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Radioimmunoassay for PMSG and its application to in-vivo studies

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Summary. A double-antibody radioimmunoassay for PMSG, especially for measuring PMSG in cattle blood after exogenous application, has been developed. A rabbit antiserum against PMSG and pure PMSG for radioiodination were used. There was a strong cross-reaction against equine LH and FSH, but the slight cross-reaction against bovine LH and FSH could be eliminated by adding bovine LH to each tube during the assay. Unspecific, interfering influences of equine or cow serum could be eliminated by adding a constant amount of PMSG-free serum to each tube. PMSG added to 200 µl of serum could be recovered by this method with a mean of 90·5 ± 9·9%. Inhibition curves obtained with pregnant mare serum or cow serum after administration of PMSG were parallel to those obtained with the PMSG standard preparation. The intra-assay coefficient of variation (CV) was 6·9%. The inter-assay CV was 12·6%. Sensitivity of the assay was 1 mi.u. PMSG/tube. Values of PMSG measured in the serum of pregnant mares by this assay were comparable with those obtained by a bioassay on the same samples. PMSG was still measurable in blood serum about 10 days after injection of 1500–3000 i.u. PMSG. After infusion of 12 000 i.u. PMSG for 3 h (2 heifers), the half-life of PMSG was found to have two components, one of 51 or 40 h and a slower one of 123 or 118 h.

Introduction

The glycoprotein, PMSG, is secreted by the endometrial cups (Clegg, Boda & Cole, 1954), local endometrial outgrowths which develop from an invasion of specialized trophoblast cells into the maternal endometrium on Day 36 of gestation (Allen, Hamilton & Moor, 1973), and is present in the serum of mares between Days 40 and 130 of pregnancy (Cole & Hart, 1930). PMSG is a single molecule possessing both follicle-stimulating hormone (FSH) and luteinizing hormone (LH) activities (see review by Papkoff, 1978). Since PMSG is commercially available in large quantities and at reasonable cost, it is widely used in veterinary therapy and for stimulation of ovulation and/or superovulation in cattle and other animals. Due to its high concentration in the serum of pregnant mares, a bioassay (Cole & Erway, 1941) or haemagglutination inhibition assay (Wide & Wide, 1963; Allen, 1969a) can be used for measuring PMSG in mares and such assays are efficient for pregnancy diagnosis. For monitoring PMSG levels in the blood of other species after administration of PMSG, a more sensitive and efficient method is necessary. For this reason, a radioimmunoassay for PMSG has been developed and is described in the present paper. Some of the data have been used in a previous paper (Schams *et al.*, 1978).

Materials and Methods

Hormone preparations

A crude preparation of PMSG (Anteron: Schering), containing 1000 i.u./mg, was used for immunization. The highly purified preparation, PMSG-O, described by Schams & Papkoff

(1972) and containing 10 000 i.u./mg was used for labelling and standard preparation. Cross-reaction experiments were carried out with the following pituitary preparations: pure equine (e) LH and FSH; bovine (b) LH–DSA (1·0 × NIH-LH-S1, prepared in our laboratory); ovine (o) LH (NIH-LH-S13, 0·93 × NIH-LH-S1); porcine (p) LH; human (h) LH (3·6 × NIH-LH-S1); ovine FSH I, II (31 × NIH-FSH-S1, prepared in our laboratory); bovine FSH (NIH-FSH-B1, 0·49 × NIH-FSH-S1); ovine LH subunits α and β; bovine thyroid-stimulating hormone (TSH); pure human chorionic gonadotrophin, hCG (13 000 i.u./mg: Serono); and bovine prolactin (NIH-P-B3).

Antisera to PMSG

For immunization, rabbits received, at 3-week intervals, 4 injections (s.c. and i.m.) each of 1000 i.u. PMSG dissolved in saline (0·154 M-NaCl) and suspended in Freund's complete adjuvant. The animals were bled after two additional booster injections of 1000 i.u. PMSG at 4-week intervals.

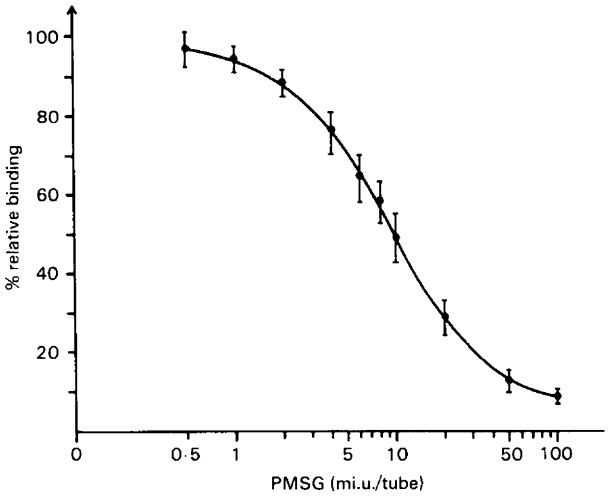
Radioiodination of PMSG

Radioiodination was carried out using a modification of the chloramine-T method of Hunter & Greenwood (1962). Labelling was performed using 0·5 mCi ^{125}I-iodide in 50 µl 0·5 M-phosphate buffer and 5 µg PMSG dissolved in 25 µl 0·5 M-phosphate buffer, pH 7·5. The reaction was initiated by the addition of 50 µg chloramine-T dissolved in 25 µl 0·05 M-phosphate buffer, pH 7·5. After an oxidation time of 20 sec, the reaction was blocked by the addition of 80 µg sodium metabisulphide dissolved in 400 µl 0·05 M-phosphate buffer, pH 7·5. Separation of labelled hormone from free ^{125}I-iodide was performed by means of gel filtration with a Sephadex-G50 column (20 × 1·5 cm). Elution was carried out with 0·07 M-veronal buffer, pH 8·6.

The labelled hormone was stored at −20°C. Immediately before use in the assay a second purification step with a freshly set-up cellulose column (about 15 ml gel, type HBS: Serva) was necessary. Hormone damage products were eluted with about 20 ml 0·01 M-phosphate buffer, pH 7·5. The undamaged, immunoreactive PMSG was finally eluted with serum from non-pregnant mares, diluted 1 : 4 with 0·5 M-phosphate buffer, pH 7·5.

Radioimmunoassay

Dilutions of standard PMSG or serum samples were prepared with 0·05 M-sodium phosphate buffer, pH 7·5, containing 0·18% (w/v) EDTA and 0·05% (w/v) human serum albumin. To small plastic tubes (10 × 65 mm) either known concentrations of standard hormone in 0·2 ml buffer or in 0·1 ml buffer plus 0·1 ml PMSG-free serum, or 0·2 ml of the unknown serum sample were added. Incubation was set up by adding 0·1 ml antiserum to a final dilution of 1 : 40 000 (for assay in bovine serum) or 1 : 80 000 (for assay in equine serum) containing normal rabbit serum at a final concentration of 1 : 1800. For measuring PMSG in bovine serum, 5 ng bovine LH were added to the antiserum. After incubation for 24 h at 4°C, 0·1 ml labelled PMSG (about 10 000 c.p.m.) was added to each tube. Separation of bound and free hormone was performed by means of the double-antibody method. A precipitating antiserum against rabbit gamma-globulins was added after an additional 48 to 72 h incubation. After 24 h 1·5 ml 0·05 M-phosphate buffer were added to each tube and the samples were centrifuged at 1500 *g* for 30 min. The supernatant was decanted and the precipitate counted in an automatic gamma-counter. Text-figure 1 shows a typical standard curve for measuring PMSG in cattle serum.



Text-fig. 1. Binding inhibition curve of standard PMSG after addition of 5 ng bovine LH and 0·1 ml PMSG-free bovine serum in the radioimmunoassay described. Each value represents a mean ± s.d. for 20 observations.

Experimental animals and blood sampling

Experiment 1. From 3 pregnant mares serum samples were taken during a period of approximately 2 months. The mares were bled once or twice a week. The serum samples were also assayed by means of the biological assay of ovarian weight gain in infantile rats.

Experiment 2. Two heifers of the local Fleckvieh breed were each given a single i.m. injection of PMSG (1500 or 3000 i.u.) dissolved in saline. Blood was collected from the jugular vein by means of an indwelling catheter and the serum obtained was stored at −18°C until further use. Sampling was initially carried out at 6-h intervals during a period before and after application of PMSG and then at 12-h intervals.

Experiment 3. For determination of the disappearance time of PMSG, 2 heifers of the local Brown Swiss breed were used. Blood samples were withdrawn via an indwelling catheter before and during a 3-h period of PMSG infusion at 30-min intervals, for 12 h after the end of the infusion at 2-h intervals, for a further 5 days at 6-h intervals and continuing for the next 12 days at 12-h intervals.

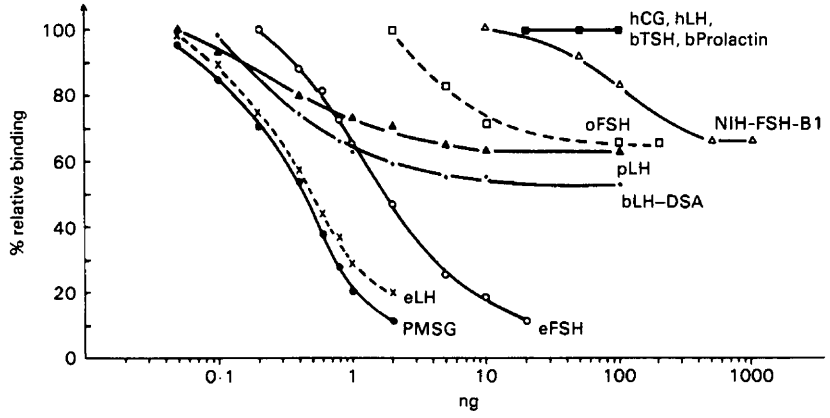
Parts of experiments 2 and 3 have been reported earlier (Schams *et al.*, 1978).

Results

Validation of the method

Radioiodination. The mean incorporation of ^{125}I into PMSG was 12·3 ± 2·7% of the total ^{125}I-iodide (n = 14). The mean specific activity was 19·8 μCi/μg protein (n = 12). Storage of the labelled hormone was possible only for a limited time due to increasing hormone damage found by means of the second purification procedure. After 2 days, iodination damage was 5 ± 1% (n = 12), increasing after 3–8 days to 16 ± 6% (n = 7) and to 26 ± 3% (n = 5) after 10–20 days. Iodination with lactoperoxidase (unpublished data) gave comparable results without any significant improvement.

Specificity. As demonstrated in Text-fig. 2, the antiserum against PMSG showed a strong cross-reaction with equine LH as well as a weaker one with equine FSH. A slight cross-reactivity could be observed with porcine and bovine LH, as well as with ovine and bovine FSH, which



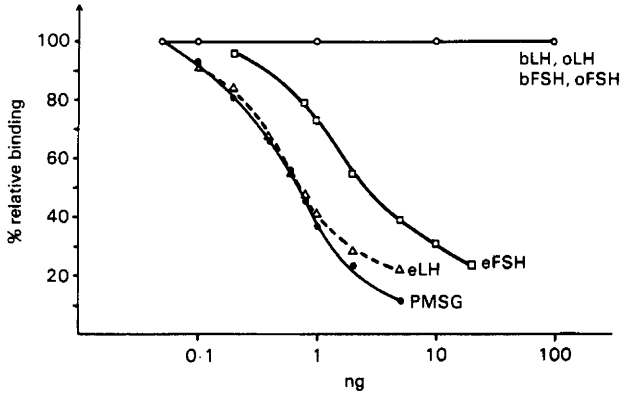
Text-fig. 2. Binding inhibition curves for all the hormones tested in the radioimmunoassay for PMSG.

reached a plateau at different concentrations. No cross-reaction could be found against bovine TSH, prolactin, human LH, hCG or the α- or β-subunits of ovine LH.

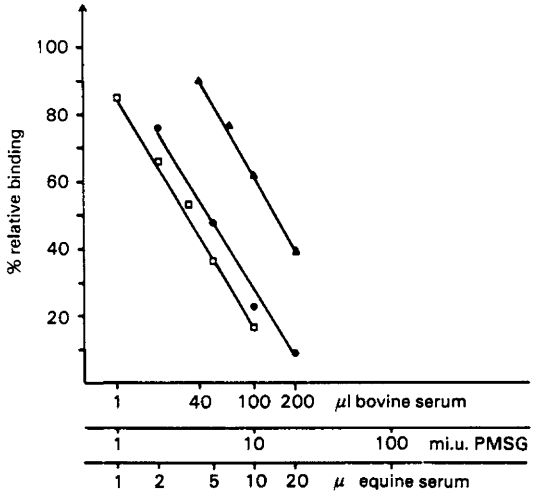
For determination of PMSG in bovine serum, this cross-reaction against bovine LH as well as FSH could be eliminated by addition of 5 ng bLH to each test-tube, as demonstrated in Text-fig. 3, but the cross-reaction against eLH and eFSH was still present. The depression of absolute binding after addition of LH had to be compensated for by a lower dilution of the antiserum. In pregnant mares, cross-reaction of LH and FSH can be neglected since a high dilution of serum samples is necessary, due to high PMSG concentrations and sensitivity of the assay.

Assay precision. The intra-assay coefficient of variation (CV), calculated from a mean value of 25 parallel tubes of two different pregnant mare serum samples (2·9 and 12·4 mi.u./0·2 ml) was 6·9%. The inter-assay variation is expressed as the mean of the variation coefficients of 6 different pregnant mare serum samples (range 5–50 mi.u./0·2 ml) running in the assay as controls for the reproducibility of the system; the mean CV was 12·6%. The 50% intercept of relative binding was estimated as 0·88 ng PMSG with a CV of 14·8% ($n = 22$). The limit of sensitivity of the assay was 0·1 ng (1 mi.u.) PMSG when 0·1 ml PMSG-free serum was added.

Dilution curves. Inhibition curves of sera of pregnant mares as well as serum samples of cattle after injection of PMSG (diluted in buffer) were parallel to the standard curve for PMSG as shown in Text-fig. 4.



Text-fig. 3. Binding inhibition curves of PMSG (●——●), equine LH (△- - -△), equine FSH (□——□), ovine and bovine LH, ovine and bovine FSH (○——○) after addition of 5 ng bovine LH per tube.

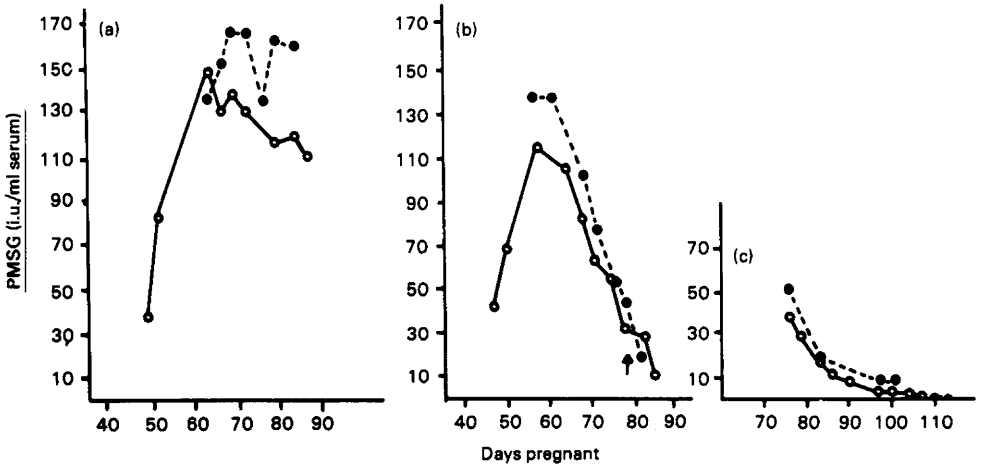


Text-fig. 4. Inhibition lines after dilution of endogenous PMSG in pregnant mare serum (□——□) and exogenous PMSG in bovine serum (▲——▲) compared to standard PMSG (mi.u./tube ●——●).

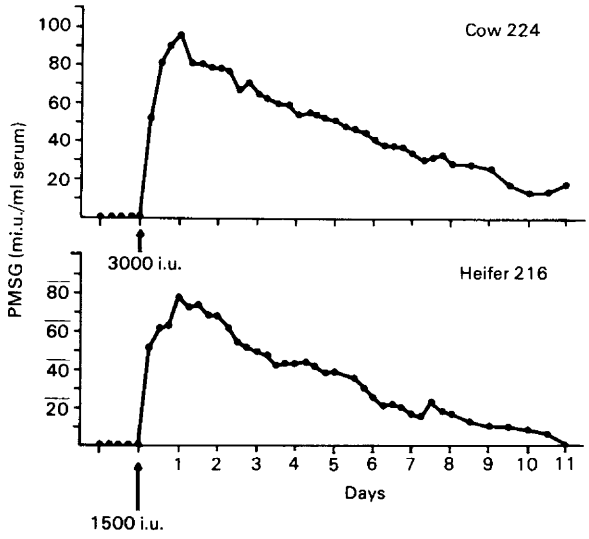
Recovery experiments. Known amounts of PMSG were added to samples of serum from non-pregnant mares or cows. As can be shown from the recovery results, equine as well as bovine serum had an influence on the assay system, resulting in an average recovery of only 54 ± 10%. By adding PMSG-free serum to the standard, especially of homologous serum obtained from the same animal, the recovery results (range of added PMSG 2–20 mi.u.) could be improved on average to 90·5 ± 9·9% with a CV of 11%.

Physiological results

PMSG determination in pregnant mare serum. As shown in Text-fig. 5, serum levels of PMSG were determined in 3 pregnant mares. The results of the radioimmunoassay were compared with those obtained for the same samples by bioassay. There was clearly good agreement between the two assays. The mean index of discrimination between bioassay and RIA was 1·2.



Text-fig. 5. Serum levels of PMSG in 3 different pregnant mares (a, b, c) when measured by radioimmunoassay (O——O) and bioassay (●---●). Arrow = abortion.



Text-fig. 6. Blood serum levels of PMSG in 2 heifers after a single i.m. application of 1500 or 3000 i.u. PMSG.

PMSG concentration in cows after administration of PMSG. The concentrations of PMSG were highest about 12–24 h after a single injection of PMSG and were still measurable after about 10 days (Text-fig. 6).

Disappearance time of PMSG in peripheral blood serum of cattle. Two heifers were given an infusion of 12 000 i.u. PMSG dissolved in 3 litres of saline over a period of 3 h. In both animals two components of the disappearance time were obvious: a shorter one of 51·2 h ($y = -10{\cdot}7x +$ 1281 expressed in mi.u.) or 40·0 h ($y = -13{\cdot}25x + 1299$) and a longer one of 123·2 h ($y = -1{\cdot}32x + 470$) or 118·4 h ($y = -1{\cdot}08x + 376$), respectively.

Discussion

With regard to the validation of the method, the inhibition curves with pregnant mare serum or PMSG in cow serum, the recovery experiments with PMSG added to bovine serum and the good reproducibility of control samples demonstrate that the radioimmunoassay developed is potentially useful for measuring endogenous or exogenous PMSG in mare or cow serum. Interference of serum components of equine or bovine serum within the assay could be eliminated by adding a constant amount of PMSG-free serum to the standard curve.

An unspecific interfering influence of equine serum has also been reported by Wide & Wide (1963) and Allen (1969a), who used a haemagglutination–inhibition asay for PMSG determination. The partial cross-reaction with bovine LH and ovine or bovine FSH (Text-fig. 2) was eliminated by addition of bovine LH to each tube (Text-fig. 3). This cross-reactivity could be also eliminated by addition of ovine FSH and is perhaps due to a small population of antibodies with lower specificity in the antiserum used. Because the structure of PMSG and eLH and to some extent that of eFSH is similar biochemically the cross-reactivity is to be expected (Papkoff, 1978). This offers the possibility of measuring radioimmunologically the total gonadotrophin activity in non-pregnant mares.

Concerning the limited PMSG data in 3 pregnant mares, the values are comparable to those obtained in more intensive studies in pregnant mares by means of the haemagglutination–inhibition assay (Allen, 1969b). The good agreement between values obtained in the immuno- and bioassays further validates the radioimmunoassay method. The possibility of measuring

PMSG levels in blood after administration of PMSG with this assay may improve the understanding and interpretation of studies in which PMSG is involved. To our knowledge, apart from our earlier publication (Schams *et al.*, 1978) no data exist about PMSG levels in cattle blood after a single administration. It was surprising that PMSG values could still be measured 10 days after injection, although a reasonably long half-life of PMSG in the cow could have been expected. The component with the longer half-life agrees well with that obtained in the mare by means of bioassay (Cole, Bigelow, Finkel & Rupp, 1967) and is much longer than that observed in ewes ($t_{\frac{1}{2}} = 21$ h: McIntosh, Moor & Allen, 1975). The prolonged half-life of PMSG is probably related to the high sialic acid content (Schams & Papkoff, 1972; Gospodarowicz, 1972).

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References

Allen, W.R. (1969a) A quantitative immunological assay for pregnant mare serum gonadotrophin. *J. Endocr.* **43**, 581–591.

Allen, W.R. (1969b) The immunological measurement of pregnant mare serum gonadotrophin. *J. Endocr.* **43**, 593–598.

Allen, W. R., Hamilton, D.W. & Moor, R.M. (1973) The origin of equine endometrial cups. II. Invasion of the endometrium by trophoblast. *Anat. Rec.* **177**, 485–502.

Clegg, M.T., Boda, J.M. & Cole, H.H. (1954) The endometrial cups and allanto chorionic pouches in the mare with emphasis on the source of equine gonadotrophin. *Endocrinology* **54**, 448–463.

Cole, H.H. & Erway, J. (1941) 48-hour assay test for equine gonadotrophin with results expressed in international units. *Endocrinology* **29**, 514–519.

Cole, H.H. & Hart, G.H. (1930) The potency of blood serum of mares in progressive stages of pregnancy in effecting the sexual maturity of the immature rat. *Am. J. Physiol.* **93**, 57–68.

Cole, H.H., Bigelow, M., Finkel, J. & Rupp, G.R. (1967) Biological half-life of endogenous PMS following hysterectomy and studies on losses in urine and milk. *Endocrinology* **81**, 927–930.

Gospodarowicz, D. (1972) Purification and physicochemical properties of the pregnant mare serum gonadotropin (PMSG). *Endocrinology* **91**, 101–106.

Hunter, W.M. & Greenwood, F.C. (1962) Preparation of iodine-131 labelled human growth hormone of high specific activity. *Nature, Lond.* **194**, 495–496.

McIntosh, J.E.A., Moor, R.M. & Allen, W.R. (1975) Pregnant mare serum gonadotrophin: rate of clearance from the circulation of sheep. *J. Reprod. Fert.* **44**, 95–100.

Papkoff, H. (1978) Relationship of PMSG to the pituitary gonadotropins. In *Control of Reproduction in the Cow.* Ed. J. Sreenan. EEC Seminar, Galway.

Schams, D. & Papkoff, H. (1972) Chemical and immunological studies on pregnant mare serum gonadotropin. *Biochim. biophys. Acta* **263**, 139–148.

Schams, D., Menzer, C., Schallenberger, E., Hoffmann, B., Hahn, J. & Hahn, R. (1978) Some studies on pregnant mare serum gonadotropin (PMSG) and on endocrine responses after application for superovulation in cattle. In *Control of Reproduction in the Cow.* Ed. J. Sreenan. EEC Seminar, Galway.

Wide, M. & Wide, L. (1963) Diagnosis of pregnancy in mares by an immunological method. *Nature, Lond.* **198**, 1017–1018.

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