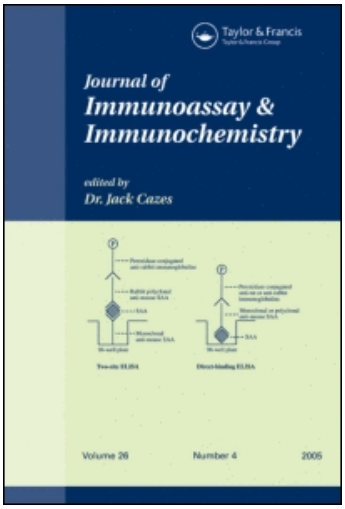

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A SOLID-PHASE RECEPTOR BINDING ASSAY FOR ^{125}I-hCG

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ABSTRACT

A solid-phase radioligand-receptor assay (RRA) to measure the binding of ^{125}I-labelled human chorionic gonadotropin (^{125}I-hCG) to target cell membranes has been developed. The binding of ^{125}I-hCG to membranes immobilized on the wells of microtitration plates reached a maximum at about 3 hours at 37°C, was saturable, displayed a high affinity ($Ka = 2.4 \times 10^{9}M^{-1}$) and was specifically inhibited by unlabelled hCG. In comparison with RRAs carried out with membranes in suspension, the solid-phase RRA is significantly simpler and much faster to perform as it avoids centrifugation or filtration procedures.

The solid-phase RRA was adapted profitably to process large numbers of samples at the same time. It proved particularly useful as a screening assay to detect anti-hCG monoclonal antibodies with high inhibitory activity for binding of ^{125}I-hCG to its receptors.

INTRODUCTION

Radioligand-receptor assays (RRAs) are of general use to measure the binding of a ligand to its receptors. As for many other ligands, human chorionic gonadotropin (hCG) RRAs are

commonly performed by incubating the hormone with suspensions of membrane fractions obtained from a target tissue. At the end of the incubation, the free and the bound ligands are then separated either by centrifugation, which usually involves one or more washes, or filtration. While adequate, both methods are cumbersome and time consuming whenever dealing with a large number of samples.

We are currently interested in various aspects of the hCG-receptor interaction, a study in which we make also use of monoclonal antibodies (Mabs). In our protocol the binding of ^{125}I-hCG to its receptors had to be assessed routinely and, therefore, we looked for a simple and rapid RRA avoiding, if possible, the centrifugation or filtration steps.

We have thus developed a suitable solid-phase RRA to measure the binding of ^{125}I-hCG to target tissue membranes immobilized on the wells of microtitration plates, in which the unbound ligand is simply removed by aspiration at the end of the incubation. The assay is easy and fast to carry out and proved particularly suited to process simultaneously large numbers of samples. For our purposes, it replaced very efficiently conventional RRAs performed with membrane suspensions.

MATERIALS AND METHODS

Hormones and reagents

hCG (CR-121, 13,450 IU/mg) and follicle stimulating hormone (NIAMDD-hFSH-2, FSH biopotency 3,925 IU/mg, luteinizing hormone (LH) biopotency 523 IU/mg) were kindly supplied by

the Center for Population Research (National Institutes of Health, Bethesda, MD). hCG was radioiodinated with ^{125}I-Na (Amersham, UK) by the lactoperoxidase method (1). The specific activity of the various batches of labelled hCG ranged from 50 to 70 μCi/μg. The active fraction of the iodinated hCG, i.e. the fraction of radioactivity associated with biologically active hormone, determined as the maximum specific binding of a sample of the tracer to an excess of testis receptors, was about 60%. Partially purified hCG (Profasi, 5,000 IU/mg) was purchased from Serono (Rome, Italy).

Affinity purified rabbit IgG anti-mouse Ig were labelled with ^{125}I-Na by the Chloramine-T method at a specific activity of 15-25 μCi/μg. Phenyl methyl sulphonyl fluoride (PMSF), N-ethyl-maleimide (NEM) and bovine serum albumin (BSA), fraction V, were obtained from Sigma. All other chemicals were reagent grade.

Preparation of testicular membranes

Porcine testes from 2-3 week-old animals were obtained at a local pig-breeding farm. Testes were kept on ice and processed within 2-3 hours. The tissue was minced with scissors, diluted 1:1 (w:v) with PBS (10 mM phosphate buffered saline, 150 mM NaCl, pH 7.4) containing 1 mM PMSF, 1 mM NEM and 10 mM NaN_3, and processed with an electric device (Ultra Turrax). The tissue to buffer ratio was then brought to 1:10 and the material was further homogenized with a glass homogenizer. The resulting homogenate was filtered through cheesecloth, centrifuged at 500 x g for 10 minutes and the pellet was discarded. The

supernatant was centrifuged at 30,000 x g for 30 minutes and the resulting pellet (referred to as membranes) was resuspended in PBS at the appropriate concentration and used in the solid-phase RRA as described below.

Solid-phase RRA

Forty μl aliquots containing 2 mg wet weight (60 μg protein) of testicular membranes were plated into 96-well microtitration plates (flexible, flat bottom microtiter plates, Dynatech). The plates were then incubated overnight at 37°C to dryness and stored at -20°C or at 4°C until used in the RRA. The wells were saturated with PBS containing 1% BSA (PBS-1% BSA) for 15 minutes at room temperature. The saturating solution was removed and the plates were incubated at 37°C with 25 μl of ^{125}I-hCG in PBS-1% BSA and (a) 25 μl of PBS-1% BSA (total binding) or (b) 25 μl of PBS-1% BSA containing 100 IU Profasi (non specific binding). At the end of the incubation time, the binding mixture was aspirated and the wells were washed three times with PBS. The wells were cut and the bound radioactivity was measured in a gamma counter. All determinations were run in triplicate. The specific binding was calculated by subtracting the non specific value from the total binding. The affinity of the hCG-receptor interaction was determined according to Scatchard (2) taking into account the active fraction of the tracer.

In RRAs carried out in the presence of anti-hCG Mabs, 25 μl of hybridoma supernatant, or 25 μl of protein A purified Mabs (3) in PBS-1% BSA, followed by 25μl of ^{125}I-hCG in PBS-1% BSA were added to the wells.

Preparation of anti-hCG Mabs

The antibodies described hereafter were derived from two separate fusions. In fusion 1 a (Balb/c x SJL) F_1 mouse was immunized subcutaneously with 100 μg hCG in PBS mixed with complete Freund adjuvant. Three weeks later 100 μg hCG in PBS were injected intraperitoneally and the animal was sacrificed three days after the second injection. In fusion 2 a Balb/c mouse was immunized with hCG according to the procedure described by Cianfriglia et al. (4) with a total of 115 μg hCG. Fusions were performed with spleen cells and mouse myeloma NS-1 cells using polyethylene glycol according to Galfré et al. (5). Cells from the fusions were plated in 96-well microtiter plates and clones were selected by growth in hypoxantine- aminopterine-thymidine medium. Culture supernatants were assayed for the presence of anti-hCG Mabs using a double antibody solid-phase radioimmunoassay (RIA): 40 μl-aliquots of 0.1 M carbonate buffer, pH 9.6, containing 1 μg hCG were plated in 96-well microtiter plates, desiccated overnight at 37°C and stored at -20°C until used. The wells were then saturated with PBS-1% BSA, incubated with 40 μl of supernatants from growing clones for 1 hour at room temperature, washed and incubated with ^{125}I-labelled rabbit IgG anti-mouse immunoglobulins again for 1 hour at room temperature. The wells were then washed and the bound radioactivity was measured in a gamma counter. The clones were considered positive if the amount of radioactivity bound was at least 10 times greater than the background value obtained with an unrelated Mab.

Other methods

The RRA with membranes in suspension was performed as previously described (6) except that 1.5 ml Eppendorf conical

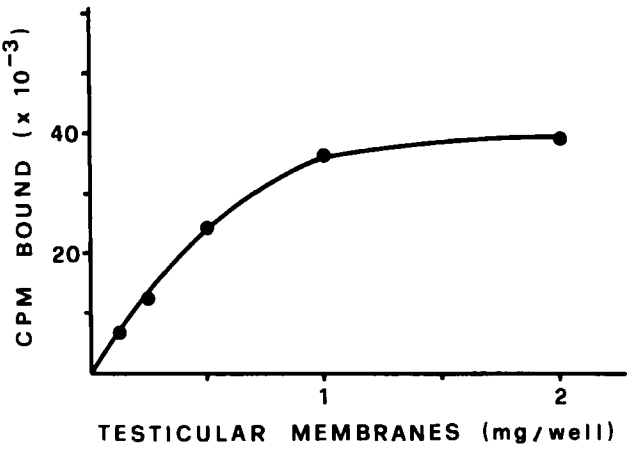

Figure 1: Specific binding of ^{125}I-hCG to testicular membranes in the solid-phase RRA. The plates were incubated at 37°C for 3 hours with ^{125}I-hCG (10^5 cpm).

tubes were used and centrifugations were carried out at 12,000 x g. The protein content of the membrane preparations was determined according to the method of Lowry et al. (7) using BSA as standard.

RESULTS

Features of the solid-phase RRA

Iodinated hCG could bind very efficiently to testicular membranes in the solid-phase RRA: an amount of 2 mg of membranes (wet weight)/well displayed a specific binding reaching approximately 40% of the added radioactivity (Fig.1).

The effects of repeated washings on the cpm bound was next investigated. Three to four washes were sufficient to reduce

TABLE 1

Binding of ^{125}I-hCG to Testicular Membranes Plated and Stored at -20°C and at 4°C for various Periods of Time.

Storage Time	Cpm Bound	
	Total	Non Specific
1) No storage	45,625 ± 938	1,923 ± 121
2) 7 Months at 4°C	45,163 ± 736	2,079 ± 92
3) 3 Months at -20°C	39,750 ± 1,803	2,463 ± 140
4) No Storage	38,435 ± 1,270	2,113 ± 127

The membranes (2 mg/well, wet weight) were incubated for 3 hours at 37°C with ^{125}I-hCG (10^5 cpm) with (non specific binding) or without (total binding) an excess of unlabelled hCG. Membranes in 1 and 2 were of the same batch but incubated with different preparations of ^{125}I-hCG. Membranes in 2, 3 and 4 were from different batches but were incubated with the same preparation of ^{125}I-hCG. The data are the mean ± SD of triplicate determinations.

the non specific binding to values around 3 to 7% of the specific binding depending on the membrane and ligand preparation (see Table 1), and further washes did not reduce appreciably the cpm bound.

The binding of ^{125}I-hCG to the immobilized testicular membranes reached the equilibrium after 3 hours incubation at 37°C (Fig.2). The process was saturable and , as determined by Scatchard analysis, displayed a high affinity (Ka = 2.4 x $10^9 M^{-1}$, Fig.3). A similar affinity (Ka = 6 x $10^9 M^{-1}$) was obtained in the classical RRA with membranes in suspension. The binding of ^{125}I-hCG could be completely inhibited by excess unlabelled hCG (Fig.4). A slight inhibition was observed with FSH only at the

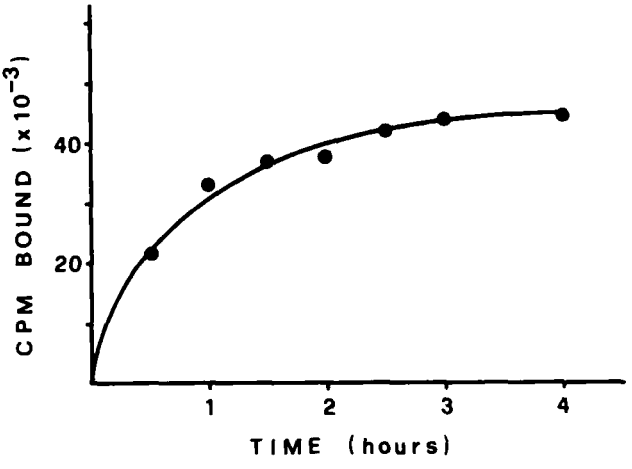

Figure 2: Time-course of ^{125}I-hCG (10^5 cpm) binding to testicular membranes (2 mg/well, wet weight) in the solid-phase RRA. Incubations were carried out at 37°C.

higher concentrations tested, consistent with the LH content of the FSH preparation used.

Furthermore, the solid-phase RRA could also be performed using luteal tissue from pig and pseudopregnant rat (data not shown).

Once immobilized on solid phase the binding activity of the testicular membranes was preserved for very long periods of time. In fact, plates stored at -20°C or even at 4°C, retained an adequate binding activity for several months (Table 1). In our experience the differences noted between stored and fresh preparations were negligible and, when detected, due to the target tissue preparations were the receptor content could vary slightly.

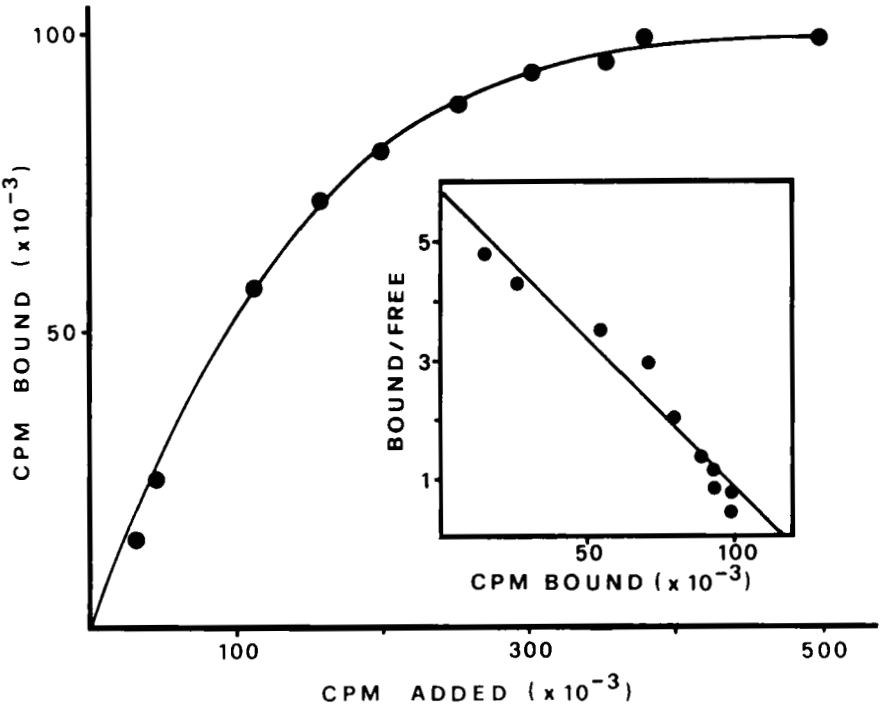

Figure 3: Saturation curve of ^{125}I-hCG binding in the solid-phase RRA. The membranes (2 mg/well, wet weight) were incubated for 3 hours at 37°C with increasing concentrations of ^{125}I-hCG with or without unlabelled hCG. Only specific binding is reported. Inset: Scatchard analysis of the binding data.

Selection of Mabs inhibiting hCG binding

We are currently interested in the preparation of antibodies directed against the hCG receptor. A possible approach to this scope is the use of anti-hCG Mabs as antigens to raise anti-idiotypic antibodies specific for the receptor molecule (8). In this respect, as discussed below, anti-hCG Mabs capable of inhibiting very efficiently the hormone-receptor binding are likely to re-

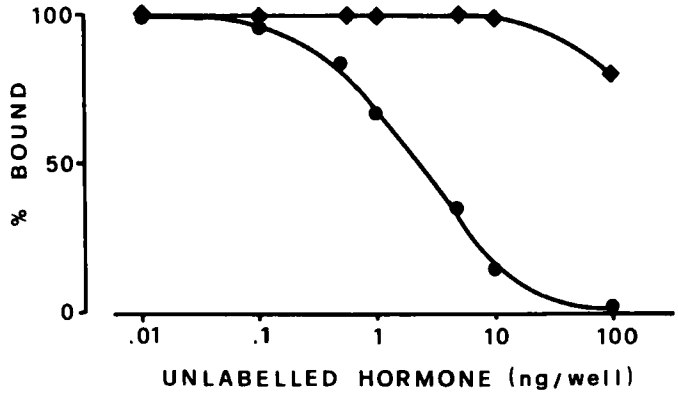

Figure 4: Inhibition of ^{125}I-hCG binding in the solid-phase RRA by increasing concentrations of unlabelled hCG (●----●) or FSH (◆----◆). The testicular membranes (2 mg/well, wet weight) were incubated with ^{125}I-hCG (10^5 cpm) and the indicated amounts of unlabelled hormones at 37°C for 3 hours.

present the most useful antigens. Therefore, we examined the suitability of the solid-phase RRA as a screening assay to detect readily anti-hCG Mabs with high inhibitory activity for the hormone-receptor binding.

The solid-phase RRA was as effective as a RRA performed with membrane suspensions to measure the inhibition of ^{125}I-hCG binding by anti-hCG Mabs (Table 2). Typical inhibition curves were obtained by performing the solid-phase RRA in the presence of increasing concentrations of affinity purified anti-hCG Mabs (Fig.5). In addition, the suitability of the solid-phase RRA to detect, directly in the screening step, cell clones producing anti-hCG Mabs which inhibited markedly (>50%) the ^{125}I-hCG binding, was demonstrated in fusion 2 where 4 out of 39 clones had strong inhibitory activity (Table 3).

TABLE 2

Inhibition by various Hybridoma Supernatants of the Binding of ^{125}I-hCG to Immobilized (Solid-Phase RRA) and Suspension (Tube RRA) Membranes.

	Solid-Phase RRA		Tube RRA		RIA
	cpm bound specific	inhibition %	cpm bound specific	inhibition %	
medium	37,585	-	34,604	-	723
161F2	22,792	39.4	21,249	38.6	37,527
163G2	311	99.2	117	99.7	38,474
164A2	104	99.7	62	100	39,781
166A11	35,682	5.1	33,508	3.2	36,430
209G2	36,559	2.7	33,666	2.7	428
209H9	36,313	3.4	35,507	0	632

Both RRAs were carried out with the same amount (2 mg/well or tube, wet weight) of testicular membranes. 161 F2, 163G2, 164A2 and 166A11, are anti-hCG Mabs obtained in fusion 1; 209G2 and 209H9 are anti-ricin Mabs (9) used as negative controls. The values obtained using the same supernatants in the hCG RIA are also reported.

DISCUSSION

The present experiments have shown that pig testis membranes can be assayed after adsorption to plastic wells with full retention of the hCG receptors' binding activity.

Indeed, the characteristics of the ^{125}I-hCG binding to immobilized membranes were found to be quite similar to those reported for its binding to suspensions of various hCG target tissue preparations (6,10,11). This made possible the development

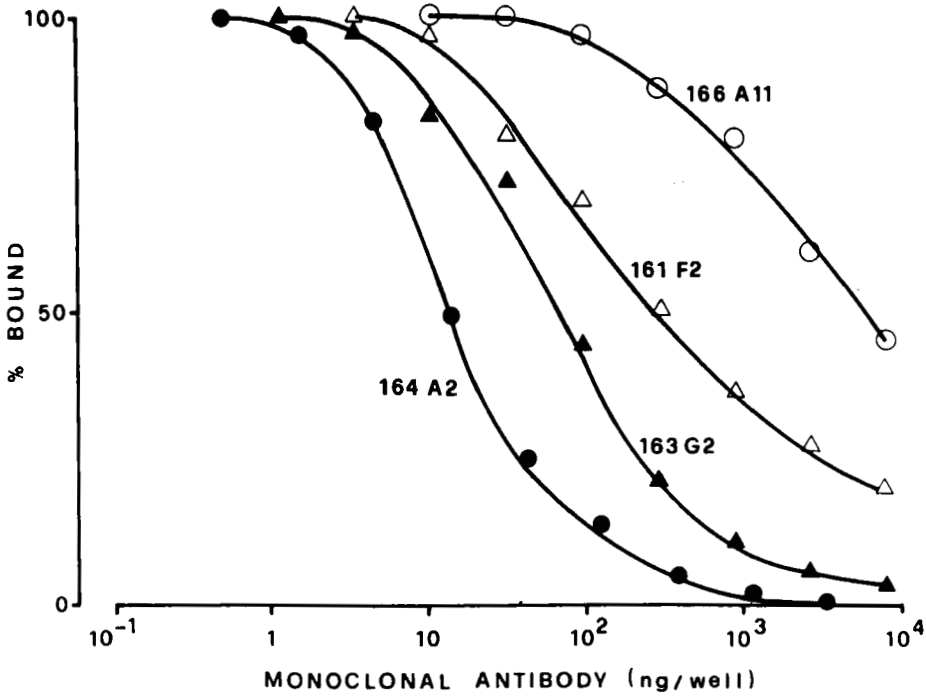

Figure 5: Inhibition by affinity purified anti-hCG Mabs of ^{125}I-hCG binding in the solid-phase RRA. Testicular membranes (2 mg/well, wet weight) were incubated for 3 hours at 37°C with ^{125}I-hCG (10^5 cpm) in the presence of the indicated amounts of Mabs.

TABLE 3

Results of Fusion 2

Wells plated	1152
Wells with growing Hybridomas	354
Wells with Supernatant positive in the hCG RIA	39
Wells with Supernatant inhibiting (>50%) the ^{125}I-hCG Binding in the Solid-Phase RRA	4

of the very simple and efficient solid-phase ^{125}I-hCG binding assay here described.

Testicular tissue of 2-3 week-old pigs was chosen for two main reasons. It displays a high hCG binding capacity, since hCG receptor-bearing interstitial cells account for more than 60% of the testicular volume at this age (12) and elevated ^{125}I-hCG binding values are obtained with the small amount of membranes which can be plated per well. Secondly, it is available at will and free of charge at a pig breeding facility.

Membranes adsorption to the wells was carried out overnight at 37°C, a procedure that might be questionable as a partial inactivation of hCG receptors at this temperature has been reported (10). If inactivation occurred in our hands it was negligible, probably due to the presence of protease inhibitors in the homogenization step. Moreover, the receptors' binding activity was retained for a long time if plated membranes were stored at -20°C or even at 4°C. This represents a very convenient feature as immobilized membranes can be prepared all in one experiment and used for the following months. The wells have only to be saturated for 10-15 minutes with BSA before use.

The various steps of the solid-phase RRA are significantly accelerated if loading of the reagents is done with a multipipette and washing steps carried out with a multi-way device. Typically, a 96-well plate can be loaded with ^{125}I-hCG in about one minute and it takes one or two minutes to wash it at the end of the incubation. Therefore, the solid-phase RRA allows a considerable time-saving in comparison to RRAs performed with membrane suspensions and its use could be introduced profitably in labora-

tories in which ^{125}I-hCG binding assays are carried out routinely.

RRAs performed with membrane suspensions have been used to measure hCG levels in biological fluids (13). In the present experiments we did not test the possibility to apply the solid-phase RRA to this purpose because this is out of the scope of our current research. However, since the characteristics of the ^{125}I-hCG binding to immobilized and suspended membranes resulted similar, it is conceivable that the solid-phase RRA should be as effective as other hCG-RRAs in this regard.

The solid-phase RRA is particularly advantageous in hybridoma technology which often requires the simultaneous processing of large numbers of samples. For example, inhibition of ligand binding represents a useful parameter to identify anti-receptor Mabs (14) and the solid-phase RRA provides a very convenient screening assay to detect such Mabs.

Anti-hormone Mabs, directed against the epitope of the hormonal molecule which combines with the receptor, can be used as immunogens to raise anti-idiotypic Mabs with specificity for the receptor (8). Anti-hCG (or anti-LH) Mabs useful for this purpose are conceivably among those which are more effective in inhibiting the hormone binding. Such Mabs were easily selected in the screening step of fusion 2 with the solid-phase RRA. This assay could also be employed to identify anti-idiotypic Mabs reacting with the receptors in the target cell membranes.

The advantage of using the solid-phase RRA instead of other RRAs is even greater in experiments requiring the combination of sequential incubations and repeated washings of the membra-

nes, such as the study of hormone-receptor complexes dissociation or the use of Mabs to study the role of different hormone or receptor epitope in the ligand-receptor binding (15).

The solid-phase hCG-RRA could then replace conveniently and effectively other RRAs performed with membrane suspensions in many applications and we feel it will be readily adaptable to other ligand-receptor systems.

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