A STUDY OF THE ANTIHORMONAL ACTIVITY OF AN ANTISERUM TO OVINE PROLACTIN USING THE LOCAL LACTOGENIC RESPONSE IN THE RABBIT

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SUMMARY

A rabbit antiserum to a purified preparation of ovine prolactin was prepared. The capacity of the antiserum to counter the biological effect of the preparation of ovine prolactin was determined. When injected intraductally before the injection of prolactin into the same duct the antiserum inhibited the lactogenic effect of prolactin on the rabbit mammary gland. The weight, nitrogen content and reducing sugar content of the mammary glands were used as criteria to judge the effect of the antiserum. The results of specificity tests on the antiserum, using the techniques of double diffusion and immunoelectrophoresis, are also reported.

INTRODUCTION

The capacity of prolactin to elicit antibody formation has been demonstrated by Kabak & Stulova (1937), Kabak (1938), Young (1938), Strangeways (1938), Rowlands & Young (1939), Bischoff & Lyons (1939), Levy & Sampliner (1961, 1962) and Emmart, Bates, Condliffe & Turner (1963). Hayashida (1962) used the pigeon-crop sac assay of Riddle, Bates & Dykshorn (1933) and showed that an antiserum to ovine prolactin, when injected concurrently with prolactin caused inhibition of the effects of the latter.

Lyons (1942) observed that the rabbit responds with lactogenesis in the injected sector of the mammary gland after the intraductal injection of preparations rich in prolactin. This observation was confirmed by Meites & Turner (1947) and by Bradley & Clarke (1956). The latter workers adapted the technique for the quantitative estimation of prolactin, using the total reducing sugar content of the mammary gland as the criterion of response. Chadwick (1962, 1963) investigated the factors which affect the local lactogenic response of the rabbit to prolactin and found that there were distinct breed differences. He also devised a method for the separate estimation of the lactose content of the mammary gland in preference to the estimation of total reducing sugar.

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In investigations concerned with the development of an immunological assay procedure for prolactin, it became necessary to demonstrate the antihormonal activity of an antiserum to prolactin. To date, we have seen no studies in which the antihormonal activity of an antiserum to prolactin has been evaluated by means of a mammalian lactogenic test. For these reasons, a procedure was designed to use the local lactogenic response in the rabbit to study the ability of an antiserum to ovine prolactin to inhibit the biological activity of a purified preparation of ovine prolactin.

**MATERIALS AND METHODS**

*Preparation of antiserum*

**Animals.** The antiserum to ovine prolactin was prepared in adult albino rabbits of mixed New Zealand–Dutch stock weighing approximately 3–4 kg.

**Antigen.** Three preparations were employed as antigen. These were ovine hypothalamic preparations (NIH-P-S 4, NIH-P-S 5 and NIH-P-S 6) containing respectively 21-0, 17-0 and 24-8 i.u. prolactin/mg, as measured by the pigeon-crop sac assay.

**Adjuvant.** The quantity of ovine prolactin required for injection was adsorbed on to an equal amount (w/w) of bentonite (British Drug Houses) in 1·0 ml. of 0-9% NaCl solution. The material was administered by the intradermal, subcutaneous and intravenous routes in various combinations (Table 1).

<table>
<thead>
<tr>
<th>Rabbit no.</th>
<th>Doses of prolactin (mg.)</th>
<th>Routes of administration</th>
<th>Schedule of injections and bleeding</th>
<th>Haemagglutination titre</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0-5</td>
<td>Intradermal (foot pad)</td>
<td>Once per week for 3 weeks. Booster dose in 4th week. Schedule repeated 4 times. Bleeding 10 days after last booster</td>
<td>1:10,000</td>
</tr>
<tr>
<td></td>
<td>1-0</td>
<td>Intravenous (ear vein)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>0-5</td>
<td>Intravenous (ear vein)</td>
<td>Once per week for 14 weeks. Bleeding 10 days after last injection</td>
<td>1:620</td>
</tr>
<tr>
<td>3</td>
<td>0-5</td>
<td>Intradermal (foot pad)</td>
<td>Once per week for 4 weeks, followed by s.c. injection once per week for 5 weeks. Booster dose 7 days after last s.c. injection. Bleeding 10 days after booster. Schedule repeated from 1st s.c. injection onwards as blood required</td>
<td>1:20,000</td>
</tr>
<tr>
<td></td>
<td>0-5</td>
<td>Subcutaneous (chest wall)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0-5</td>
<td>Intravenous</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Antibody titres.** The antibody titres of the antiserum samples were measured by the haemagglutination technique of Boyden (1951), using the modification of Butt, Crooke & Cunningham (1961) in which sheep erythrocytes stabilized with pyruvic aldehyde after the method of Ling (1960) are employed. Antiserum samples with high antibody titres were pooled and stored in 1·0 ml. glass ampoules at −15° until required.

**Specificity tests.** The double diffusion technique of Ouchterlony (1953) and the immunoelectrophoresis test of Scheidegger (1955) were employed to determine the specificity of the antiserum and the homogeneity of the antigen.
Prolactin antiserum and lactogenic response

Preparation of rabbits for local lactogenic response assay

Animals. The rabbits used were albino virgin females, 9–12 months old, of mixed New Zealand–Dutch stock, bred in a closed colony. They were maintained in a controlled environment, at 65 ± 5°F and a schedule of 12 hr. light and 12 hr. darkness. Pseudopregnancy was induced by a single intravenous injection of 50 i.u. of human chorionic gonadotrophin (Lutormone, Burroughs Wellcome and Co.) dissolved in 2·0 ml. 0·9 % NaCl solution.

Intraductal injections. The intraductal injections of prolactin and the antiserum were made on the 11th day of pseudopregnancy and the rabbits were killed by dislocation of the neck on the 7th day after the injections. The methods of anaesthesia and preparation of the rabbits were those of Chadwick (1963). In the rabbit there are four pairs of mammary glands which cover almost the whole of the thorax and abdomen. The thoracic pair were employed as uninjected controls throughout. Of the remaining three pairs, the glands on the left were each injected with 0·15 ml. undiluted and untreated antiserum, while those on the right were injected similarly with the same amount of normal rabbit serum (Plate). In each case only one sector of each gland was injected and the injected ducts were marked with a permanent dye to facilitate identification for the subsequent injections of prolactin. After 45–60 min., the ovine prolactin was injected dissolved in 0·15 ml. saline. The injections were made into the ducts which had been injected previously with antiserum or normal rabbit serum, the same dose of prolactin being administered to both glands of any one pair (Plate). Each gland was massaged gently after injection with the duct occluded in order to distribute the material and prevent leakage.

Doses of prolactin providing clear lactogenic responses were selected after preliminary experiments. Doses of 25, 50 and 75 μg. were found to be suitable. Since the inguinal mammary glands in the rabbit are always smaller than the others, the experimental design was such that in each repetition of the experiment the paired doses of prolactin were administered to different pairs of mammary glands. Three replicates were carried out of each combination, thus a total of nine rabbits was used for the experiment.

Removal of mammary glands and chemical measurements

The mammary glands were exposed by cutting the skin on the mid-line and dissecting away the two sides. After visual examination, the mammary glands were removed together and photographed. Each mammary gland was then dissected out carefully, so as not to damage the sectors filled with milk. The whole glands were weighed individually and stored at −15°C for 1–2 hr., before being divided into small pieces and homogenized in 5·0 ml. of de-ionized water in a top-drive blender (Folley & Watson, 1948). An 0·5 ml. sample of the homogenate was de-proteinized with 5 % zinc sulphate and 0·3 molar barium hydroxide (Nelson, 1944). From the supernatant, an aliquot of 2·0 ml. was taken for the estimation of the total reducing sugar against a glucose standard, using the method of Somogyi (1945). A further 1·0 ml. sample of the original homogenate was used for the estimation of the total nitrogen content by a micro-Kjeldahl technique (Sreenivasan & Sadasivan, 1939; Redemann, 1939; Bradstreet, 1954).
RESULTS

Antibody titres

All rabbits used for raising the antiserum lacked demonstrable antibodies to ovine prolactin before immunization. Quantitative haemagglutination assays showed that two rabbits had developed particularly high titres of the antibodies against ovine prolactin. Those antiserum samples which gave complete agglutination at dilutions of 1:10,000 to 1:20,000 were used.

Specificity tests

The immuno-diffusion results obtained using the double diffusion technique and immuno-electrophoresis demonstrated that the rabbit antiserum to ovine prolactin (NIH-P-S4) cross-reacted with both the ovine prolactin preparations used (NIH-P-S4; NIH-P-S5) forming broad single precipitin lines. These lines were confluent, showing the immunological identity of the two preparations. A single precipitin line was also observed as a result of reaction between the antiserum to ovine prolactin and a sheep pituitary extract obtained by dissolving the powdered gland in saline. This precipitin line was confluent with that of the purified prolactin, thus establishing the immunological identity of the two antigens. No precipitin lines were observed between the antiserum to ovine prolactin and ovine follicle-stimulating hormone (NIH-FSH-S2) or ovine luteinizing hormone (NIH-LH-S7).

The antiserum to ovine prolactin also cross-reacted with sheep serum, forming a faint precipitin line at dilutions of 1:32 and 1:64 only. This precipitin line was not confluent with the precipitin line of the purified prolactin, thus demonstrating lack of immunological identity with the purified prolactin. The faint precipitin line demonstrated a low titre of antibodies, probably against sheep serum protein.

Effect of the antiserum on the lactogenic response to prolactin

Occasionally, a small quantity of milk was observed in the ducts of mammary glands treated with the antiserum. When this occurred, traces of milk were also found in the uninjected control thoracic glands, and this effect was therefore assumed to be due to endogenous prolactin. Visible milk secretion in response to ovine prolactin was completely inhibited in mammary glands treated with the antiserum. In contrast, lactogenesis was clearly apparent in those mammary glands which were treated with normal rabbit serum and prolactin (Plate).

In demonstrating the antihormonal effect of the antiserum to ovine prolactin, it was thought adequate to use the criteria of weight, nitrogen content and total reducing sugar content of the mammary glands. The technique of Chadwick (1962) for determining the lactose content of the mammary glands is rather time-consuming and is more suitable for use in quantitative assays.

The results of the analysis of variance of the above criteria are summarized in Table 2. The antiserum produced a highly significant depressant effect ($P < 0.01$) on the response to prolactin when judged by any of the three criteria used. Increasing the dose level of prolactin over the range employed produced significant increases in response ($P < 0.05$) as judged from changes of weight and nitrogen content and a
Table 2. Mean effects of antiserum on assay criteria

<table>
<thead>
<tr>
<th></th>
<th>With antiserum</th>
<th>Without antiserum</th>
<th>With antiserum v. without antiserum</th>
<th>Effect of increasing dose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls* (means ± s.e.)</td>
<td>25  50  75</td>
<td>25  50  75</td>
<td>25  50  75</td>
<td></td>
</tr>
<tr>
<td>Mammary gland weight (g.)</td>
<td>1·57 ± 0·11</td>
<td>2·62  2·46  3·23</td>
<td>3·20  3·87  5·18</td>
<td>± 0·25</td>
</tr>
<tr>
<td>Total nitrogen content (mg.)</td>
<td>9·64 ± 0·51</td>
<td>13·87  15·06  17·02</td>
<td>20·18  25·06  29·80</td>
<td>± 1·48</td>
</tr>
<tr>
<td>Total reducing sugar content (mg.)</td>
<td>1·90 ± 0·18</td>
<td>3·09  3·59  4·63</td>
<td>5·02  9·31  12·83</td>
<td>± 0·63</td>
</tr>
</tbody>
</table>

* Each figure is the mean of 16 observations (two values missing).
† Each figure is the mean of three observations. The s.e. is calculated for all observations on injected animals.
highly significant increase \((P < 0.01)\) in the case of total reducing sugar content. The variation between rabbits was less in the case of measurements of total reducing sugar than with the other two criteria.

**DISCUSSION**

The present study demonstrates that the rabbit antiserum prepared against ovine prolactin inhibits one biological effect of purified ovine prolactin, namely lactogenesis. The results show that 0.15 ml. antiserum neutralized 50 \(\mu g\) or more of ovine prolactin (NIH-P-S5). Although visible milk secretion in response to ovine prolactin was inhibited at all three dose levels of prolactin, the weight, nitrogen content and total reducing sugar content of mammary glands treated with the antiserum were higher after 75 \(\mu g\) prolactin than at lower doses. This may indicate a lactogenic effect of non-neutralized prolactin.

In spite of the careful selection of rabbits uniform with regard to age and body weight, considerable variation was encountered between rabbits in their response to prolactin. This variation was more marked when the criteria of weight and nitrogen content were considered than in the case of estimations of total reducing sugars.

The exact mechanism of the antigen-antibody reaction in the injected sectors of the mammary glands is not clear. Presumably, the conditions within the ducts and alveoli were favourable for the reaction. The lactogenic response to prolactin was so marked on the right side of the rabbits (i.e. the side without the antiserum) that there could have been no appreciable diffusion of the antiserum across the epithelium of the alveoli to the adjacent tissues, nor could significant amounts have been present in the general circulation.

The results obtained show that the antiserum to purified ovine prolactin (NIH-P-S4) was effective in inhibiting the biological effect of prolactin, as judged by the local lactogenic response in the rabbit.

The authors are indebted to the National Institutes of Health, Bethesda, U.S.A. for supplies of the purified hormone preparations used and to Dr A. Chadwick, Department of Zoology, University of Leeds, for advice on the biological assay procedure.

**REFERENCES**


Treated with antiserum plus ovine prolactin (NIH-P-S5)

50 µg

75 µg

75 µg

25 µg

25 µg

Control
Prolactin antiserum and lactogenic response

Rowlands, I. W. & Young, F. G. (1939). The capacity of pituitary preparations containing the thyrotrophic hormone to induce the formation of antisera. J. Physiol., Lond. 95, 410–419.

DESCRIPTION OF PLATE

Mammary gland area of a rabbit after dissection, showing uninjected control glands (thoracic pair), the lactogenic response to graded doses of prolactin (three abdominal glands) and the inhibition of this response by prolactin antiserum (contralateral abdominal glands).