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Development and characterization of anti-spiroperidol antibodies*

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Ligand-specific antibodies are valuable tools for several types of applications. For example, antibodies specific for neuroleptic antipsychotic drugs are used to measure serum levels of these drugs in medicated patients [1]. Anti-ligand antibodies can also be used to induce anti-idiotypic antibodies that cross-react with receptors to which the hapten binds [2, 3], and it has been suggested [4] that the use of primary sequence information and X-ray crystallographic studies to construct three-dimensional models of the ligand binding site of an antibody may aid in constructing models of binding sites on receptors that are membrane-bound and difficult to crystallize. To measure serum levels of a drug it is necessary to have antibodies very selective for that drug, but for other applications antibodies that recognize a number of ligands are preferred. Thus, when anti-ligand antibodies are used to induce cross-reactive anti-idiotype antibodies, or as models for receptors, the primary antibody should recognize many ligands, mimicking as closely as possible the pharmacological profile of the receptors.

A panel of anti-aminospiroperidol and anti-*N*-aminophenethyl-spiroperidol (NAPS) monoclonal antibodies with high affinity for the dopamine D-2 receptor ligand spiroperidol and other butyrophenones, and high affinity for the non-butyrophenone D-2 ligand domperidone, has been produced [4]. These antibodies were elicited from mice in an early stage of the immune response by immunizing the mice once, then boosting those mice that had the highest titer 3 days before preparation of hybridomas. We now describe three monoclonal antibodies isolated from a mouse in a later stage of the immune response. These antibodies have the highest affinity for spiroperidol and related ligands of any antibodies produced to date.

Methods

NAPS and NAPS-KLH (keyhole limpet hemacyanin) were synthesized as described previously [4]. Briefly, 0.01 mmol of NAPS was reacted with 0.04 mmol of bromoacetic acid in 1.0 ml of *N,N*-dimethylformamide in the presence of 0.04 mmol of dicyclohexyl carbodiimide. The reaction mixture was incubated for 16 hr at 25°, at which time conversion of NAPS to its *N*-bromoacetyl derivative appeared to be complete. The ether-precipitated bromoacetyl derivative was dissolved in 1.0 ml of dimethylformamide and added to 4.0 ml of a solution containing 5.0 mg/ml of KLH in 0.5 M $NaHCO_3$ buffer at pH 10.0. To solubilize KLH, 0.2 ml of 10% sodium dodecyl sulfate in water was added to the protein solution. The coupling reaction was carried out for 16 hr at 4°. Uncoupled hapten was separated from the NAPS-KLH conjugate using Sephadex G-50 equilibrated in 50 mM sodium phosphate buffer containing 150 mM NaCl and 0.01% NaN_3 at pH 7.4.

Female BALB/c mice (6–8 weeks old) were immunized with equal volumes of Freund's Complete Adjuvant and NAPS-KLH (1.0 mg/ml). Mice received intraperitoneal and multiple subcutaneous injections of a total of 0.4 ml of the emulsion. The mice received subsequent injections of NAPS-KLH and Freund's Incomplete Adjuvant (0.4 ml, i.p.) at approximately 1-month intervals. One month after the third immunization one mouse was treated with NAPS-KLH in saline (0.2 ml, i.p.). Three days after this boost, splenocytes were removed for the preparation of hybridomas using the method of Kennett *et al.* [5] as described previously [4]. A charcoal adsorption assay [4] was used with minor modifications to measure the binding of [^{3}H]-spiroperidol to the monoclonal antibodies. Diluted hybridoma supernatant (50 μl) was incubated for 1 hr at 37° in 1.5-ml microfuge tubes with Tris-buffered saline (50 mM Tris–HCl, pH 7.4, and 0.9% NaCl), [^{3}H]-spiroperidol, and appropriate drugs, in an assay volume of 1 ml. Direct binding experiments were carried out using various concentrations of [^{3}H]-spiroperidol in the absence of an inhibitor. Competition experiments were carried out using six concentrations of inhibitor with approximately 1 nM [^{3}H]-spiroperidol in each assay. Nonspecific binding was determined by replacing hybridoma supernatant with control tissue culture medium. For each competing drug, values for IC_{50} and Hill coefficient were obtained from indirect Hill plots, and K_i was calculated by the method of Cheng and Prusoff [6]. Statistical comparisons of the geometric means of K_i and K_d values were made using Student's *t*-test. Light chain type and isotypes were identified by Dr. Robert Luedtke at the University of Pennsylvania.

Spiroperidol, haloperidol, pipamperone, ketanserin, and domperidone were donated by Janssen Pharmaceutica

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(Beerse, Belgium). Bromocriptine was donated by Sandoz Research Institute (East Hanover, NJ, U.S.A.). [^{3}H]-Spiroperidol (21 Ci/mmol) was purchased from New England Nuclear (Boston, MA, U.S.A.).

Results

Hybridomas designated N8-1, N8-2, and N8-3 were isolated from a mouse treated four times with NAPS-KLH. Each of the hybridomas secreted an antibody of the IgG_{2a} isotype with a kappa light chain. Monoclonal antibody N8-2 had the highest affinity for [^{3}H]-spiroperidol, as determined by saturation analysis (Fig. 1; Table 1). The K_d values of N8-2 (41 pM) and N8-3 (65 pM) for [^{3}H]-spiroperidol differed significantly ($P < 0.05$, $df = 3$) from that of N8-1 (138 pM) but not from each other.

The three monoclonal antibodies were characterized pharmacologically by inhibiting the binding of [^{3}H]-spiroperidol with a number of drugs. Competition curves were determined for haloperidol, domperidone, and pipamperone. Antibody N8-1 had an apparent affinity for domperidone of 7 nM, with higher K_i values of 43 and 234 nM for haloperidol and pipamperone respectively. Antibodies N8-2 and N8-3 had K_i values for haloperidol and domperidone of 10–20 nM, with apparent affinities for pipamperone of 60–90 nM. The K_i values of N8-1 for each drug differed significantly ($P < 0.05$, $df = 2$) from those of N8-2 and N8-3, which did not differ from each other. All competition curves had Hill coefficients close to 1.0. The binding of [^{3}H]-spiroperidol to the antibodies was not inhibited by dopamine (1 mM), bromocriptine (0.01 mM), ketanserin (0.001 mM), raclopride (0.001 mM) or sulpiride (0.001 mM).

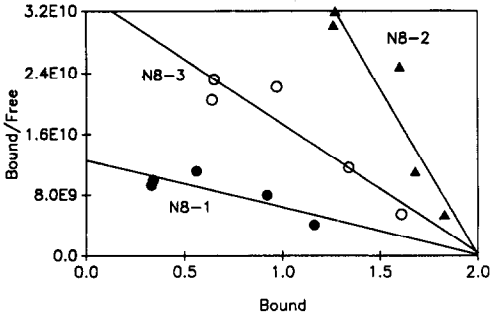

Fig. 1. Scatchard plots of the binding of [^{3}H]spiroperidol to anti-NAPS antibodies. Results shown are from an experiment representative of four independent experiments in which five to six concentrations of [^{3}H]spiroperidol from 0.03 to 0.8 nM were incubated with hybridoma supernatant. Nonspecific binding was the binding that occurred in the absence of antibody. Each point is the average of duplicate determinations. In this experiment, K_d values were 158 pM (N8-1), 23 pM (N8-2), and 58 pM (N8-3). B_{max} for each antibody was assumed to be two binding sites per molecule [7].

Discussion

The goal of the work described in this and a previous paper [4] is to develop anti-spiroperidol antibodies with as much structural similarity as possible to D-2 receptors. Such antibodies are likely to be useful for generating anti-idiotype antibodies that cross-react with dopamine D-2 receptors, and the three-dimensional structure of the combining site of such antibodies may serve as a model for the binding site of the receptors. We have made several assumptions in our approach. The first assumption is that macromolecules with similar pharmacological properties have similar structures. As an extreme example, if two proteins have precisely the same affinity for a variety of drugs, it could be assumed that the residues involved in forming the ligand binding sites of the two proteins are identical. A second assumption is that the prototypical D-2 ligand spiroperidol is the compound most likely to induce antibodies similar to D-2 receptors, because of the high affinity and selectivity of spiroperidol for D-2 receptors. A third assumption is that an antibody with a combining site that can accommodate several classes of ligands for a receptor will share more structural features with the receptor than an antibody highly selective for one ligand or class of ligands.

We isolated antibodies with high affinity for spiroperidol, with special emphasis on antibodies that also bound the butyrophenone D-2 ligand haloperidol and the non-butyrophenone domperidone. Three monoclonal antibodies with these characteristics were obtained from a mouse immunized four times with NAPS-KLH. The affinity of these antibodies for spiroperidol was at least 100-fold higher than antibodies described previously by other groups [8–10], and also higher than antibodies from an earlier stage of the immune response to NAPS-KLH [4]. The K_d values of [^{3}H]spiroperidol for antibodies N8-1, N8-2, and N8-3 were approximately the same as for D-2 receptors [4]. Of the three antibodies, N8-2 had the highest apparent affinity for haloperidol. Antibody N8-1 had the highest affinity for domperidone and lower affinity for haloperidol. All the antibodies bound the S-2 ligand pipamperone, a butyrophenone, with lower affinity than spiroperidol and haloperidol, which is consistent with the lower potency of spiroperidol at S-2 than at D-2 receptors.

Antibody N8-1 differed from antibodies N8-2 and N8-3 in affinity for each of the four drugs tested. The latter two antibodies, however, were indistinguishable and possibly identical. Hybridomas N8-2 and N8-3 are subclones of hybridomas that were in separate positive wells after fusion of splenocytes and myeloma cells (wells 11 and 72 from the same 96-well plate). It is possible that the parent splenocytes of hybridomas N8-2 and N8-3 secreted the same antibody.

In summary, monoclonal antibodies were produced by multiple immunizations with a derivative of spiroperidol, NAPS, coupled to KLH. The affinity of these antibodies for [^{3}H]spiroperidol was similar to the affinity of D-2 recep-

Table 1. Pharmacological analysis of anti-NAPS antibodies

	pK_i			pK_d
Antibody	Haloperidol	Domperidone	Pipamperone	Spiroperidol
N8-1	7.37 ± 0.02	8.16 ± 0.01	6.63 ± 0.09	9.86 ± 0.05
N8-2	7.94 ± 0.05	7.82 ± 0.09	7.06 ± 0.07	10.39 ± 0.16
N8-3	7.93 ± 0.12	7.78 ± 0.07	7.17 ± 0.03	10.19 ± 0.04

Results shown are the mean ± SEM of the pK_i values determined in three independent experiments or pK_d values determined in four independent experiments using competition (pK_i) or Scatchard (pK_d) analysis.

tors for the radioligand. These antibodies also bound several butyrophenone and non-butyrophenone D-2 ligands with high affinity and bound an S-2 ligand, pipamperone, with lower affinity. The high affinity of these antibodies for spiroperidol and other D-2 ligands, in particular their ability to accommodate domperidone, indicates that the combining sites of the antibodies share structural elements with the ligand binding site of dopamine D-2 receptors. The pharmacological profiles of the combining sites of the antibodies suggest that they mimic the D-2 ligand binding site more closely than any previously described antibodies.

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