Heteroimmunization with isolated human ova

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Summary. Rabbit antiserum raised against isolated human ova produced an intense immunofluorescence and a precipitate on the outer surface of human and porcine zonae pellucidae. The antiserum reacted with none of 22 human tissues nor with any of 4 body fluid components by immunofluorescence or immunodiffusion analysis, but the antiserum agglutinated AB erythrocytes after absorption with O erythrocytes. The anti-zona activity was achieved by higher titres for human than for porcine zonae. Immunofluorescence on porcine zonae was completely abolished by absorption with porcine ova, whereas a weak but definite fluorescence remained on human zonae. These findings indicate that the human zona pellucida consists of at least three distinct components; (1) a specific antigen(s) shared by human and porcine zonae, (2) an antigen(s) specific to human zonae, and (3) a non-specific antigen(s) associated with the blood group substances.

Introduction

Initial investigations to explore the presence of a zona-specific antigen(s) have been successful in several species, including man (Shivers & Dunbar, 1977; Sacco, 1977a), and have shown that the heteroimmune antibodies specific to ovarian extract react with the antigen(s) in the zona pellucida. The specificity of the zona-antigen(s) was further established by using, as the immunogen, cumulus-free mouse eggs (Tsunoda, 1977), isolated mouse zonae pellucidae (Gwatkin, Williams & Carlo, 1977; Tsunoda & Sugie, 1977) or isolated pig zonae pellucidae (Sacco & Palm, 1977). For the purpose of immunological regulation of human fertility, specificity of the zona-antigen(s) must be established with certainty. Mori et al. (1979) have shown that the blood group substances are present in the human zona pellucida as associated antigen(s), and therefore in the present study heteroimmunization with isolated human ova was attempted to examine the antigenic composition of the human zona pellucida.

Materials and Methods

Collection of isolated human and porcine ova. Fresh human ovaries were obtained at laparotomy for gynaecological malignancies from informed patients aged 24 and 46 years. Fresh pig ovaries were purchased from a local slaughterhouse. Ova of a uniform size of about 120 µm but of undefined maturational stage were randomly collected from follicles of various sizes and were completely freed from the cumulus cells by the methods described for porcine (Mori et al., 1978) and human (Mori et al., 1979) ovaries. Isolated human ova used for immunization were washed in several changes of 0·01 M-phosphate-buffered saline, pH 7·2 (PBS), and were stored...
frozen at \(-20^\circ C\) until use. A separate group of human ova obtained from fresh ovaries were subjected to zona precipitate or immunofluorescence tests before and immediately after being freed of the zona pellucida in the presence of 0.1\% (w/v) prasone (Pronase E: Kaken Chemical Co. Ltd, Tokyo) in PBS. Porcine ova were prepared from fresh ovaries as needed.

**Immunization.** A single mature female New Zealand White rabbit (approximately 2.5 kg) was injected intradermally at 20 different sites on the back with 20 human ova which had been homogenized in 1.0 ml PBS and emulsified in an equal volume of Freund’s complete adjuvant. The injection was repeated 4 times at intervals of 2 weeks. Having received a total of 100 ova, the rabbit was bled 2 weeks after the last injection. A control rabbit was treated similarly with the immunogen preparation but without the ova. A blood sample was collected before the first immunization injection to provide preimmune serum. The separated sera were inactivated at 56°C for 30 min and were stored frozen at \(-20^\circ C\).

**Absorption procedures.** Absorption of the serum was performed with human liver, erythrocytes of the O and AB groups, or porcine ova. Acetone powder of the liver was prepared by the method of Johnson, Holborow & Dorling (1978). Liver powder (100 mg) was mixed well with 1.0 ml serum by gentle shaking for 60 min at room temperature. The mixture was centrifuged at 12,000 \(g\) for 20 min, and the same amount of liver powder was added to the supernate. The mixture was shaken for 60 min at room temperature, kept at 4°C overnight and then centrifuged at 12,000 \(g\) for 20 min. Human O erythrocytes were used for absorption of natural heteroagglutinins and AB erythrocytes were used for absorption of immune heteroagglutinins to human AB blood group substances according to the method described elsewhere (Mori et al., 1979). After complete absorption with O and AB erythrocytes the antiserum was absorbed with porcine ova. Porcine ova (10,000) were mixed with 0.2 ml serum by gentle shaking for 60 min at room temperature. The mixture was centrifuged at 3000 \(g\) for 5 min. Another 10,000 porcine ova were added to the supernate, the mixture was kept at 4°C overnight and then centrifuged at 3000 \(g\) for 5 min.

**Characterization of the antiserum.** Anti-zona activities of the antiserum were evaluated by two tests, zona precipitate (Oikawa & Yanagimachi, 1975) and immunofluorescence (Johnson et al., 1978); each test was repeated at least twice and used 3–5 ova. For the zona precipitate test, isolated ova were incubated in 0.2 ml unabsorbed serum for 30 min at 37°C, washed thoroughly in PBS, and were examined for the presence of precipitate on the outer surface of the zona, revealed as a strong light-scattering layer under dark-field illumination. The zona precipitate was further tested for its solubility by treatment with a proteolytic enzyme (Oikawa, Yanagimachi & Nicolson, 1973; Tsunoda, Sugie & Mori, 1979). Human ova with a precipitate on their zonae were exposed to 0.5% (w/v) prasone in PBS at room temperature and the time required for complete dissolution was measured. For the immunofluorescence test, isolated zona-coated or zona-free ova which had been treated with the antiserum, as described above, were further reacted for 30 min at 37°C with pig anti-rabbit immunoglobulins labelled with fluorescein isothiocyanate (FITC) (DACO-Immunoglobulins Ltd, Copenhagen), which had been diluted 80-fold with PBS to eliminate non-specific staining. The coefficient of dilution was determined so that direct application of the conjugate did not produce any fluorescence on the human zona pellucida in a preliminary experiment. The eggs were then washed thoroughly in PBS and observed under a fluorescence microscope (Olympus Model BH-RFL, Tokyo). Kodak Tri-X Pan and Ektachrome 64 films were used to record the fluorescence.

**Tissue specificity of the antiserum** was tested by indirect immunofluorescence and immunodiffusion analysis. Human tissues (adrenal gland, adipose tissue, cerebrum, cerebellum, duodenum, heart, hypophysis, ileum, kidney, liver, lung, oviduct, pancreas, skeletal muscle, spleen, stomach, testis, thyroid gland and uterus) were obtained post mortem from a 58-year-old woman and a 60-year-old man, and frozen sections (5 \(\mu m\)) were prepared. An ovary from a 30-year-old woman was also used. Smears or suspensions of AB erythrocytes and of ejaculated spermatozoa from a man of proven fertility were also prepared after being washed several times
in PBS. The method described elsewhere (Johnson et al., 1978) was followed for staining of the 22 different human tissues. The antiserum, before and after absorption, was variously diluted and first reacted with the tissues for 30 min at 37°C in a moist chamber. The tissues were washed in PBS, incubated for 30 min at 37°C with pig anti-rabbit immunoglobulins labelled with FITC which had been diluted 10-fold with PBS to eliminate non-specific staining. In addition to the extracts of the 22 tissues human follicular fluid, plasma, seminal plasma and serum were tested against the antiserum by agar-gel double diffusion. The tissue extracts were prepared by homogenizing the tissue in an equal volume of PBS and by centrifuging at 12 000 g for 20 min. Follicular fluid was collected from an ovary of a 36-year-old woman. Plasma, serum and seminal plasma were obtained from fertile women and men.

Results

A strong immunofluorescence was observed along the contour of all the zona pellucida in a frozen section of the ovary (Pl. 1, Figs 1 and 2) as well as on the outer surface of the zona pellucida of isolated human (Pl. 1, Fig. 3) and porcine (Pl. 1, Fig. 4) ova following treatment with the unabsorbed serum or with the serum absorbed with liver as well as with AB erythrocytes, but not with the adjuvant control serum or the preimmune serum. Regardless of the absorption with liver or AB erythrocytes, the greatest dilution of the antiserum that produced a definitely discernible fluorescence remained unchanged, being 512-fold for human zonae in a frozen section of ovary and round isolated ova but 256-fold for porcine zonae (Table 1). There was no fluorescence with any of the human tissues examined after treatment with the antiserum before and after the absorption with liver or AB erythrocytes. The unabsorbed antiserum agglutinated O erythrocytes and the antiserum which had been absorbed with O erythrocytes microscopically agglutinated AB erythrocytes. No agglutination occurred after absorption with AB erythrocytes. The agglutinated O or AB erythrocytes did not show any grade of fluorescence. After repeated absorption with a total of 20 000 porcine ova, the immunofluorescence on porcine zonae was

Table 1. Anti-zona pellucida potency* of the antiserum against human ova raised in a rabbit

<table>
<thead>
<tr>
<th>Dilution of sera</th>
<th>Immunofluorescence</th>
<th>Zona precipitate</th>
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<tr>
<td></td>
<td>Frozen sections of human ovary</td>
<td>Isolated human ova</td>
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</table>
| Antiserum
| 2°       | +++ | +++ | +++ | +++ | +++ |
| 2¹       | +++ | +++ | +++ | +++ | +++ |
| 2²       | +++ | +++ | +++ | +++ | +++ |
| 2³       | +++ | +++ | +++ | +++ | +++ |
| 2⁴       | +++ | +++ | +   | +   | +   |
| 2⁵       | +++ | +++ | +   | +   | +   |
| 2⁶       | ++  | ++  | +   | +   | +   |
| 2⁷       | +   | +   | -   | -   | -   |
| 2⁸       | -   | -   | -   | -   |
| 2⁹       | -   | -   | -   | -   |
| 2¹⁰      | -   | -   | -   | -   |
| Control sera†
| 2° ~ 2¹⁰ | -   | -   | -   | -   |

* Graded as strong (+ + +), moderate (+ +), weak (+) or absent (-).
† The serum of the rabbit immunized with Freund's complete adjuvant and the preimmune serum.
completely abolished, whereas a weak but definite fluorescence remained on human zonae (Pl. 1, Fig. 5). There was no detectable fluorescence on isolated zona-free human ova after treatment with the unabsorbed antiserum.

A precipitate was produced on the outer surface of the zona pellucida of isolated human (Pl. 1, Fig. 6) and porcine ova with the antiserum before and after absorption with liver or AB erythrocytes, but not with the adjuvant control serum or with the preimmune serum. Absorption of the antiserum with porcine ova completely abolished the precipitate on the human and the porcine zonae. The highest dilution of the antiserum at which a clear precipitate was formed was 64-fold for human and 32-fold for porcine zonae (Table 1). Absorption with liver or AB erythrocytes did not affect the titre of the antiserum, as determined by the zona precipitate. The time required for complete dissolution of the zona precipitate decreased along a sigmoid curve with the increasing dilutions of the antiserum reaching to the control level at the dilution of 1024 (Text-fig. 1). No precipitin band was detected by immunodiffusion analyses against any of the tissue extracts or body fluid components.

Text-fig. 1. Relation between the dilution of the antiserum to human ova and the time required for dissolution of zona precipitate by 0-5% (w/v) pronase. Vertical bars indicate the maximum and the minimum values of the dissolution tests in which at least 5 ova were used for each dilution of the antiserum.

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PLATE 1

Fig. 1. A strong immunofluorescence on the outer surface of the zona pellucida in a frozen section of human ovary after treatment with the unabsorbed antiserum to human ova. ×200.

Fig. 2. A photomicrograph showing the same portion as in Fig. 1, not stained. ×200.

Fig. 3. A strong immunofluorescence on the outer surface of the zona pellucida of an isolated human ovum after treatment with the unabsorbed antiserum to human ova. ×100.

Fig. 4. A strong immunofluorescence on the outer surface of the zona pellucida of an isolated porcine ovum after treatment with the unabsorbed antiserum to human ova. ×100.

Fig. 5. A weak but definite immunofluorescence on a human zona pellucida remaining after treatment with the porcine ova-absorbed antiserum to human ova. ×100.

Fig. 6. A precipitate on the outer surface of the zona pellucida of an isolated human ovum after exposure to the unabsorbed antiserum to human ova. ×100.
Antibodies to human ova

Discussion

The difficulty of collecting a large number of human ova is presumably offset by the strong heteroantigenic potency of the zona pellucida, as proven with hamsters (Gwatkin et al., 1977) and mice (Tsunoda, 1977), and the present results show that a relatively small number of human ova can be used for immunization and elicit a higher titre of antibodies comparable to the titre at which in-vitro fertilization was significantly reduced in mice (Tsunoda et al., 1979).

Although tissue specificity of the human zona antigen(s) was established by double-diffusion analysis (Sacco, 1977a; Shivers & Dunbar, 1977), more sensitive methods should be introduced for examining minor cross-reactivity in antigenicity between the zona and other tissues, because immunodiffusion and/or immunoelectrophoresis tests give divergent results (Glass & Hanson, 1974; Jilek & Pavlok, 1975; Tsunoda, 1977), possibly due to the titre of the antiserum and the solubility of the antigen(s) (Tsunoda et al., 1979). Specificity of the porcine zona antigen(s) has been confirmed by a radioimmunoassay system (Palm, Sacco, Syner & Subramanian, 1979). Although there was no cross-reactivity with any of the 22 tissues examined by immunofluorescence or with any of the 4 body fluid components, including the follicular fluid (Sacco & Palm, 1977), examined by immunodiffusion, tissue specificity of the antiserum for the zona was established only when absorption with AB erythrocytes eliminated immune heteroagglutinins against the blood group substances on the zona (Mori et al., 1978, 1979), the presence of which was confirmed in the present study by the agglutination of AB erythrocytes even after removal of natural heteroagglutinins by absorption with O erythrocytes. Since immunofluorescence tests failed to visualize the presence of agglutinins, it is evident that the test against erythrocytes is essential to establish the tissue specificity of the antiserum. Production of immunofluorescence or immunoprecipitate on porcine zonae after exposure to the antiserum indicates the presence of the common antigen(s) shared by the zonae from the two species (Sacco, 1977b). The residual immunofluorescence on human zonae after rigorous absorption with porcine ova may represent another antigenic component of human zonae which is specific to this structure.

No antibody directed against the vitellus was induced, as far as could be determined, by immunofluorescence. This failure is probably due to weak antigenicity of this component, because cytotoxic antibodies against eggs were produced with zona-coated mouse eggs (Baranka, Koldovsky & Koprowski, 1970) or with mouse vitelli (Tsunoda & Sugie, 1979), although much larger number of eggs were used than in the present study. However, the detection of such antibodies is not feasible because of the limited supply of human ova. Assuming that a constant number of antigen-antibody molecules is dissolved by a certain concentration of the enzyme per unit time, the observed sigmoid curve could be related to the total number of complex molecules with decreasing number of antibody molecules as dilution of the antiserum increases. There was a considerable degree of discrepancy between the two titres of zona precipitate and immunofluorescence in the present study. As suggested by Gwatkin et al. (1977), the determinants detected by immunofluorescence must include others in addition to those responsible for binding the spermatozoa to the eggs. The biological activities of the anti-human zona antiserum in blocking fertilization await further study.

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References


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