LOCALIZATION OF TISSUE-SPECIFIC ANTIGENS IN THE RABBIT OVARY, OVIDUCT AND UTERUS BY THE FLUORESCENT ANTIBODY TECHNIQUE

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Summary. The indirect fluorescent antibody technique was used to localize the reproductive tissue-specific antigens present in the rabbit ovary, oviduct and uterus. The two ovarian antigens were localized in the zona pellucida, theca interna and atretic follicles; the six oviducal antigens in the epithelium of the mucosa; and the two uterine antigens in the endometrial stroma and lumina of uterine glands and the lamina propria of the cervix.

INTRODUCTION

Ouchterlony analysis indicates that the ovaries and reproductive tract of the female rabbit contain reproductive tissue-specific antigens (Sacco & Shivers, 1973b). A minimum of two such antigens was detected in the ovary, six in the oviduct and two in the uterus. Since these antigens may be of importance in reproductive processes, it seemed of interest to determine their location within the respective tissues. It was the purpose of this investigation to localize these antigens using the indirect fluorescent antibody technique.

MATERIALS AND METHODS

Preparation of antisera

Sheep antisera produced against rabbit ovary, oviduct and uterus were prepared and absorbed according to procedures described by Sacco & Shivers (1973b). The antisera were rendered specific to the reproductive tissues by absorption with rabbit plasma, kidney and spleen.

Antiserum to be labelled with fluorescein isothiocyanate (FITC) for the indirect fluorescent antibody technique (Goldman, 1968) was produced in rabbits against the γ -globulin fraction (ammonium sulphate precipitation) of normal sheep serum. Each of two male Californian rabbits was injected intramuscularly with an emulsion consisting of 1.5 ml sheep γ -globulin and 1.5 ml Freund's complete adjuvant. Two weekly booster injections followed.

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These injections were identical to the initial injection except that Freund's incomplete adjuvant was used. The rabbits were bled for antiserum 1 week after the third injection and bleedings were continued on a weekly basis. Such antiserum produced no precipitin bands in Ouchterlony plates when allowed to react against rabbit ovary, oviduct or uterus, thus indicating no cross-reactivity with these tissues.

Conjugation of antiserum

The rabbit anti-sheep serum was conjugated with FITC (Nutritional Biochemicals Corporation) following a modification of the procedure described by The & Feltkamp (1970). Briefly, this consisted of the following steps: (1) a crude γ -globulin fraction was obtained from the antiserum by precipitation with saturated ammonium sulphate (final concentration, 50%) and concentrated by dissolving the final precipitate to half the original antiserum volume with phosphate-buffered saline (PBS, 0.01 M-phosphate, 0.85% NaCl, pH 7.1); (2) after dialysis against PBS, the γ -globulin fraction was put on a DEAE Sephadex A-50 (medium) column (4° C) and eluted with PBS. All fractions from the γ -globulin peak with an $OD_{280} > 0.1$ were pooled and concentrated by dialysis through evaporation to a final protein concentration of 25 mg/ml (determined by UV absorption); (3) a standard solution of 1 mg FITC/ml 0.15 M- $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ (pH 9.0) was prepared. This standard solution was added to the purified γ -globulin solution (10 ml/g protein) while stirring at room temperature. The reaction was allowed to continue for 60 min, maintaining the pH at 9.5 by the addition of 0.1 M- $\text{Na}_3\text{PO}_4 \cdot 10\text{H}_2\text{O}$; (4) the free dye was removed by placing the crude conjugate on a Sephadex G-25 column and eluting with PBS; (5) the conjugate obtained from the G-25 column was then purified by placing it on a DEAE Sephadex A-50 column (4° C) and eluting the first peak with PBS followed by a gradient elution ranging from 0.15 M-NaCl to 1.0 M-NaCl (0.01 M-phosphate, pH 7.1). Fractions having a fluorescein/protein ratio between 1 and 4 were pooled, dialysed against PBS and concentrated by dialysis through evaporation to 10 mg protein/ml. The labelled antiserum was stored at -20° C in 1-ml aliquots.

Preparation and staining of tissue sections

All reproductive tissues were obtained from mature virgin doe Californian rabbits at 5 to 6 months of age. Rabbits were killed with an overdose of sodium

EXPLANATION OF PLATE 1

Cryostat sections of rabbit ovary treated with sheep anti-ovary serum and FITC-labelled anti-sheep serum. The anti-ovary serum was rendered specific to ovary by absorption with rabbit plasma, kidney and spleen. CO, cumulus oophorus; E, egg; HM, hyaline material; MG, membrana granulosa; OS, ovarian stroma; TE, theca externa; TI, theca interna; ZP, zona pellucida.

FIG. 1. Section through follicle showing localization of fluorescence in the zona pellucida and theca interna. $\times 250$. Inset: haematoxylin-and-eosin-stained section of a comparable region. $\times 90$.

FIG. 2. Section through an atretic follicle showing localization of fluorescence. Hyaline material does not fluoresce. $\times 445$. Inset: haematoxylin-and-eosin-stained section of a comparable region. $\times 128$.

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pentobarbital (Diabutal Diamond Laboratories, Inc.) and tissue sections of ovary, oviduct, uterus and cervix were prepared at -19°C with a cryostat microtome. Sections ($8\text{ }\mu\text{m}$) were placed directly on alcohol-washed cover-slips and allowed to air dry.

Techniques similar to those described by Nairn (1962) and Goldman (1968) for the indirect fluorescent antibody technique were used. The appropriate tissue section was covered with 0.04 to 0.08 ml of its specific sheep anti-rabbit serum and incubated in a moist Petri dish for 45 min at 37°C . The cover-slips containing the sections were then put into Coplin jars containing PBS (0.01 M-phosphate, pH 7.3) and placed in a shaker for three, 5-min washes to remove the antiserum which had not reacted. After the third wash, the sections were dried, covered with 0.04 to 0.08 ml FITC-labelled anti-sheep serum, placed in moist Petri dishes and incubated for 45 min at 37°C . After further washing and drying, the sections were mounted in buffered glycerol (Kawamura, 1969) on glass slides and were immediately observed for fluorescence. Control preparations were treated in a similar manner and run simultaneously with all experimental tissue sections.

Fluorescence was observed with a Zeiss microscope, using an HBO 200-W mercury vapour lamp with excitation filter UV BG12 and barrier filters 53/44 in conjunction with a dark field condenser. Kodak Tri-X Pan film was used to record fluorescence.

RESULTS

Tissue sections of ovary, oviduct and uterus were stained by the indirect fluorescent antibody technique with absorbed (plasma, kidney and spleen) anti-ovary, oviduct and uterine sera, respectively. In ovarian sections, specific staining was found to be localized within the zona pellucida, theca interna and atretic follicles (Pl. 1, Figs 1 and 2). A faint fluorescence was also visible in the membrana granulosa, but was comparable to that observed in the control sections. No staining was observed in the hyaline material of atretic follicles (Pl. 1, Fig. 2). Specific staining in oviducal sections was localized in the mucous membrane (Pl. 2, Fig. 3). The lamina propria appeared to be free of stained material. Identical results were obtained in sections of preampullary, ampullary and isthmic regions of the oviduct. Specific staining in uterine sections was

EXPLANATION OF PLATE 2

Cryostat sections of rabbit oviduct, uterus and cervix treated with the homologous sheep antiserum and FITC-labelled anti-sheep serum. The sheep antisera were rendered specific to reproductive tissue by absorption with rabbit plasma, kidney and spleen. CG, cervical gland; ES, endometrial stroma; LP, lamina propria; MUC, mucosa; MUS, muscularis; UG, uterine gland.

FIG. 3. Isthmus of oviduct showing localization of fluorescence in the epithelium of the mucosa. $\times 240$. Inset: haematoxylin-and-eosin-stained section of a comparable region. $\times 144$.

FIG. 4. Section through uterus showing localization of fluorescence in the endometrial stroma and lumina of uterine glands. $\times 240$. Inset: haematoxylin-and-eosin-stained section of a comparable region. $\times 120$.

FIG. 5. Section through cervix showing localization of fluorescence in the lamina propria. $\times 228$. Inset: haematoxylin-and-eosin-stained section of a comparable region. $\times 120$.

localized in the endometrial stroma (lamina propria) and in the lumina of glands (Pl. 2, Fig. 4). Although no staining was seen in the epithelium of the uterine glands, some stained material was occasionally observed between the epithelial cells. Sections of cervical tissue treated with anti-uterus serum showed specific staining in the lamina propria (Pl. 2, Fig. 5), but no staining was observed in the epithelial cells or lumina of cervical glands. A summary of these results is shown in Table 1.

Table 1. Indirect fluorescent antibody reactions in rabbit tissues

<i>Rabbit tissue section</i>	<i>Sheep anti-rabbit serum*</i>	<i>Result†</i>
Ovary	Anti-ovary	Strong fluorescence in zona pellucida, theca interna and atretic follicles
Oviduct	Anti-oviduct	Strong fluorescence in mucous membrane
Uterus	Anti-uterus	Strong fluorescence in endometrial stroma and in lumina of glands
Cervix	Anti-uterus	Strong fluorescence in lamina propria

* Absorbed with rabbit plasma, kidney and spleen.

† After treatment with FITC-labelled rabbit anti-sheep serum.

Table 2. Controls for establishing specificity of staining by the indirect fluorescent antibody technique in rabbit tissues

<i>Tissue section</i>	<i>Reagent for Step 1</i>	<i>Rabbit serum for Step 2</i>	<i>Result</i>
1	Sheep anti-rabbit ovary	Anti-sheep serum‡	Strong non-specific fluorescence
2	Sheep anti-rabbit oviduct	Anti-sheep serum‡	Strong non-specific fluorescence
3	Sheep anti-rabbit uterus	Anti-sheep serum‡	Strong non-specific fluorescence
4	Sheep anti-rabbit uterus	Anti-sheep serum‡	Strong non-specific fluorescence
1	Absorbed* anti-rabbit ovary	Control	Moderate yellow autofluorescence
2	Absorbed* anti-rabbit oviduct	Control	Moderate yellow autofluorescence
3	Absorbed* anti-rabbit uterus	Control	Moderate yellow autofluorescence
4	Absorbed* anti-rabbit uterus	Control	Moderate yellow autofluorescence
1,2,3,4	Phosphate-buffered saline	Anti-sheep serum‡	Weak non-specific fluorescence
1,2,3,4	Sheep control serum	Anti-sheep serum‡	Moderate non-specific fluorescence
1,2,3,4	None	Anti-sheep serum‡	Weak non-specific fluorescence
1,2,3,4	Heterologous antiserum†	Anti-sheep serum‡	Weak non-specific fluorescence
1	Anti-ovary serum absorbed with ovary	Anti-sheep serum‡	Moderate non-specific fluorescence

1 = rabbit ovary; 2 = rabbit oviduct; 3 = rabbit uterus; 4 = rabbit cervix.

* Absorbed with rabbit plasma, kidney and spleen.

† Rabbit anti-fertilized *Rana pipiens* jelly.

‡ Antiserum labelled with fluorescein isothiocyanate.

Preparations used for establishing specificity of staining are summarized in Table 2. Except for the control using unabsorbed antisera, fluorescence observed in control tissue sections was always much less intense than that observed in experimental sections. All controls treated with FITC-labelled antiserum produced uniform staining, indicating non-specificity.

DISCUSSION

The present investigation suggests that the two antigens specific to the rabbit ovary as shown by Sacco & Shivers (1973b) are localized in the zona pellucida, theca interna and atretic follicles. These findings are similar to those reported by Porter, Highfill & Winovich (1970) who demonstrated that the gonad-specific antigens detected in the guinea-pig ovary were restricted to the zona pellucida and atretic follicles. The localization of antigen(s) in the zona pellucida has been confirmed by treating ovulated ova with anti-ovary serum (Sacco & Shivers, 1973a). Such treatment resulted in the formation of a precipitation layer on the surface of the zona.

The six tissue-specific antigens of the rabbit oviduct were restricted to the epithelium of the mucosa. Although this epithelial lining consists of two major cell types, secretory and ciliary (Hafez & Blandau, 1969), further studies are necessary to determine whether the antigens are restricted to one cell type only. Because the material of the mucin coat is a product of the secretory cells and since it has been established that some of the oviduct-specific antigens are located within the mucin coat (Sacco & Shivers, 1973a), the preampullary, ampullary and isthmic regions were examined for differences in fluorescence. Although the number of mucin-rich secretory cells increases as one progresses from the preampullary region toward the isthmic end (Hafez & Blandau, 1969), no differences in localization or intensity of fluorescence could be distinguished between the three regions.

The specific antigens in the uterus and cervix have been localized in the connective tissue. Epithelial cells of both uterine and cervical glands failed to fluoresce but fluorescent material was present in the lumina of uterine glands. Similar stained material was not observed in cervical glands. The contrast in localization of antigens between the oviduct and uterus, i.e. mucosa of oviduct versus connective tissue of uterus, was surprising, especially since Sacco & Shivers (1973b) have shown by immunodiffusion analysis that one of the uterine antigens is also present in the oviduct.

The functions of these reproductive tissue-specific antigens are unknown at the present time. Their localization, e.g. in the zona, mucosa and connective tissue, however, suggests that they might be important in reproductive processes. Consequently, if any of these antigens do possess a unique reproductive function, the effect of antibodies directed against that particular antigen would be of great interest. For example, specific antibodies directed against the antigen(s) located in the zona may alter the properties of the zona to such an extent that the spermatozoa might be incapable of penetrating it during the fertilization process. Likewise, the presence of specific antibodies directed against the antigen(s) localized in the theca interna may prevent normal follicle, ovum or corpus luteum formation. Finally, inhibition or alteration of the function(s) of any of the antigens specific to the oviduct or uterus by specific antisera may modify the oviducal or uterine environment to such an extent that normal reproductive processes could not be carried out. Consequently, the presence of these tissue-specific antigens in the female reproductive tract greatly encourages the possibility of utilizing immunological methods for fertility control.

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