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Studies on the Zona Pellucida of Sheep Ova: Immune Lysis and Localization of Antibodies in Ova with, and without, Zonae

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Abstract

The susceptibility of fertilized sheep ova and oocytes, with and without zonae pellucidae, to lysis by complement plus antibodies in antisera raised against sheep ovaries, oocytes and sperm was examined *in vitro*. The presence of the zona reduced the proportion of ova lysed (8% *v.* 57%) and a large proportion of ova with zonae continued development in culture following exposure to antisera.

Antisera to ovary and to oocytes lysed a higher proportion of ova than did antisera to sperm (51 and 56% *v.* 29%). There was no effect of age of fertilized ova (day 2, 4, 6; day 0 = day of oestrus) on susceptibility to lysis.

Fluorescein-labelled antibodies from high-titre antisera to ovary and oocytes traversed the zona and attached to blastomeres. Following absorption of the antibody conjugates with sheep serum, fluorescein staining of ova with intact zonae was confined to the zona, whereas with zona-free ova the blastomeres were stained.

It appears that the zona may protect embryos from lytic antibodies and it is suggested that the zona may be important in protecting the embryo from immunological attack *in vivo*.

Introduction

In pre-blastocyst mammalian ova the presence of an intact zona pellucida appears to be necessary for continued development *in vivo*. In the sheep, complete or partial removal of the zona results in the survival of very few 2- to 16-cell ova (Moor and Cragle 1971; Trounson and Moore 1974*a*). It has been suggested that the zona might protect the developing embryo from destruction by maternal immune systems (Simmons and Russell 1966; Heyner *et al.* 1969; James 1969). Moskalewski and Koprowski (1972) found that the presence of an intact zona reduced the proportion of mice ova lysed by anti-ova serum raised in the guinea pig. On the other hand, Sellens and Jenkinson (1975) reported that zonae of mouse ova were readily permeable to antibodies and provided little protection against immune lysis.

In the present experiments, the susceptibility of fertilized sheep ova and oocytes, with and without intact zonae, to immune lysis *in vitro*, by antisera raised against sheep oocytes, ovarian tissue and ram sperm, was examined. Immunofluorescence was used to study the localization, in fertilized ova with and without zonae, of antibodies present in antisera to oocyte and ovary.

Materials and Methods

Experimental Animals

Mature Merino ewes were used to provide oocytes, fertilized ova and ovaries and a single Romney Marsh ram supplied semen used for raising anti-sperm serum and for insemination of ewes from

which the fertilized ova were collected. Three-month-old virgin female rabbits (New Zealand White) provided normal and control rabbit sera and were used for the preparation of antisera.

Preparation of Antisera

Normal serum was collected prior to immunization.

(i) *Anti-sperm serum*

Ram serum (5 ml) collected by artificial vagina was washed twice in an equal volume of phosphate buffered saline (PBS), pH 7·3, resuspended in 5 ml PBS and then homogenized at 0°C. Cellular components were removed by centrifugation (2000 *g* for 20 min at 4°C) and the supernatant used for immunization. Each of two rabbits was injected on two occasions, 7 days apart, with 2·5 ml supernatant emulsified in an equal volume of Freund's complete adjuvant (Commonwealth Serum Laboratories, Melbourne) and antisera were collected at weekly intervals for 8 weeks commencing 4 weeks after the initial injection.

(ii) *Anti-ovary serum*

Ovaries were collected from cyclic ewes immediately after slaughter. Any corpora lutea present were dissected out and discarded and the remaining ovarian tissue was finely minced and homogenized at 0°C in 5 ml PBS and then centrifuged (2500 *g* for 20 min at 0°C). The supernatant was used as described in (i) above for the immunization of two rabbits. One of the rabbits received two additional injections, 5 days apart, commencing 12 weeks after the first injection and antiserum (HT2) was collected weekly for 4 weeks commencing 4 weeks after the final injection.

(iii) *Anti-oocyte serum*

Oocytes were collected 2–4 days after oestrus from ewes that had been treated with an equine anterior pituitary extract to induce superovulation. One rabbit was injected on each of six occasions with 80–90 oocytes (enclosed in their cumulus mass) in 0·75 ml PBS to which an equal volume of adjuvant had been added. Injections were given on the day on which oocytes were collected and 7, 12, 17, 22 and 47 days later. Antiserum was collected weekly commencing 2 weeks before and concluding 8 weeks after the final injection.

(iv) *Control serum*

A further rabbit was immunized with an emulsion of Freund's complete adjuvant and PBS. It was treated and bled as described in (iii) above and the resulting serum was used as a control for all antisera.

All rabbit sera were heated and held at 56°C for 30 min and then stored at −20°C. Agglutination tests and double diffusion tests on Ouchterlony plates were used to establish the presence and titre of antibodies in antisera.

Experimental Procedure

(i) *Experiment 1*

In experiment 1 the effect of control and normal sera and the various antisera on oocytes and fertilized ova with and without their zonae was examined. Fertilized ova were collected on days 2, 4 and 6 (day 0 = day of oestrus) from ewes which had been inseminated into the uterus following treatment with an equine anterior pituitary extract. Oocytes were collected from the same ewes by aspirating ovarian follicles of $> 2 \cdot 5$ mm diameter. Zonae were removed mechanically as described by Trounson and Moore (1974*a*), or by exposure to 0·5% Pronase (Protease from *Streptomyces griseus*; Sigma) in PBS. The ova and oocytes were then incubated at 37°C for 3 h in the various sera. Prior to incubation the sera were diluted 1 : 3 with PBS containing complement (2% guinea pig serum). During incubation the ova and oocytes were examined at 15 min intervals for evidence of lysis.

A number of fertilized ova which showed no evidence of lysis after 3 h were cultured as described by Trounson and Moore (1974*b*) for 48 h in a balanced bicarbonate culture medium containing 20% heat-treated sheep serum under a gas phase of 5% O_2, 5% CO_2 and 90% N_2.

(ii) *Experiment 2*

Immunoglobulins from control, anti-oocyte and anti-ovary (HT2) sera were isolated and then conjugated to fluorescein isothiocyanate (FITC) using the procedures described by The and Feltkamp

(1970). The FITC conjugates (containing 5 mg/ml immunoglobulin) were either absorbed with heat-treated sheep serum or left unabsorbed and then used to examine the localization, in fertilized day-4 ova, of antibodies reacting with ovary and oocyte antigens.

The ova were subjected to one of the following procedures:

(1) incubation in FITC conjugates which had been absorbed with heat-treated sheep serum;

(2) incubation in unabsorbed FITC conjugates;

(3) zonae removal with 0·5% Pronase and then incubation in absorbed FITC conjugates;

(4) incubation in FITC conjugates (absorbed and unabsorbed), washing in PBS and then treatment with 0·5% Pronase to remove zonae.

All incubations were carried out at 37°C for 10 min. Ova were washed three times in PBS then transferred onto microscope slides and examined at a magnification of 160× using a Reichert Zetopan microscope fitted with dark field condenser and exciter filter UG 1/1·5 mm with u.v. blue filter GG 9/1 mm and OC 1/1·5 mm.

Results

Control and normal rabbit sera displayed extremely weak agglutinating activity (titres < 2) when tested against the various antigen preparations. Several antisera collected at particular times showed low agglutination titres (< 10) when tested with their respective immunizing antigen and they were discarded. The remaining antisera raised against particular antigens (titres 15–130) were pooled for further testing. In the double diffusion tests four precipitin lines were identified when anti-ovary serum was tested against ovary antigen and a further four lines identified when high-titre anti-ovary serum (HT2) was tested. Eight lines were identified when anti-sperm serum was tested against sperm antigen. No precipitin lines were observed when normal and control rabbit serum were tested against ovary and sperm antigens. Similarly, no lines appeared when anti-ovary serum was tested against sperm antigen, nor when anti-sperm serum was tested against ovary antigen.

The HT2 anti-ovary serum was further tested against antigens prepared from sheep kidney, heart, brain, liver, spleen, serum and spermatozoa by double diffusion tests. Unabsorbed HT2 gave between 5 and 14 precipitin lines, whilst after absorption with sheep serum the numbers of lines detected were 0 (kidney, brain, serum and spermatozoa), 1 (liver) and 2 (heart and spleen).

Insufficient oocyte preparation remained after the injection procedures to allow testing of the various sera against oocyte antigen.

Experiment 1

The proportions of ova lysed after 3 h incubation in the various antisera are shown in Table 1. Age of ova had no effect on their susceptibility to lysis, and data on age of ova were pooled for tests of χ^2. The presence of an intact zona markedly reduced the lytic effect of all antisera. Of 91 ova with intact zonae, only 7 (8%) were lysed, whereas 55 of 99 ova (57%) were lysed following removal of the zona. No lysis was observed in normal rabbit serum, but some 20–30% of ova incubated with control serum and anti-sperm serum were lysed. Antisera to both ovary and oocytes lysed more ova than did control serum (22 of 43, or 51%, and 23 of 41, or 56% *v.* 7 of 34, or 21%; $P < 0{\cdot}01$). There was no difference between anti-ovary and anti-oocyte sera and both lysed markedly more ova than did anti-sperm serum (51% *v.* 29%, $P < 0{\cdot}05$ and 56% *v.* 29%, $P < 0{\cdot}01$).

Of the 129 ova which were not lysed, 97 were subsequently cultured *in vitro* for 48 h and 63 of the 97 showed continued development during culture (Table 2).

Table 1. Proportions of fertilized ova and oocytes lysed during incubation *in vitro* in antisera to ovary, oocytes and sperm, and in control and normal rabbit sera

Age of ova	State of zona	Type of serum					
		Normal	Control	Anti-sperm	Anti-ovary	Anti-oocyte	Total
Oocytes	Present	0/4	0/4	1/4	0/4	1/5	2/21
	Absent	0/5	2/5	1/5	5/5	7/8	15/28
Day 2	Present	0/5	0/4	0/5	0/8	1/4	1/26
	Absent	0/7	1/4	4/5	6/6	4/4	15/26
Day 4	Present	0/4	0/4	0/4	0/5	1/5	1/22
	Absent	0/4	2/5	1/4	5/5	5/5	13/23
Day 6	Present	0/4	0/4	0/4	2/5	1/5	3/22
	Absent	0/5	2/4	3/4	4/5	3/5	12/23
Total	Present	0/17	0/16	1/17	2/22	4/19	7/91
Total	Absent	0/21	7/18	9/17	20/21	19/22	55/100

Source of deviation	χ^2	d.f.	P
State of zona	55·6	1	$<0{\cdot}001$
Type of serum	43·7	4	$<0{\cdot}001$
Interaction	16·7	4	$<0{\cdot}005$
Total	116·1	9	—

Overall, there was no effect of type of serum on the subsequent proportion of unlysed ova which developed in culture, but there was a minor effect of removal of the zona (47 of 65, or 72% *v.* 16 of 32, or 50%; $\chi^2 = 4{\cdot}69$, $P < 0{\cdot}05$). There was no effect of age of ova at the time of incubation with the various sera. Day-2 ova developed during 48 h culture from 2 and 4 cells to 16–20 cells, day-4 ova from 8 cells to morulae and day-6 ova from morulae to blastocysts.

Table 2. Proportions of non-lysed ova which showed continued development during culture *in vitro*

State of zona	Type of serum					
	Normal	Control	Anti-sperm	Anti-ovary	Anti-oocyte	Total
Present	9/13	8/12	12/13	12/16	6/11	47/65 (72%)
Absent	10/16	4/8	2/5	0/1	0/2	16/32 (50%)
Total	19/26	12/20	14/18	12/17	6/13	63/97 (65%)

Experiment 2

The localization and intensity of fluorescence observed following incubation with FITC conjugates are shown in Table 3. In ova with intact zonae fluorescence was localized mainly in the zona (Fig. 1*a*), but weak fluorescence was detected on blastomeres of intact ova that had been incubated with unabsorbed conjugates prepared from HT2 antiserum and from anti-oocyte serum (Fig. 1*c*). When zonae were

removed prior to incubation, fluorescence was detected on blastomeres following incubation with absorbed FITC conjugates of antibodies from anti-ovary and anti-oocyte sera (Fig. 1*b*). Removal of zonae following incubation showed that most, or all, of the antibodies failed to traverse the zona (Table 3).

Table 3. Location and intensity of fluorescence in fertilized (day-4) sheep ova following incubation with various FITC-conjugated antibody preparations

Treatment	Serum from which antibody prepared[A]	No. ova incubated	Intensity[B]	Location
Zona intact	Anti-ovary, unabsorbed	5	+++	Zona and blastomeres
	Anti-oocyte, unabsorbed	5	++	Zona and blastomeres
	Control, unabsorbed	4	+	Zona
Zona intact	Anti-ovary, absorbed	4	++	Zona
	Anti-oocyte, absorbed	4	+	Zona
	Control, absorbed	4	—	—
Zona removed before incubation	Anti-ovary, absorbed	4	++	Blastomeres
	Anti-oocyte, absorbed	4	+	Blastomeres
Zona removed after incubation	Anti-ovary, unabsorbed	5	+	Blastomeres
	Anti-oocyte, unabsorbed	3	—	—
	Anti-ovary, absorbed	3	—	—
	Anti-oocyte, absorbed	3	—	—

[A] FITC-conjugated antibody absorbed or not absorbed with sheep serum before incubation.
[B] Degree of intensity: +++ high, ++ moderate, + low.

Discussion

The results of experiment 1 showed that the presence of an intact zona pellucida reduced the susceptibility of sheep ova and oocytes to immune lysis by antisera raised against ovary, oocytes and sperm. This is consistent with the evidence of Heyner *et al.* (1969) and Moskalewski and Koprowski (1972) who found the zona to be a barrier to the cytotoxic effect of specific antisera. However, Sellens and Jenkinson (1975) found that the zona failed to protect mouse ova from immune lysis by rabbit anti-mouse serum and two of its antibody components (IgG and IgM). It may be that sera of relatively low titre, as used by Moskalewski and Koprowski (1972), are required to demonstrate a protective effect of the zona.

The zona has been shown to be permeable to large-molecular-weight compounds such as peroxidase, ferritin (Hastings *et al.* 1972) and serum proteins (Glass 1963), but appears to exclude heparin (Austin and Lovelock 1958) and colloidal thorium dioxide particles (Enders 1971). In the present experiments antibodies from unabsorbed antisera to ovary and oocyte traversed the zona and were located on blastomeres of fertilized ova. Following absorption with sheep serum, antibodies could not be detected on blastomeres of ova with intact zonae but when zonae were removed before incubation fluorescence was detected on the denuded blastomeres. Thus, it appears that the zona of the sheep ovum is impermeable to serum antibodies displaying reactivity specific for ovary and oocyte antigens. The results of experiment 1 suggest that antibodies reaching the blastomeres of ova with intact zonae during incubation with

antisera to ovary or oocytes were either inactive, or present in insufficient concen tration, to cause lysis of more than a very few ova ($< 10\%$). In this context it has been shown that antibodies specific for ovarian tissue of hamsters, rabbits and mice when exposed to oocytes become localized in the zona (Ownby and Shivers 1972; Sacco and Shivers 1973; Jilek and Pavlok 1975).

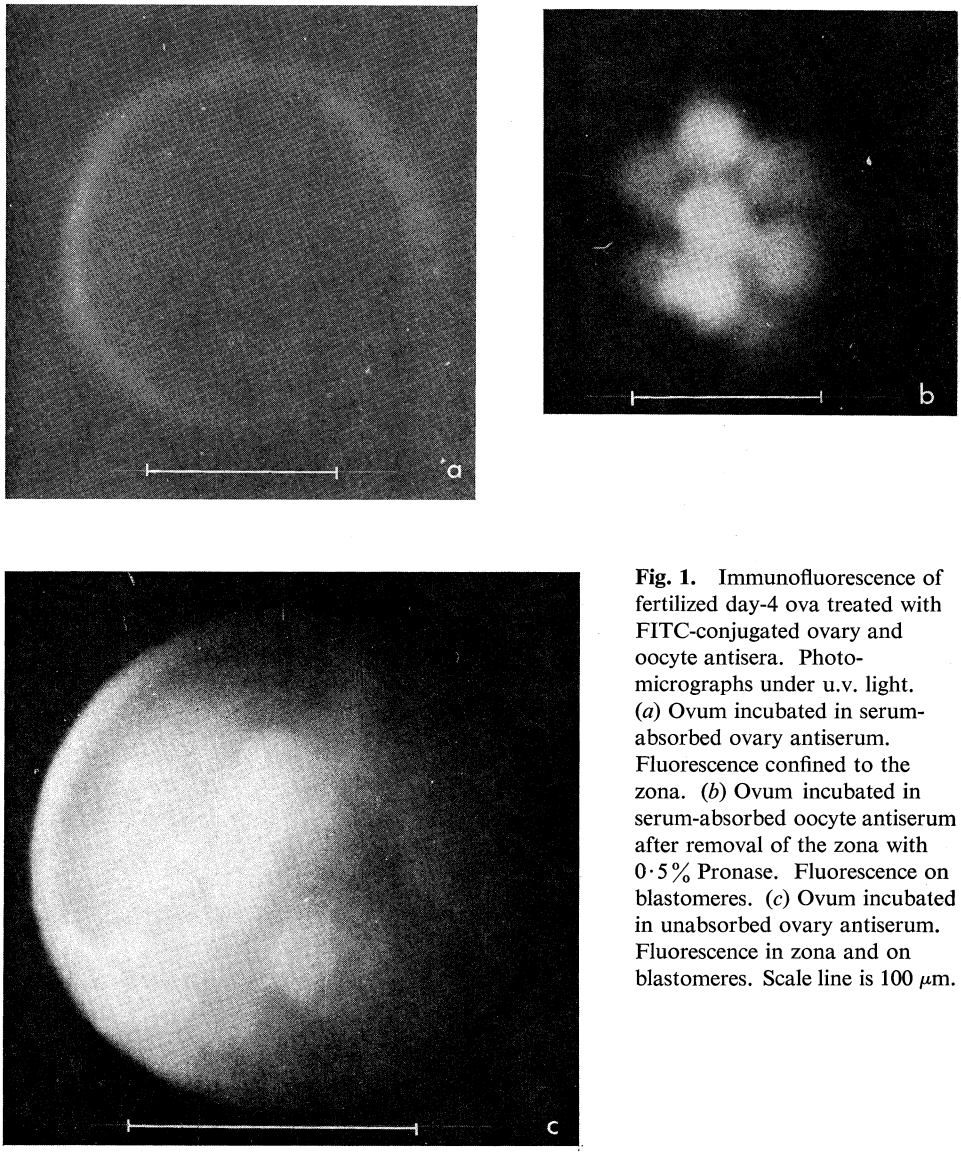

Fig. 1. Immunofluorescence of fertilized day-4 ova treated with FITC-conjugated ovary and oocyte antisera. Photo-micrographs under u.v. light. (*a*) Ovum incubated in serum-absorbed ovary antiserum. Fluorescence confined to the zona. (*b*) Ovum incubated in serum-absorbed oocyte antiserum after removal of the zona with 0·5% Pronase. Fluorescence on blastomeres. (*c*) Ovum incubated in unabsorbed ovary antiserum. Fluorescence in zona and on blastomeres. Scale line is 100 μm.

Like antisera to ovary and oocytes, anti-sperm serum had little effect on oocytes or fertilized ova with intact zonae. When zonae were removed both anti-ovary and anti-oocytes sera were effective in causing lysis of oocytes and fertilized ova, whereas anti-sperm serum appeared to be more effective in causing lysis of denuded fertilized

ova than of denuded oocytes (Table 1). The sperm used to fertilize the ova were from the ram used to produce the antiserum and it may be that the increased incidence of lysis in fertilized ova was due to the presence of paternal antigens in the fertilized ova.

There was no indication that day-6 ova were more or less susceptible to lysis than younger ova. In the mouse, Moskalewski and Koprowski (1972) reported an effect of age. They found that late blastocysts were less susceptible to lysis by antisera to oocytes than were early blastocysts and ova of 2–10 cells. In the sheep, late blastocysts are not observed until day 8 and hence the present experiment could not determine if a similar relationship exists between age of ovum and susceptibility to lysis. The ova of many mammalian species lose their zona pellucida at about the late blastocyst stage of development, thus exposing the denuded cells of the developing embryo to the uterine environment. Accordingly, it may be important to determine if development and subsequent loss of the zona is associated with loss of reactivity between ovum and anti-ovum serum.

The zona pellucida of the fertilized ovum appears to not only maintain the integrity of the devloping embryo but also to act as a barrier, to at least a low level, of immunoglobulin directed against the embryo. This may be a contributing factor to the failure of zona-free sheep ova to survive *in vivo* (Trounson and Moore 1974*a*). However, it will be necessary to demonstrate that uterine secretions in the ewe do in fact contain antibody to embryos before concluding that the zona plays an immunoprotective role in the survival and development of early embryos.

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References

Austin, C. R., and Lovelock, J. E. (1958). Permeability of rabbit, rat and hamster egg membranes. *Exp. Cell Res.* **15**, 260–1.

Enders, A. C. (1971). The fine structure of the blastocyst. In 'The Biology of the Blastocyst'. (Ed. R. J. Blandau.) p. 71. (University of Chicago Press: Chicago.)

Glass, L. E. (1963). Transfer of native and foreign serum antigens to oviducal mouse eggs. *Am. Zool.* **3**, 135–56.

Hastings, R. A., Enders, A. C., and Schlafke, S. (1972). Permeability of the zona pellucida to protein tracers. *Biol. Reprod.* **7**, 288–96.

Heyner, S., Brinster, R. L., and Palm, J. (1969). Effect of iso-antibody on pre-implantation mouse embryos. *Nature (London)* **222**, 783–4.

James, D. A. (1969). Antigenicity of the blastocyst masked by the zona pellucida. *Transplantation* **8**, 846–51.

Jilek, F., and Pavlok, A. (1975). Antibodies against mouse ovaries and their effect on fertilization *in vitro* and *in vivo* in the mouse. *J. Reprod. Fertil.* **42**, 377–80.

Moor, R. M., and Cragle, R. G. (1971). The sheep egg: enzymic removal of the zona pellucida and culture of sheep eggs *in vitro*. *J. Reprod. Fertil.* **27**, 401–9.

Moskalewski, S., and Koprowski, H. (1972). Presence of egg antigen in immature oocytes and preimplantation embryos. *Nature (London)* **237**, 167–8.

Ownby, C. L., and Shivers, C. A. (1972). Antigens of the hamster ovary and effects of anti-ovary serum on eggs. *Biol. Reprod.* **6**, 310–18.

Sacco, A. G., and Shivers, C. A. (1973). Effects of reproductive tissue-specific antisera on rabbit eggs. *Biol. Reprod.* **8**, 481–90.

Sellens, M. H., and Jenkinson, E. J. (1975). Permeability of the mouse zona pellucida to immunoglobulin. *J. Reprod. Fertil.* **42**, 153–7.

Simmons, R. L., and Russell, P. S. (1966). The histocompatibility antigens of fertilized mouse eggs and trophoblasts. *Ann. N.Y. Acad. Sci.* **129**, 35–45.

The, T. H., and Feltkamp, T. E. W. (1970). Conjugation of fluorescein isothiocyanate to antibodies. II. A reproducible method. *Immunology* **18**, 875–81.

Trounson, A. O., and Moore, N. W. (1974*a*). The survival and development of sheep eggs following complete or partial removal of the zona pellucida. *J. Reprod. Fertil.* **41**, 97–105.

Trounson, A. O., and Moore, N. W. (1974*b*). Attempts to produce identical offspring in the sheep by mechanical division of the ovum. *Aust. J. Biol. Sci.* **27**, 505–10.

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