Localization of Relaxin in the Corpus Luteum of the Rabbit.* (30843)

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Information on the source of relaxin indicates that the ovary and especially the corpus luteum may be a major source of the hormone in the sow during pregnancy(1). It has also been suggested that the placenta and uterus may be involved in the production of relaxin (2-5). This was based on the findings that a material with relaxin-like activity could be extracted from the above organs. However, identification of the active substance was not possible. Recent findings by Cohen that relaxin is antigenic(6) have made it possible to reexamine the problem of the site of relaxin formation and at least to determine whether relaxin is present in such tissues as the corpus luteum, uterus and placenta. This study is concerned with localization of relaxin by the indirect fluorescent antibody technique. Ovaries and uteri of pregnant rabbits and mice were tested with a fluorescein-labelled antibody and localization demonstrated in the corpus luteum of the pregnant rabbit.

Materials and methods. Production of antiserum. Relaxin† was dissolved in saline and suspended in an equal part of Freund's complete adjuvant at a final concentration of 1 mg of hormone per 0.1 ml. This suspension was injected subcutaneously into Dutch-belted male rabbits weighing 2-3 kg in a volume of 0.1 ml per injection. The treatment consisted of 3 injections per week for 3 weeks followed by a booster injection of 0.5 ml at week 8 and exsanguination by heart puncture at week 9.

Hemagglutination reactions. The passive red blood cell hemagglutination test was performed on the relaxin antiserum to determine the antibody titer. Sheep red blood cells were used according to the method of Stavitsky (7).

In vivo assay. The antiserum was also assayed by an *in vivo* method involving an inhibition of the action of relaxin on the pubic ligament of the mouse. Virgin female, Swiss mice, 28 days old, were primed with 5 μg estradiol cyclopentylpropionate. Seven days later relaxin was administered subcutaneously in 0.1 ml of 1% Benzopurpurin 4B in amounts of 1 or 2 G.P.U. Simultaneously 0.1 ml or 0.2 ml of relaxin antiserum were injected intraperitoneally. Twenty-four hours later, the mice were killed with ether. The abdominal cavity was opened and pubic symphysis exposed. The interpubic ligament was measured by a calibrated ocular micrometer and transilluminator(8) and the per cent of inhibition of the elongation of the pubic ligament by the antiserum was determined.

Labelling techniques. The indirect method of fluorescent antibody labelling was used (9). Sheep anti-rabbit gamma globulin was obtained by injecting rabbit serum into adult sheep according to the schedule used for production of the relaxin antiserum. The sheep anti-rabbit gamma globulin was conjugated with fluorescein isothiocyanate and then passed through a Sephadex column to purify the conjugate(10).

Frozen sections and staining procedures. Ovaries, uteri and other tissues were obtained from pregnant Dutch-belted rabbits and Swiss albino mice in the last week of their respective gestation periods. The mice were killed by ether and the rabbits were killed with an overdose of Nembutal. The tissues were removed, quickly frozen in the cryostat, and sectioned at 4 μ with the temperature maintained at $-12°$ to $-15°$C.

Fixation. The sections were air dried at room temperature, and fixed for 30 minutes in 95% ethyl alcohol maintained at 37°C. After removal from the alcohol the sections

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† Obtained under the trade name of Releasin (W1164, Lot 8) through the courtesy of Dr. R. L. Kroc, Warner-Chilcott Laboratories, N. J. The material contained 622 GPU per mg of powder.

were air dried at 37°C for 30 minutes.

Staining. According to the procedure for the indirect method of fluorescent antibody labelling a few drops of unlabelled relaxin antiserum were added to the sections and incubated at room temperature for 30 minutes to 4 hours. Precautionary measures were employed to prevent evaporation. After incubation the slides were placed in phosphate buffered saline, pH 6.8, for three washings of 6-8 minutes apiece. A few drops of fluorescein isothiocyanate labelled sheep anti-rabbit gamma globulin were then added to the sections and incubated at room temperature for 30 minutes to 4 hours. The slides were again washed in the buffered saline as above and mounted in buffered glycerol. The slides were examined with a Zeiss fluorescent microscope using excitatory filters BG 12 and UG 1 and suppression filters GG 14 (#44) and UG 14 (#65).

Tests of specificity of staining. To insure the specificity of the staining, ovaries and uteri from non-pregnant mice and rabbits as well as other tissues were used as controls. A second control experiment consisted of absorbing the relaxin antiserum with relaxin prior to the incubation procedures, and in a third experiment normal rabbit serum was used in place of relaxin antiserum.

Photography and histological staining techniques. The cellular localization of fluorescence was established first and the coordinates on the mechanical stage were recorded. Photographs were taken with a Leica-M-1 camera using Kodak Tri-X black and white film and varying exposure times. After photography, the coverslips were removed by immersing the slides into phosphate buffered saline, pH 6.8. The sections were then fixed in formaldehyde and stained with hematoxylin and eosin. The stained slides were then placed on the stage according to the exact coordinates used for photography of the fluorescence and the type of cells identified and photographs taken.

Results. The relaxin antiserum developed for use in the indirect method of fluorescent antibody labelling was subjected to immunoassay by both *in vivo* and *in vitro* procedures. The passive hemagglutination test gave a 2+ response at a dilution of 1:32 and a plus-minus response at a dilution of 1:512. In the *in vivo* assay, 0.2 ml of the antiserum produced a 34% inhibition of the interpubic ligament when the antiserum was assayed against 1 G.P.U. of relaxin.

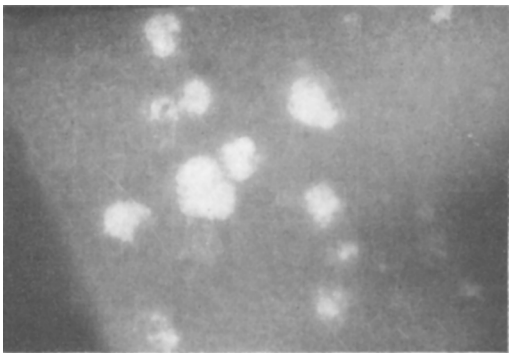

FIG. 1. Fluorescent photomicrograph of rabbit ovarian tissue section in day 26 of pregnancy. (267×)

Localization of the fluorescent antibody was observed in sections of the rabbit ovaries removed on days 23, 26, and 28 of gestation. Marked fluorescence was observed in clusters of cells which could be located in the corpus luteum with the light microscope and routine staining (Fig. 1 and 2). The results of all the control experiments were negative. Tissue sections of pregnant ovaries, when incubated with relaxin antiserum that had been previously absorbed with relaxin, showed negligible fluorescence as well as tissue sections of non-pregnant rabbit ovaries. Another control experiment using normal rabbit serum in place of relaxin antiserum gave negative results. Also, adrenal tissue from both pregnant and

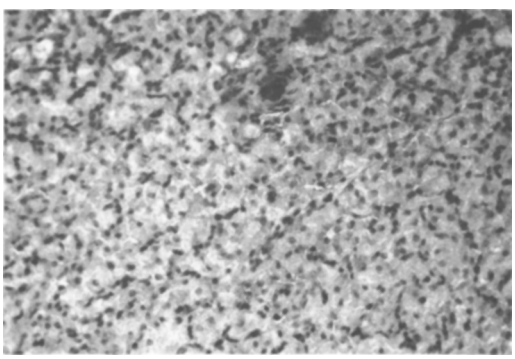

FIG. 2. Photomicrograph of same section as Fig. 1 with conventional hematoxylin and eosin stains. (267×)

non-pregnant rabbits demonstrated no localization.

No comparable cellular localization of fluorescence was observed in sections of the pregnant rabbit uterus. Some scattered fluorescence was demonstrated within cells of the endometrium, but this was inconsistent and could result from non-specific staining.

No localization of the fluorescent antibody occurred in the ovaries and uteri from mice on days 16-19 of their pregnancy. Some fluorescence was demonstrated within areas of thecal cells of the developing follicle, but this was very inconsistent.

Discussion. The present results confirm the findings of Cohen(6) that relaxin is antigenic. Although these results indicate that the hormone is a weak antigen, it is possible to demonstrate localization of relaxin in the luteal cells of the ovary of the pregnant rabbit with the indirect fluorescent antibody technique. The direct method of fluorescent localization failed to give positive results which would also confirm the weak antigenicity of the hormone.

The failure to obtain any localization in the uterus of the pregnant rabbit and mouse confirms the negative results reported for the uterus of the pregnant rat by Battista and Lisk(11), who observed localization with a fluorescent-labelled relaxin antiserum in the ovary of the pregnant rat but not in the placenta and uterus. Such results are contrary to the findings of Zarrow and Rosenberg(5) that relaxin is present in the ovary, uterus and placenta of the pregnant rabbit. Part of the explanation may be that the negative results reported here are due to the weak antigenicity of the hormone. Hence the reaction was not sufficiently sensitive to detect the small amounts of relaxin present in these tissues.

The localization of relaxin to the luteal cells of the ovary indicates that in at least 3 species, sow, rabbit and rat, relaxin is probably secreted by the corpus luteum of pregnancy. Failure to get localization in the mouse ovary will have to be checked with more sensitive techniques.

Summary. Localization of relaxin in the cells of the corpus luteum has been demonstrated in the rabbit on days 23-28 of gestation. No localization could be observed in the uteri of pregnant rabbits or the ovaries and uteri of pregnant mice.

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