days before the mice spleens were harvested 24 h after the last injection and used for cell fusion (10).

Fusion

The hybridization of the immunized spleen cells with mouse myeloma cells (X63-Ag8.653) were carried out as described by Kearney et al. (11). Cloning of hybrid cells was conducted by limiting dilution with mouse peritoneal feeder cells in microculture plates (Costar). Spleen cells (1 × 10⁶) of a mouse showing the highest titer were mixed with X63-Ag8.653 myeloma cells (1.4 × 10⁶) and fused by use of polyethylene glycol 4000 (Fusion I) or polyethylene glycol 1500 (Fusion II). The cells were distributed into the wells of flat-bottomed tissue culture plates and cultivated in Dulbecco’s modified Eagle’s medium supplemented with 15% fetal calf serum (Boehringer Mann-
Monoclonal Anti-idiotypic Antibodies to Opioid Receptors

Pristane-primed nude mice (15). The ascitic fluids were centrifuged
plates were then washed three times with PBS/Tween 20 and twice
by the addition of metabisulfite. The iodinated IgG was first separated
with distilled water. The individual wells were counted for radioactiv-
membrane. Samples were applied to a fast protein liquid chromatog-
A-Sepharose column consisting of 500 μl of gel. The adsorbed
Iodinations of 3-E7 (IgG) were performed with chloramine T at
Molecular weight standards (low molecular weight protein standards,
the generation of F(ab') 2 was controlled by hydroxylapatite chro-
Molecular weight (92,500), BSA (66,200), ovalbumin (45,000), carbonic anhydrase (31,000), soy-

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis
To monitor the proteins in the column fractions and to estimate
their molecular weight and purity, samples were separated under
reducing conditions on a 13% SDS-PAGE slab gel according to the
method of Laemmli (18). Gels were stained with Coomassie Blue.

Adaptation of Hybridoma Cell Lines to Serum-free Medium
The serum-free medium used for the production of the anti-Ids in
tissue culture flasks was that described by Kawamoto (12); it consisted
of 2:1:1 mixture of RPMI 1640, Dulbecco's modified Eagle's medium,
and Ham's F-12 supplemented with 2 mM L-glutamin, 0.01% sodium
pyruvate, 15 mM Hepes 2.0 g/liter glucose, 2.2 g/liter sodium bicar-
bonate, 40 mg/liter penicillin, 90 mg/liter streptomycin, 10 μg/ml
bovine insulin, 10 μg/ml human transferrin, 10 μM 2-mercaptoethanol,
1 mM sodium selenite, and 4 μg/ml oleic acid in complex with fatty acid-free BSA.

Anti-F(ab') 2-3-E7 ELISA
The hybridoma culture medium from wells with growing cell lines was
assayed for anti-Fab-3-E7 antibodies by a double-antibody
sandwich ELISA. Microtiter plates (Costar 2596) were coated by
preincubating each well with 150 μl of 250 μg/ml F(ab') 2-3-E7 fragments in PBS (16, 37 °C). After washing twice with PBS, 0.1% Tween 20,
the wells were blocked with 200 μl of 3% BSA in PBS (37 °C, 3 h) and
then washed with PBS/Tween 20. 100 μl of hybridoma superna-
tant were transferred to the plates and incubated overnight at room
temperature on a shaker. After intense washing with PBS/Tween 20,
100 μl of a 1:1000 dilution of rabbit anti-mouse IgM(Fc)-peroxidase
conjugate dissolved in PBS containing 0.1% horse serum were added
and incubated for 1 h at 37 °C and for 1 h at room temperature. After
washing the plates several times, 100 μl of substrate H2O2/2'-azino-
di-3-ethylbenzthiazoline sulfonic acid (13) were added to each well.
The enzyme reaction was allowed to proceed for 30-60 min, stopped
with 50 μl of 20 mM NaNO2, and the absorbance was recorded at 414
nm (Immuno Reader, Intermed, Nunc).

Solid-phase Inhibition Assay
Microtiter plates were coated with β-endorphin (100 μl/well, 300
pmol/ml in PBS) for 16 h at 37 °C. Thereafter, the plates were
blocked for 4 h at 37 °C with 3% BSA dissolved in PBS. Subsequently,
the plates were washed three times with PBS, 0.1% Tween 20 and
twice with distilled water. Samples or solutions of control IgM (MOPC 104E) in 100 μl of radioimmunoassay buffer (0.02 M phos-
phate, pH 7.4, containing 0.15 M NaCl, 1% BSA, 0.05% Tween 20,
0.1% polylysine 4000) were incubated at 4 °C overnight together with
415-labeled 3-E7 (2,000 cpm in radioimmunoassay buffer). The plates
were then washed three times with PBS/Tween 20 and twice with
PBS. Each well was then divided into two parts for radioactivity
assay.

Iodination of 3-E7
Iodinations of 3-E7 (IgGω) were performed with chloramine T at
pH 7.4 in 0.5 μM phosphate buffer according to Bolton and Hunter
(14). The molar ratios of reactants were 2:1:25:100:100 for Na125I/
IgG/chloramine T/metabisulfite. The antibody was exposed to chlor-
amine T for 30 min at room temperature and the reaction was stopped
by the addition of metabisulfite. The iodinated IgG was first separated
on a Sephadex G-25 column (0.5 × 10 cm) and further on a protein
A-Sepharose column consisting of 500 μl of gel. The adsorbed 125I-
labeled 3-E7 was eluted with citrate, pH 4.0, and immediately neutral-
ized with 1 N NaOH.

Asces Production
Hybridoma cells (5 × 108) were injected intraperitoneally into pristane-primed nude mice (15). The ascitic fluids were centrifuged
1000 × g for 10 min after dilution in appropriate buffer to remove
cells. The supernatants were applied to an A-Sepharose column
(3-E7 isolation) or treated with ammonium sulfate (50% saturation)
for precipitation and further purification of the anti-Ids (IgM class).

Purification of Anti-idiotypic Antibodies from Cell Culture
Supernatants and from Asces
The ammonium sulfate precipitate of IgM was dissolved in 0.025
m phosphate buffer, pH 6.7, and ultrafiltrated on an Amicon XM 300
membrane. Samples were applied to a fast protein liquid chromatog-
raphy system, equipped with an anion exchange column (Mono Q
HR 5/5, Pharmacia LKB Biotechnology Inc.) and eluted with a step
gradient from 0.025 m phosphate, pH 6.7, 0.05% Lubrol PX to 0.3 m
phosphate, pH 6.5, 0.05% Lubrol PX, at a flow rate of 1.5 ml/min.

Preparation of Fab Fragments from 3-E7
Papain digestion of 3-E7 was carried out by a modified method of
Mage (16) using 4 mg of papain/100 mg of IgG. After alkylation
with iodoacetamide, the Fc fragments and undigested 3-E7 were removed
from the reaction mixture by means of protein A-Sepharose. After
ultrafiltration, the flow-through containing the Fab fragments was
used for preparation of a thyroglobulin conjugate.

F(ab') 2 fragments were prepared by papain digestion as described
(17). Minor modifications. 3-E7 was dissolved in 0.1 M citrate,
pH 4.6; the optimal digestion time was determined to be 6 h at 37 °C.
The generation of F(ab') 2 was controlled by hydroxylapatite chro-
matography (0.01 M NaH2PO4, pH 6.8, to 60% 0.5 M NaH2PO4, pH
6.8). The mixture was ultrafiltrated on an Amicon YM 10 membrane
and separated on a protein A-Sepharose column as described above.
The purity of the fragments was controlled by SDS-PAGE under
reducing and nonreducing conditions.

Opioid Receptor Binding
Rat Brain Membranes—Neural membranes were prepared as pre-
viously described (19), and binding studies were conducted in a final
volume of 200 μl. Each sample consisted of 100 μl of membrane
preparation (20 μg of protein; Bio-Rad protein assay, Munich, Federal
Republic of Germany) and 50 μl of the tritiated tracer in Tris buffer
(50 mM, pH 7.4). Unless otherwise stated, incubation was conducted
at 25 °C for 30 min (maximal binding is observed within 20 min).
Total binding was measured at 0.8-1.0 nM of the tritiated drugs, and
non-specific binding was in the presence of 10 μM of the
respectively labeled substance. Thereafter, samples were submitted
to Whatman GF/B glass fiber filters which were washed twice under
reduced pressure with 4 ml of cold Tris buffer and then counted by
liquid scintillation spectrometry. The binding for non-opioid ligands followed the same procedures. The total binding measured for
the tritiated ligand was in the range of 0.4-0.7 pmol/0.1 mg of protein.
The specific binding of the compounds collectively amounted
to 96%.

For 35S-labeled β-endorphin binding assays the method described
by Toogood et al. (20) was slightly modified. Briefly, rat brain cortex
(2 g) was homogenized in 100 ml of 20 mM Hepes buffer, pH 7.4,
containing 3 mM MgSO4, and per ml, 100 μg of BSA and 50 μg of
bactracin. Binding was assayed with 2 μg of protein (cortex mem-
branes) in a final volume of 100 μl. After incubation for 20 min at
25 °C, maximal binding was observed. The tubes were centrifuged
for 4 min (12,000 × g), the supernatant was removed, and the pellet
counted for radioactivity. The total binding amounted to 38 fmol, 10
μg of protein. The specific binding was 80-85%.

Effect of Anti-idiotypic Antibodies on the Binding of Opioids and Non-opioids—Antibodies were concentrated employing Amicon Ul-
trafiltration tubes (Centricon, M, cutoff 30,000). To measure inhibi-
tion of opioid and non-opioid binding, the antibodies were incubated
with the receptor material 15 min prior to exposure to the radiolabeled
opiods (30 min). Reversibility of opioid binding by anti-Ids was
tested by preincubation of the radiolabeled opioids with the receptor
material (15 min) followed by an incubation (30 min) with the
appropriate antibody concentrations. The subsequent procedure
followed was that described in the previous section.

Immunoprecipitation
Rat brains (minus cerebellum) were prepared as described (21).
The homogenate was washed in the presence of 2% dигi-
tonins (30 min, 4 °C). Following ultracentrifugation (100,000 × g, 1 h,
4 °C) the clear supernatant was used for binding studies. Varying
amounts of purified anti-Id-135 or anti-Id-14 were added to 700 µl of solubilized opioid receptor solution and incubated at 4 °C for 48 h. Subsequently, 300 µl of goat anti-mouse IgM, conjugated to Sepharose 4B, was added and the samples shaken at 37 °C for 2 h. After centrifugation at 12,000 × g for 2 min, the supernatant was removed and [3H]DIP binding to the supernatant was measured using the opioid receptor binding assay (see above).

**cAMP Assay in NG 108CC15 Hybrid Cells**

Neuroblastoma x glioma hybrid cells (NG 108CC15) were cultured in Dulbecco's modified Eagle's medium as described by Hamprecht (26). Cells were harvested at the state of confluency, and each assay consisted of 2 × 10^6 cells. Cells were exposed to anti-Id-14 in Dulbecco's modified Eagle's medium (37 °C, final volume 80 µl). After 1 h, PGE, (10^-4 M) was added in the presence of a phosphodiesterase inhibitor (Ro 20-1724, 10^-4 M), each in 10 µl of Dulbecco's modified Eagle's medium. The stable DADL was given by the incubation system 10 min prior to stimulation. The generation of cAMP was terminated by addition of 500 µl of 0.1 N HCl (95 °C), and measured by radio-immunooassay (25).

**Materials**

Ligands purchased from Amersham Buchler (Braunschweig, Federal Republic of Germany) included [3H]DA (41.8 Ci/mmol), [3H]DHM (60 Ci/mmol), [3H]DIP (38.1 Ci/mmol), [3H]EKC (24.1 Ci/mmol), [3H]fluorotrazepam (85.8 Ci/mmol), [3H]haloxone (51 Ci/mmol), [3H]lucidamine methyl chloride (60 Ci/mmol), and [3H]-Tyroactinophilic endorphin (2000 Ci/mmol), [3H]Bremazocine (320 Ci/mmol) from Du Pont-New England Nuclear, Dreieich, Federal Republic of Germany. CNS-activated Sepharose, Sepharose 4B, protein A-Sepharose, and IgG exchange columns (M Q HR) were obtained from Pharmacia. The following chemicals were purchased from Sigma: Dulbecco's modified Eagle's medium, RPMI 1640 medium, Ham's F-12 medium, Hepes, bovine insulin, human transferrin, 2-aminoethanol, 2-mercaptoethanol, sodium selenite, oleic acid, fatty acid-free BSA, stegomycin, polystyrene, Tween 20, bovine thyroglobulin, polyethylene glycol 1500, Lubrol PX, digitonin, pristane, IgM MOPC 104E, papain, pepsin, hypoxanthine, aminopterin, thymidine, cAMP, and PGE. Polyethylene glycol of 4000 was from Merck (Federal Republic of Germany). 2,2'-azinodiethylbenzophenone was obtained from Boehringer Mannheim; goat anti-mouse IgM, rabbit anti-IgG/Fc peroxidase conjugate, and rabbit anti-IgM/Fc peroxidase conjugate were from Nordic Immunological Laboratories (Tilburg, The Netherlands) and human β-endorphin from Novabiochem (Laufelfingen, Switzerland). Naloxone was obtained from Da Pont de Nemours, and microtiter plates from Costar.

**RESULTS**

**Fusion and Tests for the Interaction of Anti-idiotypic Antibodies with Opioid Receptors—**Thirteen out of 144 growing hybridoma colonies from the first fusion proved positive in the anti-F(ab')2-(3-E7) ELISA. Four master wells were selected for limiting dilutions. Ninety out of the 1300 growing cell lines tested secreted IgM. The second fusion resulted in about 400 growing cell lines, 70 of which showed a production of anti-Fab-(3-E7) antibodies of type M. IgG antibodies could not be detected (anti-IgG ELISA) in supernatants from either fusion. Spent medium of stabilized and anti-F(ab')2-positive hybridomas from both fusions were tested for their ability to interact with the binding site of 3-E7 by the solid-phase inhibition assay. Thirty of these were found positive, as demonstrated by inhibition of T125I-labeled 3-E7 binding to the β-endorphin-coated wells (20%-70% inhibition).

The supernatants of stable cell lines that were positive in the solid-phase inhibition test were further screened in the opioid receptor assay to identify anti-idiotypic antibodies capable of interaction with the opioid receptor. The T125I-labeled β-endorphin binding to rat brain membranes was inhibited to varying degrees by 10 of the concentrated hybridoma supernatants.

Two of the most ELISA positive IgM producing cell lines (L55.153.135 (anti-Id-135) from Fusion I and L30.14 (anti-Id-14) from Fusion II) were selected for production either in culture (serum-free medium) or in nude mice (ascitic fluid) so as to obtain sufficient material for purification and further characterization.

**Purification of the Anti-idiotypic Antibodies—**Attempts to purify both anti-Ids (of IgM class) by conventional gel chromatography (Sephacryl S-300) proved unsuccessful; the strong adsorption properties of the antibodies resulted in a poor yield. Including 0.05% Lubrol PX in the elution buffer and using a Mono Q anion exchange column, we obtained a good separation of the anti-Ids from protein contaminants originating in culture medium or ascitic fluid (Fig. 1).

Further analysis of the material eluting from the Mono Q column was performed using SDS-PAGE under reducing conditions (Fig. 2). IgM of the hybridoma line L55.153.135 was eluted by the 100% step of 0.3 M phosphate buffer with only low amounts of protein contaminations. Two main bands (M, 82,000 and 25,000) were separated as described for the heavy and light chain of IgM (22). Similar results were obtained for IgM of clone L30.14 (data not shown). The remainder of the study described here deals with the two fast protein liquid chromatography-purified antibodies.

**Demonstration of Anti-idiotypic Activity—**The anti-idiotypic activity of both antibodies was examined by testing their ability to inhibit the interaction of 3-E7 with its ligand β-endorphin. Fig. 3 shows that the binding of iodinated 3-E7 to β-endorphin (coated to a solid support) can be inhibited by increasing concentrations of both antibodies. The same degree of displacement was observed when the binding of 125I-labeled β-endorphin to immobilized F(ab')2 fragments of 3-E7 was measured (data not shown). This demonstrates that both anti-Ids interact with the β-endorphin binding site of 3-E7. To obtain a 50% inhibition of binding, an antibody concentration of about 130 nM was necessary for both antibodies.

**Interaction of Anti-Id-135 and Anti-Id-14 with the Binding of Opioid Ligand to Rat Brain Membranes—**At the outset studies were conducted to examine the time course of interaction of the anti-Ids (50 nM) with opioid receptor binding at brain membranes. The data revealed a rapid inhibition of 125I-labeled β-endorphin binding, reaching maximal effects 20 min

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**Fig. 1. Purification of anti-Id-135 by ion exchange chromatography using a Mono Q column (Pharmacia).** The A260 profile of 1 ml of ammonium sulfate-precipitated IgM from ascitic fluid is shown (—). Proteins were eluted with a step gradient of 0.025 M phosphate buffer, pH 6.7, 0.05% Lubrol PX to 0.3 M phosphate, pH 6.5, 0.05% Lubrol PX (— — — —). IgM was measured by the anti-Fab ELISA (○) (see “Experimental Procedures”) and the inhibition of the binding of β-endorphin to rat brain membranes (C) determined by the receptor assay.
5856 Monoclonal Anti-idiotypic Antibodies to Opioid Receptors

Fig. 2. SDS-gel electrophoresis of anti-Id-135 purified by anion exchange chromatography (Mono Q HR 5/5, Pharmacia). Samples were separated on a 13% polyacrylamide SDS slab gel in the presence of β-mercaptoethanol. Lane a, molecular weight standards as described under “Experimental Procedures.” Lane b, control mouse myeloma IgM MOPC 104E. Lane c, unpurified ascitic fluid from hybridoma cell line anti-Id-135. Lane d corresponds to the 50% gradient peak. Lane e corresponds to the last peak eluting from the Mono Q column. Lane f = a.

Fig. 3. Inhibition of binding of $^{125}$I-labeled 3-E7 to solid-phase coated β-endorphin by purified anti-idiotypic antibodies. Anti-Id-135 (○), anti-Id-14 (▲), and control IgM (MOPC 104E) (□) were incubated together with $^{125}$I-labeled 3-E7 (20,000 cpm) for 16 h at 4 °C. In the absence of inhibitors 25% of total $^{125}$I-labeled 3-E7 was bound to the wells.

after addition of the antibody (anti-Id-14 or anti-Id-135) to the assay system. Similar results were obtained with $[^3]$H-DHM as tracer, indicating that equilibrium conditions have been established for the binding both of the opioids and the antibodies.

For detailed binding studies brain membranes were exposed to increasing concentrations of anti-Ids prior to incubation with a constant concentration of radiolabeled opioids. Fig. 4 shows that both antibodies, at concentrations up to 500 nM, failed to affect the binding of $[^3]$H-EKC while the unlabeled EKC inhibited the binding with an IC$_{50}$ of 10 nM under the same conditions (upper panel).

An opposite result was obtained with DHM. Both antibodies, anti-Id-135 and anti-Id-14, prevented the specific binding of the μ-ligand $[^3]$HDHM (lower panel) in a dose-dependent fashion. Half-maximal inhibitions were estimated to be 20 and 130 nM for anti-Id-14 and anti-Id-135, respectively; i.e. anti-Id-14 was 6 times more potent in competing for the binding site of the μ-ligand as compared to anti-Id-135. More than 200 nM of each antibody was required to completely impede specific binding of the μ-ligand. The IC$_{50}$ of DHM in the binding assay was 2 nM. Thus, the anti-Id-14 was (on a molar basis) 10-fold less effective than the alkaloid ligand. Anti-Id-14 was employed to further characterize the specificity of the interaction with opioid receptors (Fig. 5). The antibody did not affect or only moderately reduced the binding of κ-receptor ligands ($[^3]$H-EKC and $[^3]$Hbremazocine) and of $[^3]$H]DIP, an opioid antagonist with high affinity for κ-, δ-, and μ-receptors (23). In contrast, the antibody competed effectively with μ- and δ-receptor ligands. $[^3]$HNaloxone, a narcotic antagonist like DIP, displays a somewhat higher preference for μ-receptors, and its receptor binding appeared to be sensitive to the action of the antibody. The anti-Id-14 affected the binding of the opioid peptide $[^3]$H-labeled β-endorphin somewhat less (IC$_{50}$ 50 nM) than compared to the alkaloid DHM.

The inhibition of binding activity was specific for opioid receptors: the binding of $[^3]$H-flunitrazepam was not altered by anti-Id-14 even at the highest concentration employed, and similar results were obtained with $[^3]$H]scopolamine, a muscarinic antagonist (data for scopolamine not shown). Con-
Monoclonal Anti-idiotypic Antibodies to Opioid Receptors

The inhibition of opioid binding activity was observed regardless of whether the antibody was incubated with the membrane prior to, or following, the addition of radioligand, although the IC50 was 2–3 times higher in the latter case. Since the anti-Id may noncompetitively interact with opioids (Fig. 6) this limited data available does not permit to draw any firm conclusion regarding the interpretation of this observed difference.

Mode of Interaction of Anti-Id with Opioid Receptors—To examine the opioid receptor interaction of the anti-Id-14, rat brain membranes were exposed to 50 nM of the antibody, a concentration known to cause 60–70% inhibition of β-endorphin. This was followed by increasing concentrations of [3H]labeled β-endorphin. As demonstrated in the Scatchard analysis in Fig. 6, the interference of anti-Id-14 with β-endorphin binding occurred in a noncompetitive fashion. This is indicated by a decline of the Bmax value (total number of receptors) with an apparently unchanged affinity of the receptor for the ligand. Similar experiments conducted with [3H]DHM also revealed a noncompetitive interaction between the antibody and the opiate (data not given).

Immunoprecipitation of Opioid Receptors—The above demonstration of an interaction of the anti-idiotypic antibodies described here with the binding of opioid ligands to opioid receptors prompted attempts to precipitate these solubilized molecules. Fig. 7 shows the ability of both anti-Id-135 and anti-Id-14 to precipitate [3H]DIP binding sites from a solubilized rat brain membrane preparation. Anti-Id-135, which was less potent in competing for opioid binding in brain membrane preparations proved slightly less efficient than anti-Id-14 to immunoprecipitate soluble opioid receptors. No significant precipitation was obtained with control IgM (MOPC 104E).

Investigations into the Function of Anti-Id-14—To study whether the anti-Id behave like an opioid agonist or antagonist, the ability to interfere with cAMP production in NG 108CC15 cells was tested. Table I demonstrates the ability of the β-receptor selective opioid DADL to inhibit PGE2-stimulated cAMP synthesis, an effect blocked by the narcotic antagonist naloxone. The anti-Id-14 by itself, added in concentrations proven to inhibit [3H]DADL binding to the hybrid cells (data not given), failed to affect the synthesis of cAMP. On the other hand, the antibody clearly blocked in a dose-dependent manner the effect of DADL.

Table I
The effect of anti-Id-14 on cAMP of NG 108CC15 neuroblastoma-glioma hybrids*

| Material | %  |
|----------|----|
| PGE2 (100 μM) | 100 |
| PGE2 + 10 nM DADL | 9  |
| PGE2 + 100 nM DADL | 3  |
| PGE2 + 100 nM DADL + 10 μM naloxone | 94 |
| PGE2 + 10 nM anti-Id | 95 |
| PGE2 + 50 nM anti-Id | 103 |
| PGE2 + 10 nM anti-Id + 10 nM DADL | 42 |
| PGE2 + 50 nM anti-Id + 10 nM DADL | 68 |

*Data represent means of triplicate assays.
DISCUSSION

It has been suggested that an anti-ligand antibody, suitable for the generation of anti-receptor anti-idiotypic antibodies, should share with the receptor a high degree of similarity in binding properties. This requirement is partially fulfilled by the monoclonal anti-β-endorphin antibody 3-E7; the antibody recognizes virtually all known endogenous opioid peptides, but does not bind any of the acetylated, iodoinated, or N-terminal shortened peptides. Furthermore, opioids with alkaloid structure or opioid peptides bearing D-amino acids in position 2 are not recognized by the 3-E7 antibody (1). In principle, as shown in our previous study with polyclonal antibodies (4, 5) and confirmed in the present study, the 3-E7 is useful in generating both xenogeneic and syngeneic anti-idiotypic antibodies.

The reason for using a murine monoclonal antibody for the immunization of mice resides in the fact that no anti-isotypic or anti-allotypic antibodies were to be expected. The difficulties inherent in a syngeneic system (i.e. screening for anti-Id antibodies from the same species as the immunogen) was circumvented by the use of the Fab fragment of 3-E7 and measuring the binding of the anti-Id antibodies to solid phase-adsorbed Fab fragments with the aid of Fc-specific antibodies. The screening for anti-opioid receptor antibodies revealed that both fusions carried out here resulted in antibodies of different IC50 values in inhibiting opioid receptor activity. This signalizes that the receptor preference of the two anti-idiotypic antibodies is similar to that exhibited by enkephalin, an opioid peptide with affinity for µ- and δ-receptors. However, it does not completely abolish binding.

An interesting point of this study is that both antibodies displayed a broad spectrum of different IC50 values in inhibiting opioid ligand binding to µ- or δ-opioid receptor types. It demonstrates the ability of the anti-Ids to discriminate between different opioid receptor types. Both µ- and δ-ligands are inhibited with similar potencies, whereas the binding of [3H]EK or [3H]bremazocine, which have preferential affinity for the κ-receptor, is poorly affected by both antibodies. This signals that the receptor preference of the two antibodies is similar to that exhibited by enkephalin, an opioid peptide with affinity for µ- and δ-receptors. Such a result was expected on the basis of the concept that the combining site of both antibodies represents the internal image of the N terminus of β-endorphin (identical to Met-enkephalin), which was employed to generate the idiotype antibody 3-E7. In fact, although 3-E7 does not interact with non-peptide opioids, it recognizes the N-terminal pentapeptide sequence of opioids and, therefore, the resulting anti-idiotypic antibodies may be expected to carry the internal image of this sequence. Consequently, both of the anti-Ids are able to compete with opioid peptides as well as with alkaloids for the binding to the receptors. This finding is in line with Jernes (24) postulation that an anti-Id may reflect the internal image of the ligand.

A similar preference of binding to both µ- and δ-opioid receptors was recently described by Glaslack and Denton (8) for a polyclonal anti-opioid receptor antibody generated from an anti-morphine antibody. Since the idiotype was specific for the µ-ligand morphine, these authors expected anti-idiotypic receptor antibodies to be directed to the µ-receptor. However, they obtained antibodies which interacted not only with µ- but also with δ-receptors. κ-Receptors were not affected by this antibody. Similarly, the anti-opioid receptor antibody described by Bidlack and Denton (8) does interfere with µ- and δ-receptors but fails to exhibit activity on κ-receptors. The ability of our anti-idiotypic antibodies to interact with solubilized receptors is a further indication of their specificity. This is confirmed by the fact that the two IgMs can be used to quantitatively immunoprecipitate all the opioid-binding activity in solution. In view of the high molecular weight of the IgMs some constraints of their accessibility to membrane-bound receptor proteins may be expected. Nevertheless, both of the antibodies were able to completely inhibit the binding of ['3H]DHM to rat brain membranes in a way similar to that previously found by us for polyclonal anti-idiotypic antibodies (5); this is in contrast to findings with anti-opioid receptor antibodies generated by others (6–8), and suggests that the receptor binding sites are truly occupied.

The anti-Id failed to display opioid agonistic activity as is indicated by its inability to reduce adenylate cyclase activity in NG 108-15 hybrid cells. This contrasts the function of a polyclonal anti-idiotypic antibody (IgG) in the identical test system (5). On the other hand, the 3-E7 exerts opioid antagonistic activity as is indicated by its ability to reduce the action of DADL on CAMP production in NG cells. Although antagonistic activities have been reported for antibodies to non-opioid receptors (27), information regarding anti-opioid receptor antibodies have been missing so far. Although the antagonistic activity demonstrated here was incomplete, the binding data given (Fig. 5) would suggest a complete blockade of the action of DADL in the NG hybrid cells.

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REFERENCES

1. Gramsch, C., Meo, T., Riethmüller, G., and Herz, A. (1983) J. Neurochem. 40, 1220–1226
2. Meo, T., Gramsch, C., Inan, R., HölT, V., Weber, E., Herz, A., and Riethmüller, G. (1983) Proc. Natl. Acad. Sci. U. S. A. 80, 4048–4048
3. Strosberg, A. D., Guillett, J. G., Chama, S., and Abebe, J. (1985) Curr. Top. Microbiol. Immunol. 119, 92–110
4. Schulz, R., and Gramsch, C. (1984) Neuropetides 5, 221–224
5. Schulz, R., and Gramsch, C. (1985) Biochem. Biophys. Res. Commun. 122, 658–665
6. Ng, D. S. S., and Ison, G. E. (1985) Biochem. Pharmacol. 34, 2553–2558
7. Gläsle, J. A., and Myers, W. E. (1985) Life Sci. 36, 2525–2529
8. Bidlack, J. M., and Denton, R. R. (1985) J. Biol. Chem. 260, 15655–15666
9. Couraud, P. O., Lu, B. Z., and Strosberg, A. D. (1983) J. Exp. Med. 157, 1369–1378
10. Stahlh, C., Staehein, T., Miggiano, V., Schmidt, J., and Häring, P. (1986) J. Immunol. Methods 92, 297–304
11. Keesey, R. F., Radbruch, A., Lieszang, B., and Rajewscky, K. (1979) J. Immunol. 123, 1548–1550
12. Kawamoto, T., Sato, J. D., Le, A., McClure, D. B., and Sato, G. H. (1983) Anul. Biochem. 130, 445–453
13. Gallatii, H. (1979) J. Clin. Chem. Clin. Biochem. 17, 1–7
14. Bolton, A. E., and Hunter, W. M. (1979) Biochem. J. 153, 529–538
15. Brodeur, B. R., Tsang, P., and Larose, Y. (1980) J. Immunol. Methods 71, 265–272
16. Mace, M. G. (1980) Methods Enzymol. 70, 142–150
17. Lamoyi, E., and Nisonoff, A. (1983) J. Immunol. Methods 56, 235–243
18. Laennli, U. K. (1970) Nature 227, 680–685
19. Rubini, P., Schulz, R., Wüster, M., and Herz, A. (1982) Naunyn-
Monoclonal Anti-idiotypic Antibodies to Opioid Receptors

Schmiedebergs Arch. Pharmakol. 319, 142-146
20. Toogood, C. I. A., McFarthing, K. G., Hulme, E. C., and Smyth, D. G. (1986) Neuroendocrinology 43, 629-634
21. Ruegg, V. T., Hiller, J. M., and Simon, E. J. (1980) Eur. J. Pharmacol. 64, 367-368
22. Matthew, W. D., and Reichardt, L. F. (1982) J. Immunol. Methods 50, 239-263
23. Chang, K. J., Hazum, E., and Cuatrecasas, P. (1981) Proc. Natl. Acad. Sci. U. S. A. 78, 4141-4145
24. Jerne, N. K. (1974) Ann. Immunol. (Paris) 125, 373-389
25. Costa, T., Aktories, K., Schultz, G., and Wüster, M. (1983) Life Sci. 33, Suppl. I, 219-222
26. Hamprecht, B. (1977) Int. Rev. Cytol. 49, 99-170
27. Greaves, M. F. (1984) in Monoclonal Antibodies for Receptors, Series B (Greaves, M. F., ed.) Vol. 17, pp. 3-77, Chapman and Hall, London