A panel of monoclonal antibodies to ovine placental lactogen

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Abstract

A panel of 11 rat monoclonal antibodies (mAbs) has been raised to ovine placental lactogen (PL). By competitive enzyme-linked immunoabsorbent assay (ELISA), confirmed by two-site ELISA, the antibodies were shown to recognize six antigenic determinants on the ovine PL molecule, two of which overlap. One antigenic determinant (designated 1) was shared by other members of the prolactin/growth hormone (GH)/PL family in ruminants, humans and rodents. The binding of ^{125}I -labelled ovine

PL to crude receptor preparations from sheep liver (somatotrophic) or rabbit mammary gland (lactogenic) was inhibited by mAbs recognizing antigenic determinants 2–6. Both types of receptor preparation were affected similarly. In the local *in vivo* pigeon crop sac assay, mAbs directed against determinants 3 and 6 enhanced the biological activity of ovine PL.

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Introduction

Placental lactogens are a group of polypeptide hormones which are structurally related to prolactin and GH. They have evolved more than once, from the GH gene in primates (Walker *et al.* 1991) and from the prolactin gene in rodents and ruminants (see Forsyth 1994). Ovine placental lactogen (PL) is a 22 kDa protein, synthesized by trophoectoderm binucleate cells in the cotyledonary placenta of sheep (Wooding *et al.* 1992), that reaches both the maternal and fetal circulations (Chan *et al.* 1978). Its physiological role in pregnant ewes and their fetuses is still poorly understood. There are potentially three receptor types through which it may act (see Sakal *et al.* 1997), the prolactin receptor, the GH receptor and a specific PL receptor suggested to exist, on the basis of affinity binding studies, mainly in fetal tissues (Freemark & Comer 1989) and also in the sheep endometrium (Galosy *et al.* 1991).

To assist work on the biological role, the structure–activity relationships and the antigenic determinants of ovine PL, we have made rat monoclonal antibodies against the purified hormone and now report on their preparation and preliminary characterization.

Materials and methods

Materials

Ovine PL was purified and characterized as described by Colosi *et al.* (1989). It was labelled with either Na^{125}I (Amersham International, Amersham, Bucks, UK) by

the iodogen method (Salacinski *et al.* 1981) or biotin (*N*-hydroxysuccinimidobiotin, Calbiochem, Beeston, Nottingham, UK) by mixing antibody (1 mg in 1 ml phosphate buffered saline, PBS, pH 7) with biotin (1 mg in 40 μl dimethylsulphoxide), stirring for 1 h at room temperature and dialysing.

Immunization

Four male rats of the F344 strain were injected with purified ovine PL (oPL, 100 μg) in Freund's adjuvant. Complete adjuvant was used for the first of three injections, given by i.m. and/or s.c. routes at 1–2 month intervals. A small blood sample was taken from the tail vein and tested by radioimmunoassay (Thordarson *et al.* 1987) for level of humoral immunity. Two rats showing the highest antibody titre (>40% labelled oPL bound at 1:1200 dilution, v/v) were given 100 μg ovine PL in saline i.v. and killed 3 days later by exposure to rising concentrations of CO_2 .

Generation of monoclonal antibodies

Rat spleen cells and rat myeloma cell line Y3Ag1.2.3 (Galfrè *et al.* 1979) or IR983F (Bazin 1982, Table 1) were fused and hybridomas obtained after HAT (hypoxanthine, aminopterin and thymidine) selection using standard methods (Galfrè & Milstein 1981). Culture media were tested for antibody using either radioimmunoassay (Thordarson *et al.* 1987) or enzyme-linked immunoabsorbent assay (ELISA, see below). Positive hybridomas were grown on and cloned on agar. In some cases, ascitic

fluids were generated in (LOU/c x F344)F1 rats, to obtain larger quantities of particular antibodies for purification and biotinylation.

Cross-reactivity and antigenic mapping

A solid-phase ELISA method was used to obtain information on binding of antibodies to oPL and to other members of the prolactin/GH gene family. A competitive ELISA gave preliminary data on antigenic determinants on ovine PL. To confirm the results on antigenic determinants, a two-site ELISA method was used.

Solid-phase ELISA Ninety-six-well plates (Dynatech, Billingshurst, Sussex) were coated overnight at 4 °C with ovine PL (1 µg/well) in 100 µl PBS (pH 7) and blocked for 2 h at 4 °C with 10% fetal calf serum (FCS) in PBS. Other purified antigens tested at 1 µg/well for cross-reactivity were: ovine (NIH-P-S9) and bovine (NIH-P-B4) prolactin, ovine (NIH-GH-S9) and bovine (NIH-GH-B2) GH, human PL (National Hormone and Pituitary Program, Bethesda, MD, USA), bovine PL (USDA-bPL-I-1, United States Department of Agriculture, Beltsville, MD, USA), human GH (Dr A Stockell-Hartree, University of Cambridge, UK), mouse PL-I and -II (Dr G Thordarson, University of California, Santa Cruz, CA, USA) and rat PL-I and -II (Dr R Shiu, University of Manitoba, Canada). Unlabelled antibody (supernatant from hybridoma cultures at dilutions from neat to 1:64, ~5 mg/l to 80 µg/l IgG, or ascites at dilutions from 1:100 to 1:218 700, ~10 mg/l to 5 µg/l) was added and incubated for 2 h at 4 °C. The plates were washed 3 times in tap water, incubated for 1.5 h at room temperature with horseradish-peroxidase conjugated anti-rat IgG (Dako, Ely, Cambs), washed once with Tween 20 (0.05 g/l) and 10 times with tap water, developed using 3,3',5,5'-tetramethylbenzidine (Pierce and Warriner (UK) Ltd, Chester, UK) and read in a microphotometer. mAb EM1/21.9 directed against an irrelevant nematode antigen was used as a negative control; binding above mean + 1 s.d. ($n=11$) was regarded as positive.

Competitive ELISA The method was as described for the solid-phase ELISA, except that biotin-labelled antibody (25–100 ng/well in 50 µl PBS, pH 7) was added to oPL-coated 96-well plates together with dilutions of unlabelled antibody. Incubation was for 2 h at 4 °C. The plates were washed twice in tap water before incubation for 30 min at 37 °C with streptavidin-horseradish peroxidase complex (Amersham International, diluted 1:500 in 10% FCS in PBS). Plates were washed twice with Tween 20 (1 g/l in PBS) and 10 times in tap water. They were developed using 3,3',5,5'-tetramethylbenzidine (Pierce and Warriner (UK) Ltd, Chester, UK) and read in a microphotometer.

Two-site ELISA Using 96-well plates, unlabelled antibody was bound to each well by adding 100 µl of the first antibody solution (10 µg/ml) and incubating for 2 h at room temperature. The wells were washed twice with tap water, blocked for 2 h at 4 °C with 10% FCS in PBS (pH 7) and washed again. Ovine PL (0.1, 0.5, 1, 5, 10, 15 and 20 µg in 100 µl blocking buffer) was added and the plates incubated for 2 h at room temperature. The second antibody (1 µg/ml), labelled with biotin and diluted with blocking buffer, was then added and plates were incubated for 2 h at room temperature. They were washed, developed and read as described for the competitive ELISA. The second, biotin-labelled, antibody will bind to ovine PL only if it recognizes an antigenic determinant not overlapping with that recognized by the first antibody bound to the microtitre plate.

Tissue binding studies

The effect of the mAbs on interaction of ovine PL with tissue receptors was investigated by minor modification of the method of Cadman *et al.* (1982). Microsomes were prepared by homogenization and differential centrifugation (Winder *et al.* 1993) from the mammary glands of bromocriptine-treated New Zealand White rabbits in early lactation and the livers of Poll-Dorset ewes and were freeze dried (Parke & Forsyth 1975). Displacement studies using unlabelled ovine PL, ovine prolactin (NIH-P-S-12, 35 i.u./mg) and bovine GH (NIH-B-GH-2, 1.5 i.u./mg) confirmed that rabbit mammary gland contains predominantly lactogenic receptors (displaced by prolactin and ovine PL, but not by bovine GH, Kelly *et al.* 1976), while sheep liver contains mainly somatotrophic receptors (displaced by bovine GH and ovine PL, but not by prolactin, Brier *et al.* 1994). Doubling dilutions of monoclonal antibody (100 µl), assay buffer (25 mmol Tris-HCl/l, pH 7.4, 10 mmol CaCl₂/l, 0.1% (w/v) bovine serum albumin, 200 µl) and ¹²⁵I-labelled oPL (100 µl, 20 000 c.p.m.) were incubated at 4 °C for 48 h. Microsomes (1 mg in 100 µl assay buffer) were added and incubation continued overnight at room temperature. Ice-cold assay buffer (1 ml) was added and tubes spun at 1820 g for 30 min at 4 °C. Radioactivity in the pellet was counted. Parallel determinations of antibody titre were carried out by adding buffer only instead of microsomes after 48 h and, following overnight incubation, separating bound and free label using a second antibody (donkey anti-mouse IgG, Cambridge Medical Diagnostics, Billerica, MA, USA).

Bioassay

The effect of mAbs on the biological activity of ovine PL was tested using dilutions of ascitic fluids in the pigeon crop sac assay (Nicoll 1967). Birds were allocated to treatment at random and received a single injection (0.1 ml i.d.) of ovine PL (3 or 5 µg in saline or in control

Table 1 Designation and isotype of monoclonal antibodies to ovine placental lactogen

	Myeloma	Fusion number*	Isotype
mAb			
MAC 193	IR983F	1	IgG2a
MAC 215	Y3	2	IgG2b
MAC 216	Y3	2	IgG2a
MAC 217	Y3	2	IgG2a
MAC 218	Y3	2	IgG2b
MAC 219	IR983F	3	IgG2a
MAC 220	IR983F	3	IgG2b
MAC 221	IR983F	3	IgG1
MAC 222	IR983F	3	IgG2a
MAC 223	IR983F	3	IgG2b
MAC 224	IR983F	3	IgG2a

*Fusions 2 and 3 used the two halves of the same spleen.

monoclonal) over one crop sac and the same dose of ovine PL plus mAb to ovine PL over the contralateral crop. Sites could be regarded as independent, as in excess of 20 µg ovine PL would be required to affect one crop if injected over the other (Nicoll 1967). Ovine PL and mAb were pre-incubated for 60 min before injection. Dilutions of ascitic fluid were calculated by reference to antibody titre (see Aston *et al.* 1986). The pigeons were killed by cervical dislocation 48 h later and the wet weight of crop mucosa, removed from a standard 4 cm diameter area, was determined. The mAb used as control was directed against nitrate reductase of spinach (AFRC MAC 77, Notton *et al.* 1984). The statistical significance of differences was tested using Student's *t*-test, either unpaired (between birds) or paired (within birds).

Results

Antigenic mapping of ovine PL by ELISA methods

The 11 mAbs generated and their IgG class and subclass are shown in Table 1. Competitive ELISA (Fig. 1 and Table 2) indicates that the 11 antibodies recognize six antigenic determinants (summarized in Fig. 2). Determinants 4 and 5 are suggested as partially overlapping on the basis of non-reciprocal competition between MAC 222 and 224 and the ability of MAC 215 to compete with both (Table 2). Using MAC 193, 218, 220, 221, 222, 223 and 224 in the two-site ELISA (Fig. 3a, b), these conclusions were confirmed. MAC 218 and MAC 220 each interfered with the ability of the other to bind, as did MAC 224 with MAC 222.

MAC 223 (determinant 1) bound to all members of the prolactin/GH/PL family tested with the exception of mouse PLII (Fig. 4a, b). Cross-reactivity was also detected of MAC 217 (determinant 1) on bovine PL, ovine and bovine prolactin (Fig. 4a), ovine, bovine and human GH

Table 2 Competitive ELISA assay: ability of unlabelled monoclonal antibody (1 : 4 dilution, ~1.25 mg/l) to compete with biotin-labelled monoclonal antibody for binding sites on ovine PL (1 µg/well). Self-competition is underlined and competing antibodies are shown in bold type. Biotinylation of MAC 224 partially interfered with ability to bind ovine PL. Values are optical density at 490 nm

Unlabelled mAb	Labelled mAb					
	193	220	221	222	223	224
193	0.19	0.91	1.06	0.63	0.74	0.44
215	0.72	1.18	1.05	0.27	0.96	0.33
216	1.11	1.00	1.24	1.07	0.22	0.74
217	1.09	1.06	1.25	1.00	0.21	0.83
218	0.74	0.19	1.31	0.86	0.53	0.62
219	1.00	1.02	1.35	1.01	0.31	0.78
220	0.66	0.20	1.28	1.49	0.60	0.56
221	0.79	1.39	0.21	0.85	0.64	0.68
222	0.82	1.27	1.25	0.28	1.04	0.62
223	0.88	1.06	1.24	1.07	0.18	0.75
224	0.91	1.35	1.14	0.29	0.72	0.24

Figure 1 Competitive ELISA: ability of dilutions of unlabelled monoclonal antibodies to compete with biotin-labelled MAC 220 for binding sites on ovine PL (1 µg/well). Values for nine non-competing mAbs (MAC 193, 215, 216, 217, 219, 221, 222, 223 and 224) are shown as means ± S.D. (●); 220 (○); 218 (■).

(Fig. 4b), and of MAC 219 (determinant 1) on bovine PL, ovine and bovine prolactin (Fig. 4a). No other mAbs showed cross-reactivity (defined as binding not significantly greater than that shown by the negative control).

Influence of monoclonal antibodies on binding of ovine PL to microsomes prepared from rabbit mammary gland or sheep liver

The mAbs were tested for their effect on interaction between ¹²⁵I-labelled ovine PL and tissue binding sites.

Figure 2 Schematic diagram of antigenic determinants 1–6 on ovine PL recognized by rat monoclonal antibodies. Non-intersecting circles represent non-overlapping determinants. Determinants 4 and 5 are presented as partially overlapping on the basis of ELISA results (see text). Determinant 1 is shared with other ruminant and human prolactins, GHs and PLs.

The specific binding of ^{125}I -oPL was 40.8% to heterologous rabbit mammary gland microsomes (lactogenic) and 29.2% to homologous sheep liver microsomes (somatotrophic). In all cases, the effect of mAbs on binding to rabbit mammary gland receptors and to sheep liver receptors was similar (Fig. 5). Seven monoclonal antibodies, MAC 218 and 220 (determinant 2), 193 (determinant 3), 224 (determinant 4), 222 (determinant 5), 215 (determinant 4/5) and 221 (determinant 6), inhibited the binding of ovine PL (Fig. 5a, b). Potency varied and MAC 224, 222 and 215, directed against the overlapping determinants 4 and 5, were effective only when present at high concentration (Fig. 5b). In some cases (MAC 218, 193 and 221) binding recovered at higher mAb concentrations (Fig. 5a). Four antibodies (MAC 223, 216, 217 and 219), all directed against antigenic determinant 1, had little inhibitory effect on oPL binding. MAC 223 enhanced binding at higher concentrations (Fig. 5c).

Effect on biological activity

The wet weight of crop sac mucosa from a 4 cm diameter site was 10.4 ± 1.9 mg (mean $\pm$ S.E.M., $n=6$). After a single injection of ovine PL, the weight was 11.1 ± 0.7 mg (3 μg ovine PL, $n=16$, $P>0.1$) or 17.9 ± 1.0 mg (5 μg ovine PL, $n=28$, $P<0.001$). mAb to spinach nitrate reductase (MAC 77) had no effect on the response to ovine PL; crop weights in response to 5 μg ovine PL combined with 1:500 and 1:4000 dilutions of MAC 77 were, respectively, 18.2 ± 2.8 mg ($n=8$, $P>0.1$) and 21.0 ± 2.8 mg ($n=8$, $P>0.1$).

Effect on biological activity (Table 3) was tested using mAbs recognizing each of the six antigenic determinants (Fig. 2). At the dose used (2000 $\times$ ABT 50, where one ABT 50 is the dilution (titre) of mAb binding 50% of ^{125}I -labelled oPL in a liquid phase), none of the ovine PL mAbs inhibited biological activity. Enhancement was seen with MAC 193 (determinant 3, Table 3) and MAC 221 (determinant 6).

Discussion

Competitive and two-site ELISA assays indicate that the 11 rat monoclonal antibodies raised against ovine PL recognize six antigenic sites. Mouse mAbs have been prepared which recognize four distinct epitopes on bovine GH (Aston *et al.* 1987, Krivi & Rowold 1986). Human GH exhibits at least ten epitopes, grouped into five antigenic regions, of which at least one is shared with human PL and one is not (Strasburger *et al.* 1989). As analogues of ovine PL become available, further analysis of its antigenic surface will be possible. One of the determinants on ovine PL (designated 1) was shared with other members of the prolactin/GH gene family and appears to be a major determinant, recognized by four of the 11 mAbs. On the basis of *in vitro* binding studies, this determinant did not appear to be involved in binding of ovine PL to receptors on rabbit mammary gland (lactogenic) or sheep liver (somatogenic); mAbs directed against the other five determinants all interfered in the binding of ^{125}I -labelled ovine PL to receptors *in vitro*, but did not distinguish between heterologous (rabbit) lactogenic and homologous (sheep) somatogenic receptors.

Ligand-induced dimerization is a frequent mechanism to initiate signal transduction. A two-site model of sequential receptor homodimerization was developed by studying the interaction of human GH with the extracellular domain (ECD) of the human GH receptor (see Wells 1996). In this model, the hormone binds the first receptor through its binding site 1 and then a second receptor through binding site 2. There is also a substantial receptor–receptor interface. The model is believed to apply to other helix–bundle peptides.

Consistent with this model, ovine PL forms 1:2 complexes with heterologous (rabbit and rat) prolactin receptor ECD and with heterologous (human) GH receptor ECD (Sakal *et al.* 1997). With bovine prolactin ECD (Sakal *et al.* 1997), a 1:2 complex is observed only transiently, because

Figure 3 Two-site ELISA: ovine PL (0–200 µg/l) captured by unlabelled monoclonal antibody (10 mg/l) absorbed onto plastic (a) MAC 220; (b) MAC 224. Biotin-labelled MAC 193 (○), 218 (●), 220 (△), 221 (▲), 222 (■) and 223 (□) bind only if recognizing a non-overlapping determinant.

of rapid dissociation to 1:1 complexes, associated with high k_{off} constants (Gertler *et al.* 1996). By contrast, recent evidence (Herman *et al.* 1999) suggests ruminant PLs may be unable to homodimerize ruminant GH receptors, binding only through site 1, and their status as growth hormone agonists in ruminants remains unclear (see also Staten *et al.* 1993).

Figure 4 Solid phase ELISA: binding of MAC 217 (hatched bars), 219 (closed bars) and 223 (open bars) to hormones absorbed onto plastic. The hormones tested were (a) PL and prolactin (PR) and (b) PL and GH of ovine (o), bovine (b), human (h), mouse (m) and rat (r) origin (see Methods for full details: values shown are for undiluted culture supernatants, ~ 5 mg/l specific IgG). Values for the eight monoclonal antibodies showing binding only to oPL (MAC 193, 215, 216, 218, 220, 221, 222 and 224) are shown (means $\pm$ S.E.M.) as vertically striped bars. The vertical lines are mean (continuous) and mean + 1 S.D. ($n=11$, dotted) for the binding of a control monoclonal antibody (EM1/21-9). For MAC 217, 219 and 223, binding to oPL is mean $\pm$ S.E.M. ($n=4$ experiments) and means of closely agreeing duplicates for the other hormones.

The effect of mAbs on binding of ovine PL was the same for sheep liver microsomes and rabbit mammary gland microsomes, suggesting, therefore, that the binding

Figure 5 Effect of monoclonal antibodies raised against purified ovine PL on the binding of ^{125}I -labelled ovine PL to microsomes prepared from rabbit mammary gland or sheep liver. ^{125}I -labelled ovine PL was pre-incubated with dilutions of the monoclonal antibodies for 48 h. Buffer (▲), microsomes prepared from rabbit mammary gland (●) or sheep liver (○) were then added and incubation continued for a further 24 h. Binding was determined as described in the Methods. Each value is the mean of duplicate observations in one assay and all assays were replicated. (a) MAC 218; (b) MAC 224; (c) MAC 223.

assays measure site 1 interaction. If receptor binding is sequential, this would indeed be expected; subsequent site 2 binding would be reflected in percentage specific binding only if it resulted in stabilization and reduced dissociation of the hormone–receptor complex. Effect of mAbs on ability of hormone to bind to receptor may involve (i) binding to a region on the hormone making contact with receptor; (ii) binding close to a region on the hormone making contact with receptor and causing steric hindrance; (iii) binding to a region distinct from that making contact with receptor, affecting hormone conformation and changing affinity for the receptor. Each of

these mechanisms could provide explanations for inhibition of receptor binding. The third could explain the binding enhancement seen with MAC 223 (Fig. 5c) if affinity was increased. Enhancement of binding is also seen with some monoclonal antibodies to ovine GH (Aston *et al.* 1987). Inhibition followed by recovery (MAC 218, 193 and 221, Fig. 5a) is not readily explained.

Enhancement of biological activity *in vivo* by mAbs has previously been demonstrated for a number of polypeptide hormones (Mockridge *et al.* 1998), including human GH (Holder *et al.* 1985), bovine GH (Aston *et al.* 1987) and human PL (Aston *et al.* 1986). At the doses used, both

Table 3 Effects of monoclonal antibodies on the *in vivo* biological activity of ovine PL, tested in the pigeon crop sac bioassay

	Antigenic determinant	Dose of ovine PL (µg)	Mucosa, mean weight difference (mg) ± SEM	P value
mAb 223	1	3	0.2 ± 1.7	ns
		5	0.2 ± 1.4	ns
218	2	3	3.1 ± 2.6	ns
		5	0.9 ± 2.3	ns
220	2	5	3.3 ± 3.3	ns
193	3	3	33.7 ± 1.7	<0.001
224	4	5	1.9 ± 2.5	ns
222	5	5	8.8 ± 2.3	ns
221	6	3	10.9 ± 3.4	<0.05
		5	10.9 ± 1.8	<0.01

MAC 193 (antigenic determinant 3) and MAC 221 (determinant 6) showed enhancing activity in the pigeon crop sac assay, the standard *in vivo* assay for prolactin action. The mechanism(s) underlying antibody-mediated enhancement remain imperfectly understood. However, of the various mechanisms proposed (Holder 1992), prolongation of hormone half-life is now thought to play a primary role (Mockridge *et al.* 1998). This mechanism is independent of any influence on receptor interaction.

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