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GLYCOSYLATED HUMAN PROLACTIN: DETECTION BY IMMUNOBLOTTING OF BIOLOGICAL FLUIDS IS CONFOUNDED BY ANTISERA CROSSREACTIVITY WITH IMMUNOGLOBULINS

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Glycosylated human prolactin is found in several biological fluids and can only be detected by western immunoblotting. Glycosylated prolactin migrates at 25,000-27,000 molecular weight while prolactin migrates at 23,000. We have discovered that several anti-human prolactin sera crossreact with some light chain components of human immunoglobulins, which also migrate at 25,000 on electrophoretic gels run under reducing conditions. Evidence is provided for prolactin binding to immunoglobulins in biological fluids, which may explain why some polyclonal anti-prolactin sera demonstrate this crossreactivity.

Glycosylated human prolactin (G-hPRL) was first identified in pituitary extracts and later in serum, amniotic fluid, and media from cultures of late luteal phase endometrium and term decidual tissue (1-6). G-hPRL is identical in primary amino acid sequence to human prolactin (hPRL), but carries an additional carbohydrate unit at asparagine[31] where there is a consensus sequence for N-glycosylation (1). Because of the great similarity between hPRL and hG-PRL, most of the available anti-PRL antisera cannot distinguish between the two forms when used in an immunoassay (3). Therefore, almost all of the studies identifying G-hPRL and hPRL as independent species have relied on immunoblot analysis after polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate (SDS-PAGE). This detection method exploits the molecular weight difference between the variants: hPRL migrates with an apparent molecular weight of 23,000 and G-hPRL migrates with an apparent molecular weight of 25,000-27,000.

We reported previously that some G-hPRL immunoreactivity in amniotic fluid copurified with IgG (7). Subsequent experiments have shown that some, but not all, immunoreactivity

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attributed to G-hPRL in biological fluids results from hPRL antisera crossreactivity with human light chains. Some hPRL in both amniotic fluid and serum is bound to immunoglobulin, which may explain why some hPRL antisera crossreact with light chains.

MATERIALS AND METHODS

Sample preparation

All samples were obtained following protocols approved by the Human Research Committee of Brigham and Women's Hospital and the Institutional Review Boards of Children's Hospital Medical Center and the University of Cincinnati School of Medicine.

Amniotic fluid specimens were centrifuged at 11,000 x G to remove cellular debris, filtered through cheesecloth, Whatman #4 filter paper, a 5μ glass filter, and then sequentially through 1.2μ and 0.22μ nylon filters. Filtered amniotic fluid was concentrated to five times original volume and equilibrated with phosphate buffered saline (PBS; pH=7.0) using ultrafiltration across a 10K exclusion membrane (10K Omega, Filtron Corp., Northboro, MA).

Serum was separated by centrifugation at 11,000 x G from formed clot after a four hour incubation at 4C. Where indicated, serum was immunoprecipitated with 1:1000 VLS-2 anti-hPRL serum (kindly supplied by Dr. Y.N. Sinha, The Whittier Institute, LaJolla, CA) which precipitates 80-95% of the hPRL detectable by RIA and reacts with both the glycosylated and cleaved forms of hPRL.

Protein electrophoresis and immunoblotting

Samples were dissolved in sample buffer containing 2% β-mercaptoethanol and electrophoresed, using the discontinuous buffer system of Laemmli, on 12.5% polyacrylamide gels containing SDS (SDS-PAGE) (8). The samples were electrotransferred to PVDF membranes (9) and probed overnight at room temperature with one of the primary antisera described below.

Membranes probed with rabbit anti-hPRL serum (Chemicon AB960 at 1:1750; VLS-2 at 1:1000 or IC-4 at 1:1000) were layered with affinity purified peroxidase conjugated donkey anti-rabbit serum (1:12,500; Chemicon) or [^{125}I]protein A. IC-4 anti-hPRL was generously supplied by the National Hormone and Pituitary Program of the NIDDK. Membranes probed with goat anti-kappa chain serum (1:1000; Chemicon) or goat anti-lambda chain serum (1:1000; Chemicon) were layered with affinity purified peroxidase donkey anti-goat serum (1:1750; Chemicon). Membranes probed with peroxidase conjugated second antibody were treated with Amersham ECL substrate for chemiluminescence detection. Radiograms were made from the [^{125}I]protein A probed membranes using Kodak X-OMAT film.

Imunoglobulin isolation by HPLC

Immunoglobulin G (IgG) was isolated from amniotic fluid and serum samples using Protein G affinity high performance liquid chromatography on a Genex Gammabind G-HPLC column (Genex Corp., Gaithersburg, MD). Following equilibration with PBS, the column was loaded with either amniotic fluid or serum. Unbound proteins were washed through the column. The bound proteins were eluted with 0.5M ammonium acetate buffer, pH 3.0, and collected into tubes containing 1M Tris buffer, pH 10. Eluted IgG was dialyzed overnight against PBS.

Where indicated, IgG containing samples were further separated into fractions based on small charge differences. Samples were equilibrated overnight in 20 mM 2-(N-morpholino) ethane sulfonic acid (MES) buffer, pH 5.2, and loaded on a Baker Versa-ten analytical mixed ion exchange HPLC column equilibrated with MES buffer. The column was then eluted with a stepped gradient of 25 mM $(NH_4)_2SO_4$, pH 6.86.

Immunoabsorption of PRL

Polystyrene beads coated with a monoclonal anti-hPRL antibody (Hybritech) were incubated with various combinations of hPRL and IgG for 3 hours at 37C. Unbound material was aspirated and the beads washed with PBS. Beads were then treated with 0.1M acetic acid,

pH 3.0, to remove bound proteins. Beads were removed from the acetic acid and the supernatant dried under nitrogen. Pellets were resuspended in Laemmli sample buffer, electrophoresed using SDS-PAGE under reducing conditions as above and electrotransferred to PVDF membranes. Blots were immunostained with either anti-hPRL or anti-hIgG sera and a peroxidase conjugated second antibody.

Binding of hPRL to IgG

Protein-G affinity chromatography was used to isolate IgG from serum. Serum was passed through a Protein-G Sepharose column and the bound IgG was removed with 1M glycine, pH 2.6. Samples were immediately neutralized with 1M Tris, pH 10. Eluted material was dialyzed, lyophilized and resuspended in PBS. To increase specific radioactive hPRL binding to IgG, IgG was incubated in ice cold 4M $MgCl_2$ to remove endogenous hPRL. The mixture was desalted over Sepahdex G-100 and the IgG peak was collected. [^{125}I]hPRL (100,000 cpm) was added to the recovered IgG and incubated overnight at room temperature. An additional tube with [^{125}I]hPRL and buffer was set up as a control to account for nonspecific aggregation. The samples were chromatographed over Sepahdex G-100.

To determine nonspecific binding of proteins to IgG, [^{125}I]insulin was incubated overnight at room temperature with either purified human IgG (1 mg/ml; Sigma) in 0.1M PBS (pH 7.4) or in PBS alone. The incubated samples were then chromatographed over Sephadex G-100.

RESULTS

Prolactin immunoreactivity at 23,000 (23K) and 25,000-27,000 (25-27K) molecular weight is readily demonstrated in biological fluids by Western blot analysis. Figure 1 shows the PRL variants seen after immunoblotting human serum and amniotic fluid. Two bands, one at 23K and one at 25K, are specifically stained in samples of both serum (Fig. 1A) and amniotic fluid (Fig. 1B) but are not visible in the absence of the first antibody (serum control shown in Fig. 1C). The higher molecular weight bands seen nonspecifically stained in all three panels are present on blots prepared both with and without specific hPRL antiserum; they appear to be at different molecular weight positions on these gels because each was run for a different length

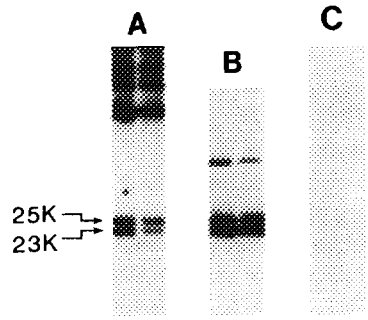


Figure 1. Western blot analyses demonstrating immunopositive hPRL at 23K and G-hPRL at 25K in human serum (**A**) and amniotic fluid (**B**). VLS-2 antiserum was used both to immunoprecipitate serum prior to electrophoresis and as the first antibody in the blot prepared from serum. Amniotic fluid was electrophoresed without immunoprecipitation and NIH IC-4 was used as the first antibody. Panel **C** is serum immunoprecipitated with VLS-2 antiserum and immunoblotted with nonimmune rabbit serum.

of time. Inclusion of intact IgG and IgG heavy chains as standards show that these higher molecular weight bands result from [^{125}I]protein A binding to the heavy chain of the immunoglobulins used to immunoprecipitate the hPRL in serum and to the immunoglobulins present in the amniotic fluid loaded directly on the gels (data not shown).

Figure 2 is a composite of Western blots of gels loaded with IgG and PRL standards and immunoblotted with either an anti-human IgG serum or one of three anti-hPRL sera. Nonimmune serum was used as a control and all 23-27K immunostaining is specific for the immune serum used. As may be seen in the middle panels, both the VLS-2 and IC-4 anti-hPRL serum show significant crossreactivity with kappa and lambda light chains. The third anti-hPRL serum used, Chemicon AB960, did not show significant staining of light chains.

Evidence for why polyclonal hPRL antisera might cross-react with light chains, an antigenically diverse group of proteins structurally unrelated to prolactin, is provided by the data in Figures 3 and 4. Figure 3 is an immunoblot demonstrating that IgG isolated from amniotic fluid has hPRL bound to it. A Western blot prepared with the Chemicon hPRL antiserum demonstrates specific hPRL immunostaining at the positions of both the hPRL and G-oPRL markers (lane 3). Kappa and lambda chains, which stain very faintly with the Chemicon AB960 antiserum, can actually be seen to occupy a position on the gel between the two hPRL bands. Calculation of molecular weights for the proteins on this gel gives apparent molecular weights

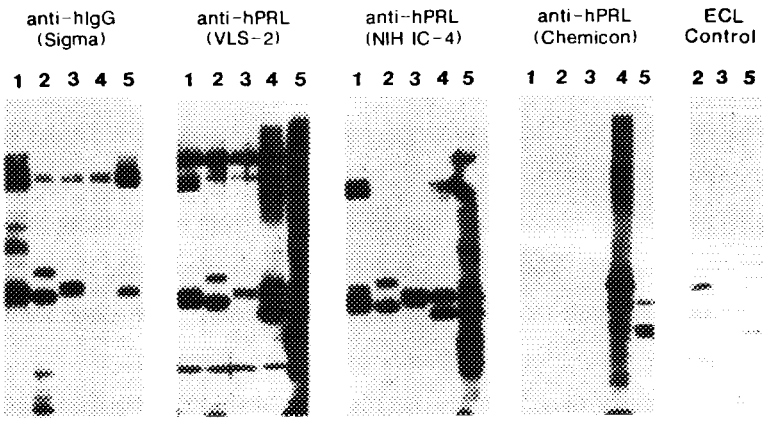


Figure 2. Immunoblots of hPRL, G-oPRL and IgG components prepared with various immune sera. Protein bands were visualized by staining with affinity purified goat anti-rabbit peroxidase conjugate and the image developed using the Amersham ECL kit.
For each gel, proteins were loaded in the following quantities:
1 = 1 μg purified human IgG (Sigma)
2 = 1 μg purified human kappa chain (ICN Immunobiologicals)
3 = 1 μg purified human lambda chain (Chemicon)
4 = 50 ng human PRL (NIH AFP9900) and 100 ng glycosylated ovine PRL (Lewis 600-5-1)
5 = 1 μl human pituitary extract (82 mg/ml protein; 13 μg/ml PRL by RIA).

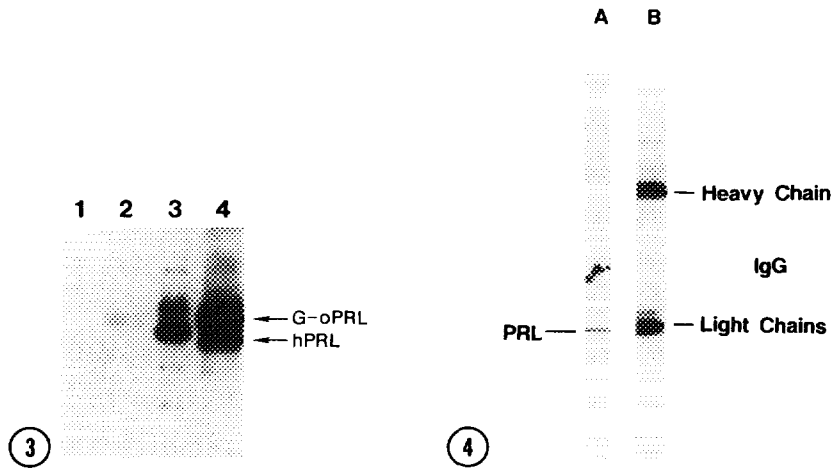


Figure 3. Immunoblot demonstrating the presence of hPRL immunoreactivity in fractions of affinity purified IgG. IgG was isolated from term amniotic fluid using protein G affinity HPLC. hPRL was detected using Chemicon AB960 anti-hPRL serum which does not cross-react with human IgG components (see Figure 2). Protein bands were visualized by staining with affinity purified goat anti-rabbit peroxidase conjugate and the image developed using the Amersham ECL kit.

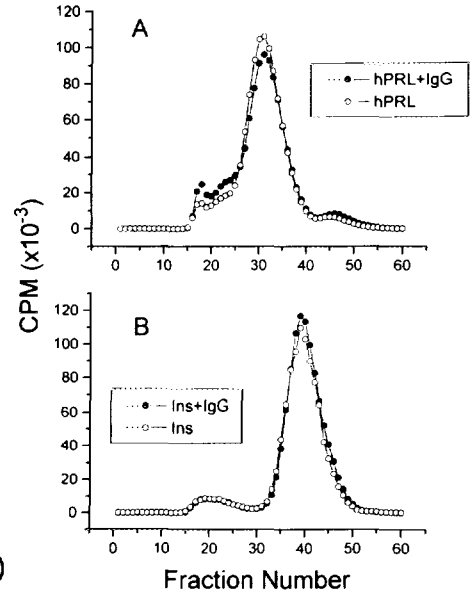
1 = 1 μg purified human IgG (Sigma)
2 = 1μg purified human kappa chain (ICN Immunobiologicals) + 1 μg purified human lambda chain (Chemicon)
3 = 120 μg affinity purified IgG from human amniotic fluid
4 = 50 ng human PRL (NIH AFP9900) + 100 ng glycosylated ovine PRL (Lewis 600-5-1).

Figure 4. Immunoblot of serum after immunoabsorption with anti-hPRL antibody coated beads. Panel **A** was immunostained with anti-hPRL serum. Panel **B** was immunostained with anti-human IgG serum.

close to 25K for the light chains and 23K and 27-28K for the hPRL bands. IgG isolated from human serum using protein G affinity chromatography also demonstrates similar specific hPRL immunostaining (data not shown).

Figure 4 is a Western blot of PRL immunoabsorbed from serum using monoclonal anti-hPRL antibody coated beads. The immunoabsorbed PRL shown in lane A was identified by western blot blot analysis with the Chemicon anti-hPRL serum. The same immunoabsorbed material is also positive when probed with anti-hIgG serum (Panel B).

To further investigate the possibility that hIgG can bind hPRL, purified hIgG was treated with 4M $MgCl_2$ to displace any endogenous PRL, incubated with [^{125}I]hPRL, and chromatographed over Sephadex G-100. Figure 5A demonstrates that incubation with IgG was able to increase the quantity of [^{125}I]hPRL in the higher molecular weight fractions compared to the monomeric hPRL fractions when compared to incubation of [^{125}I]hPRL with buffer alone. The specificity of the interaction of hPRL and hIgG is demonstrated with [^{125}I]insulin, which does not show this size shift after incubation with IgG (Figure 5B).



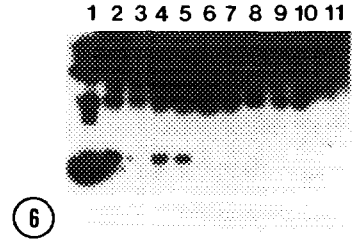


Figure 5. **A**. *Sephadex G-100 profile of* ^{125}I-hPRL *after binding to purified IgG.* Purified human IgG was treated with 4M $MgCl_2$, incubated with ^{125}I-hPRL and chromatographed over G-100. ^{25}I-hPRL incubated in buffer alone was used as the control. **B**. Sepahdex G-100 profile of ^{125}I-insulin after binding to purified IgG. Insulin was used as a control protein to determine the extent of nonspecific protein interaction with IgG.

Figure 6. Immunoblot of IgG fractions separated by weak cation exchange HPLC following affinity purification. Equal amounts of IgG were loaded in each lane. Fractions were immunostained with IC-4 anti-hPRL and antibody binding detected with ^{125}I-protein A.
oPRL+IgG = 1 μg ovine PRL (Sigma) + 2 μg human IgG (Sigma)
1 = end of peak eluted with 5 mM $(NH_4)_2SO_4$
2-5 = peak eluted with 7.5 mM $(NH_4)_2SO_4$
6-9 = peak eluted with 10 mM $(NH_4)_2SO_4$
10 = pooled peak eluted with 12.5 mM $(NH_4)_2SO4$.

Figure 6 is an immunoblot of hIgG fractions which demonstrates that only a small subset of light chain molecules cross-react with hPRL antiserum. IgG was isolated from amniotic fluid and then rechromatographed using very weak cation exchange HPLC to separate the IgGs based on small differences in charge. Following immunoblotting with IC-4 hPRL antiserum, marked differences are seen in the 25K immunoreactivity of consecutive IgG fractions.

DISCUSSION

Our results show that polyclonal anti-hPRL sera from widely divergent sources show crossreactivity to human immunoglobulin light chains in addition to the expected interaction with hPRL. That such an unusual crossreactivity went undetected for so long stems from the fact that both kappa and lambda light chains have such similar molecular weights to G-hPRL that they

co-migrate with G-hPRL in SDS-PAGE gels. The origin of this cross-reactivity between light chain and PRL may be the result of any of a number of processes. First, it is possible that spontaneous anti-idiotypic antibodies to the PRL receptor are present in the circulation. The variable regions of these anti-idotypic antibodies could bear sufficient similarity to the native PRL molecule that specific antibodies to PRL fail to distinguish between the anti-receptor antibody and the native protein. Anti-idiotypic antibodies to the human growth hormone receptor have been detected and reported to interfere with the RIA of growth hormone in treated acromegalics (10). Second, it may be that a small subset of IgG molecules act as a PRL binding protein in biological fluids such as serum and amniotic fluid. Depending upon the source of the antigen used for antibody production, immunoglobulin components could have contaminated the seemingly pure hPRL. Finally, there simply may be sufficient similarity in the tertiary or quartenary structure of a subset of immunoglobulins and hPRL that some antibodies in polyclonal antiserum cannot distinguish betwen the hPRL and some light chains.

The major impact of the crossreactivity between light chains and hPRL on previously published G-hPRL data is to make the results quantitatively inaccurate. G-hPRL does exist, along with PRL, in the biological fluids we have retested. It will be important in future studies involving PRL antisera to test for crossreactivity and to use only antisera without light chain crossreactivity for studies in which interference can give misleading results. Testing is crucial for antisera to be used for both western blots and immunohistochemistry, techniques in which the antisera dilutions are much lower than those used in radioimmunoassay (RIA). We have tested the crossreactivity of both kappa and lambda chains in a standard RIA using CII580 and no interference is detectable (data not shown). Interestingly, autoantibodies to hPRL occurring in the serum of some patients with hyperprolactinemic symptoms do interfere with accurate RIA measurements (11).

Our results also indicate that a fraction of naturally occurring PRL in normal individuals is bound to IgG. It has previously been shown that hPRL associates with immunoglobulins in certain pathological conditions. A fraction of the "big-big" hPRL circulating in patients with hyperprolactinemia appears to be hPRL-IgG complexes (12,13). Schizophrenic patients on neuroleptic medication have a fraction of circulating hPRL bound to IgG heavy chain (14). Our present study demonstrates that hPRL-IgG complexes are also a normal component of hPRL heterogeneity in both serum and amniotic fluid.

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