IN VITRO ANALYSIS OF ANTISERA TO RELAXIN

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ABSTRACT

This article describes the development of an in vitro assay to quantitate the ability of antisera to inhibit the biological action of the pregnancy hormone, relaxin. The procedure employed a modification of the in vitro assay for the ability of relaxin to inhibit spontaneous uterine contractions as initially described by Kroc et al. (1959). Several antisera were tested that showed inhibition of relaxin activity. Tests of cross-reactivity demonstrated that antiserum produced against porcine relaxin effectively inhibited the activity in relaxin preparations from cows and rabbits but was much less effective in inhibiting the activity in rat relaxin preparations. Agar double-immunodiffusion studies supported the cross-reactivity studies in that cow and rabbit relaxin preparations gave reactions of identity with the porcine relaxin while the rat relaxin preparation did not produce a precipitin line with the anti-porcine relaxin antiserum.

Since the initial studies of Cohen & Graff (1963), who demonstrated that porcine relaxin preparations were antigenic in male rabbits, numerous articles have been published that confirmed this by employing a variety of porcine relaxin preparations and immunization procedures (Steinetz et al. 1964; McClintock & Zarrow 1966; Zarrow & O'Connor 1966; Bryant 1972; Sherwood et al. 1975; Larkin et al. 1977). With the advent of radioimmunoassays to detect relaxin in

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sera (Bryant 1972; Sherwood et al. 1975; O'Byrne & Steinetz 1976), and immunohistologic techniques to detect relaxin in tissues and cells (Anderson et al. 1975; Larkin et al. 1977; Kendall et al. 1978), it has become imperative to develop a feasible method to determine if antisera produced against relaxin preparations are specific for the biologically active form of the hormone. Since porcine relaxin is the primary species that has been purified and used to produce antibodies, it is also important to determine if cross-reactivity exists between anti-porcine relaxin antiserum and the biologically active form of heterologous relaxin species.

This report deals with the development of an in vitro assay to quantitate the ability of anti-relaxin antiserum to inhibit the biological action of the hormone. Included are results from studies that examined reactivity between porcine relaxin antisera raised in rabbits and crude relaxin containing extracts from pregnant pigs, rats, rabbits and cows.

**MATERIALS AND METHODS**

**Materials** – The standard porcine relaxin preparation used in these studies was obtained from the National Institute of Arthritis, Metabolism and Digestive Diseases (NIH-R-P1), 460 U/mg (NIH 460). Oestradiol cypionate (Depo-Estradiol) was provided by the Upjohn Company. Ovaries from late stage pregnant rats were obtained from Sterling-Winthrop Research Institute, Rensselaer, New York, Abbott Laboratories, North Chicago, Illinois, and Parke-Davis and Company, Ann Arbor, Michigan. The rat ovaries were taken from animals that had been utilized in reproductive biology and fertility studies. Ovaries from pregnant pigs and cows were obtained from a local slaughterhouse. Male and pregnant New Zealand white rabbits were obtained from a local vendor. All mice used in the bioassays were of the ICR strain either raised in this laboratory or obtained from Flow Laboratories, Dublin, Virginia.

**Methods.** – Extraction procedures. A crude relaxin preparation was obtained from ovaries of pregnant pigs and cows and placentae from rabbits using the H₂O-HCl-Acetone technique initially described by Griss et al. (1967) and modified by Larkin (1974). The rat ovaries were treated similarly with the exception that they were dehydrated in acetone prior to shipment to this laboratory. Crude extracts of tissues from rabbits, rats, pigs and cows were partially purified by column chromatography with the following modification of a technique initially described by Sherwood & O'Byrne (1974). 0.2 M ammonium acetate buffer, adjusted to pH 5.0 with acetic acid (column buffer) was added to the crude extract and the supernatant was harvested by centrifugation. The supernatant was added to a Bio-Gel P-10 column (2.6 x 70 cm) and the fractions eluting at the same point as porcine relaxin were collected. Protein peaks (280 nm absorbance) were concentrated by lyophilization and dialyzed in Spectrapor TM-3 tubing against distilled water for 24 h in the cold. Protein content of these fractions was determined by the method of Lowry et al. (1951). Specific activities of the preparations were determined by using the in vitro uterine motility assay for relaxin (Kroc et al. 1959).
Antisera production. Male New Zealand white rabbits were immunized against different fractions of porcine relaxin using the polyacrylamide gel procedure developed in this laboratory (Larkin et al. 1977).

In vitro assay of antisera. The procedure was a modification of the in vitro assay for the ability of relaxin to inhibit spontaneous uterine contractions initially described by Kroc et al. (1959). Briefly, immature female mice weighing from 18 to 20 g were

Fig. 1.
Typical in vitro response of mouse uterus to relaxin in the presence of normal rabbit serum (NRS) and antiserum to relaxin (RAS). See text for description of assay technique. The relaxin extract was prepared from corpora lutea of late pregnant cows and had a potency of 71 U/mg protein at a concentration of 0.5 mg/ml water.
primed with 1 μg of Depo-Estradiol in 0.1 ml of sesame oil given subcutaneously. One week later the mice were killed by cervical dislocation and the uteri removed. One horn of a mouse's uterus was divided into two equal segments and each segment was suspended in a separate tube that contained 20 ml of aerated Locke's solution maintained at 37°C in a water bath. The initial treatment involved adding equal volumes of serum (50-200 μl) from a rabbit that had not been immunized against relaxin (normal rabbit serum, NRS) to one tube and serum from a rabbit that had been immunized against relaxin (relaxin antiserum, RAS) to the other tube. The uterine horn that was bathed by NRS served as the control and the uterine horn that was bathed by RAS was the experimental horn. Once a consistent rhythm and magnitude of contractions was attained after adding the sera, generally after at least 8 min, equal amounts of the same relaxin preparation were added to the control and experimental uterine baths so that the bath concentrations of relaxin were doubled every four minutes. Relaxin preparations were added until contractions ceased in each uterine horn (Fig. 1). In some cases, the addition of serum caused a brief stimulation to uterine contractions, however, no consistency of this phenomenon was established. The NIH 460 standard relaxin solution was employed at a concentration of 46 U relaxin per ml of distilled water (100 μg NIH 460/ml). The uterine strips, tubes, and bath solutions were changed after each assay. The contractions were recorded on a recorder with chart speed set at 2 mm/min and a heart lever apparatus working against 1 g of tension was employed that directly recorded the uterine contractions.

The ratio of the amount of relaxin needed to inhibit contractions of the uterine strip bathed by RAS to the amount of relaxin needed to inhibit contractions of the uterine strip bathed by NRS was termed the Antiseraum Inhibitory Measure (AIM) and was calculated using the following equation:

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\text{Antiserum Inhibitory Measure (AIM)} = \left( \frac{V_{ras} - V_{nrs}}{V_{nrs}} \right) \cdot \frac{50}{V_s},
\]

where \(V_{ras}\) represented the volume of relaxin solution in microliters necessary to inhibit by one-half the contractions of the uterine strip bathed by antiserum to porcine relaxin and \(V_{nrs}\) represented the volume of relaxin solution in microliters necessary to inhibit by one-half the contractions of the uterine strip bathed by normal rabbit serum. \(V_s\) represented the volumes of sera in microliters added to the organ baths. The number 50 was used to normalize the different amounts of sera added in different experiments, i.e., fifty microliters of serum was the smallest amount used in any of the experiments. Standard error of the mean of the AIM values was calculated following the procedure described by Snedecor & Cochran (1967).

Agar double immunodiffusion studies. – Agar double immunodiffusion analyses (Ouchterlony plates) were performed as described by Clausen (1969).

RESULTS

The ability of different antisera to inhibit the biological activity of porcine relaxin is shown in Fig. 2. Although some variability was noted, a difference in Antiseraum Inhibitory Measure (AIM) was seen between those sera that had low ability to inhibit the effects of relaxin (R3, R7, R9, R10) and those sera that had greater ability to inhibit the effects of relaxin (R8, R15, R19). The AIM of R15 antiserum was higher than that of R19, however, the difference was not statistically significant.
Inhibition of relaxin activity by different antisera produced against highly purified preparations of relaxin. NIH 460 standard (100 μg/ml) was used as the relaxin standard. The amount of sera used varied from 50 to 200 μl per assay, depending upon the potency of the antisera. Method for calculation of AIM is described in the text. Number in parenthesis represents the number of times the assay was conducted. Vertical line represents standard error of the mean.

Antiserum R15 was tested against relaxin preparations from ovaries of pigs, cows, and rats, as well as relaxin preparations from placentae of late pregnant rabbits (Fig. 3). Crude porcine and bovine relaxin preparations were inhibited by essentially the same measure. Although rabbit placental extract had a somewhat lower AIM value than the porcine and bovine preparations, the difference was not significant. Extracts from the rat ovary gave significantly lower AIM values than the other preparations. This was only slightly increased by incubating the rat relaxin preparation with the antiserum for one hour at room temperature prior to adding the antiserum to the organ bath. Incubating porcine, bovine, and rabbit relaxin preparations in a similar fashion had no effect on the AIM or the total amount of relaxin required to quiet either the experimental or control uterine strips.
Results of agar double immunodiffusion assays (Ouchterlony plates) indicated that antiserum R15 gave a heavy and continuous precipitin line when assayed against relaxin preparations from the pig, cow, and rabbit when equivalent biological units (0.7) of relaxin were added to each outer well (Fig. 4). No precipitin line was formed between the R15 antiserum and the rat relaxin preparation.

Inhibition of biological activity in relaxin preparations from pigs, cows, rabbits and rats by R15 antiserum. Relaxin preparations had the following potencies (U/mg protein) as determined by the mouse uterine motility assay for relaxin (Kroc et al. 1959): pig – 31; cow – 71; rabbit – 15; and rat – 12. Fifty \( \mu l \) of NRS and RAS were used in each assay. In the rat absorbance (incubated) assay, approximately one-half of the amount of rat relaxin preparation that routinely quieted uterine contractions was added to the 50 \( \mu l \) of NRS and RAS. The serum was incubated at room temperature for 30 min prior to assay. Method used to calculate AIM is described in the text. Number in parenthesis represents the number of times the assay was conducted. Vertical line represents standard error of the mean.

Fig. 3.
Agar double-immunodiffusion assay. The R15 well contained 4 µl of anti-porcine relaxin antiserum (rabbit R15) concentrated four times by lyophilization. Four µl of relaxin preparations from the rat, rabbit, cow and pig containing 0.7 units of relaxin activity were added to the peripheral wells.

**Fig. 4.**

**DISCUSSION**

Results from these studies showed that the uterine motility assay for relaxin could be modified to compare the potency of antisera produced against relaxin. Moreover, the technique was employed to check for cross-reactivity between antisera and bioactive fractions of heterologous relaxin preparations. AIM determinations were in agreement with previous studies conducted in this laboratory which showed: 1) that R8 antiserum had a higher titer of antibodies to relaxin than did R7, R9 or R10 antisera, 2) that R8 antiserum was capable of inhibiting the biological activity of porcine relaxin (Larkin et al. 1977). Results from the AIM studies agreed with the Ouchterlony plate assays in that similar amounts of relaxin (0.7 units) from the pig, cow, and rabbit gave distinct precipitin lines and showed reactions of identity between the three preparations. Moreover, AIM values were similar for the three preparations when tested against R15 antiserum. The rat relaxin preparation showed slight cross-reactivity in the uterine motility assay but did not show cross-reactivity in Ouchterlony plate studies when tested against R15 antiserum. While previous investigators have shown the feasibility of detecting relaxin in the rat and rabbit with antibodies raised against porcine relaxin (Zarrow & O'Connor 1966; O'Byrne & Steinetz 1976; Anderson et al. 1975), the current studies indicated that detection of biologically active relaxin can be accomplished by immunologic means in the cow as well.
The observations that cross-reactivity existed between antiserum produced against porcine relaxin and relaxin from the cow and rabbit indicated that relaxin isolated from rabbits and cows must have a certain degree of chemical similarity. On the other hand, rat relaxin would seem to have considerably less chemical similarities to porcine relaxin. Differing slopes of dose-response curves from interpubic ligament bioassays of crude rat and porcine relaxin preparations have been reported (Larkin 1974) and Sherwood (1979) has shown recently that rat relaxin varies in molecular structure from porcine relaxin.

The in vitro technique described in this article appears to give results that are comparable with the in vivo technique for analysis of antisera to relaxin (Steinetz et al. 1964). Studies employing the in vivo analysis identified several species of relaxin that inhibited the biological action of relaxin in oestrogen primed mice and gave a positive response in Ouchterlony plate assays, however, certain species (rat and mouse), did not demonstrate a positive reaction in Ouchterlony plates (Steinetz et al. 1964). Similarly, the in vitro technique described in this paper showed good correlation between high AIM values and positive reactions in Ouchterlony plates in tests for cross-reactivity with relaxin from the cow and rabbit. Rat relaxin gave a low AIM value in the assay against R15 antiserum and did not give a positive reaction in Ouchterlony plate assays when tested at the same level of activity. While similar results were obtained with the in vivo and in vitro assays of antiserum, certain advantages of the in vitro system should be mentioned. For example: 1) the complex responses of the whole animal to the antiserum are not involved in assaying the antigen-antibody reaction in the in vitro system and the in vitro assay is similar to immunodetection systems for measuring relaxin concentrations (e.g., radioimmunoassay). 2) Two or three mice are used to serve as uterine donors for the in vitro assay, whereas, the in vivo system requires from 80 to 100 mice to conduct a complete assay of one serum. 3) The in vitro system uses small amounts of antisera (0.25 ml for five assays of a single serum). 4) The in vitro technique is technically simple and determination of the AIM for one antiserum (4–5 assays) can be accomplished in one to two hours. Considering these features, the in vitro system described in this article would seem to be a simple and useful technique to determine if antiserum produced against relaxin is capable of binding the biologically active form of the hormone.

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