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A MONOCLONAL ANTI-IMIPRAMINE ANTIBODY WITH ANTIDEPRESSANT BINDING PROPERTIES SIMILAR TO THE MUSCARINIC RECEPTOR

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Abstract—A murine monoclonal antibody raised using imipramine conjugated to bovine serum albumin bound free imipramine with high affinity (K_D = 24 nM). The antibody had a binding affinity profile for tricyclic and other uptake-inhibitor type antidepressants which correlated highly with the affinities of the same compounds for the brain acetylcholine muscarinic receptor, but not for plasma-membrane 5-hydroxytryptamine carriers. This antibody was not similar in binding properties to polyclonal antisera produced using the same antigen but was highly similar in binding properties to a monoclonal anti-nortriptyline antibody, ANT3, raised by Marulo *et al.* (Marulo S, Hoebeke J, Guillet JG, Andre C and Strosberg AD, *J Immunol* **138**: 524–526, 1987) using a different antigen.

Tricyclic antidepressants, including imipramine, are effective and extensively used drugs. However the mechanism of action of these drugs remains unclear. Imipramine potently inhibits 5-hydroxytryptamine uptake into both brain cells [1] and platelets [2] and indeed high affinity imipramine binding to this carrier has been demonstrated in both platelets [3] and brain [4]. In addition at higher concentrations tricyclic antidepressants are antagonists of the muscarinic acetylcholine receptor [5] and have been reported to interact also with histaminergic [6], α-adrenergic [7] and opiate receptors [8].

One method of use in studying the interaction of drugs with their receptors or binding sites is to raise anti-drug antibodies. These antibodies may then be used in structure activity relationship studies or in the production of anti-idiotypic antibodies. This latter approach has been used successfully in the production of anti-idiotypic antibodies which recognise the β-adrenergic [9–11], opiate [12–14] and nicotinic acetylcholine [15] receptors. A prerequisite for the use of these anti-drug antibodies in raising anti-idiotypic antibodies is that the binding site on the anti-drug antibody exhibits a high degree of correlation with the binding site of the receptor in question. Thus although several antibodies against haloperidol have been produced the degree of similarity between the binding site on the antibodies and the dopamine D_2 receptor is not high enough to allow production of anti-idiotypic dopamine D_2 receptor antibodies [16].

We have recently reported the production of a polyclonal antibody with binding properties very similar to the antidepressant binding site of the 5-hydroxytryptamine carrier in brain and platelets [17]. We now report the production of a monoclonal antibody with binding properties similar to that at which antidepressants interact with the muscarinic receptor.

MATERIALS AND METHODS

Materials. Imipramine, amitriptyline and nortriptyline were a gift from Iropharm Ltd (Co. Wicklow, Ireland). All other drugs and [^{3}H]imipramine (20 Ci/mmol) were obtained as described previously [17]. Bovine γ-globulin (Cohn Fraction II), Freund's complete and incomplete adjuvant was supplied by the Sigma Chemical Co. (Poole, U.K.). Pristane (2,6,10,14-tetra-methyl-pentadecane) was obtained from Aldrich Chemical Co. (Gillingham, U.K.), fetal calf serum (Myoclone) was from Gibco (Uxbridge, U.K.).

The synthesis and coupling of 2-amino-imipramine to albumin was as previously described [17].

[^{3}H]*Imipramine binding assays.* Ascites fluid at a stated dilution was incubated with [^{3}H]imipramine (10 nM) and a range of imipramine concentrations (0–1000 nM) for 60 min at 4° in a final volume of 0.5 mL in 50 mM Tris–HCl buffer, pH 7.4 containing 120 mM NaCl, 5 mM KCl and 2 mM EDTA. Non-specific binding was determined in the presence of 100 μM imipramine. At the end of the incubation period, 50 μL γ-globulin (15 mg/mL) was added to each tube, followed by 30% polyethylene glycol (200 μL) in 50 mM Tris–HCl buffer, pH 7.4. Samples were immediately vortex mixed. Free radioligand was separated from bound ligand by rapid filtration through Whatman GF/B filters that had been previously soaked in 0.1% polyethylene-imine. Filters were washed twice with 5-mL aliquots of 8% polyethylene glycol in 50 mM Tris–HCl buffer, pH 7.4. The filters were added to scintillation vials and the radioactivity measured at 45% efficiency. Mean values (±SE) were obtained from triplicate tubes.

Inhibition of [^{3}H]*imipramine binding to antibody.* Inhibition assays were performed as above except

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that IMI-3D7 (1:100 dilution) was incubated in 50 mM Tris–HCl buffer, pH 7.4, containing 120 mM NaCl, 5 mM KCl and 2 mM EDTA with [^{3}H]imipramine (10 nM) and various compounds over a concentration range as shown for 60 min at 4° in a final volume of 0.5 mL.

Cell line and media. The HGPRT(−) Balb/c myeloma cell line SP2/0-Ag14 was obtained from Flow Laboratories (Irvine, U.K.). It was maintained in Dulbecco's modified Eagles medium (DMEM) supplemented with 2 mM glutamine, 10% (v/v) fetal calf serum (Myoclone) and 1% (w/v) gentamycin or 1% Kanamycin (Sigma) (complete medium).

Immunization of mice. Balb/c mice, 10–12 weeks old, were immunized with the imipramine–BSA conjugate (500 μg) emulsified with Freund's complete adjuvant (1:1, v/v) by intraperitoneal injection.

One week later conjugate (500 μg) emulsified with Freund's incomplete adjuvant (1:1, v/v) was injected intraperitoneally. This second injection was repeated 1 week later. Four days after this third injection a boost was given intravenously which consisted of conjugate (50 μg) in phosphate buffered saline (PBS Tablets, Oxoid). The spleens were removed 3 days later for fusion.

Production of hybridomas. Fusion was performed by a modification of the method of Galfre and Milstein [18]. Splenocytes were fused with the SP2 cells using 50% (w/v) polyethylene glycol (PEG 4000) in DMEM. They were then washed with 10 mM HEPES, pH 7.4 containing 116 mM NaCl, 5.4 mM KCl, 5.5 mM glucose and 1 mM $MgCl_2$ and suspended in 50% conditioned complete medium.

Aliquots (100 μL) were then distributed into 5 × 96 well plates (NUNC) with thymus feeder cells (10^7/plate). The next day 50% conditioned complete medium DMEM (100 μL) containing hypoxanthine, aminopterin and thymidine was added to each well. When the cultures became visible (10–14 days) the supernatants were screened for the presence of anti-imipramine antibodies by enzyme-linked immunosorbent assay (ELISA).

Subcloning of hybridomas. Hybrid cell cultures which were found by ELISA to produce anti-imipramine antibodies were subcloned using limiting dilution with syngenic thymocyte feeder cells. Positive cultures were subcloned at least twice.

Production of ascites fluid. Subcloned hybridoma cell lines were grown in 75 cm^2 tissue culture flasks (COSTAR) and amplified by growth as ascites in old Balb/c mice previously primed with Pristane (500 μL). Ascites fluid was centrifuged at 8800 *g* to remove cells and tested for the presence of anti-imipramine antibodies by ELISA. The ascites fluid was stored in aliquots at −20°.

Enzyme linked immunosorbent assay (ELISA). The wells of an immunoplate (NUNC) were coated with imipramine–BSA conjugate (10 μg/mL, 100 μL/well) in coating buffer (0.1 M Na_2CO_3–Na_2HCO_3 pH 9.6) for 2 hr at 37°. The wells were then washed three times with PBS containing 0.05% Tween 20 (washing buffer) and the free sites were saturated with defatted milk protein (5% Marvel) in washing buffer for 2 hr at 37°. The wells were again washed three times with washing buffer.

Supernatants (50 μL) from the hybrid cell cultures were applied to the wells and incubated at 37° for 30 min. The plates were then washed with washing buffer and then incubated with sheep anti-mouse Ig G-A-M antibodies coupled to peroxidase (100 μL) for 30 min at 37°. After a further wash the bound peroxidase activity was visualized by the addition of H_2O_2 (0.25 μL/mL, 30% v/v) and *o*-phenylene diamine (0.4 mg/mL) in 50 mM Na_2HPO_4 pH 4.5 containing 25 mM citric acid. The colour was allowed to develop for 10 min in the dark and the assay was stopped by the addition of 2.5 M H_2SO_4.

Parallel immunoassays were performed in which native BSA replaced the imipramine–BSA conjugate as the coating agent on the immunoplates. These ELISAs were performed exactly as above. Only those hybrid cell cultures whose supernatant gave positive readings against the conjugated imipramine–BSA alone were used for further study, those which gave positive readings in both systems were discarded.

Isotypic determination. The isotype was determined to be IgG_1 using Ouchterloney double diffusion gels and a series of rabbit antibodies raised against individual mouse IgG subclasses (Miles Laboratories Ltd, Slough, U.K.).

RESULTS AND DISCUSSION

IMI-3D7 was selected because it reacted with imipramine-bovine serum albumin but not bovine serum albumin. The antibody was tested for its ability to bind free [^{3}H]imipramine following its large scale production in ascites form. The antibody bound [^{3}H]imipramine with hyperbolic dependence of binding on imipramine concentration and with high affinity (K_D, 24 ± 3 nM) for [^{3}H]imipramine (Fig. 1). The affinity of IMI-3D7 for [^{3}H]imipramine was 10–20-fold lower than the affinities of polyclonal antisera raised against the same antigen conjugate and which showed binding to [^{3}H]imipramine as if to a single class of binding sites [17].

As the affinity of IMI-3D7 for [^{3}H]imipramine was high and in the range similar to those for binding of [^{3}H]imipramine to its binding sites on the 5-HT carrier and on the muscarinic acetylcholine receptor, the interaction of relevant tricyclic antidepressants and related compounds with this antibody was measured by their ability to inhibit [^{3}H]imipramine binding to the antibody. Figure 2 shows the inhibition of [^{3}H]imipramine binding to the monoclonal antibody by a variety of compounds. In all cases where inhibition occurred displacement curves fitted single-site displacement profiles. The correlation between binding affinities of these compounds for the brain and platelet 5-HT uptake carrier and IMI-3D7 was not significant (Fig. 3b) in keeping with the lower affinity of the antibody than the 5-HT carrier for imipramine. Indeed 5-HT at 10^{-4} M did not inhibit [^{3}H]imipramine binding to IMI-3D7. The correlation between the binding affinities for the histaminergic H_1 receptor and the α-noradrenergic receptor, two receptors at which these antidepressants have been reported to bind with sub-micromolar affinities [6, 7], and IMI-3D7 was also not significant (Table 1).

In contrast the correlation between the affinities for the muscarinic receptor and for the monoclonal

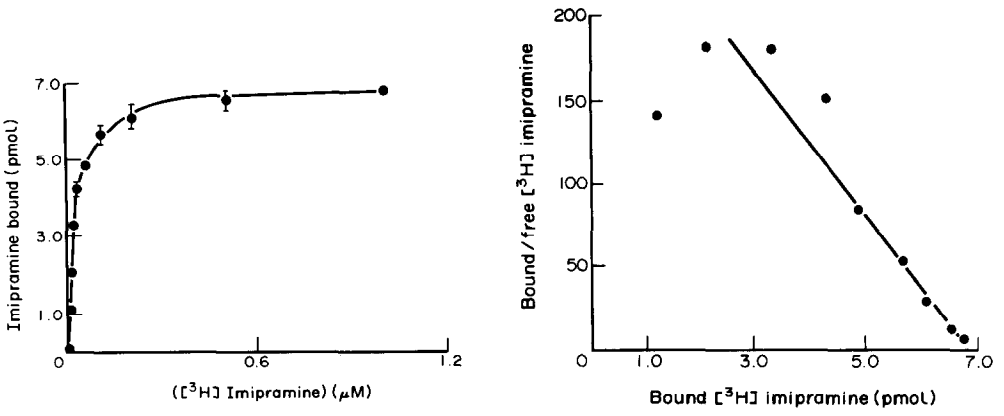

Fig. 1. Dependence of binding of IMI-3D7 to [^{3}H]imipramine. [^{3}H]Imipramine binding to IMI-3D7 (1 : 100 dilution) was performed as described in Materials and Methods. Data were fitted to a rectangular hyperbola using the computer program LIGAND [29]. The K_D and B_{max} values obtained were 24 ± 3 nM and 7 ± 0.2 nmol [^{3}H]imipramine bound/mL ascites fluid respectively. The inset shows these data plotted as a Scatchard plot.

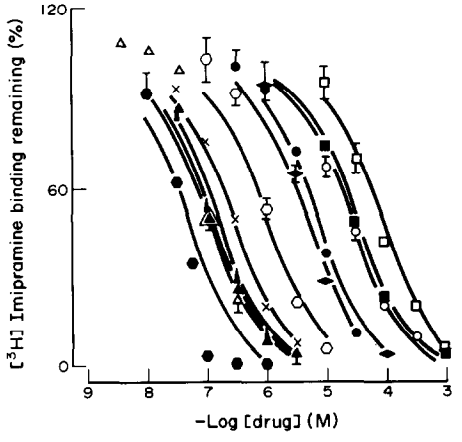

Fig. 2. Inhibition of [^{3}H]imipramine binding to IMI-3D7 by a range of compounds. The binding of [^{3}H]imipramine (10 nM) to IMI-3D7 (1 : 100 dilution) was measured in the presence of various concentrations of compounds as shown. Data are shown as the inhibition of bound [^{3}H]imipramine expressed as a percentage (±SE) of that binding in the absence of compounds. The compounds were clomipramine (●), amitriptyline (▲), desipramine (△), doxepin (×), sertraline (○), maprotiline (●), alaproclate (□), norzimelidine (○), zimelidine (■), fluoxetine (◆), and nortriptyline (▲). The inhibition curves were fitted by eye to single-site displacement curves.

antibody IMI-3D7 was highly significant (Fig. 3a). Recently a monoclonal antibody raised against the tricyclic antidepressant nortriptyline has also been shown to mimic the antidepressant binding site on the muscarinic receptor [19]. Good correlation existed (Table 1) between the affinities for antidepressants of both monoclonal antibodies, IMI-3D7 (this work) and ANT3 [19] showing the similarities in the antigen binding sites of both antibodies. The slope of the correlation curve for IMI-3D7 and the muscarinic receptor was closer to unity (1.05) than that for ANT3 (1.29) suggesting a closer similarity of the antidepressant binding site on IMI-3D7 to the antidepressant binding site on the muscarinic receptor than that for ANT3.

Similar affinities for imipramine are found for IMI-3D7 (K_D = 24 nM) and ANT3 (K_D = 60 nM). Thus in absolute values and range of affinities for antidepressants, both antibodies are remarkably similar to each other. This is despite having been raised against different antidepressants (imipramine and nortriptyline for IMI-3D7 and ANT3, respectively) with different linkage structures and positions (coupling of 2-amino-imipramine to albumin with glutaraldehyde for IMI-3D7; and carbodiimide coupling of the secondary amine of the mono-*N*-methyl aminopropyl group of nortriptyline to succinylated albumin for ANT3). These data extend those on ANT3 [19] where it was shown that the 'tricyclic backbone' of the antidepressants are important for binding (to ANT3 and presumably muscarinic receptors). The ability to raise antibody with similar properties by substitution and coupling through one benzene ring of the tricyclic structure suggests that the important feature of the tricyclic structure for immunological mimickry of the antidepressant binding site on the muscarinic receptor might be a 'half-tricyclic' backbone.

The interaction of antidepressants with muscarinic receptors is well documented (see Ref. 5) and indeed is probably responsible for many of the side effects of these drugs. It has been suggested that tricyclic antidepressants may be of use in distinguishing muscarinic receptor subtypes [20] but other work has shown non-selectivity of amitriptyline for subtypes [21]. Recently it has been proposed that the tricyclic antidepressant binding site may not be the site at which muscarinic agonists and antagonists bind [19]. These authors reported that nortriptyline decreases the B_{max} of [^{3}H]*N*-methyl-scopolamine binding to calf brain membranes without any effect on apparent K_D. This effect on B_{max} is not consistent with nortriptyline binding to the same site at which the muscarinic antagonist binds. In addition the anti-nortriptyline antibody (ANT3) raised by this group

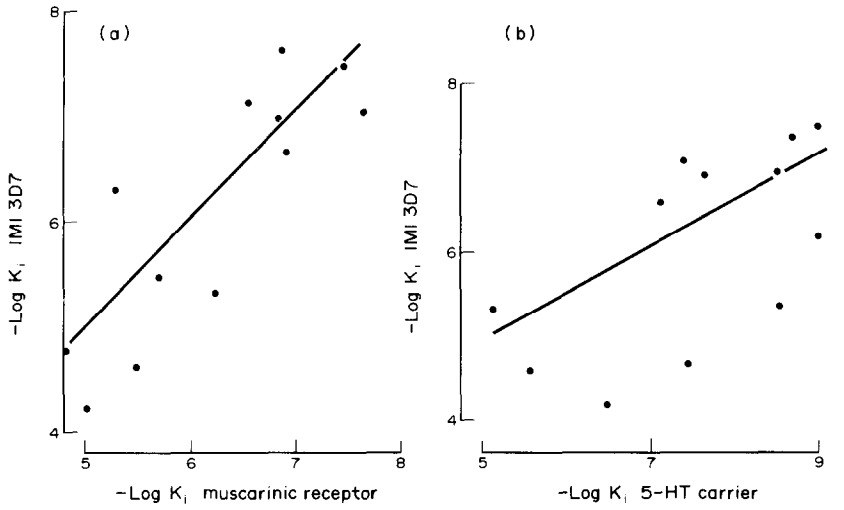

Fig. 3. Correlations between binding affinities of compounds for IMI-3D7, muscarinic receptor and 5-HT carrier. (a) The correlation between binding affinities of compounds for muscarinic receptor (MR) and IMI-3D7 yielded a correlation r of 0.8482 and a slope of 1.056 ± 0.21. From the r value, the probability P for N-2 couples of K_i were determined by using Correlation Coefficient Tables. The binding affinities for compounds to the MR were compiled from Refs 22–26. (b) The correlation between binding affinities of compounds for the 5-hydroxytryptamine carrier (5-HT C) and IMI-3D7, giving an r value of 0.6277 and a slope of 0.594 ± 0.23. The binding affinities for compounds to the 5-HT C were compiled from Refs 17 and 27.

Table 1. Relationships between binding affinities for compounds with IMI-3D7 and affinities for receptors, carriers, and ANT3

Comparison	No. of pairs	Correlation coefficient	Slope	P
3D7 vs MR	12	0.8482	1.056 ± 0.21	<0.001
3D7 vs 5-HT C	12	0.6277	0.594 ± 0.23	<0.05, >0.01
5-HT C vs MR	12	0.3096	0.4073 ± 0.4	>0.01
3D7 vs ANT3	8	0.8697	0.6154 ± 0.14	<0.01, >0.001
3D7 vs H_1	9	0.499	0.3511 ± 0.22	>0.01
3D7 vs α-A	6	0.3966	−0.4551 ± 0.52	>0.1

The correlation coefficients, slopes and P-values for the various comparisons were calculated as described in the legend to Fig. 3. The binding affinities for the compounds to the H_1-histaminergic receptor (H_1) were taken from Ref. 6, the α-adrenergic receptor (α-A) from Ref. 7 and the anti-nortriptyline antibody (ANT3) from Ref. 19. IMI-3D7 is abbreviated as 3D7 in this table.

which mimicked the antidepressant binding site on the muscarinic receptor did not interact with any of several muscarinic ligands tested again suggesting a structural dissimilarity between the muscarinic receptor binding site for the classical agonist and antagonists and that for the antidepressants. It is of interest that the monoclonal antibody (IMI-3D7) raised in this study also did not interact with either atropine (10^{-10}M–10^{-5}M) or carbachol (3×10^{-5}M – 3×10^{-3}M) at concentrations at which these compounds interact with the muscarinic receptor.

Thus these results may reflect the existence of two separate binding sites on the muscarinic receptor or it may indicate that the antibody mimics only that part of the muscarinic receptor ligand binding site to which bind tricyclic and other antidepressants.

In summary we have a monoclonal antibody against imipramine, IMI-3D7, which binds antidepressants with similar potencies to the muscarinic receptor. This antibody may be of use in the raising of anti-idiotypic antibodies against the muscarinic receptor. It may also be of use in structural activity studies of antidepressant binding to muscarinic receptors particularly those designed to limit cholinergic side effects.

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