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Induction of Autoantibodies to Zona Pellucida by Immunization with an 18mer Synthetic Peptide in Rabbits

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Abstract: *For the development of a safe contraceptive vaccine, it is necessary to define B cell epitopes which could induce fertilization-blocking autoantibodies. In this study, an 18mer peptide (CTYILDPEKLTLRVPYKA) of rabbit ZPA which was recognized by a fertilization-blocking monoclonal antibody was chemically synthesized and conjugated with diphtheria toxoid (DT). The conjugate was injected into rabbits i.d. with complete Freund's adjuvant. As a control, DT was injected into rabbits in the same way. Mice were also immunized with the same synthetic peptide to induce heteroantibodies. Antisera from both rabbits and mice reacted not only with the cognate peptide but also with the isolated intact zona pellucida of rabbits. The immunohistochemical study showed that the antibodies had remained bound to the zona pellucida of the immunized rabbits in vivo. It is therefore concluded that the synthetic peptide could induce autoantibodies reactive to the auto-antigen of the zona pellucida in rabbits.*

Key words: *Zona pellucida, Contraceptive vaccine, Autoantibody, Synthetic peptide, ZPA.*

Mammalian eggs are surrounded by a unique extracellular envelope, the zona pellucida, composed of acidic glycoproteins, whose synthesis and secretion are developmentally programmed during oocyte growth and follicular development [1–3]. The zona glycoproteins play an important role in the fertilization process including primary binding of spermatozoa followed by the induction of acrosome reaction, secondary tight binding of acrosome-reacted speramatozoa and the prevention of polyspermy.

The zona pellucida is comprised of three distinct polypeptides coded by three different genes: ZPA, ZPB and ZPC [4]. Recently, the homologue of ZPC was found not only in mammals but also in fish [5] and *Xenopus laevis* [6], suggesting important roles of the zona pellucida related proteins in reproduction.

It is well known that the zona pellucida elicits no immunological response or only a very weak response when it is used for isologous or autologous immunization [7, 8]. In contrast, heterologous immunization with zona pellucida could induce a strong antibody response [9–12]. Therefore, in early studies, attempts were made to develop a contraceptive vaccine by using the zona pellucida from heterologous species [13–16], but the immunized animals showed signs of ovarian dysfunction associated with depletion of primordial follicles resulting in premature ovarian failure[17–20].

Recently it was suggested that the zona pellucida contains two types of antigens, one to produce fertilization-blocking antibodies and another is to induce cytotoxic effects on the ovaries [21]. Because the latter T cell immunological pathway is thought to be a cause of ovarian dysfunction, for the development of a safe contraceptive vaccine, it is important to define B cell antigens to induce the production of fertilization-blocking antibodies.

For this purpose, we prepared a fertilization-blocking mouse monoclonal antibody (MAb-5H4) to pig zona pellucida (purified pZP4) and found the epitope to be present on an amino acid sequence (CTYVLDPENLTLKAPYEA) [3, 22]. An 18mer synthetic peptide corresponding to the sequence was conjugated with KLH for immunization of mice and rabbits. The resulting antisera reacted with pig zona pellucida and inhibited pig *in vitro* fertilization [23], but the rabbit antiserum showed a very poor reaction to rabbit zona pellucida, though MAb-5H4 reacted to both pig and rabbit zona pellucida [24]. It was therefore thought that the difference between the reactivity in rabbits and pigs was due to the difference in amino acids in five positions included in the 18mer peptide. In this study, an

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18mer rabbit synthetic peptide of zona protein (CTYILDPEKLTLRVPYKA) was used for immunization in rabbits and mice and examined for the production of autoantibodies reactive to rabbit zona pellucida.

Materials and Methods

1. Immunization

The synthetic 18mer peptide (CTYILDPEKLTLRVPYKA) was custom prepared by a solid phase method (Kurabo) and conjugated with diphtheria toxoid (DT) by the MCS (6-maleimidocaproic acid N-hydrosuccinimide ester) method according to the manufacturer's instructions (Wako). Three Japan White rabbits were injected i.d. with 500 μg of the synthetic peptide in emulsion with complete Freund's adjuvant at multiple sites on the back and footpads. After two weeks, the rabbits were injected with the same antigen in incomplete Freund's adjuvant followed by three additional booster injections i.p. without adjuvants at intervals of 2 days from 2 weeks after the second injection. ICR mice were subjected to the same immunization as the above schedule with 100 μg of injected dosage instead of 500 μg. Both animals were bled one week after the last booster injection.

2. ELISA

Production of the antibody to the synthetic peptide was assessed by ELISA with the cognate peptide. A microtiter plate (Falcon) was coated overnight with the peptide in carbonate buffer, pH 9.6 at 4°C in an amount of 1 μg/well. After blocking the plate with 1% BSA-PBS, the antiserum was added to each well diluted $1:2^2 \times 100$ to $1:2^8 \times 100$ followed by incubation for 1 h at room temperature. After three washing with PBS, peroxidase-labeled donkey anti-rabbit IgG or goat anti-mouse IgG (Chemicon International) was added to each well as a second antibody. Color development was carried out with 0.2 mg/ml of o-phenylene diamine and 0.01% (v/v) H_2O_2 in 150 mM citrate-phosphate buffer, pH 5.0.

3. Immunofluorescent staining

Rabbit IgG fraction purified by Protein A Sepharose (Pharmacia) was used to reduce the non-specific reaction. Normal rabbit oocytes were obtained by superovulation. A donor rabbit was injected with 70 IU of PMS s.c., followed by 80 IU of hCG. The oocytes were collected from the oviducts by flushing with 1%BSA-PBS and were treated with 0.1% hyaluronidase to remove the cumulus cells. The oocytes were treated with purified rabbit IgG (100 μg/ml) for 30 min at room temperature. After extensive washings with 1% BSA-PBS, the oocytes were treated with FITC-labeled donkey anti rabbit IgG antiserum absorbed with mouse, human and bovine IgG at a dilution of 1:100 at room temperature for 30 min. After washing, the oocytes were fixed on a slide glass for observation under a UV-microscope.

For detection of autoantibodies bound to the zona pellucida, the ovaries were taken from an immunized rabbit and mounted in OCP compound (Miles). Serial cryostat sections 6 μm thick were washed with PBS and followed by the treatment with FITC labeled donkey anti rabbit IgG antiserum in the manner described above.

Results

When the rabbit 18mer synthetic peptide conjugated with DT was injected into mice (heterologous system), high titer antibodies to the peptide were produced (Fig. 1-B), and when the same synthetic peptide was injected into rabbits (homologous system), antibodies with high titers were produced (Fig. 1-A). The antisera from animals immunized with DT as a control showed only little reactivity to the synthetic peptide. These results indicate that the 18mer peptide was immunogenic in both species.

Figure 2 shows the indirect immunofluorescent staining of rabbit oocytes with the rabbit IgG fraction purified from the antiserum. The anti 18mer peptide antibody

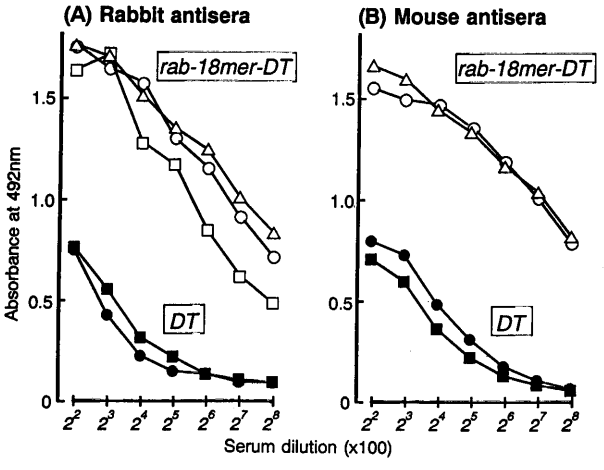

Fig. 1. ELISA with antisera to the 18mer synthetic peptide. Antisera from both rabbits (A) and mice (B) reacted with the cognate peptide. ●, ■: Control antisera from animals immunized with diphtheria toxoid. △, ○, □: The antisera from animals immunized with the 18mer peptide-conjugated diphtheria toxoid.

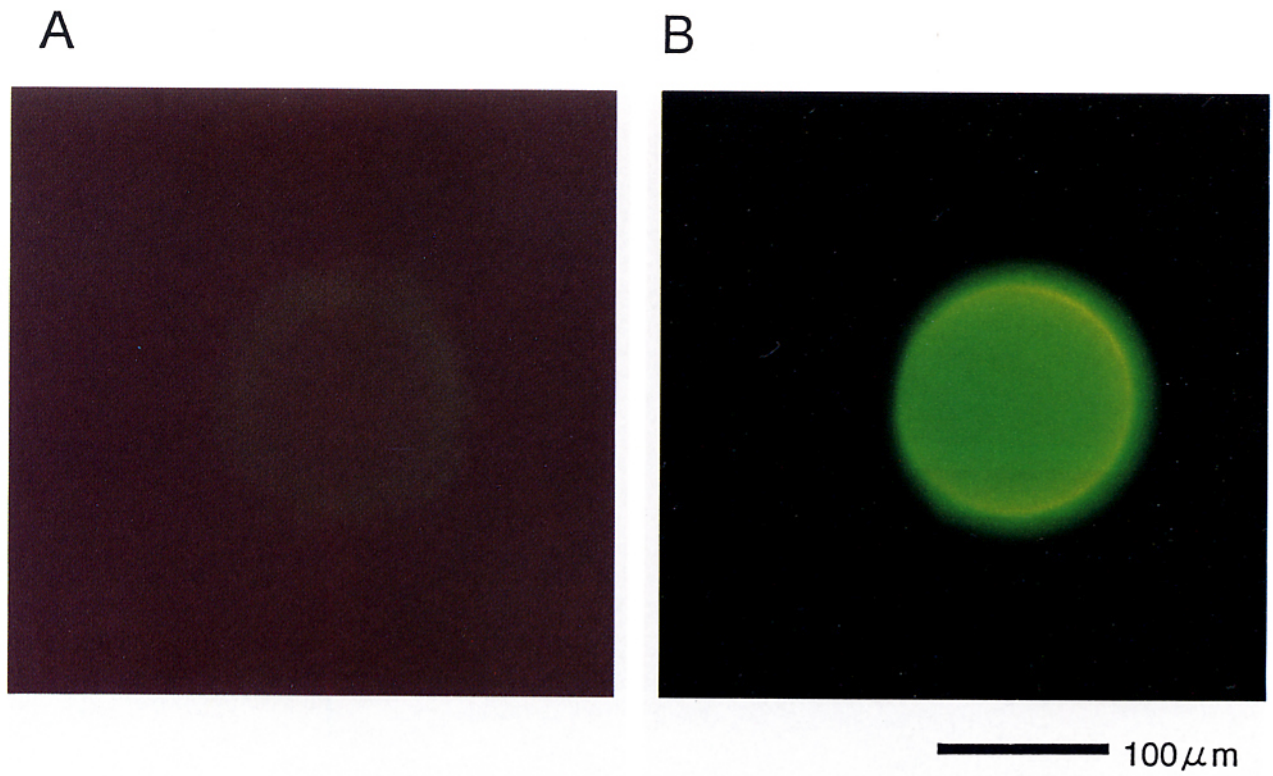

Fig. 2. Immunofluorescent staining of rabbit oocytes with the antisera by diphtheria toxoid (A) and the 18mer peptide conjugated diphtheria toxoid (B). The photographs shows that the antibody produced by the 18mer peptide recognized the native zona pellucida antigen in rabbits.

clearly stained the zona pellucida, but the anti DT antibody did not. This shows that the rabbit synthetic peptide possessed autoreactive immunogenicity and that the immunized rabbits induced autoantibodies reactive to the self-zona components. In addition, the zona pellucida in the sections of ovaries from the immunized rabbit was clearly stained by the treatment with the second antibody of the anti-rabbit IgG antiserum (Fig. 3-B), but the ovarian section from the control rabbit immunized with DT showed no specific staining (Fig. 3-A). This means that the autoantibodies produced in the immunized rabbit remained bound to the target antigen of the zona pellucida *in vivo*.

Discussion

Early studies showed that the zona pellucida possesses a strong immunogenicity against heterologous species and the raised antiserum strongly inhibits fertilization [7, 11, 25, 26]. The immunized animals produced antibodies reactive to their own zona pellucida, and they fell into infertility. On the basis of this species cross-reactivity of zona antigens [27–29], intensive studies have been done to develop contraceptive vaccines [13, 15, 16], but the animals immunized with heterologous zona antigens had ovarian dysfunction associated with depletion of primordial follicles resulting in premature ovarian failure. It is therefore necessary to segregate the epitope that inhibits the sperm-zona pellucida interaction from other epitopes that cause the induction of ovarian failure.

It has recently become possible to investigate the target antigens of zona pellucida at epitope levels, since cDNAs have been cloned in various mammalian species including mice [30, 31], hamsters [32], rabbits [33, 34], humans [35, 36], marmosets [37], pigs [4, 38], dogs, cats and cows [4]. On the basis of amino acid sequences deduced from cDNA, various core peptides were synthesized and their immunogenicity and the biological effects of the antisera raised to the synthetic peptides were examined. Yurewicz *et al.* reported that a site-directed antibody was produced against a synthetic 11mer peptide corresponding to the amino-terminal region of pZP3 α (pig ZPB), and that the antiserum

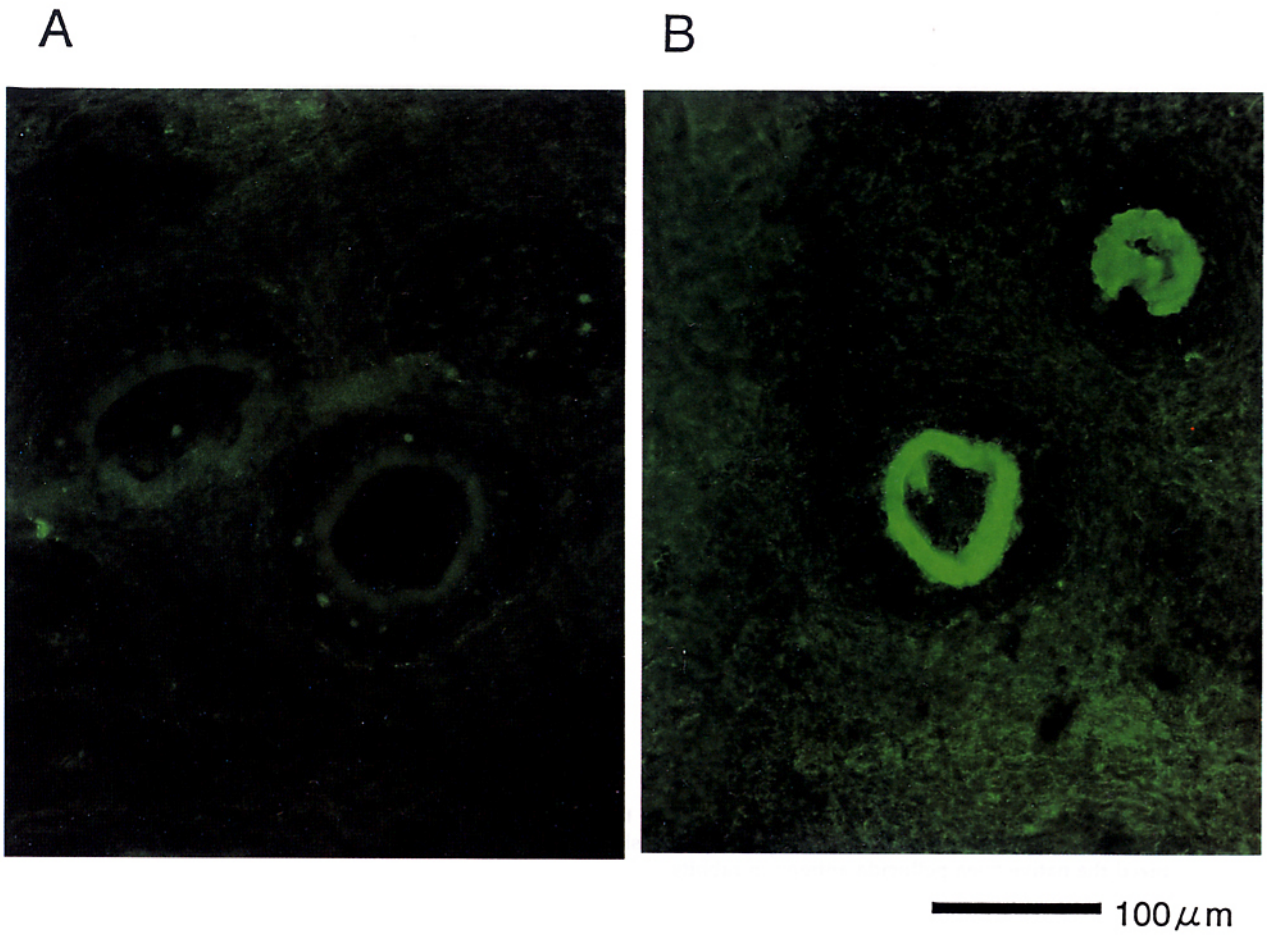

Fig. 3. Immunofluorescent staining of ovarian sections from the rabbit immunized with diphtheria toxoid (A) and the 18mer peptide-conjugated diphtheria toxoid (B). The frozen sections were treated only with FITC conjugated second antibody (1:00). The photograph shows that the antibody produced by 18mer peptide-conjugated diphtheria toxoid (B) remained bound to the zona pellucida of the immunized rabbit *in vivo*.

produced by the peptide partially interfered with boar sperm binding to porcine zona pellucida [39]. Gupta *et al.* also reported preliminarily that the synthetic peptide corresponding to the positions 323–341 of human ZP3 (ZPC) induced antibodies in hamsters, but they did not mention any biological activities of the antiserum [40]. Millar *et al.* reported that the active immunization with 16mer mouse synthetic peptide induced long-term infertility in female mice [41].

Previously we found that the 18mer pig synthetic peptide (CTYVLDPENLTLKAPYEA) conjugated with diphtheria toxoid (DT) induced good production of an antibody reactive to native pig zona pellucida in rabbits [23], but the antibody showed only a poor reactivity to rabbit zona pellucida [24]. Since it seemed that the poor reactivity of the antiserum to rabbit zona pellucida is due to the difference in the amino acid sequence of the rabbit zona protein in the corresponding region, the 18mer rabbit synthetic peptide (CTYILDPEKLTLRVPYKA) was newly prepared for rabbit immunization. The peptide conjugated with DT as a carrier protein produced antibodies reactive not only with the cognate peptide but also with the native rabbit zona pellucida. These results indicated that the 18mer homologous peptide could induce autoantibodies by conjugating a strong heterologous carrier protein such as DT.

In general, it has been believed that the antigen from homologous zona pellucida has only weak antibody production, but in this report, we demonstrated that the 18mer rabbit synthetic peptide could elicit antibodies strongly reactive to self-zona pellucida. This suggests that antibody production by the zona autoantigens is suppressed by the level of helper T cells, but that the B-cells reactive to the autoantigens are not eliminated, so

that it is possible to promote antibody production by inducing the helper T cell function with an appropriate T cell antigen such as DT. In addition, it was suggested that the antibodies induced by the amino acid sequence of the same species had a higher affinity than those induced by the cross-reactive antigens.

The present findings showing that the homologous zona antigen could induce the autoantibody reactive to the zona pellucida of the recipient encouraged us to continue the research on a contraceptive vaccine by using synthetic peptides with identical amino acid sequences to the zona protein of animals for immunization. The effect of active immunization on fertility and any adverse effects of long-term immunization are now under investigation.

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