Selective elution of immunoabsorbed anti-(human prolactin) immunoglobulins with enhanced immunochemical properties

Steven C. HODGKINSON* and Philip J. LOWRY

The Pituitary Hormone Laboratory, Department of Chemical Pathology, St. Bartholomew's Hospital, 51–53 Bartholomew Close, London EC1A 7BE, U.K.

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Specific anti-(human prolactin) immunoglobulin, isolated by immunoabsorption from a resolubilized Na_2SO_4 precipitate of a sheep antiserum, has been fractionated into antibody populations with a 10-fold range of affinity for antigen by using a new elution procedure. The procedure utilized acetonitrile (20%, v/v) in a gradient of pH, decreasing from pH 7 to pH 2. The recovered immunoglobulin (74.3%) was eluted as a single peak after chromatography on Sephacryl S-300 and on radioiodination retained high immunoreactivity (91%). One antibody population, of low abundance, had an affinity constant ($K_a = 8.5 \times 10^{10}/\text{mol}$) 5.7 times that of the unfractionated antiserum and, when used in radioimmunoassay standard curves, resulted in a 3.8-fold increase in the sensitivity of the assay. The bulk of adsorbed immunoglobulin was eluted at pH 4.3, thereby avoiding exposure to strong acid conditions and maintaining the integrity of the immunoglobulin molecule. In contrast, elution of immunoglobulin in the absence of acetonitrile could only be achieved at low pH (pH 2) with minimal fractionation, and resulted in a poor recovery (37.1%) and loss of immunoreactivity (53%).

Affinity chromatography, as a method for the isolation of specific antibodies (immunoglobulins) from an antiserum, is the normal prerequisite for the development of an immunoradiometric assay (Woodhead *et al.*, 1974; Hunter & Budd, 1981). The technique utilizes antigen, covalently linked to an insoluble solid support as immunoabsorbent, to which specific immunoglobulin molecules bind and may then be radioiodinated prior to elution under dissociating conditions. In addition to the high specificity of the antibody–antigen interaction the method is also selective, since the higher affinity antibody populations in a heterogeneous antiserum combine more tightly with insolubilized antigen and, in general, are the more difficult to desorb (Miles, 1977). Thus immunoabsorption may also be used to improve the performance of radioimmunoassay by utilizing selectively eluted antibody populations with the most desirable antigen-combining properties for assay.

Although numerous reports have described the fractionation of immunoabsorbed immunoglobulin into populations with varying specificities (Miles, 1971; Svensson, 1970), few publications have de-

scribed the isolation of immunoglobulin populations with different affinities for antigen (Weintraub & Kadesky, 1971), possibly because of the difficulty in establishing an elution procedure which is selective but mild enough to preserve the integrity of the immunoglobulin molecule and provide a high recovery. Elution of high affinity immunoglobulin with strong dissociating reagents such as guanidine hydrochloride or 0.1 M-HCl has often resulted in irreversible denaturation of the immunoglobulin molecule (Miles *et al.*, 1974).

Various methods have been described for the elution of immunoglobulins from immunoabsorbents, including manipulation of pH (Miles, 1977), exposure to denaturing solutes (Weintraub & Kadesky, 1971) or detergents (Beaumont, 1970), and electric fields (Morgan *et al.*, 1978) but dissatisfaction with the yield, fractionation or the low affinity of immunoglobulins eluted using the above methods has prompted us to develop an elution procedure which would overcome these limitations.

We have previously reported the efficacy with which aqueous acetonitrile eliminated the hydrophobic interaction of human prolactin (hPrL) with chromatographic matrices (Hodgkinson & Lowry, 1981). In view of the abundance of hydrophobic residues in the hypervariable regions of immunoglobulin molecules (Dayhoff, 1972) and the

Abbreviations used: hPrL, human prolactin; IgG, immunoglobulin G.

* To whom correspondence and requests for reprints should be addressed.

significance of hydrophobic forces in maintaining the antibody-antigen 'bond' (Feinstein & Beale, 1977), we have investigated aqueous acetonitrile as a means of dissociating immunoadsorbed antibody.

The present paper describes the use of acetonitrile in conjunction with an acid pH gradient for the fractionation of high- and low-affinity antibodies from a sheep antiserum to hPrL. The anti-hPrL antibodies were recovered in high yield and without evidence of modification by the procedure.

Materials and methods

Materials

Human prolactin (hPrL) for immunization, standards, radiolabelling, and preparation of immunoadsorbent was prepared in this laboratory by the method of Hodgkinson & Lowry (1981). CNBr-activated Sepharose CL-4B and Sephacryl S-300 were purchased from Pharmacia. The protein assay kit was obtained from Bio-Rad and acetonitrile (h.p.l.c. grade) was purchased from Rathburn Chemicals, Peeblesshire, Scotland, U.K. Horse serum 3 (unactivated) was obtained from Wellcome. Di-isopropylfluorophosphate was purchased from Sigma and all other reagents were AnalaR grade from BDH.

Methods

Antiserum to hPrL was raised in sheep by four immunizations at 1 month intervals with antigen (250 µg) emulsified in 3 vol. of Freund's complete adjuvant. The sheep were bled 14 days after each immunization, and antisera were assessed as described below.

Prolactin (4 mg) was coupled to CNBr-activated Sepharose CL-4B (1 g) by using the procedure outlined by the manufacturers. Greater than 96% of hPrL was coupled to the gel, and remaining active sites were blocked with 1 M-glycine. The hPrL-Sepharose CL-4B immunoadsorbent was stored at 4°C in 0.15 M-NaCl (10 ml) containing thiomersal (0.01%) and was washed with immunoadsorption buffer (3 × 10 ml) before use. Buffer for immunoadsorption was the phosphate/citrate/borate universal buffer (6.7 mM-citric acid, 6.7 mM-orthophosphoric acid, 11.4 mM-orthoboric acid, 68.6 mM-NaOH) as described by Teorell & Stenhagen (1938) which had been adjusted to pH 7.0 with 1 M-HCl prior to use.

The IgG fraction of a sheep anti-hPrL antiserum was isolated by a well-established procedure (Nargessi *et al.*, 1978) involving precipitation with Na₂SO₄. Antiserum (10 ml) was made 18% (w/v) in Na₂SO₄ and mixed for 15 min at room temperature before centrifugation at 3000 g for 10 min. The supernatant was retained for assessment of anti-hPrL activity and the precipitate was redissolved in

0.15 M-NaCl (10 ml) prior to re-precipitation and re-solubilization as above. The serine proteinase inhibitor di-isopropylfluorophosphate was added to the resolubilized IgG fraction to a final concentration of 0.15 mM before dialysis against immunoadsorption buffer (500 ml) at 4°C for 18 h and re-centrifugation. Phenylmethanesulphonyl fluoride (0.15 mM) which is less hazardous, may be substituted for di-isopropylfluorophosphate at this stage.

Total IgG was determined by using the Bio-Rad protein assay kit (Bradford, 1976) and was shown to correlate with A_{280} when a value of $A_{1\text{cm}}^{1\%}$ of 14 was applied. A Cecil CE 292 digital u.v. spectrophotometer (Cecil Instruments, Cambridge, U.K.) fitted with a quartz optical cell with a 1 cm path length was used to monitor A_{280} .

Immunoadsorption was carried out by mixing the dialysed IgG fraction with washed hPrL immunoadsorbent overnight at 4°C. The slurry was divided into equal portions, poured into two Bio-Rad disposable glass columns (0.7 cm × 10 cm, catalogue no. 737-1222), the supernatants were collected and the columns (labelled A and B) were washed with immunoadsorption buffer (column B, 100 ml; column A, 50 ml). Column A was further washed with the above buffer (50 ml) containing acetonitrile (20%, v/v). Immunoadsorbent-bound IgG was eluted from columns A and B by application of pH gradients (each gradient, 2 × 50 ml) from pH 7.0 to pH 2.0, in the absence of acetonitrile (column B) and in the presence of acetonitrile (20%, v/v), (column A). The pH gradients were established with the universal buffer system, the starting buffer adjusted to pH 7.0 and the finishing buffer adjusted to pH 2.0 with 1 M-HCl. The gradient is not linear with respect to pH, but the three buffering solutes with pK values in the region of the gradient combine to achieve near linearity. The gradients were developed at 3 ml/h and fractions (1.5 ml) were collected and monitored for A_{280} . The pH of eluant fractions was measured with Merck narrow range pH indicator papers (catalogue nos. 9541 and 9542) and confirmed using a Corning-Eel (model 12) pH meter fitted with a Corning-Eel combination pH electrode.

Antiserum dilution curves over a range of dilutions from 1:10 to 1:10⁶ were prepared on the unfractionated antiserum, the supernatants after Na₂SO₄ precipitation of IgG, the resolubilized and dialysed IgG fractions, the IgG fraction after immunoadsorption, and all eluted IgG fractions. Buffer for assay reagents and dilutions was 0.064 M-phosphate, pH 7.4 (0.052 M-Na₂HPO₄/0.012 M-NaH₂PO₄) containing NaN₃ (0.1%) and bovine serum albumin (0.5%). Antiserum dilution curves were prepared by incubation of ¹²⁵I-labelled hPrL (75 pg, 20 000 c.p.m., 100 µl), prepared as described previously (Hodgkinson & Lowry, 1981), with antiserum dilution (100 µl) and assay buffer

(100 μ l) for 18 h at room temperature. Antibody-bound 125 I-labelled hPrI was separated from free 125 I-labelled hPrI by precipitation in the presence of horse serum (25 μ l) by the addition of polyethylene glycol 6000 solution (20%, w/v) to a final concn. of 14% (w/v), followed by mixing and centrifugation in a MSE Mistral 6L centrifuge (30 min, 4°C, 1500 g) and aspiration of the supernatant. The precipitate was counted in a Nuclear Enterprise 1600 multiwell gamma counter. From these results dilutions of antibody fractions were chosen which would bind approx. 50% of added 125 I-labelled hPrI and the affinity constants of the antibody fractions were assessed by the preparation of standard curves. Conditions for radioimmunoassay were as described above with hPrI standards of 0, 2.0, 5.0, 10.0, and 20 ng/ml (100 μ l) and anti-hPrI fractions at the chosen dilution. Affinity constants of antibody fractions were determined by Scatchard analyses (Scatchard, 1949) of radioimmunoassay standard curves. Conventional linear regression analyses were employed, and correlation coefficients were >0.95 for all standard curves over the range of standards, which were assayed in duplicate.

Results and discussion

A_{280} indicated the presence of protein (109.6 mg) following dialysis of the resolubilized Na_2SO_4 precipitate. Antibody dilution curves of this material and the eluant following immunoadsorption demonstrated $>90\%$ adsorption of anti-hPrI IgG, of which a total of 5% was recovered in the column washes with immunoadsorption buffer (columns A and B)

and a further 11.5% in the wash with immunoadsorption buffer containing acetonitrile (column A). A_{280} also showed that 93.1% of total protein (102.1 mg) was collected either in the immunoadsorption eluant or in the column washes, indicating that anti-hPrI IgG (7.5 mg) comprises $<10\%$ of the total Na_2SO_4 precipitate.

The elution profile of the adsorbed anti-hPrI IgG obtained by using the acid pH gradient in the presence of acetonitrile is seen in Fig. 1(a). The bulk of adsorbed anti-hPrI IgG is eluted as a single major peak of A_{280} which corresponds closely to the percentage binding of 125 I-labelled hPrI to $1:10^4$ dilutions of eluant fractions. The peak of A_{280} occurred at pH 4.3. This pH is markedly nearer neutrality than that required to recover antigen-bound IgG using the pH gradient in the absence of acetonitrile (Fig. 1b) or acid conditions, either alone (Cuatrecasas, 1969), or in the presence of chaotropic solutes (Weintraub & Kadesky, 1971; Brown *et al.*, 1977). Exposure to strong acid conditions, with concomitant risk to the molecular integrity of the IgG molecule, was therefore avoided.

Scatchard analysis of antibody affinity demonstrated that a considerable fractionation of antibody populations had been achieved using the acetonitrile/pH gradient elution. Two major peaks of affinity were observed. The former represents a population of intermediate affinity ($K_a = 4.6 \times 10^{10}$ l/mol) which coincides with the peak of A_{280} (Fig. 1a, fractions 25–30), and is the predominant anti-hPrI IgG species in the antiserum. The second peak (Fig. 1a, fractions 40–50) represents an antibody population of high affinity ($K_a = 8.5 \times 10^{10}$ l/mol, fraction 43) and low

Fig. 1. Elution of immunoadsorbed anti-hPrI immunoglobulin with gradients of pH decreasing from pH 7 to pH 2 (a) in the presence of acetonitrile (20%, v/v) and (b) in the absence of acetonitrile

The gradients (total volume 100 ml) were developed at 3 ml/h and fractions (1.5 ml) were collected prior to measurement of pH and A_{280} . Antiserum dilution curves were prepared on eluant fractions prior to standard curves with anti-hPrI IgG at the chosen dilutions and Scatchard analysis of anti-hPrI IgG affinity. The broken line represents the binding of 125 I-labelled hPrI to $1:10^4$ dilutions of eluant fractions.

abundance, as judged by the reduced binding of ^{125}I -labelled hPrI to $1:10^4$ dilutions of peak fractions. The column washes and early gradient fractions contained anti-hPrI IgG of low affinity ($K_a = 8.7 \times 10^9 \text{ l/mol}$, Fig. 1a, fraction 5) and consequently the unfractionated antiserum ($K_a = 1.5 \times 10^{10} \text{ l/mol}$) was fractionated into antibody populations with a 10-fold range of affinity for antigen. Scatchard plots of the eluant fraction of highest affinity (Fig. 1a, fraction 43) and the re-solubilized IgG fraction following precipitation with Na_2SO_4 are presented in Fig. 2.

The recovery of anti-hPrI IgG from immuno-adsorbent achieved with the acetonitrile/pH gradient elution was 2.8 mg (74.3%). In contrast, elution of anti-hPrI IgG from immuno-adsorbent with the pH gradient in the absence of acetonitrile (Figure 1b) occurred at the extreme acid (pH 2) end of the gradient (fractions 48–65). The recovery was reduced (37.1%, 1.18 mg) and this may be due either to denaturation and loss of integrity of the IgG molecule resulting from exposure to the low pH required for elution, or that low pH alone is inappropriate for eluting anti-hPrI IgG. The eluant fractions demonstrated only a 2.4-fold range of affinity for antigen (Fig. 1b, fraction 5, $K_a = 8.7 \times 10^9 \text{ l/mol}$;

Fig. 2. Scatchard analysis of anti-hPrI IgG affinity. The data were derived from radioimmunoassay standard curves of the immunoglobulin fraction prior to immuno-adsorption ($\bullet$, $K_a = 1.6 \times 10^{10} \text{ l/mol}$) and of the anti-hPrI IgG of highest affinity for antigen eluted with the acid/acetonitrile pH gradient ($\circ$, fraction 43 of Fig. 1a, $K_a = 8.5 \times 10^{10} \text{ l/mol}$).

fraction 53, $K_a = 2.1 \times 10^{10} \text{ l/mol}$), and there was no evidence of the higher affinity antibody populations that were eluted with the acetonitrile/pH gradient. Continued flushing with 0.1 M-HCl failed to elute anti-hPrI IgG with increased affinity for antigen.

The effect of acid/acetonitrile conditions on the immunochemical integrity of anti-hPrI IgG was studied by exposing hPrI antiserum to acetonitrile (20%, v/v) in immuno-adsorption buffer at pH values 4, 2, and 1 for 24 h at room temperature, prior to the preparation of antiserum dilution curves as described in the Materials and methods section, (Fig. 3). The dilution curves obtained with the antiserum exposed to pH 4 and pH 2, in the presence of acetonitrile, were superimposable with a third curve of antiserum exposed only to an equal volume of assay buffer. The pH 1/acetonitrile treated antiserum alone showed decreased ability to bind ^{125}I -labelled hPrI. Therefore, IgG appears stable to aqueous acetonitrile over the range of mild acid elution conditions

Fig. 3. Antiserum dilution curves of anti-hPrI serum following exposure to assay buffer ($\square$), or acetonitrile (20%, v/v) in buffer at pH 4 ($\circ$), pH 2 ($\bullet$), or pH 1 ($\blacksquare$). Antiserum was exposed for 24 h at room temperature prior to the preparation of antiserum dilution curves. Anti-hPrI IgG dilutions ($100 \mu\text{l}$) were incubated with ^{125}I -labelled hPrI (75 pg, 20000 c.p.m., $100 \mu\text{l}$) and assay buffer ($100 \mu\text{l}$) for 24 h at room temperature prior to precipitation of antibody-bound ^{125}I -labelled hPrI with poly(ethylene glycol) (14%, w/v) and centrifugation.

employed here, but eluant fractions were neutralized by the addition of 0.5M-phosphate buffer, pH 7.4 (200 μ l) as a precaution.

The increased fractionation and recovery of anti-hPrl IgG achieved using the acid/acetonitrile elution procedure serves to illustrate the need to select elution reagents and conditions that relate to the physicochemical properties of the antigen being used. O'Sullivan *et al.* (1979) have demonstrated that elution of antibodies raised to hydrophobic haptens (e.g. cortisol) is best achieved using 'hydrogen bonding disruptive reagents' such as guanidine hydrochloride, whereas elution of antibodies to donkey anti-(rabbit Fc fragments), which are less hydrophobic, was facilitated under acid conditions. Thus, the significance of hydrophobic 'bonding' in the stabilization of the antibody-antigen interaction may be greater when the antigen is hydrophobic and the requirement of chaotropic conditions for the fractionation and high recovery of antibodies to the hydrophobic hPrl molecule comes as no surprise. The acid/acetonitrile pH gradient has also proved an efficient procedure for the fractionation and high recovery of antibodies to adrenocorticotropin (B. Allolio, unpublished work) which also has exposed hydrophobic groups.

The homogeneity of eluted anti-hPrl IgG (Fig. 1a, fraction 26) was assessed by gel filtration chromatography on a column (1.0 cm $\times$ 70 cm) of Sephacryl S-300 which had been previously equilibrated with 20 mM-phosphate buffer (16.25 mM- Na_2HPO_4 /3.75 mM- NaH_2PO_4), pH 7.4. The fraction was applied and eluted at 3 ml/h with the same buffer. Fractions (1.0 ml) were collected and monitored for A_{280} . The anti-hPrl IgG was eluted as a single peak of A_{280} (Fig. 4a, fraction 26) in the position expected for IgG (K_{av} 0.26, Pharmacia Product Handbook, Gel filtration theory and practice) and the A_{280} of non-peak fractions accounted for <5% of total absorbing material, indicating that the antibody population was free of contamination with proteins of molecular weights other than that of IgG. The peak fractions were freeze-dried.

Radioiodination, by the Iodogen method (Salacinski *et al.*, 1981) was utilized to assess the immunoreactivity of the anti-hPrl IgG. A sample of the Sephacryl S-300 peak fraction (Fig. 4a, fraction 26) of anti-hPrl IgG (0.25 nmol, 40 μ g) was iodinated with Na^{125}I (0.5 mCi, 0.25 nmol), reapplied to the same Sephacryl S-300 column as used above and eluted using the same solvent system but with added bovine serum albumin (0.5%, w/v). A single major peak of radioactivity was observed (Fig. 4b, fraction 26) eluting in the position of anti-hPrl IgG (Fig. 4a). The integrity and immunoreactivity of this peak was assessed by its ability to reassociate with excess prolactin immunoabsorbent, and was manifest by the high binding (>90%) of radioactivity achieved

Fig. 4. Gel-filtration chromatography of anti-hPrl IgG (fraction 26 of Fig. 1a) on a column (1.0 $\times$ 70 cm) of Sephacryl S-300

(a) The column had been equilibrated prior to chromatography with 20 mM-phosphate buffer, pH 7.4, and was eluted at 3 ml/h with the same solvent system. Fractions (1.0 ml) were collected and monitored for A_{280} . (b) The peak fraction (fraction 26) was then radioiodinated and rechromatographed on the column under the same conditions and the eluant fractions were monitored for radioactivity. The solvent conditions utilized here resulted in retardation of the unreacted Na^{125}I by the gel matrix. It was eluted after continual flushing of the column (3 column vol.) and is not represented on the profile. V_s , the salt volume of the column.

after an incubation of 2 h. Non-specific binding of label to Sepharose-4B was <2%. These results strongly suggest that the anti-hPrl IgG, as eluted from the immunoabsorbent by using the acetonitrile/pH gradient elution procedure, is free of non-specific contamination. However, no claim about the immunochemical purity of the anti-hPrl IgG can be made since it is conceivable that a more discriminating analytical procedure such as isoelectric focusing would resolve a multiplicity of antibody populations despite the linearity of the Scatchard plots.

Iodination of anti-hPrl IgG which had been eluted by using the pH gradient in the absence of aceto-

nitrile (Fig. 1*b*, fraction 55) yielded a preparation of reduced immunoreactivity (53%).

The increased sensitivity of a hPrI standard curve prepared using the anti-hPrI IgG of highest affinity (Fig. 1*a*, fraction 43) compared with a standard curve prepared using unfractionated antiserum is seen in Fig. 5. The assay procedure was as described in the Materials and methods section except for the use of hPrI standards of 0, 0.5, 1.0, 2.0, 5.0, and 10.0 ng/ml (100 μ l). The minimum detectable dose (the amount of hPrI two standard deviations of duplicate standard counts from zero counts) was 88 pg for the unfractionated antiserum and 23 pg for the anti-hPrI IgG, an improvement in sensitivity of 3.8-fold. Despite the low abundance of the high-affinity population (13.2% of total anti-hPrI IgG) the measurement of previously undetectable levels of hPrI would itself justify the fractionation of antisera; the careful monitoring of patients with prolactin-secreting adenomas to prevent overtreatment with bromocriptine is an example of the clinical applica-

tion of a radioimmunoassay with increased sensitivity.

Despite the widespread use of immunoadsorption as a means of purifying antigens as well as antibodies, the technique has always been limited by the inefficiency of methods for the desorption of the ligand. The use of mixed chaotropic reagents such as acid/acetonitrile may therefore have wider applicability in the desorption and purification of antigens, particularly those with labile secondary and tertiary structures, whilst simultaneously preserving the integrity of the insolubilized globulin. Indeed, we have not observed any significant deterioration of our insolubilized hPrI despite many exposures to the elution reagent. The use of acetonitrile for the fractionation of cross-reacting antibodies to steroid analogues such as testosterone and dihydrotestosterone may be another advantage of the procedure and is being investigated.

The acetonitrile/pH gradient elution procedure has proved both selective and mild, fractionating anti-hPrI IgG into populations with different affinity for antigen, whilst providing a high recovery of undamaged antibody which on radioiodination proved highly immunoreactive.

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Fig. 5. Prolactin radioimmunoassay standard curves prepared with the unfractionated antiserum (●), and the anti-hPrI IgG of highest affinity for antigen (○, fraction 43 of Fig. 1*a*)

Dilutions of anti-hPrI IgG were chosen which would bind approx. 50% of 125 I-labelled hPrI in the absence of unlabelled antigen. Standard curves were prepared by incubating 125 I-labelled hPrI (20000 c.p.m., 75 pg, 100 μ l) with hPrI (0–10.0 ng/ml, 100 μ l) and anti-hPrI IgG dilution (100 μ l) for 24 h at room temperature. B/B_0 , 125 I-labelled hPrI bound divided by 125 I-labelled hPrI bound in the absence of unlabelled antigen, expressed as a percentage.

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