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Inhibition of Acrosin Activity with a Trypsin Inhibitor Blocks Human Sperm Penetration of the Zona Pellucida

DE YI LIU[1] and H.W. GORDON BAKER

Department of Obstetrics and Gynaecology, University of Melbourne and Reproductive Biology Unit Royal Women's Hospital, Carlton, Melbourne, Victoria 3053, Australia

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ABSTRACT

To evaluate the role of acrosin in human sperm penetration of the zona pellucida (ZP), sperm-oocyte interaction was studied after acrosin activity was blocked with soybean trypsin inhibitor (SBTI). Oocytes that had failed to fertilize because of sperm pathology in a clinical in vitro fertilization program were used to assess sperm binding to and penetration into the ZP. The acrosome reaction of sperm bound to the ZP was determined using fluorescein-labeled *Pisum sativum* agglutinin after sperm were removed from the ZP. Acrosin activity, determined by a gelatin substrate film method, was severely inhibited by 2 mg/ml SBTI. Sperm motility and movement characteristics, assessed by a Hamilton-Thorn motility analyzer, were unchanged after 6-h incubation with SBTI. Inhibition of acrosin activity did not affect the number of sperm bound to the ZP but completely blocked sperm penetration of the ZP after a 5-h incubation. SBTI did not influence the spontaneous acrosome loss of sperm in culture medium after 6-h and 20-h incubations, but the percentage of acrosome-reacted sperm bound to the ZP was significantly reduced. It was concluded that acrosin activity plays a key role in sperm-zona interaction in humans. Motile sperm are unable to penetrate the ZP when acrosin activity is inhibited. This might result from interference with a phase of the sperm-ZP binding reaction or with a lytic action of acrosin. Also, acrosin may be involved in the acrosome reaction induced by sperm binding to the ZP.

INTRODUCTION

Sperm binding to and penetration through the zona pellucida (ZP) are important steps during fertilization [1]. Sperm binding to the ZP in humans is more strictly species-specific than in most other mammals such as mouse and hamster, and rhesus, squirrel, and marmoset monkeys [2, 3]. The mechanism of sperm-ZP binding and sperm-ZP penetration is not completely understood in most mammals, including humans. In the mouse, sperm bind to the ZP with the acrosome intact and the acrosome reaction occurs on the ZP, induced by one of the ZP glycoproteins, ZP3 [4, 5]. Some evidence suggests that human sperm bind to the ZP with the acrosome intact and that the physiological acrosome reaction occurs on the ZP [6–8]. Human sperm without an acrosome will not bind to the ZP [9].

It is believed that several functions such as the shape of the sperm head, motility, and acrosin activity are strongly involved in sperm penetration of the ZP. Sperm without an acrosome and immotile sperm will not penetrate an intact ZP [1, 9, 10]. The hyperactivated motility of capacitated sperm provides the sperm with strong thrusting power and may also be essential for sperm penetration through the ZP [11, 12]. Acrosin, a neutral proteinase, is a major acrosomal enzyme and is believed to play a role in sperm-ZP interaction [13, 14]. It has been reported that fertilization does not occur when this enzyme activity is inhibited [15]. Early studies by Stambaugh and Buckley [16] and Polakoski and McRorie [17] showed that acrosomal extracts or purified fractions of rabbit acrosin will dissolve the ZP slowly in vitro. However, Hartree [18] found that the crude acrosomal extracts are more effective in dissolving the zona than is pure acrosin. Tesarik et al. [19] have shown that acrosin is deposited along the path of human sperm penetrating through the ZP, though this has not been observed in other mammals. Thus this trypsin-like enzyme could play a major role in ZP penetration. However, human acrosin activity and sperm-ZP penetration have not been studied in detail, and it is still not clear whether motile sperm can penetrate the ZP after acrosin activity is completely inhibited.

We have studied human sperm binding to and penetration of the ZP, as well as the status of acrosome of sperm bound to the ZP, after inhibiting acrosin activity with soybean trypsin inhibitor (SBTI).

MATERIALS AND METHODS

Sperm Samples and Preparation

Sperm were obtained from normal fertile donors, or men with normal semen analysis. Motile sperm were selected by a swim-up technique as follows. Semen (0.5 ml) was placed in the bottom of a test tube (12 × 75 mm), the inside wall of which had been wetted with medium; then 1.0 ml human tubal fluid (HTF) medium containing 10% heat-inactivated human serum was carefully layered on the top of the semen. After incubation for 1 h, 0.8 ml of the top layer of the medium containing motile sperm was aspirated; then the motile sperm suspension was centrifuged at 600 × g for 7 min, the supernatant was removed, and the sperm pellet was resuspended in fresh HTF containing 10% serum. Only samples in which more than 90% of sperm showed progressive motility were used for subsequent experiments.

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[1]Correspondence. FAX: (61) 3-344-1761.

Human Oocytes

Oocytes that showed no evidence of two pronuclei or cleavage at 48 to 60 h after insemination within a clinical in vitro fertilization (IVF) program were used. All the oocytes were mature and none were degenerating. The project was approved by the Royal Women's Hospital Research and Ethics Committees. In the present study, all the oocytes were obtained from patients in whom ≥ 8 oocytes had been inseminated, all had failed to fertilize, and no sperm had bound to the ZP. The failure of fertilization in these patients was clearly due to sperm defects such as severe oligospermia or teratospermia. Most of the oocytes displayed regular shape and had lost the cumulus and corona cells. In the remainder, the cumulus and corona cells were removed by aspiration in and out of a glass micropipette. The majority of the oocytes observed had a polar body.

Experimental Design

Soybean trypsin inhibitor (SBTI; Sigma, St. Louis, MO) was chosen to inhibit acrosin proteolytic activity (APA) in this study because this inhibitor had no effect on sperm motility in preliminary trials. Although different inhibitors have been used by others [20, 21], we have found that a few have depressing effects on sperm motility that could interfere with sperm-ZP binding and penetration.

Motile sperm (2×10^6) in 1 ml HTF medium containing 10% human serum were incubated with 2 mg SBTI (test) and without SBTI (control) at 37°C in 5% CO_2 in air. After a 1-h incubation, 4 oocytes were added to each test and control sperm suspension and incubated for a further 5 h. To minimize the effect of oocyte variability on sperm-ZP binding and penetration, all test and control oocytes in each experiment were from the same patient. The numbers of sperm binding to and penetrating into the ZP were counted. The acrosome status of sperm in the culture medium and sperm bound to the ZP was assessed. Motility and movement characteristics of test and control sperm were measured with a Hamilton-Thorn motility analyzer (HTMA; Hamilton-Thorn Research, Danvers, MA) after 6 h of incubation. Acrosin activity of sperm after 1 h of incubation with or without SBTI was assessed by a gelatin substrate film method.

To determine whether the inhibition of APA with SBTI was reversible, sperm were incubated with SBTI for 1 h and then washed using fresh medium to remove SBTI; APA of the washed sperm was then determined.

To ascertain whether SBTI could also affect the ZP directly to alter sperm binding and penetration, oocytes exposed to SBTI-treated sperm were washed in fresh HTF to remove both SBTI and ZP-bound sperm. Then the washed oocytes were re-incubated with control sperm that had not been exposed to SBTI. After a further 5-h incubation, the numbers of sperm binding to and penetrating into the ZP were counted.

To determine whether the blocking of sperm-ZP penetration by SBTI was reversible, both sperm and oocytes exposed to SBTI were washed in fresh medium to remove both SBTI and ZP-bound sperm. (The method for removing ZP-bound sperm is described below.) Then the washed sperm and oocytes were re-incubated together for other 5 h, and the numbers of sperm binding to and penetrating into the ZP were counted.

Assessment of Acrosin Proteolytic Activity (APA)

APA of individual sperm was determined by a gelatin substrate film assay according to the methods described by Welker et al. [22]. Gelatin substrate film slides were prepared as follows. Gelatin (0.5 mg; Difco, Detroit, MI) was dissolved in 10 ml of distilled water at 50°C, and 40 μl of the warm solution was placed on one side of a clean glass slide (25 × 75 mm) and spread with a second slide as for a blood smear. The coated slides were kept horizontally for 24 h at 4°C in a humidified box. Then the slides were fixed for 2 min in 0.05% glutaraldehyde (v/v) in PBS (Commonwealth Serum Laboratory, Melbourne, Australia), rinsed twice in PBS for 20 sec, and rinsed in distilled water for another 20 sec. The fixed slides were stored vertically at 4°C and used for up to 4 wk.

Motile sperm (2×10^6) incubated with or without SBTI for 1 h were centrifuged at 600 × g for 5 min and the sperm concentration was adjusted to 20×10^6/ml. Then 40 μl of sperm suspension was evenly spread on the surface of the gelatin-coated slides with a pipette. The slides were allowed to dry in a horizontal position at room temperature for about 10 min. The slides were then placed horizontally in a humidified box and incubated at 37°C for 3 h to allow proteolysis of the gelatin film by the acrosin. After incubation, the slides were observed by means of a phase-contrast microscope at 400× magnification. A clear halo around the sperm head indicated APA (Fig. 1). The percentage of sperm showing a halo was assessed by counting 200 sperm.

Assessment of Sperm Motility and Movement Characteristics

Motility and movement characteristics of sperm were assessed after 6 h of incubation with SBTI via the HTMA. Because sperm concentration was only 2×10^6/ml when the sperm were incubated in culture medium, the concentration was adjusted to approximately 20×10^6/ml by centrifugation at 600 × g for 5 min and resuspension before motility and movement characteristics were measured. A 5-μl sample was placed in a 10-μm-deep Makler chamber at 37°C. The following movement characteristics were measured: VCL (curvilinear velocity), VAP (average path velocity), VSL (straight line velocity), LIN (linearity, VSL/VCL), STR (straightness, VSL/VAP), ALH (amplitude of lateral head displacement), and BCF (beat cross frequency). The percentages of sperm with motility (VAP > 10 μm) and progressive motility (VAP > 10 μm and STR > 70%) were also

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measured. The HTMA settings have been described previously [23]: negative phase-contrast optics, recording times of 0.4 sec at 25 frames/sec, minimum contrast 20, minimum cell size 3 pixels, low size gate 0.5, high size gate 1.5, low intensity gate 0.5, high intensity gate 1.5, non-motile head size 10, non-motile head intensity 400, medium VAP value 20 μm/sec, low VAP value 10 μm/sec, slow motile cells NO, and threshold STR > 80%. For each sperm sample, an average of 8 (7–10) fields with mean total number of 207 (range 168–277) sperm were assessed.

Assessment of Sperm Binding to and Penetration of the ZP

After incubation of motile sperm with (test) or without (control) SBTI for 1 h, 4 oocytes were added to each sperm suspension and incubated for another 5 h. Then the oocytes were transferred to fresh medium and washed by aspiration in and out of a fine glass pipette (inside diameter 250 μm) to dislodge sperm loosely adhering to the surface of the ZP. The number of sperm bound tightly to the ZP was counted with an inverted phase-contrast microscope at 250× magnification. It was impossible to count the number of sperm accurately when there were more than 100 sperm bound to one ZP. Numbers above this level were recorded and analyzed as 100.

Because large numbers of sperm were bound to ZP, it was difficult to determine which sperm had penetrated into the ZP or perivitelline space (PVS). Therefore, we developed a new technique to remove all the sperm bound to the surface of the ZP, i.e. vigorous aspiration in and out of a narrow-gauge micropipette (inside diameter about 120 μm). The number of sperm penetrating into the ZP and into the PVS was then determined by microscopy. With this technique the oocyte is aspirated in or out of a pipette of inner diameter slightly smaller than the size of the oocyte, and the sperm on the surface are sheared off by force. Only sperm with their heads either partially or wholly embedded in the ZP remain, as confirmed by histological examination of serial cross sections of some oocytes (Fig. 2). After this pipetting procedure, sperm with their heads in the ZP or PVS could be seen clearly under an inverted phase-contrast microscope (Fig. 3).

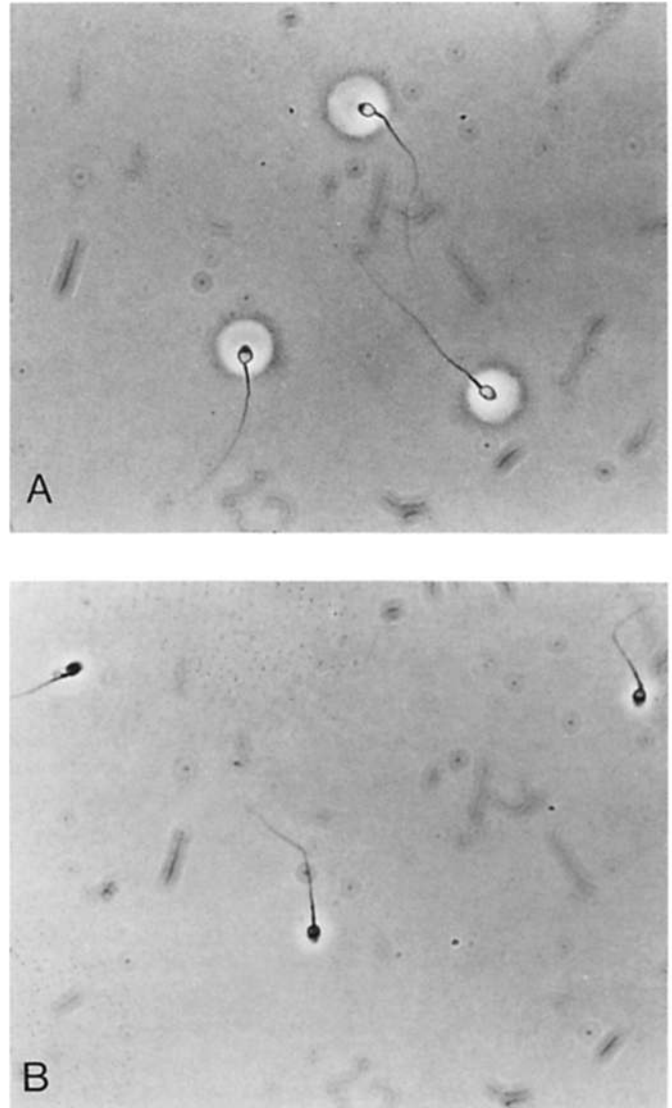

FIG. 1. Acrosin activity of control and test sperm on a gelatin substrate slide. A) Control sperm incubated without SBTI; all the sperm had digested the gelatin substrate to form a clear protein-free halo. B) Test sperm incubated with 2 mg/ml SBTI; no sperm formed halos. ×600.

Assessment of Acrosome Status

The acrosome status of sperm in culture medium and sperm bound to the ZP was determined with fluorescein-labeled *Pisum savitum* agglutinin (PSA, Sigma) [24]. Sperm in the culture medium were washed with 0.9% NaCl and centrifuged at 600 × *g* for 10 min; then the sperm pellet was resuspended in 40 μl of 0.9% NaCl and smeared on a glass slide. Sperm bound to the ZP were removed by aspiration with the narrower-gauge micropipette in PBS, pH 7.4, containing 2 mg/ml of BSA. These sperm were fixed on microscope slides in 95% ethanol for 30 min after air-drying and then stained in 25 μg/ml of PSA in PBS for 2 h. Each slide was washed and mounted with distilled water, and 200 sperm were counted via a fluorescence microscope and oil immersion at 400× magnification. When more than half the head of a sperm was brightly and uniformly fluorescing, the acrosome was considered to be normal and intact [24]. Sperm with a normal acrosomal pattern but with very weak fluorescence were scored as partially acrosome-reacted. Sperm without fluorescence (a rare pattern) or with a fluorescing band at the equatorial segment were considered to be completely acrosome-reacted. The viability of acrosome-intact and acrosome-reacted sperm both in medium and bound to the ZP was not determined since more than 90% of sperm in the medium had progressive motility and only motile sperm could bind to the ZP.

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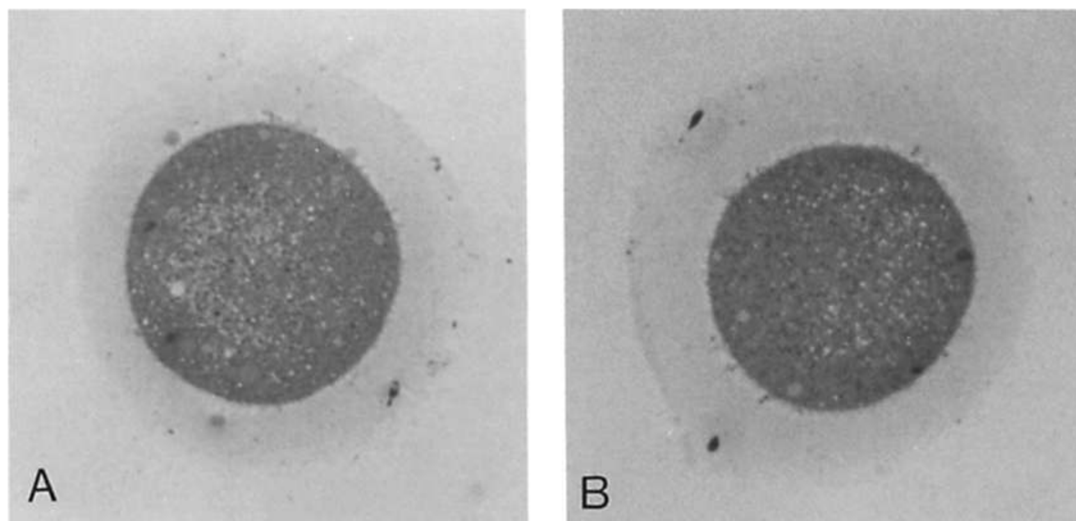

FIG. 2. Serial cross section of an oocyte with three sperm penetrating into the ZP, observed after removal of all the sperm bound to the surface of the ZP by pipetting through a small-bore pipette. A) One sperm completely penetrating into the ZP; B) two sperm heads penetrating into the surface of the ZP (toluidine blue stain). ×370.

Statistical Analysis

The significance of differences between test and control samples for sperm motility, movement characteristics, acrosome status, numbers of sperm bound, and numbers of sperm penetrating into the ZP was examined by paired *t*-test. Differences between the proportions of ZP-bound sperm with various acrosome statuses were determined by chi-square test.

RESULTS

Inhibition of Acrosin with SBTI

An average of 93% of control sperm formed protein-digested halos on the gelatin substrate film (Fig. 4). But none of the test sperm had halos, suggesting that the APA of test sperm was completely inhibited by SBTI. After test sperm were washed to remove SBTI, the percentages forming halos were similar to those of control sperm, but the diameters of the halos for washed test sperm were slightly smaller than for control sperm in some samples. This indicated that inhibition of the APA with SBTI after 1 h of incubation is reversible.

Effect of SBTI on Sperm Motility and Movement Characteristics

Because sperm motility is important for sperm binding to and penetration of the human ZP, motility and movement characteristics of sperm after incubation for 6 h with or without SBTI were measured. There were no significant differences in motility, various velocities, or other movement characteristics between test and control sperm (Table 1). Similarly, continued incubation of sperm for 20 h did not affect the percentage of sperm with progressive motility as assessed with a phase-contrast microscope. Therefore SBTI inhibits APA but does not affect sperm motility and velocities.

Effect of Inhibition of Acrosin on Sperm Binding to and Penetration of the ZP

There was no significant difference in the number of sperm bound to the ZP between test and control samples (Table 2). However, no test sperm penetrated into the ZP, since all the sperm bound to the ZP were completely removed by aspiration in and out of the fine-bore pipette. In contrast, an average of 89 control sperm penetrated into each set of 4 oocytes and there were 13 sperm in the PVS per 4 oocytes. An average of 94% (34 out of 36) of the ZP were penetrated with sperm in the PVS. These results indicate that inhibition of APA with SBTI does not affect the numbers of sperm bound to the surface of the ZP but completely blocks sperm penetration into the ZP.

To demonstrate reversibility of the effect of SBTI on sperm penetration of the ZP, oocytes incubated for 5 h with sperm and SBTI were washed to remove the inhibitor and all the ZP-bound sperm. The washed oocytes were re-incubated with control (inhibitor-free) sperm for a further 5 h. The results are summarized in Table 3. Similar numbers of sperm were bound to and penetrating into the washed ZP as into the control ZP. All (16) of the washed ZP were penetrated by one or more sperm. The number of sperm in the PVS of washed oocytes was similar to that in control oocytes. This result suggests that SBTI blocks sperm penetration into the ZP because of inhibition of acrosin in sperm and that SBTI has no additional or permanent effect on the ZP. Also, the difference in sperm-ZP penetration between test and control sperm is not due to oocyte variability. SBTI was

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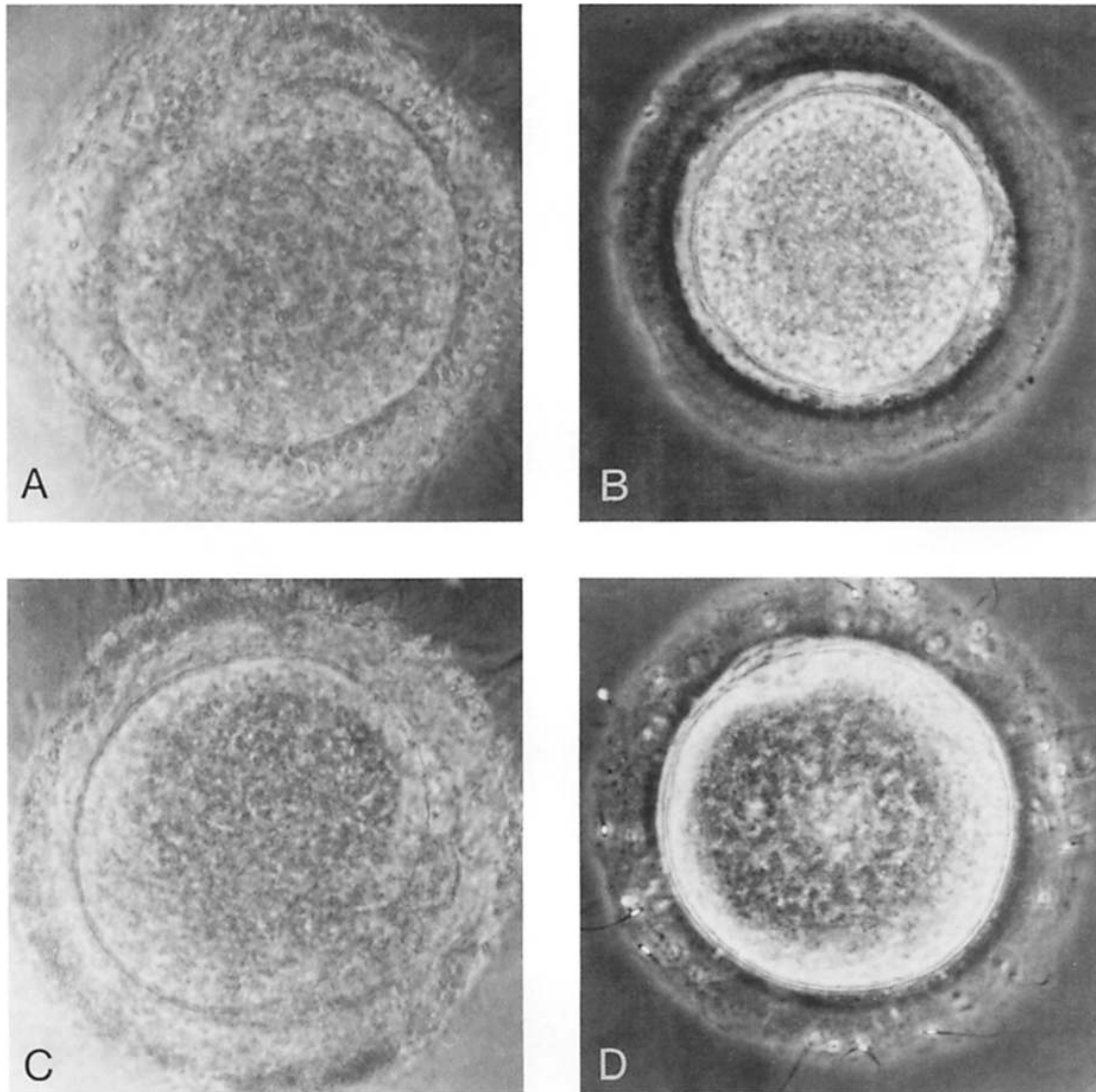

FIG. 3. One oocyte incubated with SBTI-treated sperm (A and B) and one oocyte incubated with control sperm without SBTI (C and D). In the oocyte incubated with SBTI-treated sperm, a large number of sperm were bound to the ZP (A) but no sperm had penetrated into the ZP after removal of all ZP-bound sperm by pipetting with a small-bore pipette (B). In the oocyte incubated with control sperm without SBTI, a large number of sperm were bound to the ZP (C), and many sperm had penetrated into the ZP after removal of all the ZP-bound sperm (D). ×500.

removed by washing test sperm and ZP from which all bound sperm had been removed via the narrow-gauge micropipette, and the washed sperm and ZP were re-incubated in the inhibitor-free medium for a further 5 h; the results (numbers of sperm bound to the ZP) were similar to control and pre-wash results (Table 4). Sixty-nine percent (11 out of 16) of the ZP were penetrated by one or more sperm, but the number of sperm penetrating into the ZP and PVS was significantly lower than for the controls (Table 4). This result suggests that the inhibition of APA and sperm penetration into the ZP is still partially reversible after 5-h incubation of sperm and ZP with SBTI.

Effect of Trypsin Inhibitor on the Acrosome Reaction

There was no significant difference in the percentage of sperm losing the acrosome in culture medium after incubations of 6 h or 20 h (mean ± SEM, 16 ± 1.7 vs. 20 ± 1.8) with or without SBTI. However, there was a significant ($p < 0.001$) reduction in acrosome-reacted sperm bound to the ZP after exposure to SBTI (Table 2). This result indicates that inhibition of acrosin with SBTI does not affect the loss of the acrosome in culture medium but does reduce the occurrence of the acrosome reaction of sperm bound to the ZP. Interestingly, there were significantly more partially acrosome-reacted and fewer completely acrosome-

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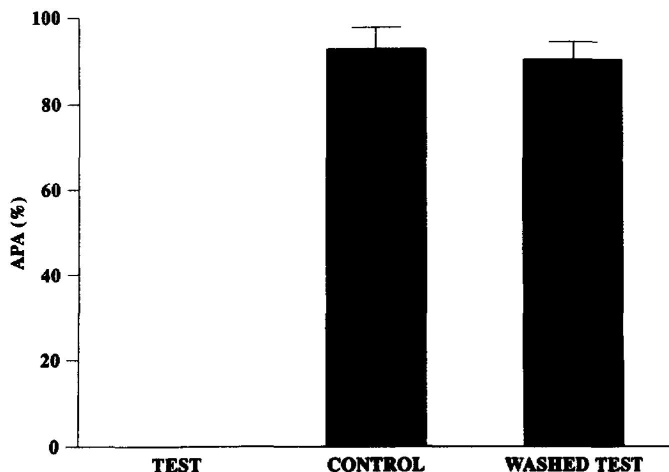

FIG. 4. Inhibition of APA with SBTI. Test, sperm incubated with SBTI; Control, sperm incubated without SBTI; Washed Test, sperm from test washed to remove SBTI (n = 15, mean ± SEM; test compared with control or washed test, $p < 0.001$).

TABLE 2. Effect of SBTI on sperm-ZP binding, sperm-ZP penetration, and proportion of acrosome-reacted (AR) sperm in culture medium and bound to the ZP after 6-h incubation (mean ± SEM, 9 experiments).

	Test	Control
Number of sperm bound/4 ZP	331 ± 23	371 ± 18
Number of sperm in ZP/4 ZP	0	89 ± 23**
Number of sperm in PVS/4 ZP	0	13 ± 3**
% ZP penetrated[a]	0	94
AR (culture medium, %)	14 ± 2	15 ± 2
AR (bound to the ZP, %)	30 ± 8	45 ± 9*

[a]Test, 0/36 oocytes penetrated; control, 34/36 oocytes penetrated.
*$p < 0.01$; **$p < 0.001$; PVS, perivitelline space.

reacted sperm after exposure to SBTI (Table 5). However, there was some variation in ZP-induced acrosome reaction between individual sperm samples. Partially acrosome-reacted sperm were rarely seen in culture medium. The partial acrosome reaction in SBTI-treated sperm may have been due to slow release of acrosomal contents because of inhibition of APA by SBTI.

DISCUSSION

The present study shows that inhibition of acrosin activity with SBTI blocks sperm penetration into the ZP but does not influence the number of sperm bound to the ZP. This suggests that acrosin plays a key role in human sperm-ZP interaction. Because SBTI does not affect sperm motility or movement characteristics, the blocking of sperm penetration of the ZP is likely to be due only to the inhibition of the trypsin-like enzyme. Although different types of acrosin inhibitors have been used by others, some of these impair sperm motility (for example, butyl *p*-hydroxybenzoate) and therefore were not suitable for the present study [20, 21]. Sperm motility and high velocity are important for sperm binding to and penetrating into the ZP; immotile sperm cannot bind to or penetrate through the ZP [1, 6]. Although the actual number of sperm bound to the ZP was not determined accurately since the majority of the oocytes from both test and control incubations had more sperm than could be counted accurately (> 100/ZP), it is clear that SBTI did not severely reduce the numbers of sperm on the ZP that could not be dislodged by wide-bore pipetting.

Because it is difficult to determine which sperm have penetrated into the ZP when large numbers of sperm are bound to the ZP, we developed a new technique to remove all the sperm bound to the surface of the ZP. Oocytes were repeatedly aspirated through a fine-bore (approximately 120 μm) micropipette with a diameter slightly smaller than the size of the oocyte. This sheared sperm off the ZP surface, leaving only those with part of the head or the entire head embedded in the ZP or perivitelline space. The effectiveness of this procedure was confirmed by histological examination of serial cross sections of oocytes following the fine-bore pipetting.

SBTI can completely inhibit acrosin activity as assessed by the gelatin substrate film method; but the inhibition is reversible, since a similar proportion of sperm could digest the gelatin substrate and form a halo with similar diameter after the inhibitor was removed by washing the sperm. This result suggests that the inhibitor must be acting at the sperm surface. Also, washed ZP previously exposed to SBTI-treated sperm were penetrated normally by control sperm. However, the SBTI effect was only partially reversible by wash-

TABLE 1. Effect of SBTI on sperm motility and movement characteristics (mean ± SEM, 9 experiments).*

	Test	Control
Manual Motility (%)		
1 h	92.0 ± 1.7	92.0 ± 1.6
6 h	89.0 ± 2.2	89.0 ± 2.3
HTMA (6 h)		
Motility (V > 10 μm, %)	88.0 ± 1.1	87.0 ± 1.8
Prog Motility (V > 20 μm, %)	67.0 ± 3.2	65.0 ± 3.7
VAP (μm/sec)	73.0 ± 5.3	71.0 ± 5.7
VCL (μm/sec)	84.0 ± 5.7	83.0 ± 6.8
VSL (μm/sec)	69.0 ± 5.3	66.0 ± 5.3
LIN (%)	81.0 ± 1.8	79.0 ± 2.3
STR (%)	92.0 ± 0.8	91.0 ± 1.1
ALH (μm)	3.2 ± 0.2	3.3 ± 0.4
BCF (Hz)	12.0 ± 0.9	12.0 ± 1.3

*No significant differences between test and control by paired *t*-test.

TABLE 3. Number of sperm bound to and penetrating into the ZP or PVS after incubation of sperm with (test) or without (control) SBTI and after re-incubation of the washed test ZP (WTZP) with control sperm (mean ± SEM, n = 4).

	Test	Control	WTZP
Number of sperm bound/4 ZP	348 ± 24	368 ± 32	364 ± 36
Number of sperm in ZP/4 ZP	0*	100 ± 33	92 ± 17
Number of sperm in PVS/4 ZP	0*	18 ± 3	17 ± 2
% ZP penetrated	0	100	100

*$p < 0.001$, test compared with control or WTZP.

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TABLE 4. Number of sperm bound to and penetrating into the ZP or PVS after incubation of sperm with (test) or without (control) SBTI and after re-incubation of washed test sperm with washed test ZP (WTSZP) (mean ± SEM, 4 experiments).

	Test	Control	WTSZP
Number of sperm bound/4 ZP	296 ± 40	369 ± 32	302 ± 40
Number of sperm in ZP/4 ZP	0*	91 ± 3**	22 ± 10
Number of sperm in PVS/4 ZP	0*	10 ± 3**	3 ± 1
% ZP penetrated	0	87	69

*$p < 0.001$, test compared with control or WTSZP.
**$p < 0.01$, control compared with WTSZP.

ing of sperm after 5-h incubation, since the number of sperm penetrating into fresh ZP was lower than for sperm not exposed to SBTI.

While these results make it clear that blocking the action of acrosin prevents sperm penetration into the ZP, the mechanism of this effect is uncertain and requires comment. Acrosin and trypsin will slowly digest rabbit ZP [16, 17]. Ghosts of the acrosome are left at the entrance of the sperm penetration slit in the ZP [7]. Acrosin is found along the penetration slits [19]. If it is assumed that human sperm bind to the ZP and undergo the acrosome reaction on the surface of the ZP [6], exposing acrosin on the inner acrosomal membrane to help digest a track through the zona, it is tempting to assume that the main effect of SBTI in these experiments was interference with a lytic effect of acrosin on the ZP. However, Bedford [25] argued from the perspective of comparative reproductive physiology that zona lysis may not be important in eutherian mammals. To take one of several examples, the ZP in sheep is resistant to homologous acrosin. Also, Saling [26] reported that the trypsin inhibitors have no influence on penetration once sperm bind to the mouse zona. Thus the current findings may not be attributable only to inhibition of lysis of the ZP. There is now increasing evidence that proacrosin and acrosin may have multiple functions in sperm-ZP recognition and binding, in the acrosome reaction, and in dispersal of the contents of the acrosome.

During the early interaction between sperm and oöcytes, two types of sperm adherence to the ZP are recognized. The initial loose association between sperm and the oocytes is termed attachment and is not species-specific. This attachment usually occurs within a few minutes. Attached sperm can be removed easily from the hamster or mouse ZP surface by gentle washing of the oocytes with a broad-bore micropipette [27–29]. The tight functional association between sperm and the oocytes is termed binding and is species-specific. In rodents, this tight binding usually occurs about 20 to 30 min after sperm and oocytes are mixed. Bound sperm cannot be removed easily from the rodent ZP surface by gentle washing with a broad-bore micropipette [27–29]. In the present study, pipetting through the wide pipette would have removed attached sperm, leaving only sperm tightly bound to the surface of the ZP or penetrating into its substance. We have used this technique in a previous study of cross-species gamete interactions and shown that sperm attached in a non-species-specific manner were removed [3]. Therefore, all the sperm remaining on the ZP after washing with a broad-bore micropipette are considered to be tightly bound.

Proacrosin and acrosin may be involved in sperm binding to the ZP [30, 31]. Hartmann and Hutchinson [27] found that exposure of hamster sperm to *p*-aminobenzamidine (pABA) did not alter sperm "attachment" but that after the oocytes were washed there were no sperm on the ZP. Benau and Storey [32] also showed in the mouse that trypsin inhibitors, including SBTI, prevented sperm binding to the ZP during 20-min incubations. Trypsin inhibitor-sensitive sperm-ZP binding sites have also been described in other species [30]. These results are quite different from our finding that there was no significant reduction in the number of sperm, bound to the surface of the ZP, that could not be removed by wide-bore pipetting of the oocytes. We believe that this pipetting would be at least as vigorous as the washing steps used by Hartmann and Hutchinson [27].

Recent work suggests that sperm interact with the ZP with at least two types of binding sites: one situated on the plasma

TABLE 5. Comparison of acrosome-intact and partially (PAR) or completely (CAR) acrosome-reacted sperm bound to the ZP after 6 h incubation with (test) or without (control) SBTI (9 experiments with different sperm samples).*

	Test			Control		
Sperm samples	Intact	PAR	CAR	Intact	PAR	CAR
1	86	11	3	65	5	30
2	70	28	2	42	2	56
3	68	30	2	27	3	70
4	5	90	5	0	1	99
5	74	24	2	58	3	39
6	90	0	10	85	0	15
7	90	6	4	75	2	23
8	68	28	4	60	12	28
9	83	14	3	83	5	12
Mean	70	26	4	55	4	41

*Comparison of proportions of sperm with intact or reacted (PAR and CAR) acrosomes between test and control results $\chi^2 = 124.3$ $p < 0.001$, and for PAR or CAR between test and control $\chi^2 = 845.8$ $p < 0.001$.

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membrane overlying the acrosome, and another connected to the acrosomal contents or inner acrosomal membrane. The importance of these sites may vary between species [30]. The sperm binding sites on the ZP in the mouse involve initial binding to oligosaccharide chains of the ZP glycoprotein ZP3, the primary sperm receptor, and secondary binding to another ZP glycoprotein, ZP2 [4]. Jones [30] has reviewed the various primary and secondary oocyte binding sites that have been reported for sperm from a variety of species. The primary sites may be glycosyl transferases or other ligands that thus far have been identified only by monoclonal antibodies. Interestingly, Jones [30, 31] has found that proacrosin/acrosin may serve as the secondary sperm-ZP binding site, perhaps binding to heavily sulphated oligosaccharide chains of the glycoprotein that is homologous with mouse ZP2. There are similarities between this binding function of proacrosin and that of the sea urchin sperm protein, bindin. Jones [30, 31] speculates that this secondary binding with proacrosin/acrosin may occur after the acrosome reaction has been initiated and that it may be intimately involved with sperm penetration of the zona: that the polysulphate binding properties of proacrosin and acrosin are decreased by autoproteolysis to low-molecular-weight proteins that bind less well and therefore release the sperm to penetrate further into the ZP. Furthermore, Eberspaecher et al. [33] have shown that conversion of porcine proacrosin to acrosin and its subsequent degradation are greatly enhanced in the presence of heat-solubilized ZP. Thus there could be a cascade of binding, activation, and breakdown of acrosin during sperm penetration of the ZP. As the sulphated polysaccharide binding of proacrosin/acrosin is not affected by proteolytic activity, trypsin inhibitors would not be expected to interfere with sperm-zona binding through this mechanism. Blocking acrosin activity could prevent sperm penetration into the ZP either by blocking of autoproteolytic degradation of proacrosin/acrosin to release sperm for penetration or by inhibition of lysis of the ZP. Both mechanisms may operate.

There is other evidence for the possibility that acrosin is present on the plasma membrane overlying the acrosome in the active form before the acrosome reaction. Tesarik et al. [34] reported that acrosin could be detected with a specific monoclonal antibody on the human sperm plasma membrane before the acrosome reaction; they raised the possibility that activation of proacrosin exposed on the surface of sperm could be an early event in the physiological acrosome reaction. Acrosin inhibitors have been reported to inhibit acrosome reactions induced by follicular fluid or the calcium ionophore A23187 [20, 35]. Pillai and Meizel [36] reported that trypsin inhibitors blocked the progesterone-initiated acrosome reaction but did not block the ionophore A23187-initiated acrosome reaction in human sperm. They suggested that the progesterone-initiated increase in intracellular calcium required for the human sperm acrosome reaction was prevented. Since in the present study there was a significant reduction in the proportion of sperm undergoing the acrosome reaction on the ZP, it is possible that the putative surface proacrosin sites may be involved in triggering the acrosome reaction after initial binding, perhaps in association with the secondary sperm-ZP binding reaction.

Finally, acrosin may act within the acrosome to facilitate the acrosome reaction as well as to catalyze the conversion of proacrosin [20, 36, 37]. Thus another explanation for the reduced acrosome reaction in the presence of SBTI—particularly the reduced completeness of the acrosome reaction as detected with fluorescein-labeled PSA, which binds to components in the acrosomal matrix—could be a blockade of the autocatalytic effect of acrosin in the acrosome, resulting in reduction of dispersal of the contents of the acrosome.

Spontaneous acrosome loss of human sperm in culture normally occurs at a very low rate, for example about 10% after 20 h. These sperm may lose the acrosome because of sperm senescence rather than a physiological acrosome reaction [8]. In the present study, SBTI did not affect the spontaneous acrosome loss of sperm in culture medium. We have shown that the proportion of sperm with an intact acrosome in the insemination medium is related to fertilization rates in vitro [24]. Thus the spontaneous loss of the acrosome of sperm in culture may be disadvantageous.

In clinical IVF, the majority of patients with complete failure of fertilization show failure of sperm binding to the ZP [38, 39]. However, some patients with failure of fertilization show a large number of sperm bound to the ZP but none penetrating into the ZP [38, 39; Liu and Baker, unpublished data]. Sperm from such patients often have good motility and morphology. Thus it is possible that these patients with failure of sperm-ZP penetration may have defects of the ZP triggering mechanism for the acrosome reaction or acrosin release. While measurement of total acrosin activity of sperm in semen does not aid in the prediction of sperm fertilizing ability in vitro [22, 40], further studies of the ZP-induced acrosome reaction in such patients may provide additional information about the cause of their infertility.

In summary, inhibition of acrosin activity with SBTI does not affect sperm motility or movement characteristics but reversibly prevents sperm penetration of the ZP and alters the acrosome reaction of sperm bound to the ZP. Thus acrosin plays crucial role in human sperm-ZP interaction. However, the mode of action remains to be determined. The present results could stem from inhibition of the function of sperm bound to the ZP, of the physiological acrosome reaction induced by the ZP, or of the formation of the sperm penetration slit in the ZP.

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