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Radioimmunoassay (RIA) of Relaxin in Sera of Various Species Using an Antiserum to Porcine Relaxin (39377)

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Recent work from our laboratory led to the development of a radioimmunoassay (RIA) for porcine relaxin utilizing [^{125}I]polytyrosyl relaxin (1). Addition of tyrosine to the relaxin molecule proved to be necessary as the purified native hormone does not contain tyrosine or histidine (2) and fails to iodinate when subjected to the Hunter and Greenwood method (1). An impure relaxin extract was used by other workers who claimed successful iodination of the hormone (3).

We have previously demonstrated cross-reactions of antisera to porcine relaxin with relaxin-containing tissues of several species including rats, mice, chickens, whales, and human beings (4). Because of this broad range of immunological cross-reactivity of relaxin from different sources, it was of interest to develop a RIA for determination of blood levels of relaxin in species other than the pig. We have now assayed relaxin levels throughout pregnancy in rats, mice, and guinea pigs, and near term in dogs and primates.

Materials and methods. Radioimmunoassay. The relaxin RIA has been modified since the publication of the original report (1).

Reagents. Two fractions of porcine relaxin prepared according to the procedure of Sherwood and O'Byrne (2) were used in the development of the method. A moderately pure fraction (G-50:1000 U/mg) was used for immunization and a highly purified fraction (CM-A + B: 2500 to 3000 U/mg) was used for standards and preparation of polytyrosyl-relaxin. The assay was equally sensitive and linear when we used a purified relaxin prepared by a different method and kindly supplied by Dr. Christian Schwabe, Medical College of South Carolina. Thus, the antigenic determinants of porcine relaxin do not appear to be altered by differing routes of biochemical purification. However, reduced-alkylated relaxin as well as isolated α and β subunits of relaxin did not compete with [^{125}I]polytyrosyl-relaxin for binding sites on the antibody. Although relaxin precursors and/or metabolic products may be immunoreactive, these observations suggest a requirement for the overall structure of two peptide chains connected by disulfide bonds for antibody recognition. Polytyrosyl-relaxin, [^{125}I]polytyrosyl-relaxin, and relaxin antisera were prepared as previously described (1). Goat anti-rabbit gamma globulin was purchased from Antibodies Incorporated, Davis, California.

RIA procedure. 1. One-hundred microliters of relaxin standard solutions [10 to 2500 pg relaxin in phosphate buffered saline (PBS = 0.01 *M* sodium phosphate, pH 7.0, 0.14 *M* sodium chloride)–1% egg albumin] plus a volume of control serum or plasma (male or castrated) equivalent to that of the unknown samples were added to standard curve tubes.

2. Unknown serum or plasma samples were added to other tubes.

3. Sufficient PBS–1% egg albumin was added to each tube to bring the volume to 500 μl.

4. One-hundred microliters of [^{125}I]polytyrosyl-relaxin in PBS–1% egg albumin (30,000 to 60,000 cpm) were added to each tube.

5. One-hundred microliters of relaxin antiserum diluted in 0.05 *M* EDTA-PBS containing 6% male rabbit serum were added to each tube. (A 1:50,000 working dilution of our antiserum binds 30–40% of the [^{125}I]polytyrosyl-relaxin when no competing unlabeled relaxin is present in the tube).

6. The tubes were then incubated at 4° for 24 hr.

7. Antibody-bound [^{125}I]polytyrosyl-relaxin was precipitated by one of the following two methods. (a) Double-antibody: Two-hundred microliters of diluted goat

anti-rabbit gamma globulin (50 μl + 150 μl PBS) were added to each tube. The tubes were incubated at 4° for an additional 24 hr and then centrifuged. (b) Polyethylene glycol: A sufficient volume of 25% w/w aqueous solution of polyethylene glycol (Carbowax 6000) at 4° was added to each tube to make the final concentration of the polymer 12.5% (5). The tubes were shaken and then centrifuged. Polyethylene glycol precipitates gamma globulin (5) and thus offers an economical and fast method for separating antibody-bound hormone from free hormone. However, as the volume of serum assayed is increased the number of nonspecific counts precipitated by polyethylene glycol is increased, thereby reducing the sensitivity of the assay. When relaxin levels were so low that more than 100 μl of serum or plasma were needed for a measurable response, the double-antibody procedure became the method of choice. This was actually the case with the samples of serum obtained from pregnant women.

8. The supernatants were aspirated and the precipitates were counted in an automatic gamma counter (Packard Auto-Gamma Spectrometer).

9. Standards and unknowns were assayed in triplicate. Logit and log transformations (6) were used to obtain linear dose response curves.

RIA of porcine relaxin added to sera of other species. Standard curves were constructed using known amounts of porcine relaxin added to serum or plasma obtained from male rats and ovariectomized women.

Porcine relaxin standards were added to 0.5-ml samples of pooled plasma obtained from ovariectomized women then incubated at 37° for 24 hr prior to RIA to determine if human serum contained a "relaxinase" or otherwise destroyed the hormone.

Porcine relaxin was injected im in a baboon and blood samples were taken at frequent intervals for RIA. The hormone was administered at a dose of 1 mg in saline solution, then 6 hr later 1 mg in a gelatin repository solution.

Relaxin levels in sera of pregnant individuals. Groups of timed pregnant mice (Charles River CD-1), rats (Charles River CD), and guinea pigs (Charles River Hartley) were bled at frequent intervals throughout pregnancy and after parturition, and relaxin levels were determined by RIA.

RIA relaxin was also determined in blood samples obtained from late pregnant dogs, java and rhesus monkeys, baboons, and human beings. Control blood samples were drawn from nonpregnant individuals and males of the above species.

Results. Addition of 100 μl of male rat serum to each sample tube did not significantly alter the linear regression of the standard curve for porcine relaxin prepared in PBS–1% egg albumin (Fig. 1). Similarly, pooled plasma obtained from ovariectomized (ovx) women did not affect the standard curve obtained with porcine relaxin. Incubation of porcine relaxin in ovx human plasma at 37° for 24 hr did not influence the reactivity of the hormone with the antiserum (Fig. 2).

Following im injection of a saline solution of 1 mg of purified porcine relaxin into a female baboon, the serum concentration of RIA relaxin rose to a peak at 2.5 hr and then declined by 4.5 hr (Fig. 3). A second im injection of 1 mg of porcine relaxin was given in a 20% gelatin retardant vehicle 6 hr after the initial injection. A much smaller but protracted increase in serum RIA relaxin was observed for the duration of the experiment (Fig. 3).

RIA relaxin was first detected in serum on Day 9 of pregnancy in rats (Fig. 4). The

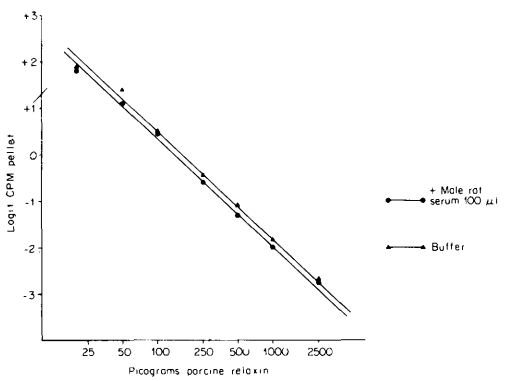

FIG. 1. Porcine relaxin standard curves in buffer or in buffer containing 100 μl of male rat serum. The linear regression for the two curves is as follows. Correlation coefficient: buffer = −0.9989; buffer + male serum = −0.9984. Slope: buffer = −1.0213; buffer + male serum = −1.0273. Logit intercept: buffer = 5.2570; buffer + male serum = 5.1382.

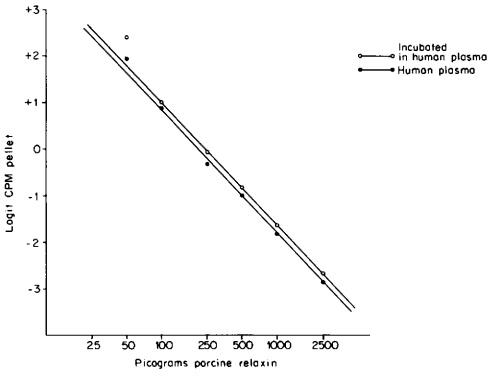

FIG. 2. Porcine relaxin standard curves. Open circles indicate samples which were incubated at 37° for 24 hr in human plasma. Closed circles are samples which were added to human plasma just prior to assay.

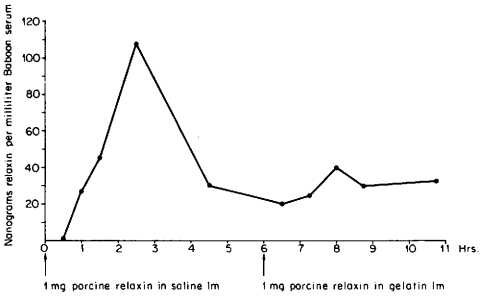

FIG. 3. RIA relaxin in serum of a baboon which received im injections of porcine relaxin in saline solution (time 0) or 20% gelatin (6 hr). Values greater than 20 ng/ml are significantly different from 0.

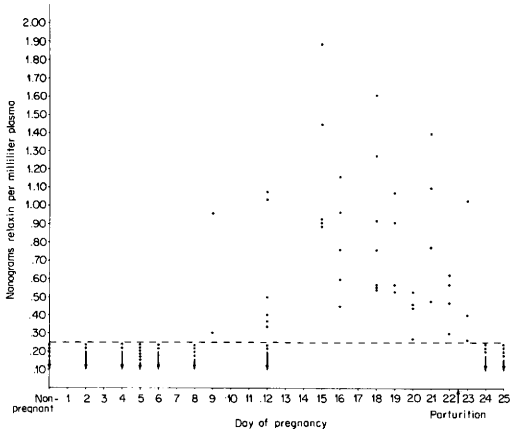

FIG. 4. RIA relaxin levels in sera of pregnant rats. Dotted line indicates sensitivity of the assay. Arrows indicate "less than."

levels of immunoreactive hormone increased until Day 15 of pregnancy and remained elevated until term, whereafter they fell to unmeasurable levels by 2 days postpartum (Fig. 4). A similar pattern of RIA relaxin was observed in sera of pregnant and postpartum mice (Fig. 5).

In guinea pigs, serum RIA relaxin rose gradually throughout the course of gestation and was still elevated on the day following parturition (Fig. 6).

Serum samples taken from a pregnant bitch 4 days prior to whelping and 1 month postpartum were assayed using serum from a male dog as control. A level of RIA relaxin equivalent to 2.4 ng/ml of serum was detected in pregnancy. No RIA relaxin could be detected in the postpartum sample.

Plasma samples taken from 19 women in the third trimester of pregnancy were assayed at 2 volumes each and calculated using standard curves containing equivalent volumes of pooled human male plasma.

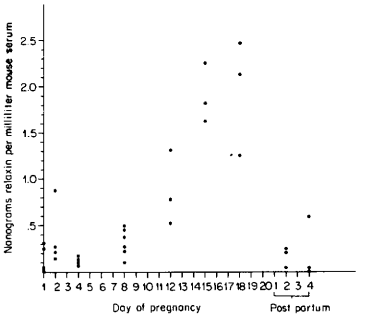

FIG. 5. RIA relaxin levels in sera of pregnant mice.

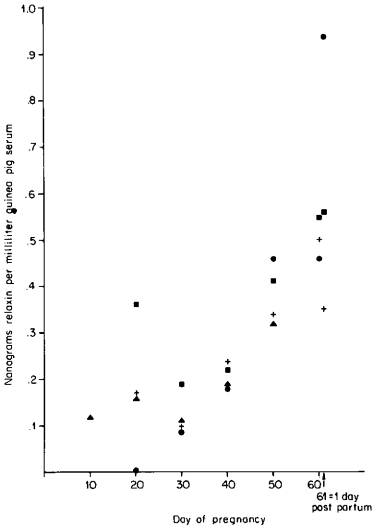

FIG. 6. RIA relaxin levels in sera of pregnant guinea pigs.

Hormone crossreacting with the antibody equivalent to 0.41 ± 0.03 ng of porcine relaxin/ml plasma was detected in these human samples. Relaxin levels in pregnant individuals of other primate species were as follows: rhesus monkey (Day 83) 0.27 ng/ml; java monkey (Day 76) 0.23 ng/ml; baboon (five individuals, Days 27–147) 0.27 ± 0.02 ng/ml.

Discussion. Control serum obtained from males or castrated individuals was added to the tubes containing the porcine relaxin standards to compensate for nonspecific effects of the volume of serum used to assay unknowns. This procedure yielded a linear relationship between the volume of serum assayed and the amount of relaxin detected.

Incubation of porcine relaxin standards in human plasma did not destroy the immunoreactivity. Thus, human plasma does not appear to have "relaxinase" activity.

When porcine relaxin in saline solution was injected into a baboon the serum level rose rapidly to a peak at 2.5 hr and then declined by the time the next blood sample was obtained at 4.5 hr. This observation suggests that the hormone was rapidly absorbed, metabolized, and/or excreted. When the relaxin was injected in a gelatin vehicle, serum levels of RIA relaxin rose much more slowly, suggesting a slower absorption from the site of injection.

RIA relaxin was first detectable in serum on the ninth day of pregnancy in the rat. The levels rose between the twelfth and fifteenth day of gestation and remained elevated until after parturition. Anderson *et al.* (7) reported a similar increase in ovarian relaxin (bioassay) between Days 14 and 20 of pregnancy in rats. The rise in serum and ovarian relaxin thus immediately precedes the first detectable increase in cervical dilatability on Day 13 of pregnancy in rats (8). Cervical dilatability then increases linearly until parturition, whereupon the cervix firms up dramatically the first 2 days postpartum (8). Similarly in mice, serum RIA relaxin levels rose precipitously between the twelfth and fifteenth days of gestation and remained elevated until after parturition. In mice, the cervix progressively softens from Day 15 until term while interpubic ligament formation proceeds from about Day 16 until term (9). These data support the view that relaxin is secreted by the rodent ovary during the last third of pregnancy and is responsible for the changes observed in the connective tissue of the pubic symphysis and uterine cervix. Likewise in guinea pigs, which have a much longer gestation period, serum RIA relaxin was observed to increase from about Days 40 to 60 of pregnancy. Many workers have described the remarkable "relaxation" of the pelvic girdle which occurs during the last third of pregnancy in this species (see 10). In addition, Porter (11) has suggested that relaxin rather than progesterone may be the major myometrical regulating factor in the guinea pig. The various pregnancy-associated changes can be duplicated in nonpregnant rodents by injected porcine relaxin (8–11). Thus when past and present data are considered together, they strongly suggest that the serum factor identified by RIA in the present study is the physiologically active hormone, relaxin.

The relaxin levels measured in serum of pregnant animals by the present RIA reflect cross-reactions of their hormones with an antibody to porcine relaxin. The cross-reactions of pregnant rats and mice were equivalent to 1–2 ng of porcine relaxin/ml serum. The levels found in pregnant guinea pigs were less than 1 ng/ml. The pregnant dog serum was equivalent to 2–3 ng/ml in comparison to 0.2 to 0.3 ng/ml detected in pregnant non-human primates. In pregnant human beings RIA relaxin levels were about 0.4 ng/ml. These apparent species differences in absolute levels of RIA relaxin may actually be due to differences in structure rather than number of the various relaxin molecules. Those structures which are most similar to porcine relaxin would presumably cross-react more strongly with the anti-porcine relaxin antibody than those structures which differ significantly from the homologous hormone. In order to prove this point it would be necessary to isolate relaxin from tissues of the various species in sufficient mass to permit molecular weight determination and the quantitative preparation of solutions. However, we believe that the fluctuations seen during the course of pregnancy in any one species (e.g., rat) do reflect accurately the relative amounts of hormone being produced.

It is of interest that Sherwood *et al.* (1) were unable to detect RIA relaxin in pregnant guinea pig serum and found a difference in slope between "pregnant rat relaxin" and their porcine standards. These differences may be due to their use of different antisera and to their failure to add control sera of the species at assay to the standard tubes.

Summary. We have investigated the application of a RIA for porcine relaxin to the assay of relaxinlike substances in the blood of various other mammalian species. The cross-reactivity between antiporcine relaxin antibody and the relaxinlike substances in the blood of other mammals during pregnancy was sufficiently high to permit the assay of 0.1–0.5 ml of serum or plasma samples. Nonspecific reactivity was controlled by adding similar volumes of serum or plasma obtained from ovariectomized female or intact male subjects to RIA tubes containing known porcine standards.

RIA relaxin levels rose markedly during the last third of pregnancy in rats, mice, and guinea pigs. RIA relaxin was also found in late pregnancy in dog, rhesus and java monkeys, and human beings. The apparent blood levels of hormone found in each species will depend upon their degree of cross-reactivity with the antiporcine relaxin antibody as well as upon their actual concentration. Thus, absolute blood level values should not be taken literally. However, the fluctuations in RIA relaxin observed during the course of gestation in any given species would appear to reflect accurately the relative blood concentrations of the hormone.

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