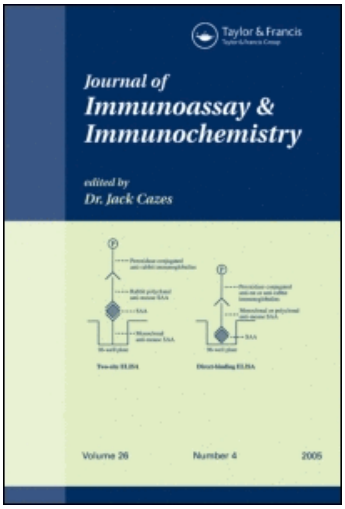

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The Production and Characterisation of Monoclonal Antibodies Against Human Prolactin and the Development of a Two-Site Immunoradiometric Assay

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THE PRODUCTION AND CHARACTERISATION OF MONOCLONAL ANTIBODIES AGAINST HUMAN PROLACTIN AND THE DEVELOPMENT OF A TWO-SITE IMMUNORADIOMETRIC ASSAY

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ABSTRACT

Monoclonal antibodies against human prolactin (PRL) have been produced and characterised and used to develop a sensitive two-site immunoradiometric assay (IRMA). Nine anti-PRL monoclonal antibodies were assessed for reactivity in immunoblotting experiments with PRL, hPL, hGH and pituitary gland extract. There was no detectable crossreactivity with hPL or hGH. In liquid phase radioimmunoassay (RIA) studies using three of the antibodies there was no detectable crossreaction from hPL or hGH. Five antibodies were positive in immunocytochemical studies using sections of human pituitary gland. Using FPLC purified monoclonal antibodies, a two-site IRMA was developed that could assay PRL over the range 17.5 - 3500 mIU per litre and was readily adapted to assaying serum samples from patients. The two-site IRMA could be performed within one day without loss of sensitivity and has potential as a rapid and simple method for screening clinical samples.

'Keywords: Prolactin Monoclonal antibody Two-site IRMA'.

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The advent of monoclonal antibodies (1) has enabled the development of two-site immunoradiometric assays (IRMAs) whereby one antibody which is immobilised on a solid phase, binds to one epitope on the antigen and a second radiolabelled antibody is bound to a different epitope on the same antigen.

In man prolactin (PRL) belongs to a group of hormones including placental lactogen (hPL) and growth hormone (hGH) which have the same fundamental structure. The main form is a single chain non-glycosylated polypeptide of molecular weight 22 kD. Glycosylated prolactin also occurs, in the decidua for example, where it represents the main form. There are 3 intramolecular disulphide bridges and approximately 20% homology between PRL and hGH and hPL (2). A number of reproductive disorders are associated with increased secretion of PRL and its assay is therefore of clinical importance particularly in infertility. PRL also occurs in several molecular forms of different sizes which may be present in biological fluids including serum samples from patients (3, 4).

It was the aim of this study to produce and characterise monoclonal antibodies specific for PRL that could be utilised in a sensitive and rapid two-site IRMA for the quantitation of PRL in sera from patients with PRL related disorders, and for physiological and research studies.

MATERIALS AND METHODS

Production of monoclonal antibodies against human PRL

A Balb/c mouse was immunised subcutaneously in 4 sites with 100 µg of human PRL (code SFK7; a gift from Dr S Lynch, The Birmingham and Midland Hospital for Women) emulsified with

Freund's complete adjuvant. Four weeks later the mouse was immunised subcutaneously with 50μg of PRL in complete adjuvant and again after 4 weeks using the same dose. A final 50μg intraperitoneal boost was administered 3 weeks later in phosphate buffered saline (PBS). Splenocytes from the immune donor were fused 3 days later with the myeloma cell line NSO as described by (5). Culture supernatants were screened in a liquid phase radiobinding assay utilising polyethylene glycol (PEG) 6000 and bovine gamma globulin to separate free and bound antigen (6). Hybridomas from positive wells were cloned twice in soft agar and specific clones were either cryopreserved, grown in culture to produce antibody containing supernatant or injected into pristane primed Balb/c mice to produce ascitic fluid. The immunoglobulin class and subclass of the monoclonal antibodies were identified by double immunodiffusion in agar using 100 times concentrated culture supernatants and polyclonal class and subclass specific sera (OLAC).

Purification and iodination of monoclonal antibodies

Three of the monoclonal antibodies (coded C63, C107 and C108) were purified from ascitic fluid by a combination of precipitation with ammonium sulphate (45% saturation) and Fast Protein Liquid Chromatography (FPLC) using a mono Q ion exchange column (Pharmacia) as described by (7). The purified material was dialysed against phosphate buffer (pH 7.4; 0.1M) and the protein concentration adjusted to 2mg ml^{-1}. Aliquots (30μl) were radioiodinated using 200μCi Na ^{125}I (Amersham International) by the chloramine T method. Free iodine was removed using a Dowex 1 - X8 ion exchange column equilibrated with phosphate buffer (pH 7.4; 0.1M) containing 2% BSA w/v (bed volume 1ml). The

radiolabelled antibody was collected in 2ml of equilibration buffer and stored at 4°C after the addition of 0.02% sodium azide. An affinity purified goat anti-mouse $F(ab')_2$ antibody was radioiodinated using the same method (8).

Immunoblotting

PRL (15μg) in reducing sample buffer was heated at 100°C for 2 minutes prior to electrophoresis. SDS polyacrylamide gel electrophoresis (SDS-PAGE) was carried out using the discontinuous buffer system described by (9). After electrophoresis the reduced protein was transferred to nitrocellulose sheets using a transblot apparatus (BioRad Laboratories) as described by (10). After transfer the unoccupied protein binding sites on the nitrocellulose sheets were blocked by incubation for 30 minutes with 3% bovine haemoglobin dissolved in PBS (Hb - PBS). Blots were incubated overnight with 50μl of ascitic fluid containing anti-PRL monoclonal antibody diluted in 25ml Hb-PBS; washed for 30 minutes with 5 changes of Hb-PBS; and incubated for 3 hours with radioiodinated goat anti-mouse $F(ab')_2$ (1×10^6 cpm per track) diluted in 25ml Hb-PBS. After washing with 5 changes of PBS (25 ml each) the blots were dried and exposed to X-ray film (Kodak X-OMAT) for 72 hours in a cassette equipped with a fast tungsten intensifying screen (11). Exactly the same procedure was carried out using hPL (1st international reference preparation, code number 73/545) hGH (code number 86/710) and an acid/acetone extract of human pituitary gland.

Liquid phase radioimmunoassay studies

Monoclonal antibodies (ascitic fluid) were diluted in RIA buffer and tested for specificity using a liquid phase double

antibody RIA. Standard curves of the 2nd international standard (code number 83/562) were constructed using a rabbit polyclonal anti-PRL serum and 3 monoclonal antibodies, C63, C107 and C108. hPL (code number 73/545) and hGH (code number 86/710) were used up to 10 times the highest concentration of PRL.

Immunocytochemistry

Sections (5 μ) of human pituitary gland were cut, air dried and fixed in acetone for 10 minutes. The sections were washed with tris buffered saline (TBS, pH 7.2) and incubated with monoclonal antibody (C63, C65, C103, C105 and C108, 1/200 dilution of ascitic fluid in TBS) for ½ hour. The sections were washed 3 times in TBS and incubated with alkaline phosphatase conjugated goat anti-mouse immunoglobulin (Sigma) diluted 1/10 in TBS for ½ hour. After washing 3 times in TBS the sections were incubated with substrate (Napthol - AS phosphate) for 15 minutes. Finally sections were washed 3 times in TBS as before, counterstained in neutral red for 2 minutes, washed briefly with distilled water and mounted in glycerine jelly.

Incubation with a monoclonal antibody (NIBSC 63/72/33) which reacts with human β-lipotropin, served as a positive control. A negative control was provided by a monoclonal antibody (NIBSC 63/17/2) raised against porcine β-lipotropin which does not crossreact with human β-lipotropin (12).

Two-site IRMA

Purified monoclonal antibodies (C63, C107 and C108) were either prepared for radioiodination as described above or dialysed against PBS and adjusted to a protein concentration of 5μ gml^{-1}. These latter solutions were used to coat the wells of

flexible 96-well microtitre plates (50µl per well). After overnight incubation at 4°C the wells were washed 3 times with PBS and then incubated at room temperature with Hb-PBS (200µl per well) for 30 minutes to block unoccupied protein binding sites on the well surface. Various concentrations of PRL standard (code number 83/562) were made in Hb-PBS (0.35 - 3500 mIU per litre) and 50µl of each dilution of these were added to the plates in duplicate for each of the monoclonal antibodies and incubated overnight at 4°C. Control wells consisted of 50 l of Hb-PBS alone. Plates were washed 6 times with Hb-PBS and 50µl (10^5 cpm) of radioiodinated monoclonal antibody (C63, C107, C108) was added to appropriate wells and incubated for 2 hours at room temperature. This procedure results in a 3 x 3 matrix enabling the 'capture' and 'detect' properties of the monoclonal antibodies to be determined. The plates were washed 6 times with Hb-PBS and individual wells cut out using a hot nichrome-wire plate cutter. The counts bound to the wells were estimated using a gamma counter.

Two-site IRMA with human sera

The overnight two-site IRMA described was used to assay in duplicate coded serum samples (50µl) from patients attending the infertility clinic at the Chelsea Hospital for Women, London. All sera had been previously assayed using a conventional RIA. The two site IRMA was also performed as a one day assay (3hr incubation at 37°C) to see if the procedure could be shortened without loss of sensitivity.

Two-site IRMA of pituitary PRL after chromatographic fractionation

A preparation of human pituitary PRL was fractionated as follows: 500µl of phosphate buffer (0.05M, pH 7.2 containing 0.5%

BSA and 0.1% sodium azide) was added to 1 ampoule of PRL (code number 81/541). This preparation contains approximately 3μg of purified product which is known to contain a proportion of dimer and aggregated PRL. The sample was applied to an Ultragel AcA 54 column (0.9 X 60 cm) equilibrated with the same buffer; and 15 drop (approximately 0.35ml) fractions were collected at a flow rate of 5 - 6 ml per hour. The fractions were further diluted with 2ml buffer, aliquoted and stored at -20°C until use. Aliquots were assayed in duplicate in the two-site IRMA and the PRL content of each fraction estimated from a calibration curve. All fractions were separately assayed in a conventional RIA and were coded before testing in the two-site IRMA.

RESULTS

Monoclonal antibodies against human PRL

A total of nine monoclonal antibodies were produced that bound radiolabelled PRL in a liquid phase binding assay. All antibodies were of the IgG class and IgG1 subclass and were designated C63, C65, C66, C101, C103, C104, C105, C107 and C108.

Immunoblotting

All monoclonal antibodies were tested by immunoblotting for binding to PRL, hPL, hGH and pituitary extract. All antibodies bound to PRL following SDS-PAGE except C63 and C65 (Fig. 1.(a), Table 1.) and C66 (Table 1.). Similarly C63, C65 and C66 did not bind to any proteins present in the pituitary extract whereas the remaining antibodies bound to 2 bands of molecular weight 40 kD and 22 kD respectively (Fig 1.(b), Table 1.). None of the antibodies tested bound to hPL or hGH (Table 1.). The

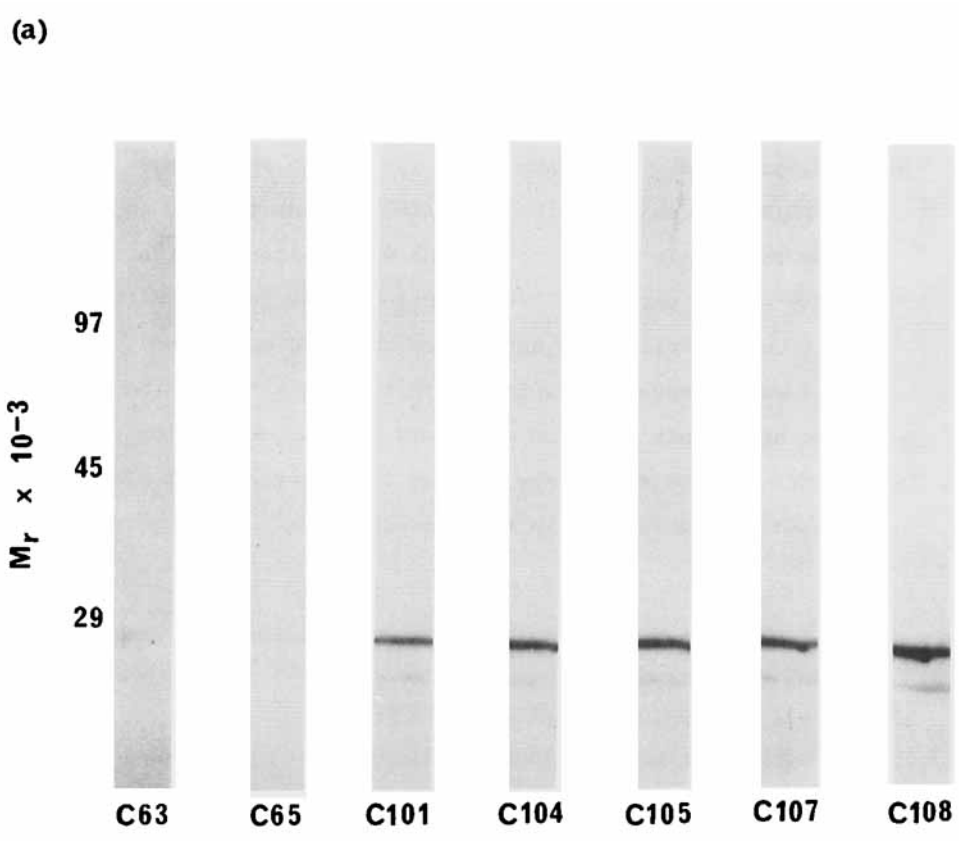
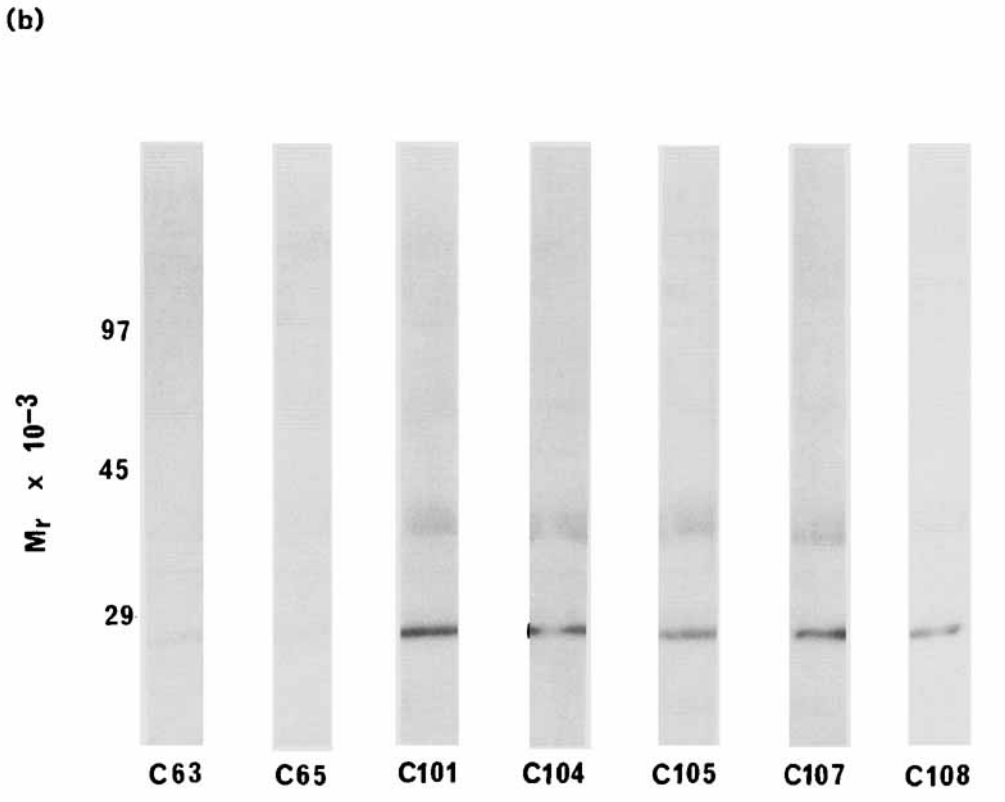


Figure 1: Immunoblot of antibodies C63, C65, C101, C103, C105, C107 and C108 using (a) pure human PRL and (b) an acid/acetone pituitary gland extract. Both preparations were run under reducing conditions on a 5% - 15% linear gradient SDS polyacrylamide gel. C63 and C65 do not react with PRL in immunoblotting experiments. The remaining antibodies bind purified PRL and detect PRL in an extract of human pituitary gland.

immunoblotting data imply that monoclonal antibodies C63 and C65 recognise different epitopes from those recognised by the remaining anti PRL monoclonal antibodies. Pairs of antibodies recognising different epitopes are requisite for the development of two-site IRMAs.



Radiobinding and radioimmunoassay studies

The monoclonal antibodies were compared with a polyclonal rabbit antiserum in a conventional RIA. The ED_{50} of the polyclonal serum was 24 ng per ml and the ED_{50} of C63 monoclonal antibody was 1800 ng per ml. hPL and hGH did not displace radiolabelled PRL at concentrations up to 50 gml^{-1}. Similar results were obtained for monoclonal antibodies C107 and C108.

TABLE 1

Immunoblotting of anti-PRL monoclonal antibodies: + indicates a positive response in immunoblotting; - indicates a negative response in immunoblotting

Anti-PRL Monoclonal Antibody	Immunoblotting Antigen			
	PRL	hGH	hPL	acid/acetone pituitary gland extract
C 63	-	-	-	-
C 65	-	-	-	-
C 66	-	-	-	-
C101	+	-	-	+
C103	+	-	-	+
C104	+	-	-	+
C105	+	-	-	+
C107	+	-	-	+
C108	+	-	-	+

Immunocytochemistry

All monoclonal antibodies tested showed binding to PRL present in sections of human pituitary gland using the alkaline phosphatase immunocytochemical technique. Fig. 2. shows the results for one monoclonal antibody (C63) compared with the positive and negative control.

Two-site IRMA

Using the matrix approach it was shown that monoclonal antibodies C107 and C108 were unreactive with each other in the two-site IRMA but both could be used as 'detecting' antibodies when monoclonal antibody C63 was used as 'capturing' antibody. Antibody C63 could not however be used to detect PRL when C107 or C108 were used to capture. Using C63 as 'capturing' antibody and C107 as 'detecting' antibody PRL could be detected in

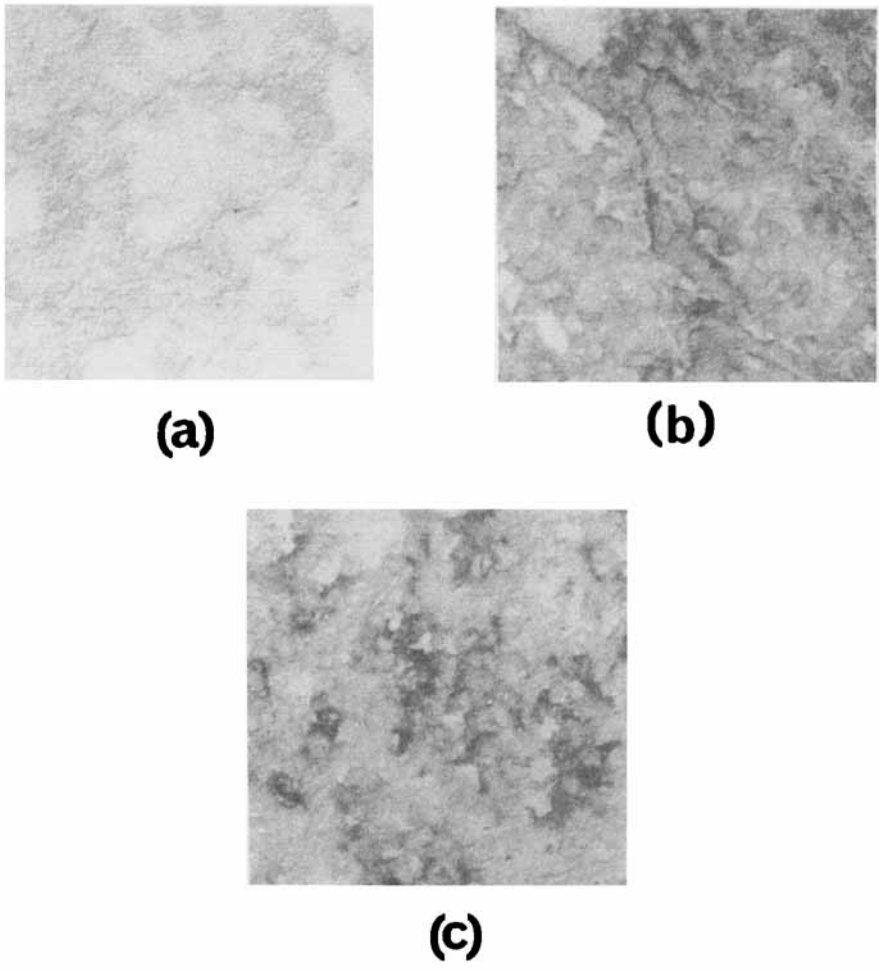


Figure 2: Sections of human pituitary gland incubated with (a) monoclonal anti-porcine β-lipotropin (negative control), (b) monoclonal anti-human β-lipotropin (positive control) and (c) monoclonal anti-PRL (C63); using the alkaline phosphatase immunocytochemical technique.

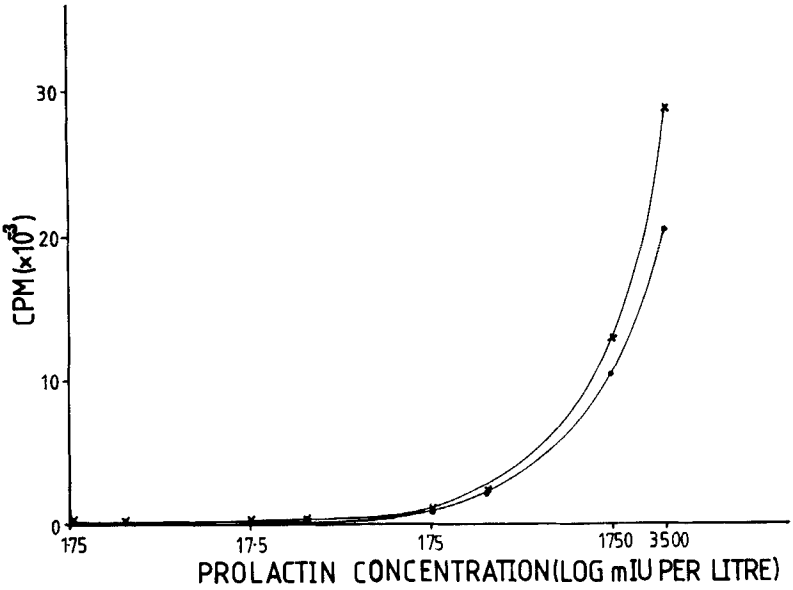


Figure 3: Standard curves for the two-site IRMA using C63 as capturing antibody and C107 as detecting antibody. ✕——✕ represents the standard curve for the two day IRMA and ●——● represents the standard curve for the one day IRMA.

concentrations as low as 17.5 mIU per litre with a useable range between 17.5 - 3500 mIU per litre in both the one day and two day IRMA (Fig. 3.). Similar curves were obtained using C108 as 'detecting' antibody (data not shown).

12 coded sera from patients attending an infertility clinic at the Chelsea Hospital for Women, London were assayed in the two-site IRMA using C63 as 'capturing' antibody and C107 as 'detecting' antibody. Table 2 shows the PRL values measured by liquid phase RIA and the values obtained in the two-site IRMA for both one day and two day incubations. Similarly coded fractions of a gel filtration separated PRL (code number 81/541) were

TABLE 2

Comparison of PRL values (mIU per litre) in serum from patients attending an infertility clinic; using liquid phase RIA and one day and two day IRMAs.

Sample Number	Liquid Phase RIA	IRMA (two day)	IRMA (one day)
O 070	500	380	380
O 075	240	160	120
O 089	420	350	240
O 192	1200	1400	1300
N 412	90	10	20
N 413	230	180	160
N 417	4300	3200	
N 431	190	140	100
N 434	330	300	220
M 495	2200	3100	2400
N 816	680	390	560
N 960	120	40	60

tested in the two-site IRMA and compared with the same fractions assayed by conventional RIA (Fig. 4.). Both IRMA and RIA detected aggregated and dimeric forms of PRL indicating that the IRMA method is no more specific in this respect than RIA.

The inter-assay precision expressed as the coefficient of variation (CV) for 3 independent two day IRMAs ranged from 7% in the linear region of the dose response curve to 28% at the extremes of the dose response curve. In the one day IRMA there was insufficient data to calculate a precision index.

DISCUSSION

A number of anti-PRL monoclonal antibodies have been produced which bind radiolabelled PRL in a liquid phase

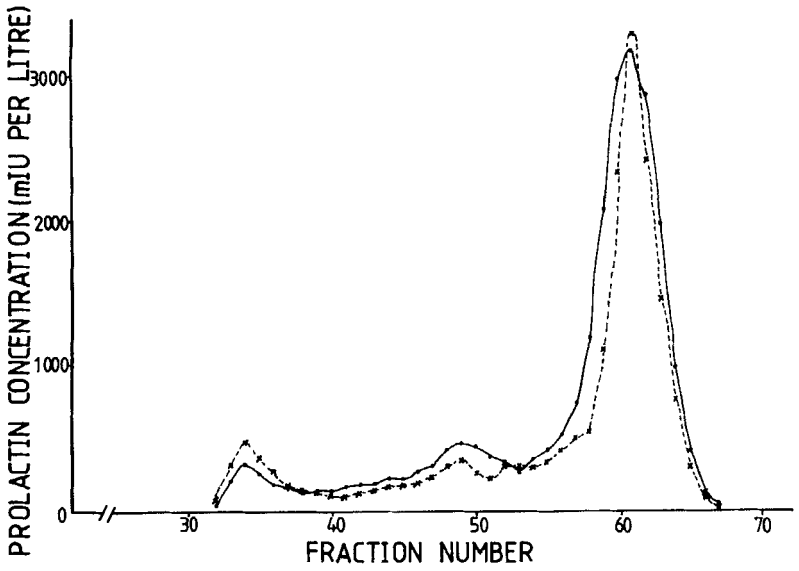


Figure 4: Comparison of liquid phase RIA (●——●) and two-site IRMA (✕------✕) to detect aggregated, dimeric and monomeric PRL fractionated by gel filtration. Aggregated PRL appears in fractions 33-35, dimeric PRL in fractions 47-51 and monomeric PRL in fractions 58-64.

radiobinding assay. Ascitic fluid preparations of these antibodies were then tested in immunoblotting experiments for specificity using PRL, hPL and hGH. Antibodies C63, C65 and C66 failed to recognise PRL using this method possibly as a result of loss of conformational determinants of PRL when subjected to reduction and treatment with SDS (Fig. 1.(a), Table 1.). None of the antibodies tested recognised determinants present on hPL or hGH. Similarly all monoclonal antibodies were tested by immunoblotting using an acid/acetone extract of human pituitary gland. Monoclonal antibodies C63, C65 and C66 failed to recognise proteins present in the pituitary gland extract whereas all other antibodies recognised 2 protein bands of molecular

weights of approximately 40 kD and 22 kD respectively (Fig. 1(b)). Monoclonal antibodies C63, C107 and C108 were also assessed using a liquid phase RIA. There was no observable crossreactivity with hPL or hGH by liquid phase RIA thus confirming the immunoblotting data.

Five monoclonal antibodies were used in an immunocytochemical method to investigate the presence of PRL in sections of human pituitary gland. All antibodies tested were positive in these experiments.

Three different techniques have been used to characterise a number of anti-PRL monoclonal antibodies which have established their specificity and indicated that in some cases the monoclonal antibodies are recognising different epitopes on the PRL molecule (C63 and C107 for example). Pairs of monoclonal antibodies such as these are well suited to the development of a two-site IRMA.

In these experiments only FPLC purified monoclonal antibodies C107 and C108 could be used as 'detection' antibodies with C63 as 'capturing' antibody. The reason why C63 could not detect when using C107 or C108 to capture is probably because radioiodination obscured or altered the binding site of this antibody. A two-site IRMA was set up that could detect PRL down to 17.5 mIU per litre and that was readily adapted to screening sera from patients attending a gynaecological clinic. The assay could be performed in one day without a significant loss of sensitivity for the detection of PRL. The two-site IRMA detected both aggregated and dimeric forms of PRL fractionated by gel filtration from an ampouled PRL sample as did conventional RIA. This shows that the IRMA technique is no more specific than RIA in this respect.

We have described the production and characterisation of anti-PRL monoclonal antibodies and the development of a sensitive two-site IRMA which can be used to screen clinical samples with an accuracy equivalent to conventional RIA procedure. RIAs generally require overnight incubations and a separation stage both of which are avoided using the IRMA technique. The assay range (17.5 - 3500 mIU per litre) obtained for the IRMA in these experiments enables the direct measurement of samples over a wide range of PRL concentrations without any need to alter the parameters of the assay.

The two-site IRMA described possesses adequate sensitivity for the quantitation of PRL and is easily adapted for use as a rapid clinical assay. It also has the potential to be used to establish a non-isotopic immunoassay.

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Footnotes

Abbreviations Used: BSA, Bovine Serum Albumin; CV, Coefficient of Variation; FPLC, Fast Protein Liquid Chromatography; Hb-PBS, 3% bovine haemoglobin in PBS; hGH, Human Growth Hormone; hPL, Human Placental Lactogen; kD, kilodaltons; PBS, Phosphate Buffered Saline; PEG, Polyethelene Glycol; PRL, Prolactin; RIA, Radioimmunoassay; SDS-PAGE, Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis; TBS, Tris Buffered Saline.

REFERENCES

1. Köhler, G. & Milstein, C. Continuous cultures of fused cells secreting antibody of predefined specificity. Nature, 1975; 256: 495-497.

2. Cooke, N.E., Coit, D., Shine, J., Baxter, J.D. & Martial, J.A. Human prolactin cDNA structural analysis and evolutionary comparisons. Journal of Biological Chemistry, 1981; 256: 4007-4106.

3. Suh, H.K. & Franz, A.G. Size heterogeneity of human prolactin in plasma and pituitary extracts. Journal of Clinical Endocrinology and Metabolism, 1974; 39: 928-935.

4. Sinha, Y.N., Gilligan, T.A. & Lee, D.W. Detection of a high molecular weight variant of prolactin in human plasma by a combination of electrophoretic and immunologic techniques. Journal of Clinical Endocrinology and Metabolism, 1984; 58: 752-754.

5. Galfrè, G., Howe, S.C., Milstein, C., Butcher, G.W. & Howard, J.C. Antibodies to major histocompatibility antigens produced by hybrid cell lines. Nature, 1977; 266: 550 - 552.

6. Desbuquois, B. & Aurbach, G.D. Use of polyethylene glycol to separate free and antibody bound peptide hormones in radioimmunoassay. Journal of Clinical Endocrinology and Metabolism, 1971; 33: 732-738.

7. Clezardin, P., McGregor, J.L., Manach, M., Boukerche, H. & Dechevenne, M. One step procedure for the rapid isolation of mouse monoclonal antibodies and their antigen binding fragments by fast protein liquid chromatography on a mono-Q anion-exchange column. Journal of Chromatography, 1985; 319: 67-77.

8. Johnstone, A. & Thorpe, R. Immunochemistry in Practice (2nd edition). Blackwell Scientific Publications, Oxford, 1987.

9. Laemlli, U.K. & Favre, M.F. Maturation of the head of bacteriophage T4. Part 1 - DNA packaging. Journal of Molecular Biology, 1973; 80: 575-599.

10. Towbin, H., Staehelin, T. & Gordon, J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proceedings of the National Academy of Sciences. U.S.A., 1976; 76: 4350-4354.

11. Laskey, R.A. & Mills, A.D. Enhanced autoradiographic detection of ^{32}P and ^{125}I using intensifying screens and hypersensitized film. FEBS Letters, 1977; 82: 314-320.

12. Austen, B.M., Lorenz, R., Spitz, M. & Thorpe, R. Elucidation of the antigenic determinant in lipotropin for two monoclonal antibodies. Biochemical and Biophysical Research Communications, 1983; 116: 527-533.