

Generation of monoclonal antibody to ovine FSH and its application in immunoneutralization and enzymeimmunoassay

K. M. Henderson, M. Camberis and A. H. M. Hardie

AgResearch, Wallaceville Animal Research Centre, PO Box 40063, Upper Hutt, NZ

Introduction

Although monoclonal antibodies against human FSH are widely available, monoclonal antibodies generated against FSH from non-human species are less common, and presently none against ovine FSH are available. Monoclonal antibodies against different epitopes on FSH are useful in studies of the relationship between FSH structure and activity, for purifying FSH by immunoaffinity chromatography, and for developing homologous, nonradioactive, noncompetitive immunoassays for FSH. The purpose of this study was to produce monoclonal antibodies to ovine(o) FSH.

Materials and Methods

The following reagents were provided by the National Hormone and Pituitary Program (NIADDK, NIAMDD), University of Maryland School of Medicine, MD, USA: NIADDK-oFSH-17, NIADDK-oLH-25, NIH-P-13, NIH-GH-S11, NIH-TSH-S8 and an oFSH radioimmunoassay (RIA) kit which uses NIAMDD-oFSH-I-1 as the tracer for radioiodination, NIAMDD-anti-oFSH-I for the primary antibody and NIAMDD-oFSH-RP-2 for the reference standard. Iodination grade oFSH and the α and β -subunits of oFSH were purchased from Bioscan Continental Inc. (St Eustache, Quebec) and Ovagen oFSH from ICP Ltd, (Auckland). Unless stated otherwise, all other reagents were purchased from the Sigma Chemical Co. (St Louis, MO).

Standard methods were used to produce monoclonal antibodies against oFSH (Campbell, 1984). The immunogens tested were Bioscan oFSH, NIADDK-oFSH-17 and Ovagen oFSH. The host animals were BALB/c mice. The fusion partner was the mouse myeloma cell line NS1. Binding of radioiodinated NIAMDD-oFSH-I-1 in conjunction with second antibody separation was used to monitor production of antibodies to oFSH. Hybridomas secreting antibody to oFSH were cloned twice by limiting dilution, expanded in tissue culture, and injected into BALB/c mice that had been given an i.p. injection of 0.5 ml pristane 10 days before. Ascites fluid containing a high concentration of antibody was collected from the mice 10-14 days after injection of the hybridomas. Cells from the selected FSH-antibody-secreting clone were cryopreserved. A purified IgG fraction of the FSH monoclonal antibody was prepared from ascites fluid by protein G affinity chromatography. The isotype class of the antibody was determined using an Immuno-Type kit (Sigma Chemical Co.). The equilibrium dissociation constant (K_d) of the antibody-Bioscan oFSH complex was determined as described by Friguet *et al.* (1985). This method was also used to determine the specificity of the antibody, relative to Bioscan oFSH. The substances tested for crossreactivity were the Bioscan α - and β -subunits of oFSH, ovine prolactin, oLH, oGH, oTSH and vasopressin.

The ability of the selected FSH monoclonal antibody to neutralize FSH stimulation of cAMP production by granulosa cells *in vitro* was examined using cells obtained from the ovaries of cows slaughtered at a local abattoir. Granulosa cells harvested from large (> 10 mm in diameter), nonatretic follicles were incubated at a density of 50 000 cells in a volume of 1 ml at 37°C with Bioscan oFSH or NIADDK-oLH-25 (0-100 ng ml⁻¹) that had been preincubated for 2 h at room temperature

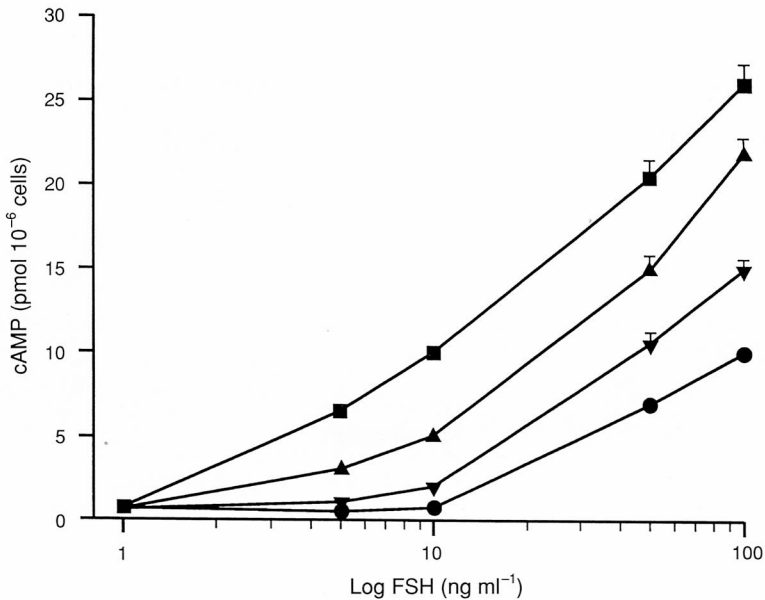


Fig. 1. Immunoneutralization of FSH stimulated cAMP production by bovine granulosa cells *in vitro* with monoclonal antibody against FSH. Granulosa cells were incubated with Bioscan oFSH (1–100 ng ml⁻¹) that had been preincubated with monoclonal antibody against FSH in the form of ascites fluid (■ 0; ▲ 0.4; ▼ 2; ● 10 μl). Values are arithmetic means for $n = 3$. Standard errors are shown by vertical lines when larger than the size of the symbols. The coefficient of variation at each point was < 6%. Mean cAMP production in response to 5–100 ng FSH ml⁻¹ was reduced significantly by 0.4 μl, 2 μl and 10 μl ascites fluid at each concentration of FSH ($P < 0.01$, analysis of variance in conjunction with the Newman–Keul's multiple range test).

with monoclonal antibody against FSH (0–10 μl ascites fluid). The incubation medium was Dulbecco's phosphate-buffered saline containing 0.1% (w/v) BSA, pH 7.2. After 1 h the reaction was stopped by heating the reaction tubes in a water bath at 80°C for 15 min. The concentration of cAMP in the incubation medium was determined by enzymeimmunoassay using cAMP antibody coated microtitre plate wells in conjunction with a cAMP–horseradish peroxidase conjugate, as described by Linden *et al.* (1992). The primary antibody used was a specific rabbit polyclonal anti-cAMP antibody generated at AgResearch (Wallaceville). The intra- and interassay coefficients of variation were < 8.5%.

The oFSH monoclonal antibody was incorporated into a noncompetitive sandwich enzyme-immunoassay (Tijssen, 1985). The oFSH monoclonal antibody served as the capture antibody, and its IgG fraction was coated onto wells of microtitre plates (Maxisorp C12; Nunc, Kamstrup) by overnight incubation at 4°C. The tracer antibody was the polyclonal antibody NIAMDD-anti-oFSH-1. It was added together with oFSH standard (NIAMDD-oFSH-RP-2 or Bioscan oFSH) or sample to capture antibody-coated wells, and incubated overnight at 4°C. The amount of bound tracer antibody was quantitated by incubation with anti-rabbit IgG–horseradish peroxidase conjugate and subsequent reaction with the chromogen o-phenylenediamine. Colour intensity was quantitated by reading the absorbance at 490 nm, and a standard curve was generated by plotting absorbance versus the logarithm of the FSH standard concentration. The FSH concentration of samples was calculated by interpolation. Checkerboard titrations were undertaken to optimize the concentrations of capture and tracer antibodies to achieve the lowest assay sensitivity. The suitability of this enzymeimmunoassay for measuring FSH in biological fluids was assessed by using it to measure FSH concentrations in sheep pituitaries extracted into 5 mmol phosphate l⁻¹ buffer containing 0.1% (w/v) BSA, pH 7.4, in serum samples from intact and

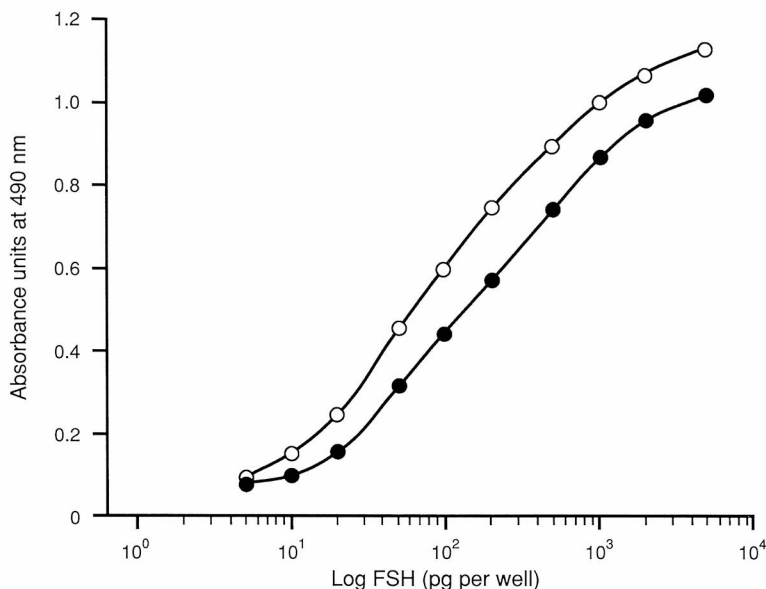


Fig. 2. Semi-logarithmic standard curves for ovine FSH from a non-competitive enzymeimmunoassay. Values are means of quadruplicates. Standard errors were smaller than the size of the symbols. The capture antibody was the monoclonal antibody against ovine FSH used at a coating concentration of 1 µg per well. The tracer antibody was the polyclonal antibody against ovine FSH (NIAMDD-anti-oFSH-1) used at a dilution of 1/5000. (●) Bioscan oFSH; (○) NIAMDD-oFSH-2.

ovariectomized ewes, and in spent culture medium from sheep pituitary cells cultured as described by Henderson *et al.* (1989). Values obtained by the enzymeimmunoassay were compared with those obtained by radioimmunoassay using the oFSH radioimmunoassay kit.

Results

Some mice having high plasma antibody titres ($> 1/1000$) were produced with each of the three oFSH immunogens tested. However, despite several fusion attempts with splenocytes from such mice, only one stable hybridoma secreting a high affinity monoclonal antibody to oFSH could be generated. This resulted from an immunization with Bioscan oFSH. The oFSH monoclonal antibody produced was of the isotype IgG2b, had a dissociation constant (K_d) of 2×10^{-10} mol l^{-1} , and crossreacted 100% with Bioscan oFSH, $> 500\%$ with the β -subunit of oFSH and $< 0.1\%$ with the α -subunit of oFSH, LH (NIADDK-oLH-25), prolactin (NIH-P-13), GH (NIH-GH-S11), TSH (NIH-TSH-S8) and vasopressin. It was concluded that the monoclonal antibody recognized an epitope on the β -subunit of oFSH.

The FSH monoclonal antibody (in the form of ascites fluid) effectively inhibited, in a dose-dependent manner, the stimulatory effect of Bioscan oFSH on cAMP production by bovine granulosa cells *in vitro* (Fig. 1). As little as 0.4 µl ascites fluid significantly reduced the stimulatory effect of 5–100 ng FSH ml^{-1} on cAMP production ($P < 0.01$). In the presence of 10 µl ascites fluid, the stimulatory effect of 10 ng FSH ml^{-1} was completely abolished, and the effect of 100 ng ml^{-1} reduced by 62%. However, the FSH monoclonal antibody, at doses of up to 50 µl ascites fluid, could not inhibit the stimulatory effect of NIADDK-oLH-25 (0.1–100 ng ml^{-1}) on cAMP production by granulosa cells.

The FSH monoclonal antibody, when used as a capture antibody in conjunction with an appropriate tracer antibody (NIAMDD-anti-oFSH-1), enabled a non-competitive sandwich enzymeimmunoassay to

Table 1. FSH concentrations in ovine biological samples as measured by enzymeimmunoassay (EIA) and radioimmunoassay (RIA)

Sample	Concentration of FSH measured by:	
	EIA	RIA
Pituitary extract (10)	18 ± 3 µg g ⁻¹ pituitary	24 ± 3 µg g ⁻¹ pituitary
Ovariectomized ewe serum (6)	13 ± 2 ng ml ⁻¹	16 ± 2 ng ml ⁻¹
Intact ewe serum (14)	1.5 ± 0.2 ng ml ⁻¹	1.6 ± 0.1 ng ml ⁻¹
Pituitary cell culture medium (12)	34 ± 4 ng 10 ⁻⁶ cells	44 ± 5 ng 10 ⁻⁶ cells

Values are means ± SEM of (*n*) samples. Mean values for each sample type did not differ significantly between the EIA and RIA ($P > 0.05$; Wilcoxon signed rank test).

be established. Standard curves using Bioscan oFSH and NIAMDD-oFSH-RP-2 as standards are shown in Fig. 2. The sensitivity of the enzymeimmunoassay, defined as the concentration of FSH producing an absorbance two standard deviations above that of the zero standard, was 10 pg per well and 17 pg per well for the RP-2 and Bioscan FSH standards, respectively. These values are comparable to those obtained by radioimmunoassay using the polyclonal antibody alone. Values obtained for the FSH concentrations measured in several different biological samples using the enzymeimmunoassay were similar to those obtained by RIA (Table 1).

Conclusion

This is the first report of the generation of a monoclonal antibody to ovine FSH. Although it had been hoped to generate several monoclonal antibodies recognizing different oFSH epitopes, only one antibody, which recognized an epitope on the β -subunit, could be generated. A similar difficulty was encountered by Miller *et al.* (1987), who could generate only one distinctive monoclonal antibody to bovine FSH, which also recognized a β -subunit epitope. The ovine FSH monoclonal antibody effectively neutralized the action of FSH *in vitro*. This antibody may therefore be useful for *in vivo* immunoneutralization studies of the biological actions of oFSH, particularly as large quantities of monoclonal antibody can be generated relatively easily. Alternatively, co-administration of the antibody together with exogenous oFSH may enhance the activity of FSH *in vivo*, as has been shown for complexes of bovine FSH and other hormones with their respective monoclonal antibodies (Aston *et al.*, 1989; Glencross *et al.*, 1993). This might then provide a novel means of reducing the number of repeat injections required for superovulating farm animals with FSH.

A sensitive noncompetitive enzymeimmunoassay for oFSH was established using the FSH monoclonal antibody in conjunction with an appropriate polyclonal antibody to oFSH. This assay provides a nonradioactive alternative to radioimmunoassay and has proved useful for measuring FSH concentrations in tissue culture media and pituitary extracts, and for monitoring FSH during purification. The assay is currently being evaluated for monitoring plasma FSH concentrations in intact ewes. Finally, the oFSH monoclonal antibody may have a potential use in simplifying oFSH purification through immunoaffinity chromatography in a manner similar to that described for purifying bovine FSH by Miller *et al.* (1987).

References

- Aston R, Cowden WB and Ada GL (1989) Antibody-mediated enhancement of hormone activity *Molecular Immunology* **26** 435–446
- Campbell AM (1984) Monoclonal antibody technology. In *Laboratory Techniques in Biochemistry and Molecular Biology* vol 13 pp 127–165 Eds RH Burdon and PH van Knippenberg. Elsevier, Amsterdam
- Friguet BF, Chaffotte AF, Djavadi-Ohaniance L and Goldberg ME (1985) Measurements of the true affinity constant in solution of antigen–antibody complexes by enzyme-linked

- immunosorbent assay *Journal of Immunological Methods* **77** 305–319
- Glencross RG, Lovell RD and Holder AT** (1993) Monoclonal antibody enhancement of FSH-induced uterine growth in Snell dwarf mice *Journal of Endocrinology* **136** R5–R7
- Henderson KM, Ellen RL, Savage LC and McNatty KP** (1989) Studies of the effectiveness of gonadotrophin-releasing hormone, steroids and follicular fluid in modulating ovine gonadotrophin output *in vivo* and *in vitro* *Journal of Reproduction and Fertility* **86** 105–117
- Linden J, Vandenhoff GE, Taylor D and Finkelstein GL** (1992) Solid phase enzyme immunoassay of cyclic adenosine 3',5'-monophosphate *Journal of Immunological Methods* **151** 209–216
- Miller KF, Goldsby RA and Bolt DJ** (1987) Immunoaffinity chromatography of bovine FSH using monoclonal antibodies *Journal of Endocrinology* **115** 283–288
- Tijssen P** (1985) Practice and theory of enzyme immunoassay. In *Laboratory Techniques in Biochemistry and Molecular Biology* vol 15 pp 329–384 Eds RH Burdon and PH van Knippenberg. Elsevier, Amsterdam