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SPECIFIC ANTIBODIES AGAINST THE IRREVERSIBLE α-ADRENERGIC ANTAGONIST, PHENOXYBENZAMINE

MICHELE GOODHARDT, GEORGES GUELLAEN and JACQUES HANOUNE
Unité de Recherches INSERM U-99, Hôpital Henri Mondor, 94010 Creteil, France

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Abstract—Antibodies with a high affinity and specificity for the irreversible alpha-adrenergic antagonist, phenoxybenzamine (POB), have been raised in rabbits immunized with POB conjugated to thyroglobulin. The antigen was prepared by first reacting POB with the sulfhydryl group of cysteine after which the POB-complex was coupled to thyroglobulin in the presence of a water soluble carbodiimide. POB-specific antibodies were first detected 10 days after the first booster injection and rose steadily in titer over the initial 10–30 weeks of immunization. Concentrations as high as 5.4 mg specific immunoglobulin per ml antiserum were reached 30 weeks after immunization. Dissociation constants for POB of 0.2–12 nM were obtained in antisera studied in detail and there was evidence of restricted heterogeneity of binding site affinities. A high degree of specificity was demonstrated. Slight cross-reactivity occurred only with compounds structurally related to POB, such as dibenamine and nitrogen mustards, while catecholamines and α-adrenergic compounds not structurally related to POB did not cross-react, even when present in 100,000-fold excess. A rapid and convenient radioimmunoassay procedure for POB was developed using these antibodies. Competition between tritiated POB tracer and unlabeled POB for specific antibody sites allowed the measurement of as little as 10–50 pg POB. These antibodies may be a useful tool in the purification of the α-adrenergic receptor of rat liver covalently linked to POB.

The haloalkylamine, phenoxybenzamine (POB), is a potent and long-acting α-adrenergic blocker, used clinically in the treatment of cardiovascular disorders [1–4] as well as certain peripheral neurological dysfunctions [5, 6]. Recent work from our laboratory has shown that tritiated POB, radiolabeled to a high specific activity, binds specifically and irreversibly to the α-adrenergic receptor of rat liver plasma membranes [7, 8]. In contrast to attempts with reversible ligands, solubilization of the hepatic α-adrenergic receptor has been made possible using tritiated POB as an irreversible marker [8].

The aim of the present study was to obtain specific antibodies against POB, to be used as a means of selectively purifying the α-adrenergic receptor-POB complex. This report describes the use of POB conjugated to thyroglobulin as antigen in the production of such antibodies. The antisera obtained were characterized in terms of affinity, heterogeneity, specificity and time course of titer rise. A specific and convenient radioimmunoassay was also developed, which permits the measurement of picomolar concentrations of POB.

MATERIALS AND METHODS

Chemicals. The following compounds were obtained as gifts from the indicated sources: chlorambucil (Techni-Pharma, Monaco), clonidine (Boehringer Ingelheim, West Germany), melphalan (Wellcome, NC, U.S.A.), prazosin (Pfizer, Sandwich, U.K.), (-)propranolol (Imperial Chemical Industries, Macclesfield, U.K.), dibenamine, *N,N*-dibenzylaminoethanol [SKF 689-A], *N,N*-dimethyl β-chloroethylamine [SKF 7834-A], (+)phenoxybenzamine and phentolamine (Smith, Kline and French, Philadelphia, U.S.A.). (−)Norepinephrine, 1-ethyl-3(3-dimethylaminopropyl)-carbodiimide, bovine serum albumin(BSA) and thyroglobulin were obtained from Sigma (St Louis, U.S.A.). Protein A-Sepharose CL-4B was from Pharmacia (Uppsala, Sweden), goat antiserum to rabbit gamma-globulin was from Institut Pasteur Production (Paris, France) and Freund's complete adjuvant from Difco (Detroit, U.S.A.). All other chemicals were from Merck (Darmstadt, West Germany) and of analytical grade. [^{35}S]-Cysteine (650 Ci/mmol) was supplied by New England Nuclear Corporation (Boston, U.S.A.). Benzyl[^{3}H]phenoxybenzamine (15.1 Ci/mmol) was prepared at the Radiochemical Centre (Amersham, U.K.) as previously described [8]. The purity of the radiolabeled material was routinely assessed by thin layer chromatography on silica gel plates, using ethanol as eluting buffer.

Preparation of POB-thyroglobulin antigen. In common with other haloalkylamine adrenergic blocking agents, POB reacts particularly readily with sulphydryl groups via an ethylenimonium intermediate [9, 10]. The antigen was therefore synthesized by first reacting POB with the sulphydryl group of cysteine followed by carbodiimide-promoted coupling of the POB-cysteine complex to thyroglobulin (Fig. 1).

(i) Synthesis of POB-cysteine complex. POB-cysteine was formed essentially according to the method of Harvey and Nickerson [11]. Seven milliliters of 0.029 M POB solution (in 95 per cent ethanol) and 2 ml 0.1 M cysteine were incubated in a final volume of 10 ml, containing 0.07 M $NaHCO_3$ (final pH 8.0), for 5 hr at 37° under an atmosphere of nitrogen. The

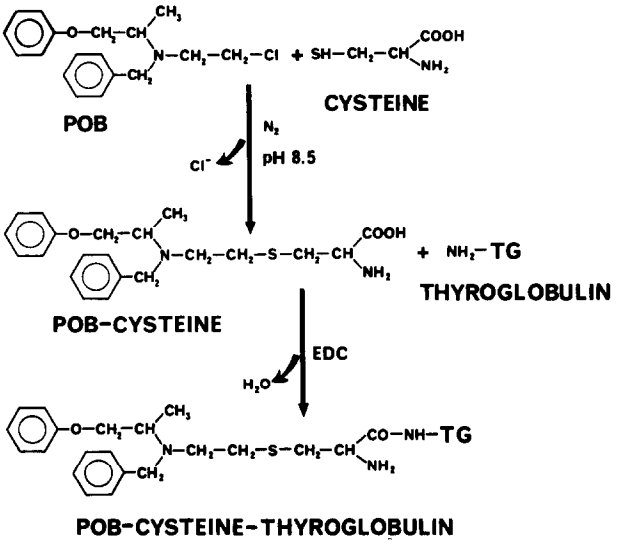


Fig. 1. Reaction scheme for the synthesis of the POB-thyroglobulin antigen. EDC: 1-ethyl-3(3-dimethyl-aminopropyl)-carbodiimide HCl, TG: thyroglobulin.

incubation mixture was then diluted with two volumes of distilled water and kept at 4° until a white gelatinous precipitate appeared. The mixture was centrifuged at 10,000 × *g* for 20 min, the supernatant discarded and the precipitate redissolved in 1 ml of 1 M HCl.

(ii) Synthesis of protein conjugate. The coupling was carried out using the method of Hoare and Koshland [12]. The pH of the POB-cysteine complex was adjusted to 4.0 with 2.5 M NaOH and 60 mg of thyroglobulin were added in 0.3 ml. The water soluble reagent 1-ethyl-3(3-dimethyl-aminopropyl)-carbodiimide was then added to a final concentration of 0.01 M, the volume was adjusted to 3.0 ml and the pH to 4.75. After 15 hr at 4°, the reaction was stopped by extensive dialysis against 0.15 M NaCl.

To determine the quantity of POB and cysteine coupled to thyroglobulin, tracer amounts of tritiated POB (50,000 cpm) and [^{35}S]cysteine (75,000 cpm) were added at the begining of the synthesis. Analysis of the radioactive content of the dialysed POB-cysteine-thyroglobulin product showed that an average of 180 moles of POB and cysteine were coupled per mole of carrier.

Immunization procedure. The POB-thryroglobulin antigen was thoroughly emulsified in an equal volume of Freund's complete adjuvant and injected intradermally at multiple sites on the back of 5 female Charles River HY/CR rabbits, according to the procedure of Vaitukaitis *et al.* [13]. Each rabbit received 1–2 ml of POB-thyroglobulin antigen containing 100 μg of POB, together with an intramuscular injection of *Bordetella Pertussis* (Institut Pasteur Production). The injections were repeated once after 4 weeks then at 6–8 weekly intervals. Control rabbits were similarly injected with cysteine-thyroglobulin complex. Blood samples were collected 7–10 days after each booster injection, and the serum separated and stored at −20°. An immunoglobulin rich fraction was prepared from some sera by precipitation in 40 per cent saturated ammonium sulphate. After washing with 38 per cent saturated ammonium sulphate, the precipitate was dissolved in distilled water, dialysed extensively against phosphate-buffered saline (0.01 M sodium phosphate buffer, pH 7.4 containing 0.15 M NaCl) and stored at −20°.

Characterization of antisera. All immunoassays were carried out in 50 mM sodium phosphate buffer, pH 7.0 containing 0.1 per cent BSA and 0.01 per cent sodium azide. Each assay contained 100 μl of appropriately diluted antisera in 1 per cent normal rabbit sera, 300 μl of phosphate buffer (with or without unlabeled competing drug) and 100 μl of tritiated POB (containing 0.7 ng POB, 10,000 cpm). The contents were routinely incubated for 16 hr at 4°. Antibody-bound POB was separated from the free drug by the addition of 100 μl goat anti-rabbit γ-globulin appropriately diluted to give maximum precipitation of the rabbit antibodies. Following a second incubation of 20 hr at 4°, the immune precipitate was pelleted by centrifugation at 3,000 × *g* for 20 min and an aliquot of the supernatant counted to determine the amount of tritiated POB remaining unbound. Nonspecific binding of tritiated POB was determined from parallel incubations containing control sera. The fraction of tritiated POB specifically bound by the antisera was defined as 1 minus the ratio of radioactivity remaining in the experimental supernatant to the radioactivity in the control supernatant. The titer of an individual antiserum was defined as the dilution at which 33 per cent of total tracer counts are bound.

RESULTS

Production of antibodies

All five animals immunized with POB-antigen produced POB-specific antibodies. The antibodies were

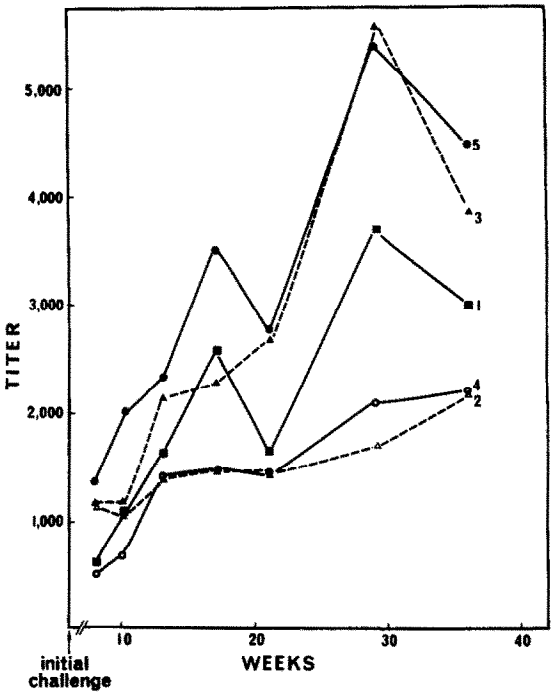


Fig. 2. Time course of appearance of POB-specific antibody response in rabbits 1–5. Sera were tested as described under Materials and Methods. The titer of an individual antiserum was defined as the dilution at which 33 per cent of the total tracer counts are bound.

first detected 10 days after the first booster injection and rose steadily in titer over the initial 10–30 weeks of immunization (Fig. 2). The immunoglobulin nature of the POB binding activity in these immune sera was indicated by the fact that the binding activity

Table 1. Effect of temperature and time on dissociation of tritiated POB binding to antibody

Temperature (°C)	Time (min)	[^{3}H] POB binding (per cent bound)
30	0.5	71
	1	48
	3	4
	5	2
	15	1
0	1	89
	3	67
	5	45
	10	22
	30	6

Tritiated POB (4 nM) was incubated with 100 μl of 1:500 dilution of antisera (rabbit 5, week 20) in a final vol of 500 μl for 20 hr at 4°. Dissociation of bound tritiated POB was assessed after addition of 2 μM POB. Antibody-bound POB was separated from the free antigen by addition of goat anti-rabbit γ-globulin as described under Materials and Methods. The results are expressed as per cent of binding in the absence of unlabeled POB (100 per cent binding was 1.1–1.3 pmoles), each value being the mean of triplicate determinations.

was quantitatively precipitated by goat anti-rabbit γ-globulin, by protein A-Sepharose, as well as by 40 per cent saturated ammonium sulphate (Fig. 3).

Characterization of antibodies

Antisera from rabbit 5 at weeks 12, 20 and 30 were selected for more detailed study. Unlike the binding of tritiated POB to the α-adrenergic receptor, POB binding to the immune sera was fully reversible. At 30°, over 90 per cent of the tritiated

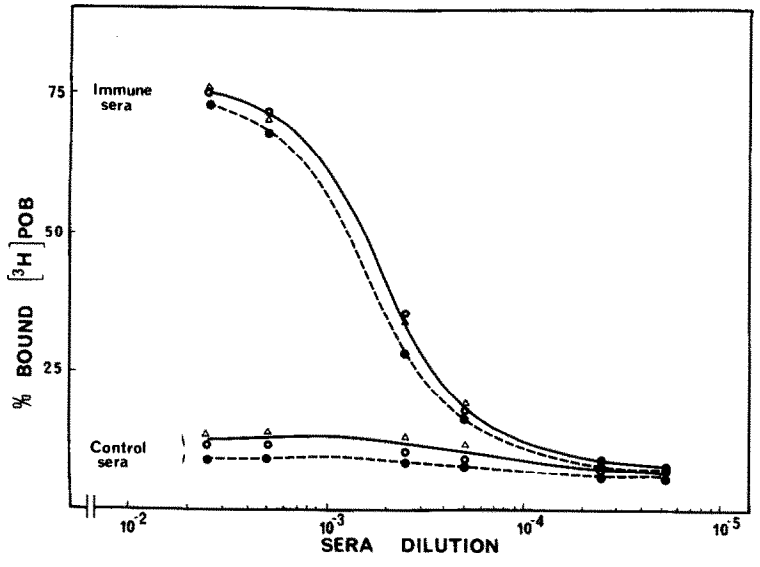


Fig. 3. Comparison of binding of tritiated POB to whole sera and a 40 per cent ammonium sulphate precipitate of sera. Tritiated POB (4 nM) was incubated with varying dilutions of antiserum from rabbit 5 at week 20 (——) or reconstituted 40 per cent ammonium sulphate precipitate of that serum (– – –). The immune complex was either precipitated with goat anti-rabbit antiserum (○, ●) as described under Materials and Methods, or by incubating with 50 μg protein-A Sepharose (△) for 1 hr at 25°. Corresponding data for control serum is also shown. Each value is the mean of triplicate determinations.

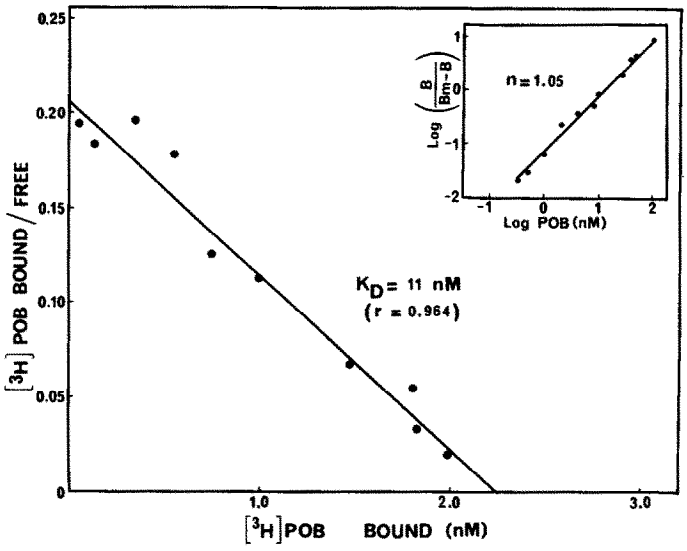


Fig. 4. Scatchard plot of tritiated POB binding to antiserum from rabbit 5 at week 12. Antiserum at a final dilution of 1/12,500 was incubated with increasing concentrations of tritiated POB (0.1–100 nM) as described under Materials and Methods. Results shown are representative of two experiments. Inset: Hill plot of POB binding to antisera ($r = 0.99$); B represents the amount of tritiated POB bound at a given concentration of POB and B_m the amount of tritiated POB bound at equilibrium. The Hill coefficient, n, is given by the slope of the curve.

POB bound to the antisera was displaced in 3 min on addition of unlabeled POB (Table 1).

Scatchard analysis of the equilibrium binding data for tritiated POB binding to serum from the early bleed (week 12) indicated a saturable, monophasic process and gave a K_D value of 11 nM (Fig. 4). From these data, the binding site concentration in the undiluted serum was calculated to be 29 μM. Assuming a mol wt of 75,000 per antibody binding site, this corresponds to 2.1 mg of POB-specific immunoglobulins per ml of antiserum. From a Hill plot of the data, a Hill coefficient, $n = 1.05$ was obtained (inset Fig. 4) indicating relative homogeneity of the binding site affinities.

The concentration and affinity of the anti-POB antibodies increased during the course of the immunization. From Scatchard analysis of tritiated POB binding, a K_D of 6 nM was obtained for sera at week 20 (data not shown) and a K_D of 0.2–12 nM at week 30 (Fig. 5). A greater degree of heterogeneity of binding site affinities in the serum from the later bleed was indicated by a non-linear Scatchard plot

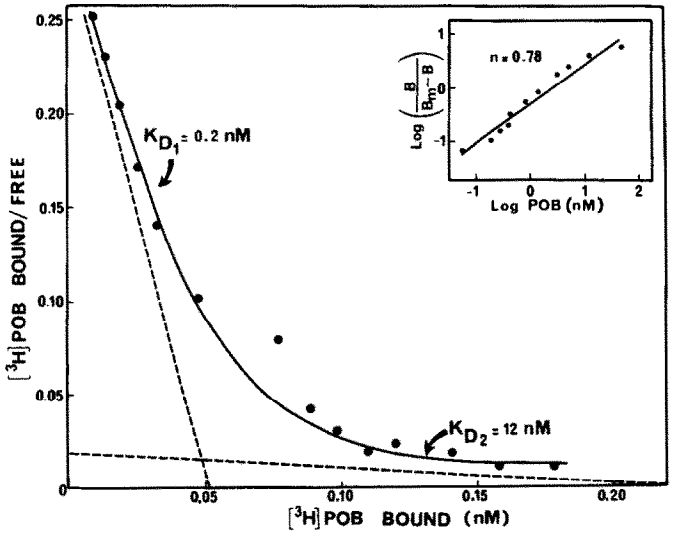


Fig. 5. Scatchard plot of tritiated POB binding to antisera from rabbit 5 at week 30 (final dilution, 1/250,000). For conditions, see legend Fig. 4. The binding parameters were determined according to Rosenthal [23]. Inset: Hill plot of tritiated POB binding to antisera ($r = 0.93$). Results shown are representative of two experiments.

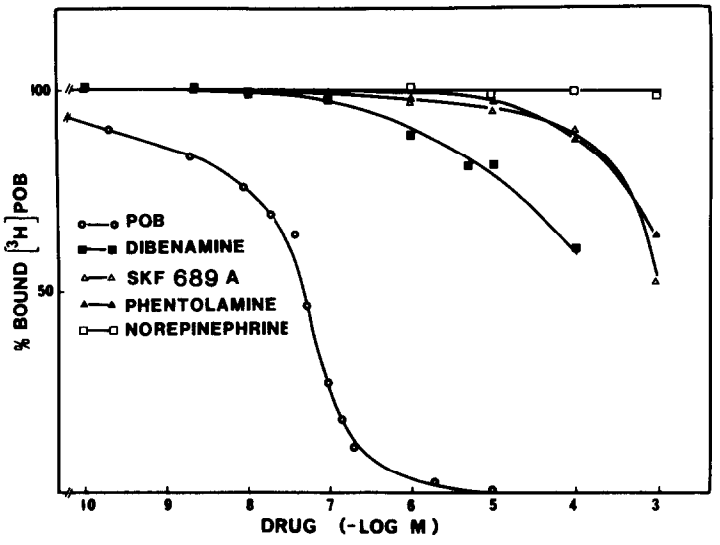


Fig. 6. Displacement of tritiated POB from anti-POB antiserum by various haloalkylamines and α-adrenergic agonists and antagonists. Antiserum from rabbit 5 at week 12 (100 μl of a 1/500 dilution) was incubated with 10 nM tritiated POB in the presence or absence of increasing concentrations of the drugs shown (final volume, 500 μl). Assays were carried out as described under Materials and Methods. The results, expressed as per cent of binding in the absence of competing drug (100 per cent binding was 0.9–1.2 pmoles/assay), are from an experiment in triplicate and are representative of two such experiments.

and a value for the Hill coefficient of 0.78. By week 30, the average antibody binding site concentration in the undiluted serum had risen to 72 μM, corresponding to a POB-specific immunoglobulin concentration of 5.4 mg/ml.

Specificity of POB antisera

To examine the structural requirements of the antibody binding sites, a series of POB analogues as well as adrenergic agonists and antagonists were tested for their ability to inhibit tritiated POB binding. As shown in Fig. 6, apart from POB, which caused 50 per cent inhibition of tracer binding at 50 nM, cross-reactivity with other drugs tested only occurred at millimolar concentrations. In order to estimate the relative potency of the various competing drugs, the per cent inhibition of tritiated POB binding obtained in the presence of 1 mM competitor was compared (Table 2). POB caused 100 per cent inhibition, while dibenamine and the analogue SKF 689-A decreased the binding of tritiated POB by only 48 per cent. The structurally related nitrogen mustards, chlorambucil and melphalan, displayed an intermediate potency, causing an inhibition of 35

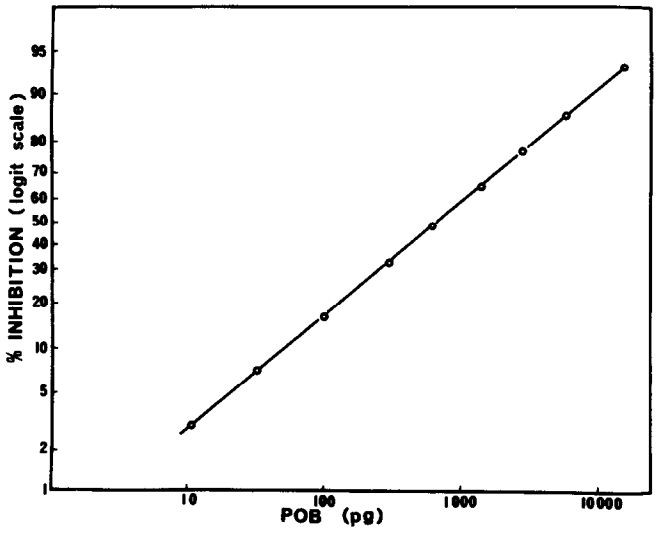
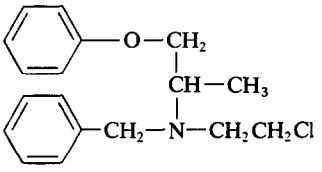
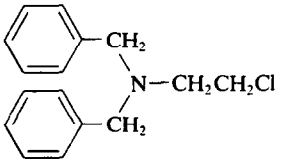
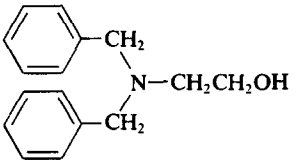
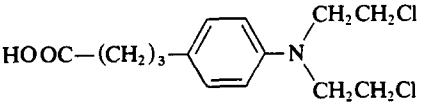
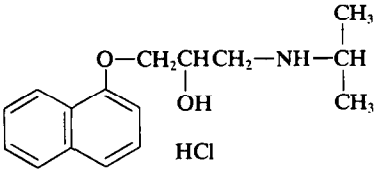
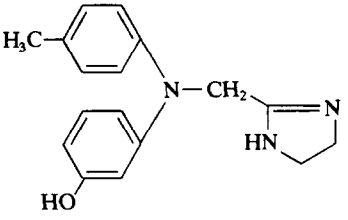
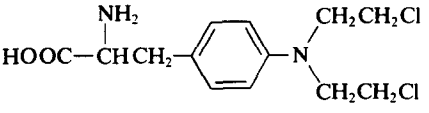
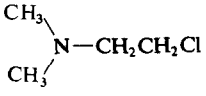


Fig. 7. Standard curve for POB radioimmunoassay. Antisera (1/12,500 final dilution) from rabbit 5 at week 30 was incubated with 1.0 nM tritiated POB in the presence of varying concentrations of unlabeled POB. Per cent inhibition values were transformed to logits and plotted against log concentration of unlabeled POB added.

Table 2. Competition with tritiated POB for antibody binding sites*

Compound	Structure	Per cent inhibition (at 10^{-3} M)
Phenoxybenzamine		100
Dibenamine		48
SKF 689-A		48
Chlorambucil		35
Propranolol		30
Phentolamine		22
Melphalan		22
SKF 7834-A		12
Norepinephrine Dopamine Prazosin Clonidine	Less than 5 per cent	

* Incubations were performed with 10 nM tritiated POB and antisera from rabbit 5 at week 12 (final dilution 1/2,500) as described under Materials and Methods, in the absence and presence of 8–10 concentrations of the drugs shown. Results indicate the per cent inhibition obtained in the presence of 1 mM competing drug and are from one or two experiments performed in triplicate.

and 22 per cent, respectively. Adrenergic agonists and antagonists, with the exception of phentolamine and propranolol, showed very little cross-reactivity, inhibiting the binding of tritiated POB by less than 5 per cent. From this order of potency it is clear that the specificity of the antibody binding sites is strikingly different from that of the α-adrenergic receptor site, as determined by tritiated POB binding [7, 8].

The results shown in Fig. 6 and Table 2 indicate that the antibodies obtained are highly specific for POB. Even dibenamine which differs from POB only in the replacements of the phenoxyisopropyl moiety by a benzyl group, showed considerably less activity than POB. The anti-POB antiserum recognized dibenamine and the dibenamine analogue, SKF 689-A (*N,N*-dibenzylaminoethanol), to the same degree. In contrast, the analogue, SKF 7834-A (*N,N*-dimethyl β-chloroethylamine), in which the aromatic groups are replaced by two methyl groups, was considerably less potent (inhibiting tritiated POB binding by only 12 per cent). These results suggest that a tertiary aromatic amine structure is of importance for recognition by the antibody binding site, while alterations to the >N—CH_2CH_2Cl group are better tolerated.

Sensitivity of the radioimmunoassay

Competition between tritiated POB tracer and unlabeled POB for POB-specific antibody binding resulted in highly reproducible semilogarithmic curves of the sort shown in Fig. 6. Using 1 nM (150 pg) tritiated POB and a final antibody dilution of 1/12,500, 10–50 pg of POB could be detected, depending on the sera used (Fig. 7). Half maximum inhibition was obtained at 200–500 pg. The intraassay variation was 5–10 per cent over the range 10–10,000 pg. Interassay variation, evaluated by repeated measurements over a 6 months period, was less than 15 per cent.

DISCUSSION

Low mol. wt compounds, not in themselves immunogenic, can be rendered so by covalent linkage to a carrier protein [14, 15]. In the present paper, we report that POB-specific antibodies have been raised in rabbits, using POB conjugated to thyroglobulin as antigen. The coupling was first effected by reacting POB with the sulphydryl group of cysteine, which was subsequently coupled to thyroglobulin using a water soluble carbodiimide. By this method, a high degree of substitution was obtained (on average 180 moles of POB per mole of thyroglobulin). Conjugation of POB via cysteine was chosen as a means of forming the antigen due to the particularly high reactivity exhibited by POB towards sulphydryl groups [11]. In addition, there is evidence to suggest that a sulphydryl group is involved in the binding of POB to the α-adrenergic receptor [7]. It could therefore be anticipated that the POB molecule of the POB-thyroglobulin antigen would be orientated in such a way that the antibodies elicited on immunization would still be capable of recognizing the drug once bound to the α-receptor.

The results obtained show that the POB binding site on the antibody differs in major respects from the α-adrenergic receptor site. Firstly, the binding of tritiated POB to the antisera is rapidly reversible, which is in marked contrast to the irreversible nature of the POB-α-adrenoreceptor interaction [8, 16]. This indicates that unlike the binding of POB to the α-adrenergic receptor, binding to the antibody does not involve an -SH group.Secondly, the pharmacological specificity of the antibody binding site is not reminiscent of that of the α-adrenergic receptor. The antisera showed very restricted specificity. Slight cross-reactivity was only observed by closely related compounds, such as haloalkylamines of the dibenamine series of nitrogen mustards. Catecholamines and α-adrenergic agonists and antagonists not structurally similar to POB showed no significant cross-reactivity, nor was there any evidence of stereospecificity. From the data obtained, it appeared that a tertiary aromatic amine group may be necessary for recognition by the antibody binding site. In contrast to POB binding to the antisera, the specificity of binding of tritiated POB to rat liver plasma membranes, determined from protection experiments using various adrenergic agonists and antagonists, followed an order of potency typical of an α-adrenergic receptor [7, 8]. Similar, although less striking differences, have been observed between the specificity of the β-adrenergic receptor and that of antibodies raised against catecholamines [15, 17] and the β-adrenergic antagonists, propranolol [18] and alprenolol [19].

The immune sera exhibited a relatively high affinity for tritiated POB (K_D values varied from 0.2 to 12 nM) and POB- specific antibody concentrations as high as 5.4 mg/ml of sera were obtained. Using these antisera, a radioimmunoassay for POB was developed which has the advantage of being technically simple, highly specific and sensitive. With this procedure as little as 10–50 pg of POB could be detected. The radioimmunoassay could be of potential value in determining the concentration of free drug present in the plasma or urine of patients treated with POB. For POB is used as a long acting α-adrenergic blocker in clinical investigations and in the management of cardiovascular disorders [1–6]. Information concerning the amount of circulating POB is all the more important since this drug acts in an irreversible manner.

POB-specific antibodies may also be potentially useful in the localization of α-adrenergic receptors. Until now two approaches have been used to localize the receptor: firstly by autoradiography [20] and secondly by the use of fluorescent blockers [21]. However these methods have only met with limited success [22]. The use of fluorescent-tagged anti-POB antibodies after pretreatment with POB, either *in vivo* or *in vitro*, may shed light on the cellular or subcellular localization of the receptor, as well as dynamic processes such as the turnover rate of the receptor. Finally, these antibodies could be a powerful resolving tool in the purification of the α-adrenergic receptor prelabeled with tritiated POB.

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