

Mouse Monoclonal Antibodies Against Ovine Prolactin

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Hybridoma producing antibodies against ovine prolactin have been obtained by fusion of spleen lymphocytes of Balb/c mice immunized with ovine prolactin and SP2/O Ag 14 mouse myeloma cells. Anti-Prolactin IgG producing cultures were identified in a solid phase ELISA using alkaline phosphatase conjugated sheep anti-mouse IgG. Cultures producing specific antibodies were subcloned and expanded. The produced immunoglobulines were isolated by ammonium-sulfate precipitation and chromatography on protein-A-Sepharose and characterized by isoelectric point and interaction with other pituitary hormones including prolactin from other species. All 32 anti-prolactin IgG producing cultures cross-reacted strongly with bovine and weakly with human prolactin but not with mouse prolactin, human LH, human TSH, human CG, human GH or bovine thyrotropin. Despite the weak cross-reactivity it is expected that monoclonal antibodies against ovine prolactin can be used as markers for different subregions of human prolactin.

During the last decade an enormous body of information has been gained about prolactin. As in the case of other pituitary hormones, prolactin was first defined by its biological properties such as mammatrophic and lactotropic effects in mammals.¹ Isolation and purification of human pituitary prolactin was achieved by several investigators.^{2,3} It is a single-chain polypeptide, with a relative molecular mass of about 22000 and an isoelectric point of 6.5 (for review, see Ref. 4). Despite much work many questions concerning especially the biological action and the role of prolactin in pathological conditions remain to be answered. Serum levels are routinely measured using different immunological methods, (for re-

view, see Ref. 5). The occurrence of prolactin of apparently larger molecular size was already described in 1974.⁶ Despite the fact that the different forms can be separated due to different Stoke's radii and isoelectric points,^{7,8} no routine method has been developed by which the biological role and clinical use of these different forms of human prolactin can be evaluated. The hybridoma technique offers a possibility to obtain specific monoclonal antibodies which might serve as markers for different parts of the molecules. By using such specific markers it might be possible to develop assays specific for the different isohormones of human prolactin. This paper describes the raising of monoclonal antibodies against prolactin. As it was impossible to obtain enough quantities of human prolactin we tried to raise monoclonal antibodies against ovine prolactin as there is a remarkable homology, about 92 %, between human and ovine prolactins⁹ and as there has been reported cross-reactivity in radioimmuno assays¹⁰ and immunochemical staining.¹¹ The obtained monoclonal antibodies were then tested against human prolactin.

MATERIALS AND METHODS

Eight-twelve week old Balb/c mice (Anticimex, Stockholm) were immunized with 40 µg of ovine prolactin (NIASMDD-OPRL-14, Pituitary Hormones and Antisera Ctr. Ca USA) in Freund's complete adjuvant (Gibco, Scotland). Half of the volume was given intraperitoneally and half subcutaneously. A boost of 40 µg of prolactin in Freund's complete adjuvant was given twice 4 and 8 weeks later and following positive testbleeding 3 weeks later another boost

of 100 μg of prolactin in FCA was given on each of the three days preceding fusion.¹²

One day preceding fusion, peritoneal macrophages were removed aseptically and after being harvested by centrifugation resuspended in DMEM (Gibco) supplemented with HAT and distributed into 96 wells microtiter plates giving a concentration of at least 10^4 cells per well.

On day of fusion animals were sacrificed, the spleen aseptically removed and cells separated from stroma. Fusion of spleen cells with SP2/O cells was performed as follows.¹³ About 5×10^7 cells were mixed with 5×10^7 myeloma cells and following centrifugation at $1000 \times g$ the pellet was resuspended in 1 ml of PEG 4000 (50 % w/v in serum free DMEM). After an incubation for 1 min at 37 °C, 10 ml of serum free DMEM was slowly added and the cell suspension was further incubated at 20 °C for 5 min. The cells were then harvested by centrifugation, resuspended in DMEM supplemented with HAT and distributed into 4, 96 wells microtiter plates (0.1 ml). The cells were fed every 2–3 d by changing 0.1 ml of the medium. Plates were scanned for clones on day 5 until 3 weeks following fusions. Culture fluids from wells containing clones were tested for presence of anti-ovine prolactin IgG in a solid phase enzymelinked immunoassay as follows: microtiter plates were coated with ovine prolactin 0.2 μg /well in carbonate buffer (pH 9.6) and after several washes in PBS-Tween buffer (pH 7.2), 100 μl of culture media were applied into each well. Following incubation for 90 min and a thorough wash with PBS-Tween buffer, rabbit anti mouse IgG conjugated with alkaline phosphatase (Sigma) was added and the bound conjugate was, after washing, identified on the basis of the enzymatic reaction.

Culture fluids were usually tested in duplicates using dilutions 1:1, 1:10, 1:100, 1:1000 and 1:10000. Specificity was tested by coating the microtiter plates with 0.2 μg of hormones to be tested as follows: Bovine Prolactin B5 (NIAMDD), human prolactin (NIAMDD), mouse prolactin (NIAMDD), human LH (NIAMDD), human TSH (NIAMDD), human PL (Pharmacia Diagnostics, Uppsala, Sweden), human Cg (Biodata, Rome), human GH (Crescorman, Kabi-Vitrum, Stockholm) and Thyrotropin (NIAMDD). Positive hybrids were cloned by limiting dilutions. Cells were harvested by centrifugation and following counting of an aliquot,

cells were spread at a concentration of 0.3, 1 and 10 cells per well. These wells had been coated with macrophages as described above. Further work was directed to wells from the concentration of cells where less than 20 % of the wells showed culture. Clones selected for further work were expanded to culture flasks and aliquotes were frozen in liquid nitrogen. Withdrawn culture media were stored at -20 °C until further analyzed.

Analyses of immunoglobulins. Twenty ml of culture medium was made 40 % of saturation with respect to ammonium sulfate. Following centrifugation the precipitate was redissolved in 3 ml 0.5 M phosphate-buffer pH 8 and chromatographed on a one ml protein-A-Sepharose column. The column was washed with phosphate buffer until no protein could be detected in the eluate. The immunoglobulin fraction was eluted using 0.1 M acetic acid in 0.15 M NaCl.

Following lyophilization and redissolving an aliquot was analyzed using isoelectric focusing on polyacrylamid plates, pH 3.5–9.1, essentially performed as earlier described.¹⁴ Proteins were visualized using Coomassie blue dye and *pI* was calculated from standard proteins (Pharmacia Fine Chemicals, Uppsala, Sweden).

RESULTS

Following three fusions culture medium from 62 wells showed antiovine prolactin activity but just 6 of these were positive at a dilution of 1:1000. Cells from these wells were cloned by limiting dilutions and 32 cell cultures from cell concentration giving less than 20 % cultures and showing antiovine prolactin activity at dilution of 1:1000 were further cultivated and studied. All these 32 cultures showed interaction with bovine prolactin at a dilution of about 1:1000. However, these immunoglobulin-containing media did interact with human prolactin only when undiluted. No culture media showed any interaction with mouse prolactin, human LH, human TSH, human PL, human Cg, human GH or Thyrotropin. Analyses of immunoglobulins using isoelectric focusing was performed on one culture derived from each of the six primary cultures. These analyses showed one major immunoglobulin band but in 3 media 2 weak additional bands were seen. The isoelectric points were found to

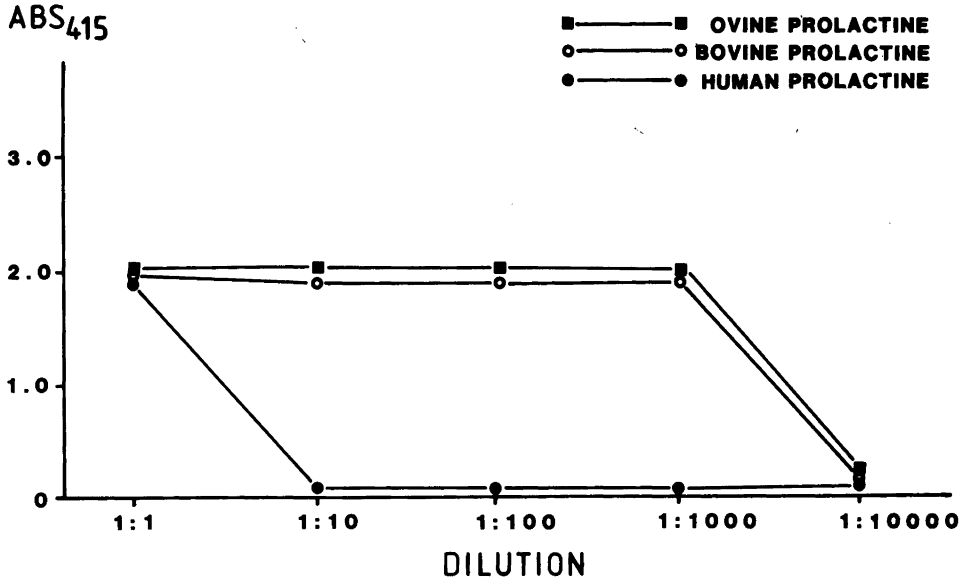


Fig. 1. Analyses of anti-ovine prolactin monoclonal antibodies interaction with prolactin from different species. Ovine, human and bovine prolactin was absorbed to microtiter plates and increasing dilutions of culture media from hybridoma E II F8 were tested. Following analyses the absorbance was recorded using a Titertech instrument. Maximal absorbance was set to 2.0.

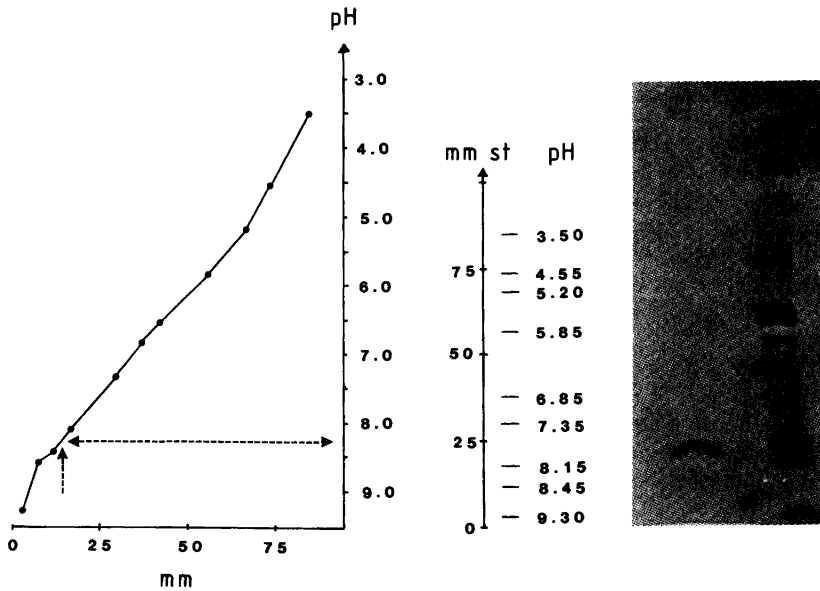


Fig. 2. Analyses of culture media from hybridoma E II F8. Following two days culture without added calf sera, the immunoglobulin fraction was partly purified by ammonium sulfate precipitation and chromatography on Protein-A-Sepharose. The obtained fraction was analyzed by isoelectric focusing using commercially available standard proteins as references. As can be seen from the chart, the obtained monoclonal antibody focused at pI 8.3.

be 8–9. A representative analysis is shown in Fig. 2.

The obtained antibodies were not able to interact and precipitate human ^{125}I -prolactin. No precipitation occurred when second antibodies rabbit anti-mouse IgG was added. (Sac-cel cellulose. The Wellcome Foundation Ltd.).

DISCUSSION

The present study shows that monoclonal antibodies raised against ovine prolactin do interact with human prolactin and hopefully can be used as markers for different subunits of human prolactin. However, probably because of low affinity, the obtained antibodies cross-reacted with human prolactin only when culture media were used undiluted, and therefore these sera should not be used for routine measurement of human prolactin. It is somewhat surprising that all obtained antibodies show the same interaction with human prolactin. As high homology exists between human and ovine prolactin, it was expected to see similar immunological reaction and we had hoped to obtain antibodies that interacted well with human prolactin and could be used for immunological measurements of different subunits of human prolactin. The obtained data can be explained by less homology between human and ovine prolactin than explored by amino acid analyses or by high antigenic activity by one or a few determinants. In the latter case the obtained antibodies should be directed against the same determinant. As no interaction with other hormones was detected, the monoclonal antibodies showed high specificity. The present study has shown that monoclonal antibodies can probably be used as markers for different subunits of prolactin. However, in order to obtain monoclonal antibodies that could be used for measurement of different subunits of human prolactin, it is probably an advantage to use human prolactin as antigen. When reliable assays for quantitation of the different isohormones of prolactin have been established, the biological role of these isohormones can be studied.

Acknowledgements. This work was supported by *Svenska Läkaresällskapet*, the Swedish Medical Research Council and Leo Research Foundation.

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Received December 27, 1983.