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Measurement by Radioimmunoassay of Casein Content in Rabbit Mammary Gland during Pregnancy and after Prolactin Stimulation in Organ Culture (42440)

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Abstract. A specific homologous radioimmunoassay was developed to measure rabbit β-casein in rabbit mammary gland with a sensitivity of 0.5 ng/ml protein. It was used to measure casein concentration during pregnancy and in organ culture of mammary gland explants. Casein was detectable in virgin mammary glands, showed a small increase during the first half of pregnancy, increased more than 20-fold between Days 21 and 27, and diminished somewhat on the first days of lactation. After 24 hr of culture, mammary gland explants had no detectable casein, but the addition of increasing concentrations of prolactin to a culture medium which contained insulin (5 μg/ml) and cortisol (0.5 μg/ml) induced a regular increase in the casein content of the tissue. Casein started to increase when 10 ng/ml of prolactin was present and maximal values were achieved for 100 ng/ml of the hormone.

The activity of the mammary gland is regulated by multiple interactions between peptide and steroid hormones, as well as by the state of the differentiation of the gland (1). Prolactin is essential for the initiation of synthesis of milk proteins in the rabbit, and its effects are amplified by glucocorticoids and insulin, and inhibited by progesterone, both *in vivo* and *in vitro* (2). Synthesis of milk proteins (caseins and α-lactalbumin) and their mRNAs provides a good biochemical index to study the biosynthetic activity of the mammary gland.

The synthesis of caseins, the major milk proteins, has been estimated for years using the Ca^{2+}–rennin precipitation method (3). Later, casein synthesis was estimated by immunoprecipitation of ^{14}C-labeled proteins synthesized after adding ^{14}C-labeled amino acids to mammary gland explants (4, 5) or in a cell-free translation system after adding casein mRNA (6, 7). More recently a radioimmunoassay for the measurement of rat β-casein (8–10) and for rabbit α-casein and α-lactalbumin (11) has been developed successfully. The first method is not strictly specific and it may reflect phosphorylation of casein rather than true biosynthesis. This drawback is not encountered with the second method, but the latter may not be very sensitive and reflects more the translational capacity of casein mRNAs than the true casein accumulation in the tissue.

In the present study, we have developed a specific radioimmunoassay for rabbit β-casein, and we have used this method to analyze casein synthesis during pregnancy and after culture of mammary gland explants in response to prolactin.

Materials and Methods. *Purification of casein and preparation of anti-rabbit casein.* Crude rabbit caseins obtained by precipitation at pH 4.6 were fractionated on DEAE-cellulose in the presence of urea, essentially as described by Testud and Ribadeau-Dumas (13). The peak corresponding to fraction IV, as defined by these authors, was repurified to homogeneity in similar conditions on DEAE-cellulose. The resulting protein was identified as β-casein by the primary sequence of its signal peptide (14) and of its NH_2-terminal part. Anti-rabbit β-casein was obtained in a sheep by monthly immunization with Freund's complete adjuvant. The sheep serum was lyophilized and stored at −20°C. Before use, it was dissolved in distilled water at a concentration of 50 mg/ml.

Anti-sheep immunoglobulin was prepared in rabbits and used as a second antibody for precipitation of the casein-anticasein complex.

Iodination of casein. Purified β-casein was iodinated by a modification of the method of

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Hunter and Greenwood. Casein (5 μg) was incubated with 800 ng of Chloramine T and 1 mCi of ^{125}I-labeled Na for 3 min at room temperature. The reaction was stopped by adding sodium metabisulfite (1 μg) and by dilution in assay buffer. The iodinated protein was purified in a column of acrylamide–agarose (ACA 54, LKB, 0.9 × 20 cm). The tubes containing the radioactive protein were pooled and stored at −20°C after the addition of an equal volume of glycerol.

Radioimmunoassay. Samples of standard β-casein were diluted in assay buffer (0.15 *M* NaCl, 0.1% NaN_3, 0.1% Triton X-100, 0.05 *M* sodium phosphate, pH 7.6) containing 0.1% BSA, to a volume of 300 μl. Antiserum (200 μl) diluted in assay buffer containing 0.25% normal sheep serum was added, followed by 2–3 × 10^4 cpm of ^{125}I-labeled casein in 100 μl. A 1/2000 initial dilution of the antiserum was found to specifically bind approximately 50% of the labeled casein in the absence of unlabeled casein. The mixture was incubated overnight at room temperature. To separate bound from free ^{125}I-labeled casein, 200 μl of appropriately diluted rabbit anti-goat γ globulin serum was added and the tubes were incubated for 1 hr at room temperature. Afterward 1 ml of a solution of 12.5% polyethyleneglycol 6000 was added to accelerate precipitation and the tubes were centrifuged for 30 min at 5000*g* (15). The supernatants were discarded and the radioactivity was counted in a gamma counter (LKB) with an efficiency of 50%.

Validation of the assay. The specificity of the antiserum was analyzed for cross-reaction with different dilutions of purified rabbit, bovine, rat, human, and goat caseins; sheep casein; and rabbit α-lactalbumin. In another experiment tissue specificity was determined using extracts of different rabbit tissues (muscle, adipose, heart, and ovary). To demonstrate parallelism of the immunoreactivity between the purified casein and endogenous casein in the assay samples, serial dilutions of mammary gland homogenates were tested. The accuracy of the assay was checked by measuring the recovery of four different concentrations of unlabeled casein added to mammary gland homogenates.

Preparation of samples. Approximately 20–30 mg of tissue obtained from mammary glands of pregnant or lactating rabbits or cultured mammary explants were homogenized in 2 ml of cold assay buffer with a Polytron homogenizer at a setting of 5 for 30 sec. The homogenates were centrifuged for 30 min at 5000*g* at 4°C and the supernatants were stored at −20°C until assay. An aliquot of the supernatant was taken for protein determination by the method of Lowry *et al.* (16). Afterward, the homogenates were diluted with a quantity of assay buffer adequate to provide 10 μg protein in 50 μl.

Animal studies. Nulliparous New Zealand rabbits were mated and then killed at the designated days of pregnancy and lactation. The mammary glands were removed and a portion of tissue was processed as indicated above.

Organ culture. Explant cultures of mammary tissue from 12- to 14-day pseudopregnant rabbits was performed essentially as described (12). Briefly, explants (approximately 2 mm^3) were cultured for 24 hr in TC 199 medium (GIBCO) with added essential amino acids and 5000 IU/ml penicillin and 10 μg/ml streptomycin in an atmosphere of 57% O_2, 5%CO_2, 38%N_2. The hormones added were porcine insulin (5 μg/ml), hydrocortisone (5 μg/ml; Roussel UCLAF), and different concentrations of ovine prolactin (30 IU/mg; NIH-P-S13). At the end of the culture the explants were collected, weighed, and frozen at −20°C until processed for casein assay.

Results. *Casein radioimmunoassay.* As shown in Fig. 1, increasing concentrations of purified unlabeled rabbit β-casein displaced binding of ^{125}I-labeled casein with the typical sigmoidal curve with a range from 0.5 to 500 ng/ml. Increasing dilutions of lactating mammary gland homogenates displaced ^{125}I-labeled casein in a way parallel to the purified β-casein curve. In contrast, homogenates of other rabbit tissues prepared in the same way as mammary gland were completely unable to compete with the radioactive casein. A recovery assay performed by adding 3, 6, 12, or 24 ng/ml casein to mammary gland homogenates showed a 99 ±5% recovery that was constant for all the quantities added. The antiserum showed very weak cross-reactivity with rabbit lactalbumin only at the highest concentration (10 μg/ml), which was probably due to a slight contamination of this preparation with rabbit β-casein (Fig. 2). There was no cross-reactivity

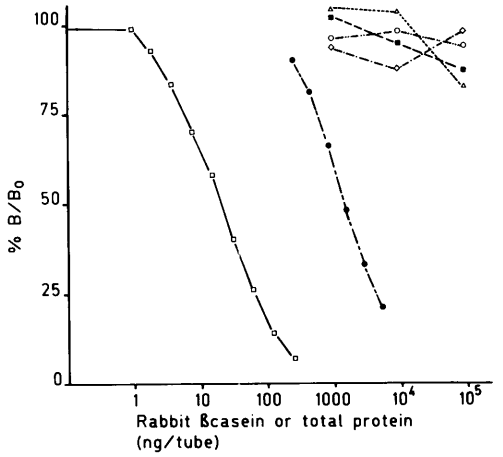

FIG. 1. Displacement curves of purified unlabeled rabbit β-casein (□) and dilutions of homogenates from lactating rabbit mammary glands (●), muscle (○), adipose tissue (◇), heart (△), and ovary (■).

with bovine, rat, human, goat, or sheep caseins (Fig. 2).

Casein content of rabbit mammary glands during pregnancy and early lactation. Figure 3 shows that casein can already be detected in virgin rabbit mammary glands (Day 0 of pregnancy). Casein concentrations remained low and unchanged until Day 13, and then showed a small increase on Day 21 and increased more than 20-fold between Days 21 and 27. After parturition there was a small decrease.

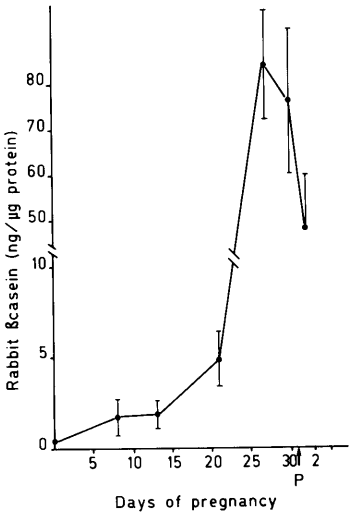

FIG. 3. Determination of β-casein in rabbit mammary gland during pregnancy and early lactation. Casein was determined in mammary gland homogenates by radioimmunoassay as described in the text. Results are the means ± SEM values of groups of four rabbits.

Prolactin stimulation of casein synthesis in culture of mammary gland explants. Mammary explants were cultured for 24 hr in the presence of different concentrations of prolactin, 5 µg/ml of insulin, and 0.5 µg/ml of hydrocortisone. Figure 4 shows that 10 ng/ml ovine prolactin stimulated casein accumulation significantly and the maximal effect was

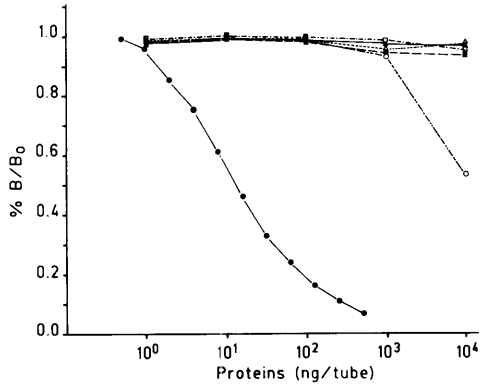

FIG. 2. Specificity of the radioimmunoassay for rabbit casein. Displacement curves of rabbit α-lactalbumin (○), ovine (□) and bovine (◇) β-casein, rat (△), human (■), and goat (▲) total caseins in comparison with standard rabbit β-casein (●). Sheep casein and rabbit β-lactalbumin were gifts of Dr. J. C. Mercier.

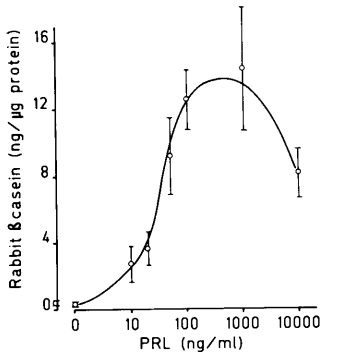

FIG. 4. Influence of prolactin on casein production by pseudopregnant rabbit mammary gland explants in culture. Explants were cultured for 24 hr in the presence of different concentrations of ovine prolactin, insulin (5 µg/ml), and hydrocortisone (0.5 µg/ml). Results are the means ± SEM of 11 independent cultures.

seen at 100 ng/ml. At 10 μg prolactin/ml, the concentration of casein in the tissue decreased.

Discussion. We have developed a sensitive, specific, and reliable radioimmunoassay for rabbit β-casein which can detect 0.5 ng casein/ml, or as little as 0.05 ng/μg protein in the mammary gland or explants under the standard conditions of sample preparation. The dilution curve of mammary homogenates was parallel to the rabbit β-casein standard curve, and this protein was only detected in mammary gland homogenates, demonstrating tissue specificity. The antibody was specific for rabbit casein, showing no reaction with caseins from other species and only a slight cross-reaction with the α-lactalbumin preparation.

This radioimmunoassay proved to be much more sensitive and accurate in detecting casein in the mammary gland than the immunoprecipitation method previously used (4, 5). However, several points should be kept in mind. First, as opposed to immunoprecipitation of newly synthesized [^{14}C]casein, the radioimmunoassay measures the total casein accumulated in the tissue. This contributes to an amplification of the response and to an enhancement of the sensitivity of the determination. By contrast, other parameters such as casein mRNA concentration or immunoprecipitation of [^{14}C]casein more precisely reflect the dynamic state of the tissue when the sample is taken. The radioimmunoassay of casein, therefore, appears preferable to the immunoprecipitation method, if the conditions of the above-mentioned restrictions are considered. In a previous study (7), it was shown that the onset of casein synthesis, estimated by immunoprecipitation, takes place by Day 18 of pregnancy in the rabbit, which coincides with the visible appearance of milk in the gland and with accumulation of casein mRNA. The results described in this paper using radioimmunoassay confirm that a marked casein synthesis occurs after Day 20 of pregnancy. However, a small but significant accumulation of casein, which could not be detected by immunoprecipitation, was seen in the early stages of pregnancy. Interestingly, the first increase in casein takes place after the first week of pregnancy, at a time when the first alveolar cells appear and when casein mRNA concentrations also increase (7). The casein mRNAs present in early pregnancy are therefore translated, albeit to a low extent, as opposed to the earlier conclusion (7). This is compatible with the fact that 33% of the casein mRNAs are recruited on polysomes at midpregnancy (20). This is also in agreement with observations made in other species (6, 9, 7, 16–19).

The measurement of casein content in mammary explants cultured in the presence of increasing concentrations of prolactin gave results in agreement with those obtained when casein synthesis was measured by immunoprecipitation (12), but the amplitude of the phenomenon was much higher with the radioimmunoassay (60-fold compared to 5-fold with the immunoprecipitation technique) and more sensitive, since this method was able to detect a stimulatory action of prolactin at a concentration as low as 10 ng/ml.

In conclusion, the radioimmunoassay of casein is a sensitive and accurate method for studying the hormonal response of the mammary gland in small amounts of tissue, and particularly for precisely analyzing the modulatory effects of different hormones on prolactin induction of casein synthesis in organ culture.

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