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Blocking Effect of Sperm Immobilizing Antibodies on Sperm Penetration of Human Zonae Pellucidae

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To investigate the mechanism of the blocking effect of sperm immobilizing antibodies on human fertilization, an in vitro zona penetration test was carried out using media containing the IgG fraction extracted from sperm immobilizing antibody-negative or -positive serum. The sperm penetration rate of the test was 100% (6/6) when spermatozoa were treated with the IgG fraction derived from sperm immobilizing antibody-negative serum, whereas it was only 17% (1/6) when spermatozoa were treated with the IgG fraction derived from sperm immobilizing antibody-positive serum. Electron microscopic observation of the sperm immobilizing antibody-negative and -positive serum-treated spermatozoa showed that the number of acrosome-reacted spermatozoa was significantly greater in the sperm immobilizing antibody-negative serum than in the antibody-positive serum. Therefore, it appears that one of the blocking mechanisms of the spermatozoal penetration of the zona pellucida by sperm immobilizing antibodies may be due to inhibition of the acrosome reaction in the spermatozoa.

KEY WORDS: sperm immobilizing antibody; zona pellucida; acrosome reaction; highly concentrated salt solution.

INTRODUCTION

Antisperm antibodies have been shown to be the cause of unexplained infertility in certain women (1,2). Moreover, it is well known that antisperm antibodies inhibit the penetration of spermatozoa through the cervical mucus by trapping or immobilizing them (3,4). On the other hand, recent reports state that pregnancy was established in a patient with antisperm antibodies in her serum by in vitro fertilization (IVF) using donor serum or human cord serum instead of the patient's own serum (5,6). Therefore, antisperm antibodies appear to block human fertilization at the site of fertilization besides at the site of the cervical mucus. Among antisperm antibodies, it is apparent that sperm immobilizing antibodies (SIAs) are closely related to human infertility (4,7). We have reported that SIA-positive serum has a blocking effect on sperm penetration of human zonae pellucidae (8). However, it has not been confirmed that the blocking effect is due to the participation of SIA itself. In this study we used the IgG fraction derived from SIA-positive serum to investigate the role of SIAs in this effect.

MATERIALS AND METHODS

Sperm Immobilization Test

The sperm immobilization test (SIT) as described by Isojima *et al.* was carried out with sera obtained from infertile outpatients at Kyoto University Hospital to detect complement-dependent SIAs (7). These sera were inactivated at 56°C for 30 min before the SIT. The quantitative sperm immobilization test for the sera judged as SIA-positive (SIA = ∞) was carried out to find accurate antibody titers (9).

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Immunobead Binding Assay

Freshly ejaculated semen of a healthy fertile donor was mixed with phosphate-buffered saline (PBS) containing human serum albumin (HSA) (Sigma Chemical Co., St. Louis, MO), 5 mg/ml, at 1:5 (vol/vol), and the sperm pellet (5×10^6 spermatozoa) was obtained after centrifugation at 250*g* for 5 min. After 0.1 ml of SIA-positive serum from patient 112, who had the highest titer in the quantitative sperm immobilization test, was diluted with 0.3 ml of PBS, 5×10^6 spermatozoa were suspended in the diluted serum and were incubated at 37°C for 30 min. After incubation the sperm suspension was centrifuged at 250*g* for 5 min. The spermatozoa were resuspended in PBS containing HSA, 5 mg/ml. Immunobeads (Bio-Rad Laboratories, Richmond, CA), coated with anti-human IgG or anti-human IgM, were suspended in PBS (2 mg/ml). Five microliters of the sperm suspension were mixed with 50 μl of the immunobead suspension on the slide glass. After reaction for 10 min, the number of spermatozoa attached by the immunobeads and the attached sites were examined under a phase-contrast microscope (10). The attached sites were classified as head, tail, head and tail, and bead-free, and the judgment was made for every spermatozoon (Fig. 1).

Extraction of the IgG Fraction and Preparation of the Medium

The IgG fraction was extracted from SIA-positive serum of the same patient. Ten milliliters of the serum was mixed with the same volume of PBS. The mixture was applied to a column containing 1.5 g (5 ml) of protein A–Sepharose (Pharmacia Fine Chemicals, Uppsala, Sweden) at the rate of 0.5 ml/min. Then the column was washed with PBS (pH 7.4). After washing the absorbance was confirmed to be $OD_{280} < 0.01$. The IgG bound to protein A in the column was eluted with 0.1 *M* glycine–HCl (pH 2.8) and the elutants were condensed by ultrafiltration; the concentration of IgG was estimated by the immunodiffusion method. HSA (3.3 mg/ml) and IgG (1.2 mg/ml) were added to the modified Biggers–Whitten–Whittingham (BWW) medium (8,11).

Preparation of Zonae Pellucidae

Immature oocytes were collected from the ovaries removed in operations for uterine cancer under informed consent and incubated under 5% CO_2 in air at 37°C for 48 hr to bring about maturation (12). After incubation only the perfectly intact zonae pellucidae as confirmed under a dissecting microscope were stored at 4°C in a highly concentrated salt solution [0.5 *M* $(NH_4)_2SO_4$ + 1.0 *M* $MgCl_2$ + 0.1% Dextran (Nakarai Chemical Co., Kyoto, Japan)] (13). The zonae pellucidae were desalted by washing before use.

Zona Penetration Test

Motile spermatozoa were collected from the freshly ejaculated semen obtained from a healthy fertile donor after 1 hr of incubation in modified BWW medium containing the above-mentioned IgG fraction derived from SIA-negative or -positive serum, and a sperm suspension of 10×10^6 sperm/ml was prepared. The salt-stored zonae pellucidae were desalted by washing and incubated in 300 μl of the above-mentioned sperm suspension under 5% CO_2 in air at 37°C for 24 hr (8). After incubation the zonae pellucidae were washed several times with a micropipette and the number of spermatozoa in the perivitelline space was counted under a Nomarski interference-contrast microscope (Olympus IMT-2-21NR, Tokyo, Japan).

Acrosome Reaction

Motile spermatozoa obtained by the layering method after 1 hr of incubation in modified BWW medium containing the IgG fraction derived from SIA-negative or -positive serum were further incubated for 3 hr in the same medium (14). After incubation, spermatozoa were prefixed in 2% glutaraldehyde, postfixed in 1% osmic acid, dehydrated, and embedded in Epon (15). After embedding the acrosome, reaction in the sperm head was examined with a transmission electron microscope (Hitachi HU 12A, Tokyo, Japan).

RESULTS

Immunobead Binding Assay

The SIT was positive in 13 (6.1%) of the 212 infertile outpatients. Additionally, the quantitative sperm immobilization test was made for the SIA-

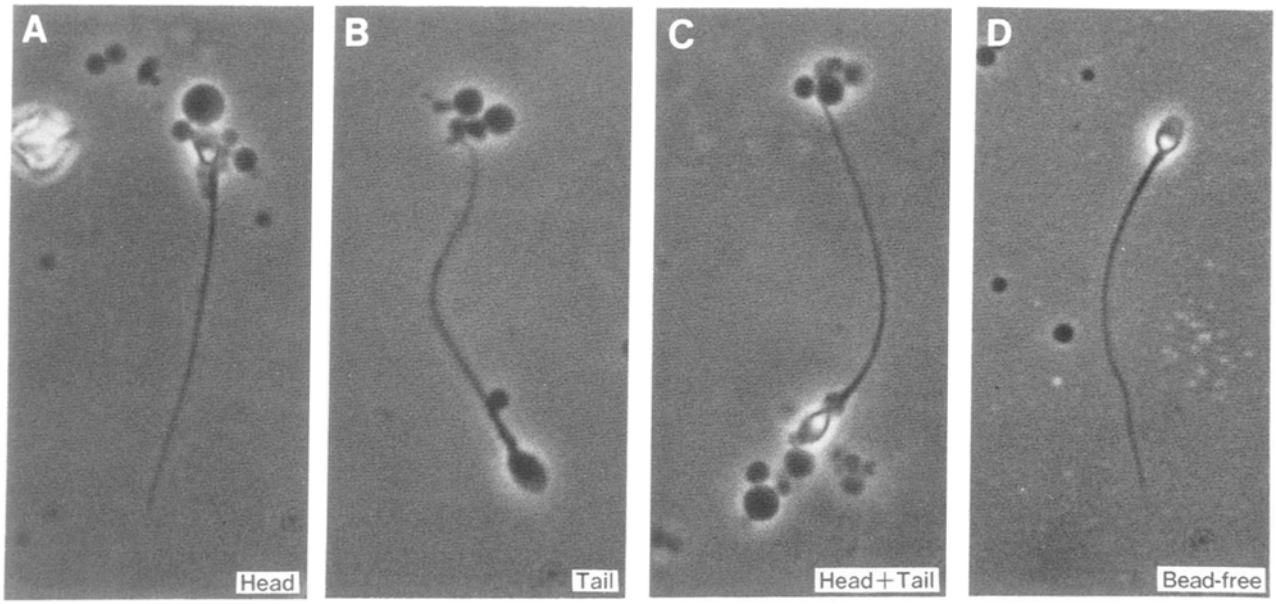

Fig. 1. Four types of immunobead-binding sites on spermatozoa.

positive (SIV = ∞) sera. The antibody titer was the highest (i.e., SI_{50} = 32.0) in the serum of patient 112. When the spermatozoa were treated with the SIA-positive serum of this patient, immunobeads coated with anti-human IgG adhered to 90% of the spermatozoa in the immunobead binding assay, but those coated by anti-human IgM adhered to only 33% of the spermatozoa (Table I). Therefore, mainly the IgG fraction had an antisperm antibody activity in this case. The immunobeads coated with anti-human IgG adhered almost uniformly to the sperm head and tail, and those coated with IgM adhered mainly to the sperm head.

Zona Penetration Test

When the spermatozoa were treated with the IgG fraction derived from SIA-negative serum as shown in Table II, the penetration rate of the zona pellucida was 100%. In contrast, the penetration rate of the zona pellucida by spermatozoa treated with IgG derived from SIA-positive serum was only 17%, significantly lower than the former rate when calculated by chi-square analysis with Yate's correction. In such complement-free medium, sperm motility was well maintained throughout the incubation period even in the medium supplemented with IgG derived from SIA-positive serum. There was no remarkable difference between the two kinds of media in sperm motility. However, at the end of incubation, when 50 μl of guinea pig serum as a complement was added to 300 μl of the medium including IgG derived from SIA-positive serum, complete immobilization of spermatozoa in the medium occurred immediately. On the contrary, the sperm motility rate did not change when the same serum was added to the medium including IgG derived from SIA-negative serum.

Table I. Immunobead Binding to Human Spermatozoa Pretreated with Sperm Immobilizing Antibody-Positive Serum

Immunobead	Case No. of supplemented serum (SI_{50})[a]	No. of sperm		% bound
		Bound (head, tail, head + tail)	Free	
Anti-human IgG	Control	3 (1, 2, 0)	29	9
	112 (32.0)	45 (16, 17, 12)	5	90
Anti-human IgM	Control	2 (1, 1, 0)	27	7
	112 (32.0)	12 (9, 3, 0)	24	33

[a] Fifty percent sperm immobilization unit.

Table II. Effect of Sperm Immobilizing Antibodies (IgG) on the Penetration of Sperm Through Zonae Pellucidae

Origin of supplemented IgG	No. of experiments	No. of penetrated ova	Mean No. of penetrated sperm
SIA[a]-negative serum	2	6/6 (100%)	2.7
SIA-positive serum	2	1/6 (17%)*	0.5

[a] Sperm immobilizing antibody.
* Significantly different from SIA-negative serum ($\chi^2 = 5.5$; $P < 0.02$).

Acrosome Reaction

As shown in Fig. 2, spermatozoa with an entirely vesiculated acrosome cap were regarded as "acrosome reacted"; those with partial vesiculation, as "modified acrosome reacted"; and those without any vesiculation, as "acrosome intact." Those which could not be evaluated were designated "unknown." Spermatozoa with any vesiculation, however, whose vesiculation could not be determined as spontaneously occurring or not, were included in this group. Of the spermatozoa treated with the IgG fraction derived from SIA-negative serum, 35% were acrosome reacted, whereas of those treated with the IgG fraction derived from SIA-positive serum, 18% were acrosome reacted. As for acrosome-intact spermatozoa, the rates were 21% in the former and 49% in the latter. Therefore, the number of acrosome-reacted spermatozoa was significantly lower in the SIA-positive serum than in the SIA-negative serum, and the reverse was true in the case of acrosome-intact spermatozoa when calculated by chi-quare analysis with Yate's correction (Table III).

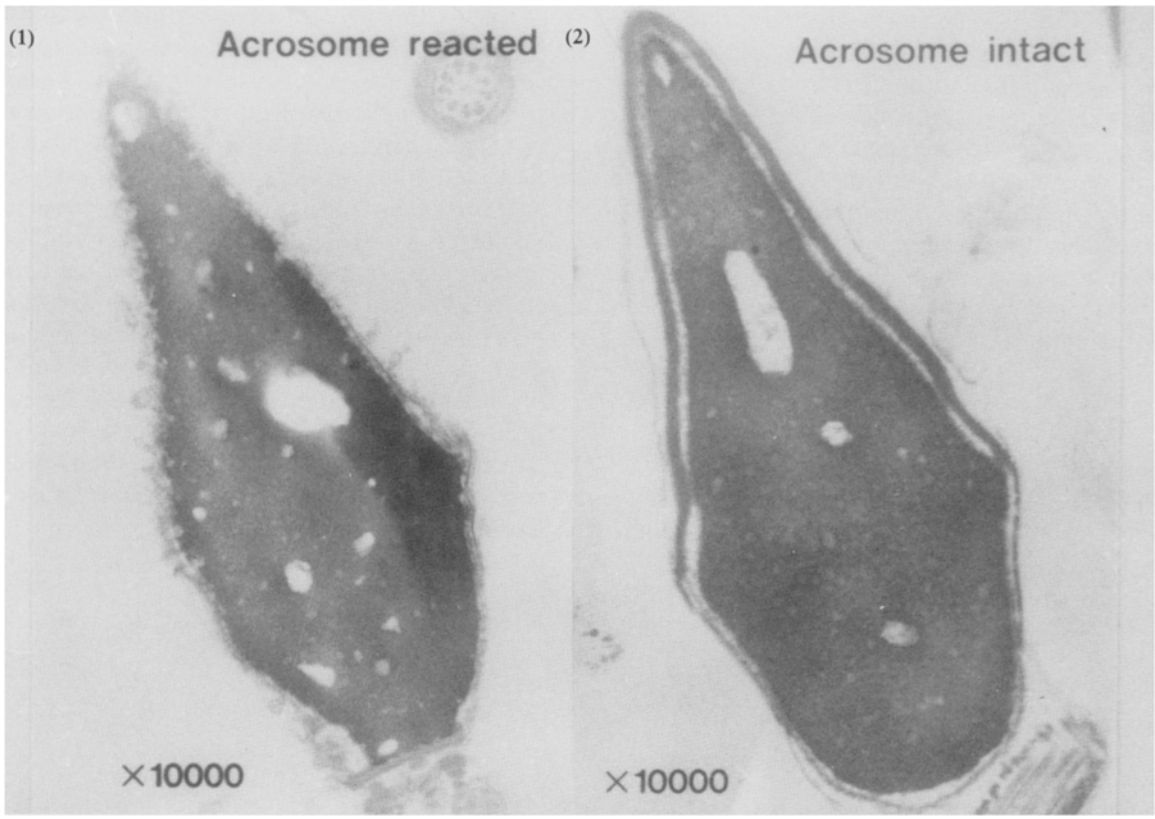

Fig. 2. (1) Spermatozoon incubated with the IgG fraction derived from sperm immobilizing antibody-negative serum. (2) Spermatozoon incubated with the IgG fraction derived from sperm immobilizing antibody-positive serum.

DISCUSSION

Yanagimachi *et al.* reported that the biological properties of the zona pellucida can be maintained in a highly concentrated salt solution (13). As mentioned earlier, by using these properties, we have reported that the zona penetration by spermatozoa is blocked in the presence of SIA-positive serum in the zona penetration test (8). In this study we performed the zona penetration test by replacing the serum with HSA and IgG fraction to eliminate any effects of unknown factors present in the serum. And similar results could be obtained as reported previously, although the number of zonae pellucidae used in this experiment was very small, which was due mainly to the difficulty in obtaining zonae pellucidae.

On the other hand, the IgG fraction used in these experiments is considered to have SIA activity, because complete immobilization of spermatozoa occurred immediately when complements were added. Moreover, the sperm motility did not affect the zona penetration rate, because according to our observation, there was no remarkable difference in sperm motility between the two kinds of media throughout the incubation period. Therefore, the blockade of the zona penetration is considered to be the effect of SIA itself. Although the mechanism of such blockade is unknown, it is thought to be due to (i) inhibition of capacitation, (ii) inhibition of the acrosome reaction, and (iii) inhibition of sperm–zona interaction. We examined with an electron microscope 148 spermatozoa which were treated with the IgG fraction derived from SIA-negative serum or SIA-positive serum. Electron microscopic examination showed that spermatozoa regarded as "acrosome reacted" were significantly more numerous when spermatozoa were treated with the IgG fraction derived from SIA-negative serum than when they were treated with the IgG fraction from SIA-positive serum. However, there were relatively large numbers of spermatozoa in the unknown group. It is somewhat difficult to conclude that vesiculation in the sperm head occurred spontaneously because artificial vesiculation in the sperm head occurs occasionally in the course of fixation.

Therefore, spermatozoa whose vesiculation was not concluded to occur spontaneously were included in the unknown group. This is the reason why there were relatively large numbers of spermatozoa in the unknown group. Stock and Fraser (16) examined over 25,000 spermatozoa with an electron microscope. They reported that after 24 hr of incubation, a mean of 15.4% spermatozoa had initiated the acrosome reaction; this figure included 9.7% which had completed it. According to this report, the acrosome reaction rate increases significantly with time but is relatively lower than that in our report (16). Therefore, it is necessary for us to examine more spermatozoa to obtain more precise data.

There have been some reports that the acrosome reaction is inhibited by antisperm antibody (IgG) and a report that SIA does not inhibit capacitation (17–19). As reported previously, when only the zona pellucida is treated with SIA-positive serum, zona penetration by spermatozoa is not blocked (8). It is, therefore, likely that the fertilizing ability of spermatozoa is blocked at the level of the acrosome reaction. This idea was supported by the results of the present study. At the moment, the mechanism of the inhibitory effect of SIA on the acrosome reaction is unknown. Friend *et al.* reported that particles in the plasma membrane of spermatozoa migrate in the plasma membrane of spermatozoa preceding the acrosome reaction and that the acrosome reaction originates in the par-

Table III. Effect of Sperm Immobilizing Antibodies (IgG) on the Acrosome Reaction of Human Spermatozoa

	No. (%) of sperm				
Origin of supplemented IgG	Acrosome reacted	Modified acrosome reacted	Acrosome intact	Unknown	Total
SIA[a]-negative serum	26 (35)	19	16 (21)	14	75
SIA-positive serum	13 (18)*	19	36 (49)**	5	73

[a] Sperm immobilizing antibody.
* Significantly different from SIA-negative serum ($\chi^2 = 4.6$; $P < 0.04$).
** Significantly different from SIA-negative serum ($\chi^2 = 11.5$; $P < 0.002$).

ticle-free area (20). Johnson reported that when macromolecules such as lectin and antibodies are bound to the plasma membrane, particles in the lipid bilayers of the plasma membrane do not move easily and then the acrosome reaction is considered to be inhibited (21). Taking account of these findings, one of the blocking mechanisms of SIA may be due to inhibition of particle migration in the sperm plasma membrane.

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