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ENZYME IMMUNOASSAY OF PROGESTERONE AT THE PICOGRAM LEVEL USING β-GALACTOSIDASE AS LABEL

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Summary

We report here the first sensitive enzyme immunoassay of a hapten. A progesterone β galactosidase conjugate was prepared using carbodiimide as a bifunctional reagent. Rabbit progesterone antisera were previously obtained. The separation of the bound from the free fraction of the label was performed with the help of polymerized anti rabbit γ-globulins. The enzyme activity of the bound fraction was determined with *O*-nitrophenyl-β-D-galactoside as substrate. Specificity and sensitivity (≈15 pg) of this enzyme immunoassay can be successfully compared with radioimmunoassay performances. It thus provides a non radioactive, inexpensive and reliable method of small molecule quantitation.

Introduction

Hapten immunoassays require specific antisera of high affinity and strongly labelled haptens. Such antisera can be now obtained, thanks to hapten-carrier coupling in privileged locations and appropriate schedules of immunization. On the other hand, labelling of hapten with radioactive atoms (^{3}H, ^{125}I) is now extensively used for radioimmunoassays, but it has some disadvantages: (a) radiolabelled compounds are expensive, (b) their use can offer some dangers, (c) the introduction of radioactive atoms into small molecules can strongly modify their immunoreactivity, (d) radiolabelled haptens can be unstable and therefore require purification before use; moreover, radioiodinated molecules have a short half-life requiring frequent labellings.

Abbreviation: NPhGal, *o*-nitrophenyl-β-D-galactoside.

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In order to eliminate the disadvantages of radiolabelling, several authors tried to set up, during the last four years, immunoassays of haptens using other kinds of labelling. In this perspective, we examined the possibility of using hapten bacteriophage conjugate as label [1-4]. This kind of viroimmunoassay allowed us to quantify hapten at a lower threshold than radioimmunoassay but some aspects of this immunoassay hamper its use to a large extent. In the same way, some investigators [5,6], following the work of Avrameas [7] with antigen-enzyme conjugates, tried to realize enzyme immunoassays of small molecules. Their assays worked but the threshold of hapten detection has not approached that of conventional radioimmunoassays.

In this paper, we describe a sensitive progesterone enzyme immunoassay using β-galactosidase coupled to progesterone as label.

Materials and Methods

Buffer

Phosphate buffer at pH 7 containing 70 mM $Na_2H\,PO_4$, 30 mM NaH_2-PO_4, 1 mM $MgSO_4$, 0.2 mM $MnSO_4$, 2 mM Mg titriplex (ethylendiamine-tetraacetic acid magnesium dipotassium salt) was used throughout.

Steroids

Progesterone and other non-labelled steroids were purchased from Roussel Uclaf (France). [^{3}H]Progesterone, 110 Ci/mmol came from New England Nuclear. 11α-Hydroxy-4-pregnene-3,20 dione-hemisuccinate from Steraloids (U.S.A.).

β-Galactosidase

The β-galactosidase (EC 3.2.1.21) preparation contained 160 000 enzyme units per mg. The enzyme activity was determined by incubation at 45° C of 2 ml of a β-galactosidase solution with 0.5 ml of 0.4% σ-nitrophenyl-β-D-galactoside (NPhGal, Touzart et Matignon, France) containing 0.7% β-mercaptoethanol. After 3 h the reaction was stopped with 1 ml of 1 M sodium carbonate. The absorbance (A) was then measured at 420 nm with the help of an UNICAM SP 1 800 spectrophotometer. The calculation of the enzyme units number was done according to the following formula:

$$\text{Enzyme units number} = \frac{A \times d}{\epsilon \times t}$$

where A is the absorbance of the aliquot at 420 nm, d is the dilution factor of the aliquot, t is the NPhGal-β-galactosidase reaction time (min) and ϵ is the molar extinction coefficient of NPhGal in 1 M Na_2CO_3: $5 \cdot 10^{-3}$.

Progesterone antiserum

The conjugate, 11α-hydroxy-4-pregnene-3,20-dione hemisuccinyl-bovine serum albumin was realized as an immunogen using the mixed anhydride method [8]. The number of mol of progesterone per mol of bovine serum albumin was estimated to be around 20.1 mg of this immunogen (1 ml) emulsified in Freund's complete adjuvant (1 ml) was injected intradermally at 40 sites on

both flanks of female rabbits (3 kg) [9]. Then, Bordetella Pertussis (Perthydral, Institut Pasteur, Paris) was subcuteneously injected. 2 months later and then every month, a subcutaneous booster with 0.3 mg of immunogen in Freund's complete adjuvant was given. Rabbits were then bled weekly by central ear artery punction. After clotting, each serum was separated and examined by a radioimmunological procedure using [^{3}H] progesterone for its titre, affinity and specificity. The best antisera were pooled and use throughout this work.

Insolubilized sheep anti-rabbit γ-globulin

A sheep was primed with 10 mg of rabbit gamma globulins (fraction II, Miles, U.S.A.) according to the schedule described above. It was boostered following the same procedure with 2.5 mg of immunogen. The bleedings were tested by microprecipitation and the best titre ones were pooled. Their total IgG fraction was then isolated by a combination of $(NH_4)_2SO_4$ precipitation and DEAE-cellulose chromatography [10]. Afterwards, insolubilization of the preparation was obtained with the help of ethylchloroformate [11]. The precipitating capacity of this anti γ-globulin preparation was tested by mixing 0.1 ml of different dilutions of progesterone antiserum with increasing concentrations of this double antibody preparation. After equilibrium (30 min at room temperature), the mixture was centrifuged and the supernatant was examined for its progesterone antibody contents with [^{3}H]progesterone. This method allowed us to evaluate the insolubilized double antibody dilution enabling the total precipitation of progesterone antibodies at any concentration.

Experimental conditions

Experiments were performed in standard Kahn plastic tubes. Each tube contained either 0.1 ml buffer or 0.1 ml of free progesterone and 0.1 ml of a dilution of progesterone antiserum. After 30 min equilibrium at room temperature, 0.1 ml of a progesterone derivative, β-galactosidase conjugate solution was added and allowed to react at room temperature for different periods of time. At the end of these periods, 0.1 ml of the adequate insolubilized double antibody dilution required for a complete precipitation of progesterone antibodies was added for 30 min at room temperature. After centrifugation at 4000 rev./min, the pellets were washed three times and resuspended in 2 ml of buffer, this allowing the measurement of the enzyme activity as described above.

Radioimmunoassay of progesterone

A radioimmunoassay of progesterone was set up using the same pool of progesterone antiserum (dilution, D = 1/60 000) and [^{3}H]progesterone (8000 dpm) in order to compare the performances of both immunoassays. The separation of bound from free progesterone was performed with dextran-coated charcoal [5].

Results

11α-Hydroxy-4-pregnene-3,20-dione hemisuccinyl-β-galactosidase conjugate.

11α-Hydroxy-4-pregnene-3,20-dione hemisuccinate was covalently linked

to β-galactosidase by means of 1-ethyl-3-(3-dimethylaminopropyl hydrochloride) carbodiimide (Touzart et Matignon, France) according to the following procedure: 0.5 mg of 11α-hydroxy-4-pregnene-3,20-dione hemisuccinate in 1 ml was activated by 0.1 ml of 1 M carbodiimide. The pH was adjusted to 4.7 and the reaction mixture was allowed to stand at room temperature for 30 min. 5 mg of β-galactosidase in 1 ml of 0.1 M citrate buffer were then added and allowed to stand at pH 5.5 for 12 h at 4°C. At the end of this period the reaction was stopped by addition of 10 ml of phosphate buffer. This mixture was dialyzed overnight against 2 l phosphate buffer and then chromatographed on a Sephadex G-25 column (internal diameter = 10 mm, vol. = 10 ml) in order to eliminate any free non-reacting small molecules; the number of mol of hapten per mol of β-galactosidase have not been directly calculated. Different tests were performed in order to control the conjugation. The percentage of active enzyme surviving the coupling process was first estimated; it was near 90% (142 000 enzyme units/ml recovered against 160 000 enzyme units/ml put in reaction). A second test was performed in order to evaluate the percentage of immunoreactivity of the progesterone-β-galactosidase conjugate: 15 units (0.1 ml) of the progesterone-β-galactosidase preparation were mixed with concentrated progesterone antiserum (D = 1/500). This mixture was allowed to stand at room temperature for 24 h to make sure the equilibrium was reached. A volume of 0.1 ml of the double antibody preparation (D = 1/5) was then added for 30 min at room temperature. After centrifugation and washing (× 3), the enzyme activity was measured on the pellet where 4 out of 15 enzyme units originally put in reaction were found. This result allowed us to assume that only 26% (4/15) of the active enzyme was immunoreactive, so in the

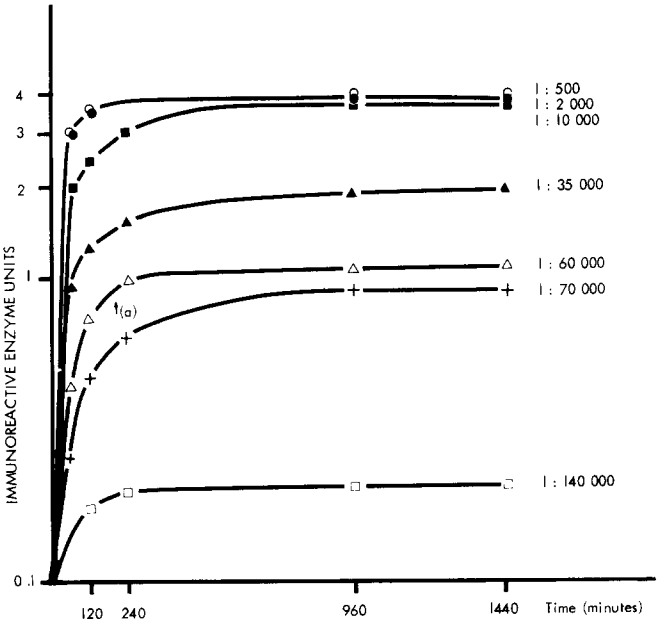

Fig. 1. Binding kinetics of progesterone-β-galactosidase conjugate for increasing dilutions of progesterone antiserum. (a) Antiserum dilution and incubation time used for the enzyme immunoassay.

further experiments only the pellet measurements will be taken into account. In last resort, the non-specific binding of the progesterone-β-galactosidase preparation was appreciated using normal rabbit serum at different dilutions instead of progesterone antiserum. Beyond a dilution of 1/500, this non-specific binding (blank) was found to be less than 0.1 enzyme unit which can be neglected.

Binding kinetics measurements

0.1 ml of increasing dilutions of progesterone antiserum (from D = 1/500 to D = 1/140 000) were added to a rack of tubes containing 0.1 ml of buffer and 0.1 ml of a progesterone-β-galactosidase preparation. These reaction mixtures were allowed to stand at room temperature from 30 min to 24 h. After addition of the double antibody preparation (0.1 ml), centrifugations and washings, the pellet enzyme activities were measured as described above. The results of this experiment were summed up in Fig. 1 where the log of the enzyme units contained in the pellet (i.e. immunoreactive enzyme units) was plotted versus time for dilution of the progesterone antiserum. According to this figure the binding reaction followed a first-order kinetics process for 1/500 to 1/2000 antiserum dilutions. For greater dilutions, the equilibrium was reached after a second-order kinetics process.

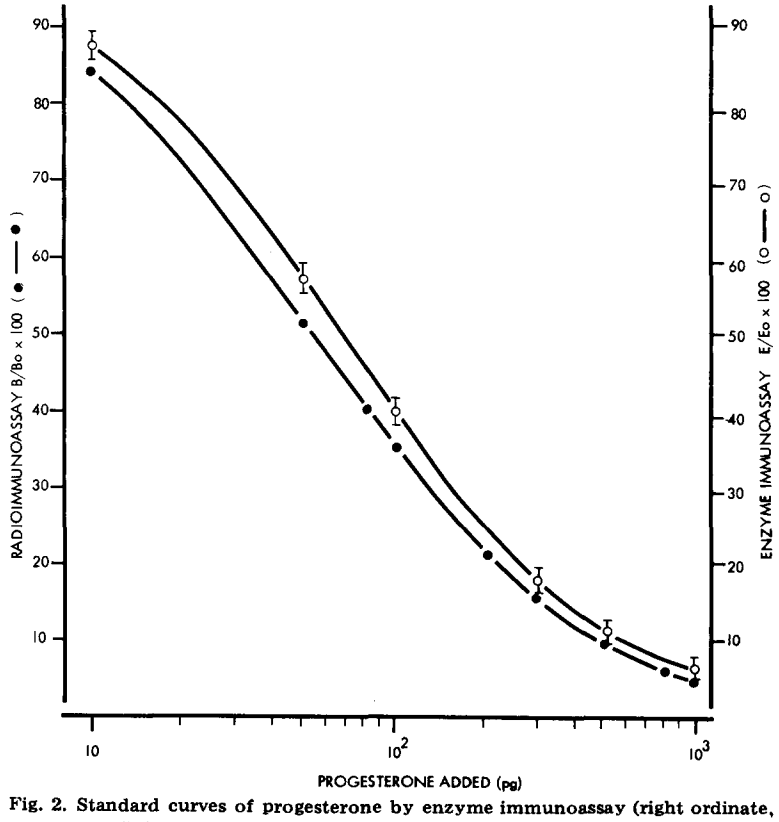

Fig. 2. Standard curves of progesterone by enzyme immunoassay (right ordinate, ○———○) and radioimmunoassay (left ordinate, ●———●). E/E_0 × 100 is the percentage of bound enzyme with and without progesterone added. B/B_0 × 100 is the percentage of bound radioactivity with and without progesterone added. $\overset{\frown}{\underset{\smile}{}}$ = ±1 S.D. on 10 samples.

TABLE I

PERCENTAGE OF CROSS REACTIVITY OF RELATED STEROIDS BY ENZYME-IMMUNOASSAY AND RADIOIMMUNOASSAY

$E/E_0 \times 100$ = percentage of bound enzyme activity with and without steroid added. $B/B_0 \times 100$ = percentage of bound radioactivity with and without steroid added.

	Enzyme-immunoassay ($E/E_0 \times 100 = 50\%$)	Radioimmunoassay ($B/B_0 \times 100 = 50\%$)
Progesterone	100	100
Deoxycorticosterone	10	5
6β-Hydroxyprogesterone	3	1.7
Corticosterone	1.4	1
5β-Pregnane-3,20-dione	1.2	2

Inhibition of binding of progesterone-β-galactosidase conjugate with progesterone antiserum by free progesterone and related steroids

These experiments were performed with the dilution of progesterone antiserum (D = 1/60 000) enabling the binding of about one out of the four immunoreactive enzyme units put in reaction within 3 h. Increasing amounts of progesterone and related steroids in 0.1 ml were preincubated at room temperature with 0.1 ml of the dilution chosen above for 30 min (equilibrium time). A volume of 0.1 ml of progesterone-β-galactosidase was then added to each tube for 3 h. Fig. 2 shows the dose/response curve (mean ± S.D.) of the progesterone enzyme-immunoassay where the percentage of bound immunoreactive enzyme in the presence and in the absence of inhibiting steroid ($E/E_0 \times 100$) is plotted versus increasing amounts of free progesterone added. 15 pg of progesterone can be measured with this enzyme immunoassay at E/E_0 = 80%. The curve obtained by radioimmunoassay is plotted on the same figure. It allowed us to measure 12 pg at B/B_0 = 80%. Table I compares for both methods, the percentage of cross reactivity of related steroids which is the amount of steroid which displaces half of the bound label (either radiolabel or enzymolabel) compared to the amount of progesterone giving the same displacement.

Discussion

Requirements for a useful assay of small molecules of endogenous or exogenous origin in biological fluids are (a) specificity and sensitivity adapted to the molecule to quantify, (b) good intra-assay and inter-assay reproducibility, (c) technical ease, (d) use by the greatest possible number of laboratories, (e) inexpensiveness. Radioimmunoassay of small molecules satisfies only the first three items mentioned above, because radioactively labelled compounds are available only to a few laboratories and the technical assistance required is still too expensive. In order to satisfy the two last items, we set up this immunoassay using a hapten-β-galactosidase conjugate as label.

We chose β-galactosidase for several reasons: (a) it is not present in human or animal biological fluids, (b) it can be obtained from *Escherichia coli* in large amounts, (c) it has a high catalytic number, (d) it can be obtained in a highly

purified and stable form. The easiness of the coupling reaction with the help of carbodiimide and the very high percentage (90%) of surviving active enzyme through the process have to be emphasized. Furthermore, this hapten-β-galactosidase conjugate when stored at 4°C was very stable: we have been using the same preparation for more than a year.

For the enzyme immunoassay, we chose the highest antiserum dilution, which within a definite period of time (3 h), enabled us to bind an easily measurable number of enzyme units. For this antiserum dilution (D = 1/60 000), the equilibrium was not completely reached within 3 h (Fig. 1 arrow) but the slowness of the binding kinetics process allowed us to stop the reaction by the double antibody addition without great risk of error within a relatively large period of time (10 min). This slowness of the kinetic process seems to be due to the high molecular weight of the β-galactosidase (M_r = 50 000). This weight provokes an important steric hindrance for the binding of progesterone antibodies to the few progesterone molecules (probably no more than three after indirect calculation) linked per molecule of β-galactosidase.

The performances and the validity of this enzyme immunoassay were compared to the radioimmunoassay of progesterone using [^{3}H]progesterone as tracer. The dose/response curves of both assays have the same slope and almost the same detection limit (Fig. 2). Furthermore the percentage cross reactions against the most related steroids are similar (Table I). Finally in order to test the immunoassay on biological fluids, we measured progesterone in the plasma of 20 pregnant rats by both assays. After petroleum ether extraction, there was no significant difference between the mean values (correlation coefficient = 0.984 $Y = 0.965X + 1.060$ (±5.521)).

The main conclusion from this study is that by using the progesterone-β-galactosidase conjugate, it is possible to set up a quantitative enzyme immunoassay of which the performance successfully competes with radioimmunoassay. In addition such an assay can be easily realized without expensive technology. This model of enzyme immunoassay could be extended to many haptens by their linkage to β-galactosidase and thus might be used on a large scale in medical laboratories.

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