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ANTIBODIES AGAINST HIGHLY PURIFIED B-SUBUNIT OF THE CHICK OVIDUCT PROGESTERONE RECEPTOR

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SUMMARY : A rabbit was immunized with the highly purified B-subunit (110kDa) (20 to 50 µg per injection) of the chick oviduct progesterone receptor (PR). Specific antibodies (IgG-RB) were observed 2 weeks after the first booster injection and high antibody titers in the serum were found after the second and third booster injections (with Kdeq of interaction ∿ 2 nM). IgG-RB were tested by immunoprecipitation, immunoblotting, density gradient ultracentrifugation and protein A-sepharose assay methods. They recognized not only the B-subunit but also the A-subunit (79K), the nuclear PR, the mero-receptor (proteolytic cleavage product) and the "non-activated" molybdate-stabilized "8S" PR. However, IgG-RB did not interact with the 90K non hormone-binding component of this 8S-PR. IgG-RB did not affect the binding of the hormone to PR, whether incubated with the receptor before or after labelling with tritiated progesterone. They did not cross-react with glucocorticosteroid receptor of the chick oviduct. Weak interaction was observed with estrogen receptor of the chick oviduct and with KCl activated "4S" forms of the rabbit and human uterus PR.

Although it has been possible to purify the chick oviduct progesterone receptor (PR) to homogeneity for some years (1-3), there have been difficulties to raise specific antibodies against this receptor (4). We have recently obtained both polyclonal (IgG-G3) (5) and monoclonal (BF4) (6) antibodies against purified "8S"-PR. IgG-G3 recognized both the hormone-binding and non-binding components of the receptor, and BF4 recognized only the non-hormone-binding component (90K protein) of the 8S-PR (7). We have recently purified the progesterone-binding 110kDa "B-subunit" of the chick oviduct (1) to apparent homogeneity (8) and used it to immunize a rabbit. Here we report the preliminary characteristics of the antibodies obtained.

MATERIALS AND METHODS

Steroids and buffers : Tritiated progesterone (2,4,6,7-^{3}H-progesterone, 104 Ci/mmol, ^{3}HP), (1,2,4,6,7-^{3}H-dexamethasone (89 Ci/mmol), 6,7-^{3}H-estra-

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diol (99 Ci/mmol), H-Org 2058 (42 Ci/mmol) were from Radiochemical Centre, Amersham (England). Buffers (TE) contained 0.01 M Tris-HCl, 1.5 mM EDTA ; 10 % (v/v) glycerol, 12 mM 1-thioglycerol, pH 7.0 at 25°C. Some buffers contained, in addition, 0.3 M KCl or 0.02 M Na_2MoO_4.

Cytosols : The cytosol of chick oviduct homogenates was prepared as described earlier (9). New Zealand rabbits (1 kg) received daily 100 µg diethylstilboestrol subcutaneously for 3 days. The uteri were removed and frozen in liquid N_2. Human Fallopian tubes were also kept frozen in liquid N_2 until homogenization. Cytosols were prepared as the chicken cytosols except for the ratio 1:3 (tissue:buffer) for the rabbit uteri.

Purification of B-subunit of chick oviduct PR : The purification procedure is described elsewhere (8). The purity of the B-subunit preparation was usually more than 90 % and in no case it contained demonstrable amounts of A- nor 90K-components of PR. For the last two booster injections, the two-step purified B-preparation was further purified in a 10 % slab gel electrophoresis containing 0.1 % SDS according to Laemmli (10). The 110K band was cut out, minced, and homogenized with Freund's incomplete adjuvant.

Immunization of the rabbit : A three-month-old male Fauve de Bourgogne rabbit (1.5 kg) was immunized with 50 µg of purified B-subunit (51 % pure in terms of specific activity) in 0.5 ml of the TE buffer containing 0.3 M KCl, and 0.5 ml of complete Freund's adjuvant (DIFCO, Detroit, MI). The major contaminants seen in polyacrylamide slab gel electrophoresis migrated in the positions of ovalbumin and myosin, but no A-subunit wad detected. The injections were made under each hind toepad and in the inguinal lymphatic nodal area. One month after the rabbit received a booster of 30 µg of 93 % pure B-subunit preparation in 1 ml of the buffer emulsified with 1 ml of Freund's incomplete adjuvant. The blood sample was collected 14 days after the booster. The third and fourth injections (about 30 µg each) were made 1.5 and 4.5 months after the second injection with the 110K band cut out of the SDS gel homogenized with Freund's incomplete adjuvant. The blood samples were collected at days 14, 16 and 18 after the respective injection and combined for IgG precipitation with 35 % ammonium sulphate. The corresponding IgG-fractions are numbered IgG-RB-1,2 and 3. The preimmune serum is IgG-R0. The concentration of IgG was adjusted to 30 mg/ml (stock solution).

Detection of anti-B subunit antibodies (IgG-RB) : For double immunoprecipitation (6), receptor samples (25 µl) were incubated with 1 or 5 µl of IgG fraction in 175 µl at 0°C for 2 h. The complexes were incubated for another 2 h with goat anti-rabbit IgGs (50 µl). The suspension was layered on 100 µl of 0.25 M sucrose and centrifuged. The bottom of the tube was cut out after freezing in liquid nitrogen. The Western blotting was performed according to Burnette (11) as described earlier (8). Glycerol (10-35 %) gradients were performed as in (5). Protein A-sepharose (12) was diluted 1:20 with sepharose CL-4B. This mixture (125 µl) was pipetted into a mini-column. Diluted serum (50 µl, dilutions 1:20-1:1000) was incubated with the gel for 2 h at room temperature and the column was then washed with 5 ml of 10 mM Tris-buffer pH 7.0. Receptor samples (50 µl) were then added to the column and incubated at 0-2°C for 2-4 h. The column was then placed over a hydroxylapatite (HAP) column (1 ml) and the non-bound receptor was washed with Tris-buffer onto HAP. HAP was washed with 20 ml of 3 mM potassium phosphate buffer pH 7.0 and counted to determine the amount of receptor not bound to the IgG's. The column of protein-A-sepharose was counted to determine the amount of IgG-bound receptor.

RESULTS

Titer and affinity : Specific antibodies were detected already after two injections of B. The titer of the serum increased considerably after the second booster injection. In the protein A assay (50 % recognizition of PR 100 pmoles), the titer of IgG-RB-1 was 1:20, whereas for IgG-RB-2 it was

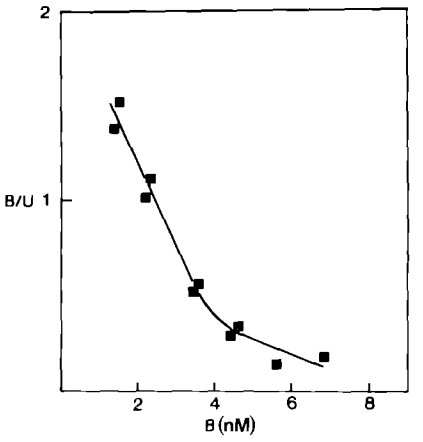

Figure 1. Scatchard plot of IgG-RB-2-PR binding. Five-fold concentrated cytosol PR was prepared by 30 % saturation with $(NH_4)_2SO_4$. The pellet was dissolved in 1/5 of the original volume. Serum dilution was 1:500. All incubation volumes were 50 µl. IgG-RB-2 bound ^{3}H-PR was determined by binding to protein-A-sepharose ; unbound ^{3}HP-PR was assayed by HAP.

1:200. Scatchard plot analysis suggested heterogeneity of IgG-RB-2 with a major high affinity population (Kd ∿ 2 nM) and a low affinity population (Kd ∿ 13 nM) (Fig. 1).

Effect on the ligand binding of PR : When unlabelled cytosol was incubated 2-4 h with varying amounts of preimmune serum or antiserum (0.1-30 µl) or of IgG-R0 or IgG-RB-2 (3-150 µg) at 0°C and thereafter postlabelled with 20 nM ^{3}HP for 2 h at 0°C, no significant effect on steroid binding was observed (data not shown).

Interaction with chick oviduct PR : IgG-RB-2 recognized the pure B-subunit as demonstrated by double immunoprecipitation (Table I), glycerol gradient

TABLE I. Double immunoprecipitation of the chick oviduct PR (results are expressed as percentage of the total receptor bound radioactivity)

	PR added	IgG-RB-2		IgG-RO		specific precipitation [IgG-RB-2]-[IgG-RO]	
	fmoles	1 µl	5 µl	1 µl	5 µl	1 µl	5 µl
Cytosol*	269	17.1	48.5	7.0	12.3	10.1	36.2
A_{as}	16	9.3	30.3	3.5	11.4	5.8	18.9
B_{as}	107	27.4	66.4	4.7	16.9	22.7	49.5
B_{as}	43	7.4	48.1	2.7	19.1	4.7	29.0
B_D	269	0.8	3.7	0.1	0.2	0.7	3.5
Cytosol* with the first antibody only							

*Cytosol prepared in TE-buffer without KCl. A_{as}, B_{as}, partially purified A and B-subunits as in (13). B_D : highly purified B-subunit as in (8). The calculated purity of B-subunit was 93 % assuming one binding site per the subunit.

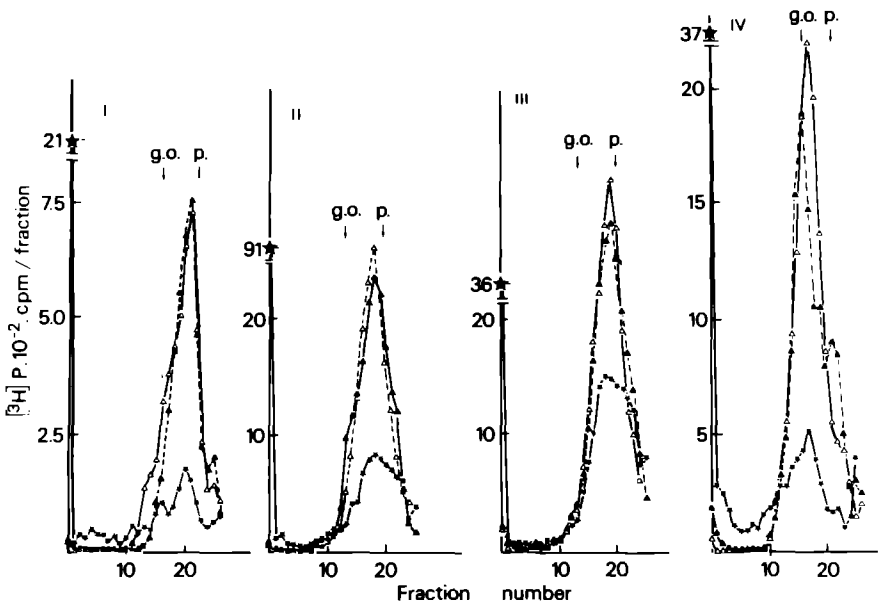

Figure 2. Glycerol (10 → 35 %) density gradient ultracentrifugation. Different amounts of ^{3}HP-PR after various purifications steps were incubated without (▲---▲) or with 200 µg of IgG-RB-2 (★—★) or 200 µg of pre-immune IgG-RO (△—△). I : Affinity eluate (first step in the purification of ^{3}HP-B (8)) 40 µl (purity ∿ 25 %) ; II : 20 µl of ^{3}HP-PR-B-subunit (purity ∿ 90 %) ; III : 40 µl of labelled A-subunit (purity ∿ 25 %) ; IV : 20 µl of molybdate-stabilized ^{3}HP-8S-PR eluted from the affinity gel (2). Purity of PR samples was calculated according to 1 binding site per 100K polypeptide chain. Internal markers : peroxidase (p, ∿ 3.6S) and glucose-oxidase (g.o, 7.9S).

ultracentrifugation (Fig. 2, panel II), Western blotting of SDS gels (Fig. 3, lane 5) and binding of ^{3}HP-B-IgG-RB-2 complexes to protein A-sepharose (Fig. 4). In Western blots, an ∿ 70K band was visualized in addition to the 110K band of the B by both IgG-RB-2 (Fig. 3, lane 5) and IgG-G3 (Fig. 3, lane 7) ; it may be a cleavage product of B. The A-subunit (79K) was also recognized (Fig. 2, panel III and Fig. 3, lane 4), but not the 90K protein (Fig. 3, lane 4). The molybdate-stabilized 8S-PR (which contains A and B) interacted with IgG-RB (Fig. 2, panel IV). Also nuclear PR, prepared as in (14) and the "mero-receptor" prepared as in (15,16), were recognized by IgG-RB-2 (Fig. 4). Protein A method was less reliable to study the A-subunit due to non-specific binding (up to 50 %) (Fig. 4).

Cross-reactivity with other steroid receptors : Fig. 4 shows that IgG-RB did not bind to chick glucocorticosteroid receptor labelled with ^{3}H-dexamethasone. According to the results obtained by the protein A assay, IgG-

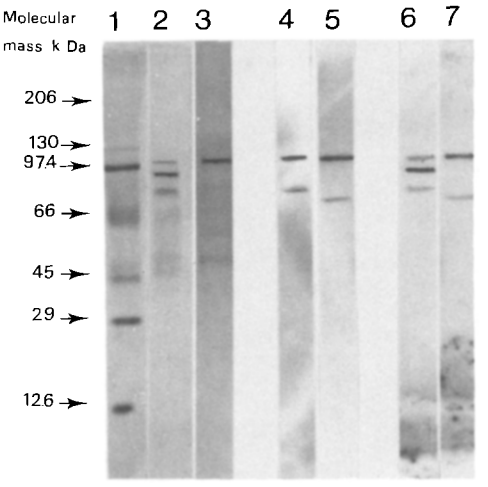

Figure 3. Western blotting of purified 8S and 4S-PR preparations with IgG-RB-2 and IgG-G3. Lanes 1-3 : silver staining of 10 % acrylamide slab gel of the 8S-PR purified by affinity chromatography purified as in (2) (lane 2), and of the B-subunit (81 % pure) (lane 3). Arrows indicate the position of markers (lane 1). Corresponding immunoblots with IgG-RB-2 (lanes 4 and 5) and IgG-G3 (lanes 6 and 7).

RB-1 and -2 cross-reacted weakly with chick estrogen receptor labelled with ^{3}H-estradiol and with the human and the rabbit PR (Fig. 4). These preliminary observations will be the matter of further investigation.

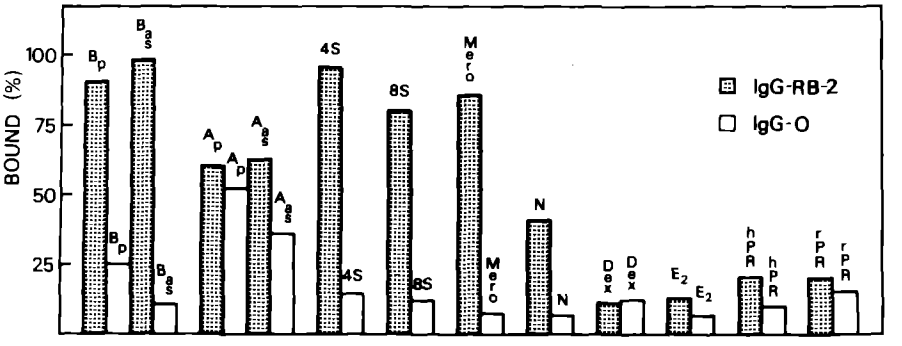

Figure 4. Binding of PR to protein-A-sepharose. The unbound PR is assayed by HAP. The results are expressed as percentage of bound PR. 50 µl of serum dilution (1:40) was used. A_p : 160 fmoles of affinity chromatography, and DEAE-sephacel, purified A-subunit (8) ; B_p : 430 fmoles (see Table I) ; B_{as} : 550 fmoles (see Table I) ; A_{as} : 70 fmoles (see Table I) ; 4S : 1130 fmoles of PR treated with 0.3 M KCl at 0°C for 2 h ; 8S : 1130 fmoles of PR with 20 mM sodium molybdate ; Mero : 65 fmoles of PR treated with 100 mM Ca^{2+} at 0°C for 1 h and fractionated with agarose gel filtration. N : 15 fmoles of nuclear PR prepared as described in (14). Dex : 160 fmoles of ^{3}H-dexamethasone labelled chick oviduct cytosol receptor (preincubated with 1 µM unlabelled progesterone). E_2 : 5 fmoles of ^{3}H-estradiol labelled chick oviduct cytosol receptor. hPR : 270 fmoles of human Fallopian tube cytosol PR. rPR : 540 fmoles of rabbit uterus cytosol PR.

Reactions with other components of chick oviduct cytosol : In the Western blotting (not shown), IgG-RB-2 revealed weak bands at the myosin and ovalbumin level. These bands, however, were also seen with IgG-RO. It is not clear whether they revealed non-specific interactions in the blots, or whether they indicated cross-reactivity of rabbit IgGs with chicken proteins. Immunodiffusion studies showed no precipitation lines with ovalbumin or lysozyme, nor with the chicken serum.

DISCUSSION

Several attempts made to produce antibodies in the rabbit and in the sheep against purified A- and B-subunits of PR have been unsuccesful (4, 17). We have obtained rabbit antibodies against the purified B-subunit. Anti-B-subunit antibodies were also present in IgG-G3, obtained in the goat against the purified 8S-PR (7). The titer of the present sera were relatively high already after the first booster injection. The antibodies persisted for a long time, since their titer decreased only to about half in 4.5 months.

IgG-RB not only recognized the B, but also the A-subunit, suggesting structural similarities between the two subunits. Such similarities between A and B have already been suggested on the basis of peptide mapping (18,19). It was carefully checked that A was lacking from the B preparations used for immunization. The main practical difference between IgG-RB and IgG-G3, raised against B-subunit and 8S-PR respectively (which contains A, B and the 90K protein), is demonstrated on Fig. 3 : IgG-G3 recognized, as expected, the 90K protein in contrast to IgG-RB.

It is therefore not surprising that IgG-RB reacts with the 8S-PR and the nuclear PR, and it is of interest that it still interacts with the so called "mero-PR" (15,16). Finally the cross-reactivity with mammalian PRs, suggests that there is some similarity between PRs of avians and mammals.

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