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Acrosin activity in spermatozoa from sterile t^6/t^{w32} and fertile control mice

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Summary

Spermatozoa from sterile t^6/t^{w32} and control fertile $+/+$, T/t^{w32}, T/t^6 mice were compared for their abilities to hydrolyse protein matrices and for their levels of acrosin activity. The data show that the immature and mature gametes from both the experimental and control males hydrolyse protein matrices. The quantitative acrosin assays show, however, that the mature gametes from the intercomplement males have significantly less total acrosin activity than any of the control groups of gametes. These findings suggest that this reduced acrosin activity is an additional phenotypic expression of the intercomplement genotype which results in male sterility.

1. Introduction

Male mice which carry the complementing *t*-haplotypes, t^6 and t^{w32}, are sterile when insemination takes place either *in vivo* or *in vitro* (McGrath & Hillman, 1980*a*). The cause of this sterility is unknown. However, gametes obtained from the caudae epididymides and vasa differentia of these males, unlike those from corresponding areas of control fertile males, exhibit neither forward motility nor hyperactivated movement upon their release into, or following their incubation in, medium which supports capacitation and fertilization (McGrath & Hillman, 1980*a*). The lack of forward motility by spermatozoa from male mice carrying two complementing *t*-haplotypes has been noted by others (Bryson, 1944; Bennett & Dunn, 1967).

The male gamete must exhibit hyperactivated movement and release proteolytic enzyme(s) to be able to penetrate and pass through the zona pellucida. While the acrosome contains numerous enzymes (McRorie & Williams, 1974; Mack *et al.* 1983; Georgiev, 1983), the results of most studies suggest that the serine proteinase, acrosin [E.C. 3.4.21.10], is the major enzyme required for hydrolysis of the zona (e.g. Hartree & Srivastava, 1965; Stambaugh *et al.*, 1969; Stambaugh & Buckley, 1969; Zaneveld *et al.* 1970, 1971; Polakoski & McRorie, 1973; Bhattacharyya *et al.* 1979; Fraser, 1982). Brown (1983) demonstrated conclusively that acrosin is the only proteolytic enzyme required for this hydrolysis in mice. During fertilization the hydrolysis produces a slit in the glycoprotein investment, providing a passageway through which the spermatozoon can swim to reach the perivitelline cavity.

Although the earlier studies (McGrath & Hillman, 1980*a*) noted that the gametes from t^6/t^{w32} males exhibit aberrant motility, it was not determined if these gametes also differ from wild-type gametes in either their quantitative levels of proacrosin, the zymogen form of acrosin, or in the conversion of proacrosin to the active proteolytic enzyme. Two series of studies were undertaken to determine the comparative levels of acrosin activity in experimental and control spermatozoa. In the first, immature and mature experimental and control gametes were examined for their abilities to hydrolyse protein matrices. In the second, the acrosin activities of mature experimental and control spermatozoa were quantitated and compared.

2. Materials and methods

The t^6/t^{w32} males were obtained from crosses between T/t^6 males and T/t^{w32} females. The genotype of the embryos from these crosses are T/T which die *in utero* (Chesley, 1935), T/t which are viable and tailless, and t^6/t^{w32} which are viable and have tails of normal lengths. Spermatozoa from BALB/c($+/+$), T/t^{w32}, and T/t^6 males were used as controls. The T/t^6 animals had been brother-sister mated for 41 generations, the T/t^{w32} animals for 47 generations, and the BALB/c for 73 generations.

The fertility level of each control and experimental

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(t^6/t^{w32}) male was determined by mating it with two fertile BALB/c females for a period of one month (Lyon, 1987). During this period none of the t^6/t^{w32} males sired offspring. Each of the control males sired at least 10 offspring and were judged to be fertile.

(i) *Obtaining gametes*

Immature spermatozoa were collected from the caput epididymides and mature spermatozoa from the caudae epididymides and the vasa differentia of 18- to 24-week-old males (Brackett *et al.* 1978). The extirpated ductules from each male were placed separately into 1 ml modified Tyrode's medium (Fraser & Drury, 1975), minced and the gametes allowed to disperse for 20 min. Since the caput spermatozoa from all males and the mature spermatozoa from the t^6/t^{w32} animals lack forward motility, pressure was applied to the minced tissue to force the gametes into the medium.

(ii) *Protein digestion*

For the gelatin digestion studies, the collected mature and immature gametes from the experimental and control males were centrifuged at 70 *g* to pellet the debris. The supernatant fluid containing the spermatozoa was then applied to autoradiographic film (AR10; Kodak) supported on microscope slides using a camel hair brush (Penn *et al.* 1972). Each slide was placed into a Petri dish containing filter paper moistened with deionized water. The covered Petri dish containing the slide was placed into a 37 °C incubator and subsequently examined at varying time intervals ranging from 10 min to 6 h. Phase-contrast optics were used to examine each slide and representative samples of each series were photographed with Kodak Tri-X film.

(iii) *Enzyme activity assay*

The protocol of Bhattacharyya *et al.* (1979) was used to compare the activities of acrosin in mature spermatozoa from the control and experimental mice. This assay measures the esterolytic activity of acrosin using the spectrophotometric protocol of Schwert & Takenaka (1955) as modified by Polakoski *et al.* (1977). Acrosin activity is measured by the hydrolysis of a synthetic substrate, α-*N*-benzoyl-L-arginine ethyl ester (BAEE, Sigma Chemical Co.). The hydrolysis is measured by a change in absorbance at 253 nm at ambient temperature (Whitaker & Bender, 1965). One milliunit (mU) of acrosin is defined as the amount of enzyme hydrolysing one nanomole of BAEE per minute per 1×10^8 gametes.

For each assay, spermatozoa were pooled from three mice of each genotype. Each experiment was repeated at least three times. Spermatozoa were obtained from the caudae epididymides and vasa differentia as described above and collected in 1 ml modified Tyrode's medium containing 50 mM benzamidine (Sigma Chemical Co.) which prevents the conversion of proacrosin to acrosin. The number of spermatozoa in each suspension was determined by haemocytometry. Each suspension was then centrifuged at 70 *g* for 5 min to pellet the debris. The supernatant fraction was centrifuged at 600 *g* for 15 min and the pellet, containing the gametes, was dispersed in 10% glycerol containing 50 mM benzamidine. The gamete sample was extracted overnight with 0·1 M HCl, pH 3 at 4 °C, and was subsequently centrifuged at 27000 *g* for 30 min at 4 °C. The supernatant fraction (extract fraction) was dialysed against 1 mM-HCl (pH 3) to remove the benzamidine.

An aliquot (0·1 ml) of the extract was added to 1·4 ml of the assay mixture (0·05 M Tris buffer, pH 3·0, containing 0·5 mM BAEE and 0·05 M-$CaCl_2$) and the baseline activity (free, non-complexed acrosin) was measured. The pH of the extract was then adjusted to pH 8, the maximal pH for the conversion of proacrosin to acrosin. After 30 minutes, the acrosin activity of the aliquot was again determined. This second measurement includes all free acrosin activity, both the baseline and the activity of the enzyme converted from proacrosin. During the 30 min incubation period, however, some of the free acrosin complexes with an endogenous acrosin inhibitor (Goodpasture *et al.* 1981). The level of acrosin activity after the 30 min incubation period is thus referred to as the 'inhibited' activity. In order to determine the total activity, the pH of the aliquot was again adjusted to pH 3·0 to dissociate the acrosin-inhibitor complex and immediately assayed for the hydrolysis of BAEE. This measurement includes the total acrosin activity for each population of gametes.

The data from these studies are presented as baseline (BA), inhibited (IA), and total acrosin (TA) activities. These three activities were used to calculate the percentage of acrosin present in the zymogen (proacrosin) form and the percentage of acrosin complexed to inhibitor using the formulae given by Bhattacharyya *et al.* (1979). Significant differences ($P < 0{\cdot}05$) among the enzyme activities were determined by Student's *t* test and among the percentages, by Student's *t* test using arcsine transformed data.

3. Results

Both the immature and mature control and experimental gametes release proteolytic enzymes which are able to digest the gelatin membranes. The patterns of proteolysis are the same among all control and experimental mature gametes. Digestion of the substrate progresses from the convex to the concave surface of the head. The digested area around the convex surface appears as a bright region which conforms to the shape of that surface of the acrosome. This well-defined area appears within 10–20 min

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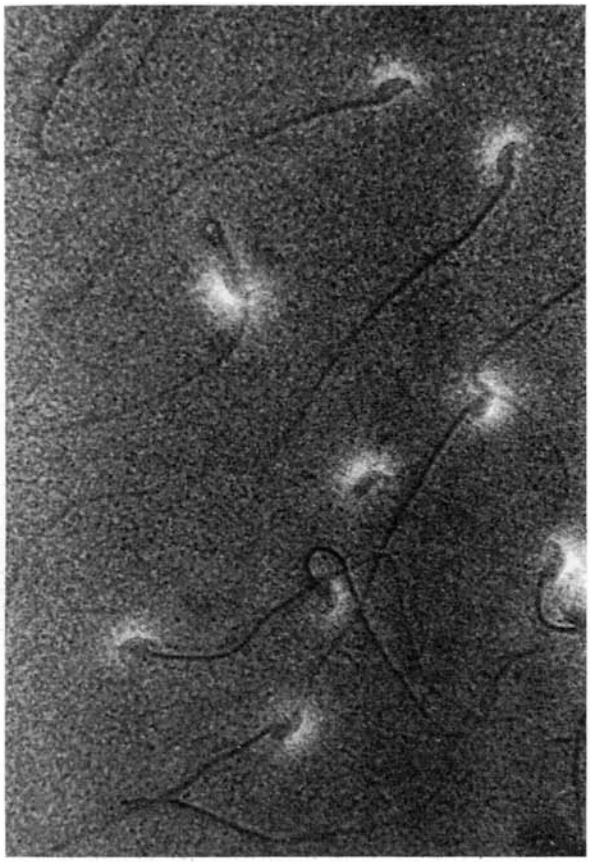

Fig. 1. A typical pattern of matrix hydrolysis by both experimental and control gametes after 20 minutes of incubation. Note that the digested region is associated with only the convex surface of the head. × 1500.

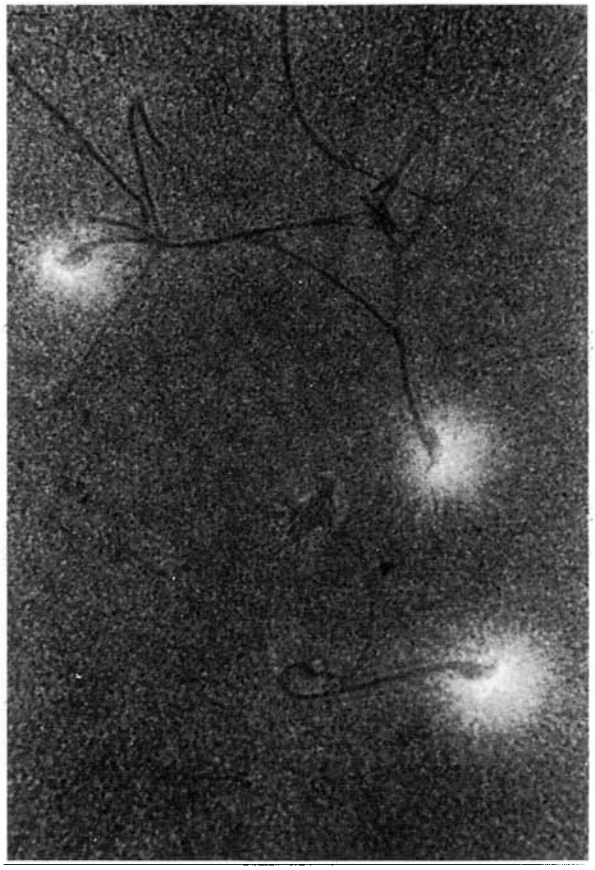

Fig. 2. A representative pattern of protein digestion by both experimental and control gametes after 5 hours of incubation. × 1500.

following the beginning of incubation (Fig. 1). If the gametes are allowed to incubate for longer periods of time, the digested area enlarges, becomes more diffuse and spreads to the concave surface of the head, creating a halo-like, digested area (Fig. 2). The reaction reaches a maximum size after approximately 5 h. The digested area around the convex side remains larger and more intense regardless of the length of the incubation. Experimental and control gametes obtained from the vasa differentia exhibit a pattern of digestion and a level of proteolytic activity comparable to that of spermatozoa obtained from the caudae epididymides. Also, the experimental and control caput epididymal gametes are comparable in their abilities to hydrolyse the matrix. Often, the immature gametes appear to digest the protein to the same extent as the mature experimental and control gametes.

A comparison of the data from the quantitative assays (Table 1) shows no significant differences between the mature gametes from T/t^{w32}, T/t^6, and $+/+$ animals in their levels of BA, IA, or TA. Also, there are no significant differences between these three groups of spermatozoa in either their percentages of acrosin in the proacrosin form or in their percentages of the enzyme complexed to the inhibitor.

The experimental gametes do not differ from the three control groups of spermatozoa in IA. Although their BA activity is lower than, but not significantly different from, that of T/t^6 spermatozoa ($0{\cdot}50 > P > 0{\cdot}20$), they do have significantly less BA than either the $+/+$ ($0{\cdot}05 > P > 0{\cdot}02$) or T/t^{w32} ($0{\cdot}05 > P > 0{\cdot}02$) gametes. The TA of the experimental gametes is significantly lower than the TA of any control group (t^6/t^{w32} vs T/t^{w32} and $+/+$, $0{\cdot}005 > P > 0{\cdot}002$; t^6/t^{w32} vs T/t^6, $0{\cdot}01 > P > 0{\cdot}005$). A comparison of the percentages of acrosin in proacrosin form shows no significant differences between the experimental and control gametes. The experimental gametes do, however, contain a significantly lower percentage of acrosin-inhibitor complex than the spermatozoa from any control group.

4. Discussion

Based on their abilities to hydrolyse protein matrices, the immature and mature spermatozoa obtained from both control fertile and experimental sterile males appear to release equivalent amounts of proteolytic enzyme. This gelatinolytic activity is caused by the activation of proacrosin to acrosin which occurs only after acrosomal membrane disruption (Brown & Harrison, 1978; Green, 1978). Studies show that

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Table 1. *Acrosin activity of control and experimental mouse spermatozoa*

Genotype	pH of extract	mU acrosin activity/min/10^8 spermatozoa, mean ± SE	Acrosinin proacrosin form (%)	Acrosin complexed to inhibitor (%)
T/t^{w32}	3·0 BA	435·6 ± 15·8		
	8·0 IA	527·7 ± 8·8	59	51
	3·0 TA	1071·6 ± 12·5		
T/t^{w32}	3·0 BA	435·6 ± 15·8		
	8·0 IA	535·9 ± 9·3	63	50
	3·0 TA	1072·8 ± 28·9		
+/+	3·0 BA	430·1 ± 6·9		
	8·0 IA	554·8 ± 11·7	62	51
	3·0 TA	1125·2 ± 21·1		
t^6/t^{w32}	3·0 BA	330·3 ± 34·4		
	8·0 IA	509·4 ± 16·2	59	37
	3·0 TA	804·3 ± 41·8		

acrosin, a diffusible substance (Green, 1978; Harrison *et al.* 1982), is found only in the acrosome of mammalian spermatozoa (Morton, 1975; Garner & Easton, 1977; Green & Hockaday, 1978; Müller-Esterl & Fritz, 1981; Harrison *et al.* 1982). Consequently, its topical localization and diffusion produces a halo-like digested area around the sperm head. Using an indirect immunofluorescence staining technique, Phi-Van *et al.* (1983) found that proacrosin is first detected in early spermatids and increases in amount with subsequent development. In these cells, the zymogen is also localized in, and limited to, the acrosome.

Although Gaddum-Rosse & Blandau (1972) and Penn *et al.* (1972) found that the proacrosin of mature gametes from numerous mammalian species is activated when the gametes are placed on protein matrices, there have been no previous reports showing that the zymogen from immature gametes is also activated upon acrosome disruption. In spite of the fact that the immature gametes from fertile male mice are unable to fertilize eggs *in vitro* (McGrath & Hillman, 1980*a*), the present study shows that they do release the active form of the enzyme.

Overall, the protein hydrolysis studies suggest that the acrosin activities of the control and experimental gametes are equivalent. However, the quantitative assays show that the latter contain significantly less total acrosin activity and a significantly lower percentage of acrosin complexed to inhibitor. The percentage of acrosin in the acrosin-inhibitor complex is a function of the inhibited and total acrosin activities (TA-IA/TA × 100 = % of acrosin in complex; Bhattacharyya *et al.* 1979). Since the mutant gametes do not differ significantly from the control gametes in IA, the decreased percentage of acrosin-inhibitor complex in the mutant gametes is a result of their significantly lower total acrosin. These data also show that the experimental gametes, while having baseline activity significantly lower than those of two of the control groups, have baseline activity which is not significantly lower than that in spermatozoa from T/t^6 males. Therefore, only the decreased total acrosin activity is unique to the experimental gametes.

A comparison of the control gametes shows that there are no significant differences between their acrosin systems (BA, IA, and TA activities) and consequently, no significant differences among their percentages of acrosin either in the zymogen form or complexed to inhibitor. It should be noted that there are no significant differences between the acrosin systems of these control spermatozoa and of spermatozoa from DDY mice (Bhattacharyya *et al.* 1979). There are, however, significant differences in the percentages of acrosin in the proacrosin form and in the complexed form between those reported here for control spermatozoa and those reported for DDY spermatozoa. The larger variance of the acrosin activities of the DDY spermatozoa may account for these differences.

The control groups of males used in the present study can be considered to be separate strains. Each line had an independent origin and was maintained in isolation by brother–sister matings for a large number of generations. The current data, together with that from DDY spermatozoa, suggest that spermatozoa from different strains of mice contain equivalent acrosin systems and that the significant reduction in the total enzyme activity in gametes from intercomplement males should be considered to be an additional aberration associated with the sterility phenotype. The overall effect of the reduced level of acrosin activity in the mutant gametes is not known. Although Katz & deMestre (1985) suggest that even limited proteolysis of this matrix may be sufficient to reduce its resistance to the thrust generated by hyperactivated gametes, it is not known how much acrosin is required for a single gamete to produce a slit in this investment.

Although our earlier studies showed that the

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spermatozoa from t^6/t^{w32} mice are unable to fertilize zona-surrounded eggs, the experiments were not designed to determine if they were able to traverse this structure (McGrath & Hillman, 1980*a*). Consequently, we recently examined eggs obtained from (C57BL/6J × BALB/c) F_1 females and coincubated with mature gametes from experimental (t^6/t^{w32}) and control (T/t^6) males for the presence of spermatozoa in the perivitelline cavities. The gametes were coincubated for six hours (McGrath & Hillman, 1980*b*). The eggs were then fixed, dehydrated and embedded as previously described (Nadijcka & Hillman, 1974). Serial thick sections were prepared and stained with methylene blue-Azure II (Richardson *et al.* 1960). Of the 55 eggs inseminated with sperm from the control males, 54 were fertilized and had an average of 4·1 spermatozoa (ranging from one to eleven) in the perivitelline cavities. None of the 64 eggs inseminated with gametes from t^6/t^{w32} males was fertilized and none had spermatozoa in either the perivitelline cavity or zona pellucida. The eggs were scored as being fertilized if they contained two pronuclei and the sperm tail (Wolf *et al.* 1976). This definitive study shows that mutant gametes are unable to traverse the zona pellucida.

The acrosin studies and the *in vitro* fertilization studies strongly suggest that both defective motility and the reduced total acrosin activity are involved in the mutant gametes' inability to traverse the zona pellucida. However, these defects are not solely responsible for the sterility phenotype. Gametes from t^6/t^{w32} males, unlike gametes from control males, are unable to fertilize eggs from which the zonae are mechanically removed. The mutant spermatozoa are able to bind with, but not fuse with, the oolemma (McGrath & Hillman, 1980*a*). These combined observations show that heterozygosity for two complementing *t*-haplotypes has multiple deleterious effects on the spermatozoa.

The exact physiological and molecular aberrations which result in intercomplement male sterility are not known. Recent studies by Lyon (1986, 1987) show that there are multiple genetic factors associated with the *t*-haplotypes which interact both cis and trans to affect the fertility levels of *t*-bearing mice. Neither the products nor the functions of these factors are known. Additional comparative physiological studies on the gametes from *t*-bearing and control males will be necessary in order to correlate the genetic factors with the various deleterious phenotypic expressions of *t*-haplotypes which result in the sterility of intercomplement male mice.

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