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Ultrastructural Observations on Gamete Interactions Using Micromanipulated Mouse Oocytes

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Cumulus-free mouse oocytes were subjected to zona opening by cracking with microhooks (ZC) or acid drilling (ZD) and fixed 30–90 min after insemination (10^5 pre-capacitated motile sperms/ml). Ultrastructural observations were made on serially thin-sectioned oocytes: 15 ZC and 12 ZD. The zona lesion in ZC oocytes was a clean cut, whereas in ZD oocytes it formed a patchy area of partial zona loss, with reduced microvillar height on the underlying oocyte surface. Spermatozoa were observed within the perivitelline space and partially fusing with the oocyte after 30 min in both situations. Only acrosome-reacted sperm heads were observed to fuse: acrosome intact forms were generally in contact with the zona pellucida, either with the inner or outer surface. Acrosome-intact spermatozoa were also observed deeply embedded in the zona matrix, possibly indicating surface enzyme activity preceding the membrane fusion events of the acrosome reaction proper. The observations are consistent with the need for spermatozoa to make contact preferentially with the zona pellucida during the course of the acrosome reaction.

Key words: zona pellucida, spermatozoa, micromanipulation

INTRODUCTION

Gamete micromanipulation has been shown to assist fertilization for couples with a history of poor or no penetration *in vitro* due to inferior sperm quality [Cohen et al., 1988; Gordon et al., 1988; Ng et al., 1988]. Several techniques have been described that have achieved fertilization with human and mouse gametes: 1) microinjection of a single spermatozoon or even sperm nucleus into the ooplasm [Uehara and Yanagimachi, 1976, 1977; Markert, 1983; Lanzendorf et al., 1988]; 2) microinjection of small numbers of spermatozoa subzonally [Metka et al., 1985; Barg et al., 1986; Lasalle et al., 1987; Laws-King et al., 1987]. However, evidence of oocyte damage, low fertilization rates, and concerns about the physiological normality of spermatozoa selected to be injected are drawbacks to these procedures.

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An alternate form of gamete micromanipulation for assisting fertilization is to weaken or open a small area of the zona pellucida to allow direct access by spermatozoa to the oocyte surface. Zona opening techniques using glass microhooks [Odawara and Lopata, 1987; Odawara et al., 1988], zona cutting [Depypere et al., 1988], partial dissection [Cohen et al., 1989], and zona drilling with acidified medium [Gordon and Talansky, 1986; Depypere et al., 1988; Gordon et al., 1988; Talansky and Gordon, 1988] have given improved fertilization rates and embryo growth with human gametes and also birth of live mice following transfer of embryos produced in this way to surrogate mothers.

Although there is no doubt that these techniques can achieve fertilization and embryonic development, a certain amount of controversy still surrounds the question of the need for spermatozoa to interact with specific oocyte investments such as the zona pellucida during the fertilization process [Yanagimachi, 1981; Meizel, 1985; Cummins and Yanagimachi, 1986]. The use of micromanipulative techniques would appear to bypass many of these specific interactions. With these points in mind, the present study aimed at elucidating the fine structure of gamete interactions in mice using the zona cracking or drilling approaches.

MATERIALS AND METHODS

Oocyte Recovery

Female F1 (CBA × C57BL) hybrid mice were superovulated with i/p injections of 5 iu of pregnant mare serum gonadotrophin (PMSG, Intervet) followed 53–54 h later with 7.5 iu human chorionic gonadotrophin (hCG, Organon Laboratories). The oocytes in cumuli were collected from the oviducts 13–14 h after hCG injection, and the cumulus matrix was dispersed using 0.1% (w/v) hyaluronidase (H3506, Sigma Chemical Co.) in 10 mM Hepes-buffered (Flow Laboratories) culture medium (HTFM, [Quinn, 1985]). Cumulus-free oocytes were washed in enzyme-free culture medium and then transferred to bicarbonate-buffered HTFM containing 3 mg/ml bovine serum albumin (BSA fraction V, Sigma).

Sperm Preparation

Both epididymides from an F1 hybrid male were used, and sperm were squeezed out from the ends of the vasa deferentia into protein-free HTFM. After allowing the sperm to disperse, aliquots containing c0.1 $\times 10^6$ cells were transferred into 1 ml of medium supplemented with 5 mg/ml BSA, in a 4-well culture dish (Nunc) previously equilibrated with 5% CO_2:5% O_2:90% N_2. The suspension was incubated for 1.5–2.0 h before being used to inseminate oocytes. Sperm counts were carried out using a haemocytometer.

Micromanipulation of Oocytes

Groups of 5 to 6 oocytes were transferred into drops of Hepes-buffered HTFM with 3 mg/ml BSA in a glass cavity dish and covered with paraffin oil (Merck low density). Each oocyte was stabilized using a glass holding micropipette. The zona pellucida was gently opened either using glass microhooks (zona cracking, ZC) or by application of nanolitre volumes of acidic (pH 2.0) Tyrodes medium through a glass microneedle (zona drilling, ZD), using a Picospritzer II instrument (GV General Valve Corporation). Following micromanipulation, the oocytes were washed and placed in the insemination medium.

Fertilization and Culture In Vitro

After culturing in the insemination medium for 5 h, the oocytes were washed and transferred into fresh HFTM medium with 3 mg/ml BSA. They were checked the next day, and fertilized 2-pronuclear zygotes were further cultured to the blastocyst stage over 3–4 days.

Electron Microscopy

At 30, 60, and 90 min after insemination, representative oocytes were fixed in 3% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.3). After post-fixing in 1% osmic acid in the same buffer, the oocytes were dehydrated in an ethanol-propylene oxide series and embedded in Epon 812 for electron microscopy. The oocytes were serially thin-sectioned, stained with lead and uranium salts, and examined under a Phillips 301 electron microscope. As controls, some oocytes were exposed to the micromanipulation environment but not zona opened; some had the zona completely removed by microhooks before insemination. Acrosomes were classified as intact, reacting (in the process of membrane vesiculation) or reacted (inner acrosomal membrane fully exposed). Acrosomes that could not be classified, thus, because not enough of a profile was present in the section were excluded from the analysis.

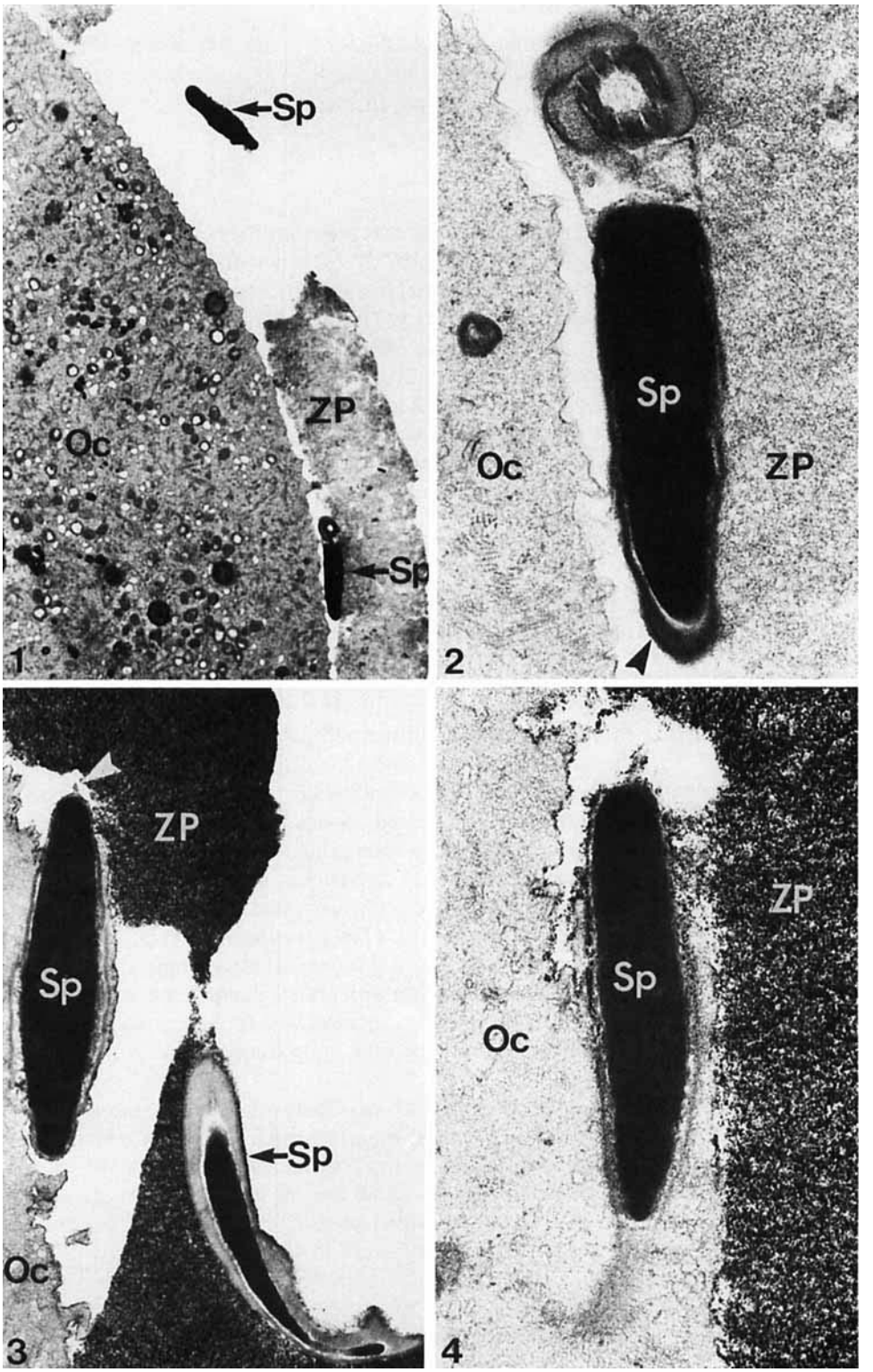
RESULTS

Observations are based on 15 ZC, 12 ZD, two zona-intact controls, and four zona-free oocytes that were all serially thin-sectioned.

Zona opening using glass microhooks resulted in a clean, sharp-edged lesion (Fig. 1). By contrast, zona drilling produced a diffuse and patchy degradation of the zona matrix (Figs. 5, 6). The oocyte cytoplasm underlying drilled regions in many cases appeared slightly modified, with reduced microvilli (Fig. 5). No such modification was seen over the rest of the oocyte or in zona opened oocytes (Fig. 1). This could indicate a direct detrimental effect of low pH on the exposed vitellus.

At 30 min after insemination using both approaches, spermatozoa were observed within the perivitelline space (Fig. 1). All the acrosome-intact spermatozoa were tightly adhering to the zona matrix, either on the inner (Fig. 2) or the outer (Fig. 3) surface. A few acrosome-reacted spermatozoa were seen at this time to have partially fused with the ooplasm (Fig. 4). At 60 min after insemination with both techniques, acrosome-reacted spermatozoa were more common, particularly in contact with the zona material (Fig. 6). Some spermatozoa were able to penetrate the zona away from the region of drilling or cracking (Fig. 7).

A common observation was of apparently acrosome-intact spermatozoa partially (Fig. 3) or wholly embedded (Fig. 8) in the zona pellucida. Table 1 summarizes the observations on sperm acrosomal status for control oocytes, zona-cracked oocytes 30 min after insemination, and zona-drilled oocytes at 60 and 90 min with zona cracking to make valid comparisons. It is clear that with increasing time from insemination to fixation, the proportion of spermatozoa that were in the process of, or which had completed the acrosome reaction, increased from less than 5% to a maximum of 67% (6/9) at 90 min with zona drilling. Most of these were within the zona matrix, on the inner surface of the zona, or in the perivitelline space or vitellus. Only at 90 min (zona drilling) were reacting and reacted sperm seen outside the zona. The control oocytes



Figs. 1–4.

fixed at 60 min had only 1/42 acrosome reacted, and this was within the zona. With such low numbers, it is difficult to ascribe statistical significance to the findings.

Control (zona intact) oocytes had acrosome-intact spermatozoa on the surface 30 and 60 min after insemination, but no penetration or acrosome reactions were observed at this time. Zona-free oocytes at 30 min also had acrosome-intact spermatozoa adhering, but the quality of fixation was poor in this series.

Successful fertilization and embryo growth was observed in this series for both ZC and ZD oocytes, and live young have been born following embryo transfer to pseudopregnant recipients [Edirisinghe et al., in preparation].

DISCUSSION

The two procedures used here for assisted fertilization produced very different lesions in the zona pellucida. Cracking with microhooks produced a clean-edged opening, whereas acid drilling resulted in a ragged hole, with many fragments of undissolved zona matrix remaining. Similar observations were made by Depypere et al. [1988]. At present, there is little evidence that either technique is superior in terms of allowing fertilization with low numbers of spermatozoa. Zona drilling, from a practical point, is preferable in that only two micromanipulators are involved (holding pipette and microneedle) as compared to three for zona opening (holding pipette and two microhooks). Although zona drilling appeared to cause changes to the oocyte surface (Fig. 5), these do not appear to have any major lasting effect on fertilization or embryo development, again corroborating the observations of Depypere et al. [1988]. Talansky and Gordon [1988] observed accelerated zona hatching and developmental abnormalities in mouse embryos produced after zona drilling, but they were using an "aggressive" drilling approach, deliberately producing larger holes in the zona than in their original report [Gordon and Talansky, 1986]. However, Nichols and Gardner [1989] have recently reported anomalous development in micromanipulated mouse embryos, so caution is clearly needed in applying such techniques to human fertilization.

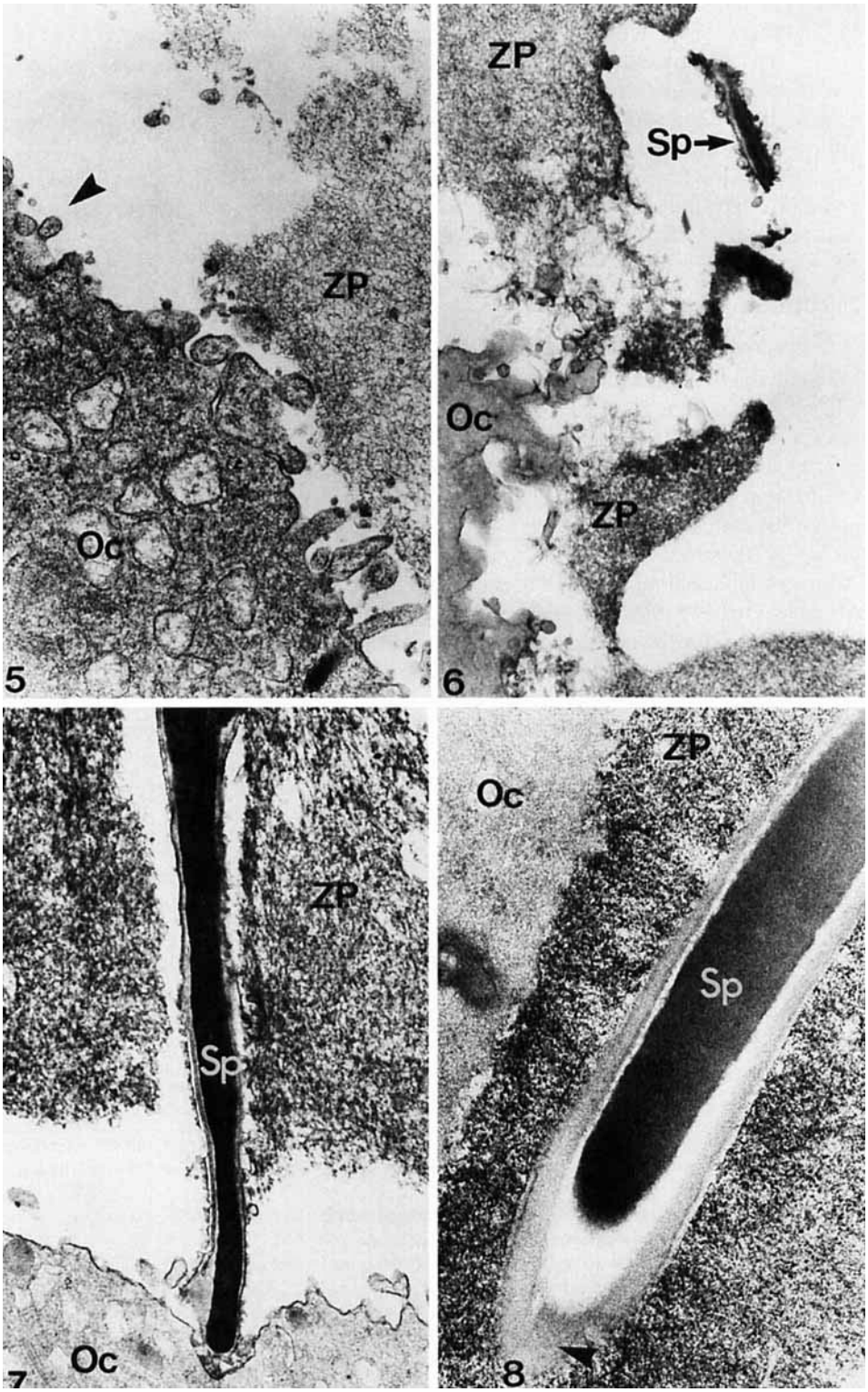
Although the numbers of spermatozoa that could be evaluated here by the time-consuming process of serial thin-sectioning were few (Table 1), the observations are consistent with models of the mouse sperm acrosome reaction that invoke a period of contact with the zona pellucida [Wassarman, 1987; Macek and Shur, 1988]. The acrosome-intact spermatozoa observed contacting the egg were all closely bound to or embedded in the zona matrix (Figs. 1, 3, 8; Table 1). Interestingly, several spermatozoa bound to the inner surface (Figs. 1, 2; Table 1). By contrast, all spermatozoa that had commenced fusing with the oocyte had completed the acrosome reaction (Figs. 3, 4, 7; Table 1). Although zona-binding might be the optimal or preferred pathway during the

(Figs. 1—4) Fig. 1. A zona-cracked oocyte 30 min after insemination shows one sperm in the perivitelline space (lower arrow) with one more in the opening (upper arrow). Oc = oocyte cytoplasm; Sp = sperm; ZP = zona pellucida. ×3,290.

Fig. 2. The same specimen as in Figure 1 shows that the spermatozoon is acrosome-intact (arrow) and appears to be adhering to the inner surface of the zona pellucida. ×24,510.

Fig. 3. Detail of the cracked zona 30 min after insemination. In this case, one sperm has entered the perivitelline space and made contact with the oocyte cytoplasm (Oc). The acrosome has disappeared as evidenced by the presence of the subacrosomal material or perforatorium (arrowed). The second sperm head is acrosome-intact but, nevertheless, apparently partially embedded in the zona matrix. ×15,270.

Fig. 4. In a zona-cracked oocyte 30 min after insemination, this spermatozoon has partially fused with the oocyte cytoplasm. ×36,130.



Figs. 5–8.

TABLE 1. Position of Sperm and Acrosomal Status at Different Times Following Insemination

	Sperm position					
Procedure/ time	Outside zona	Within zona	Inner zona surface	Perivitel-line space	Vitellus	Total
Control, 1 h						
Intact	41	0	0	0	0	41 (98%)
Reacting	0	0	0	0	0	0
Reacted	0	1	0	0	0	1 (2%)
Zona cracked, 30 min						
Intact	74	2	9	1	0	86 (96%)
Reacting	0	0	1	1	0	2 (2%)
Reacted	0	0	0	0	2	2 (2%)
Zona drilled, 60 min						
Intact	14	1	0	0	0	15 (79%)
Reacting	0	2	0	2	0	4 (21%)
Reacted	0	0	0	0	0	0
Zona drilled, 90 min						
Intact	2	0	1	0	0	3 (33%)
Reacting	1	1	0	0	0	2 (22%)
Reacted	2	0	0	0	2	4 (45%)

fertilization process in mice, it is clearly not absolutely essential as zona-free oocytes can be fertilized successfully *in vitro* by spermatozoa undergoing spontaneous acrosome reactions [Wolf, 1978; Thadani, 1982]. Moreover, fertilization of zona-drilled mouse oocytes is possible even in the presence of a monoclonal antibody to ZP3, the protein implicated in sperm-zona binding and induction of the acrosome reaction [Conover and Gwatkin, 1988].

The implications of these studies for assisted fertilization in human systems are not yet clear. Although the human zona material—or components closely associated with it—can induce the acrosome reaction *in vitro* [Cross et al., 1988], this is perhaps not obligatory *in vivo* as potent stimulatory factors are also found associated with the cumulus oophorus [Siiteri et al., 1988a] and follicular fluid [Siiteri et al., 1988b, Yudin et al., 1988]. Furthermore, human spermatozoa capacitated *in vitro* readily penetrate zona-free hamster oocytes, and this cannot occur unless the acrosome reaction has occurred [Yanagimachi, 1987].

One interesting observation was of acrosome-intact spermatozoa wholly or partially embedded in the zona matrix (Figs. 3, 8). It could be argued that the zona material had been partially softened by exposure to acrosomal enzymes from dead or spontane-

(Figs. 5–8) Fig. 5. Detail of a zona-drilled oocyte 60 min after insemination shows an apparent flattening of the oocyte surface microvilli (arrowed) under the drill site. ZP = zona pellucida; Oc = oocyte cytoplasm. ×26,870.

Fig. 6. The zona matrix shows patchy thinning rather than a discrete hole after acid drilling. In this specimen 60 min after insemination, a sperm head in the process of the acrosome reaction (arrowed) is in close association with remnants of the zona pellucida. Sp = sperm. ×13,110.

Fig. 7. Spermatozoa are also capable of penetrating the zona pellucida in areas away from the drill site. In this specimen (60 min after insemination), the sperm has clearly completed the acrosome reaction and the rostral region has made contact with the oocyte. ×17,490.

Fig. 8.—Spermatozoa were occasionally observed deeply embedded in the zona matrix with an intact acrosome (arrowed). Sixty minutes after insemination; zona drilled specimen. ×36,930.

ously reacted spermatozoa in the insemination medium. However, this situation would more probably cause non-localized thinning or dissolution of the whole zona rather than invaginations around bound spermatozoa. It seems unlikely, too, that mechanical penetration could account for such embedding, as sperm are not thought to be capable of exerting sufficient thrust to penetrate the zona without enzymic assistance [Green, 1987a]. The sequence of enzyme release and activation during the acrosome reaction is now realized as being complex [DiCarlantonio and Talbot, 1988]. There is evidence in several mammalian species that significant enzyme release might precede the membrane fusion events of the acrosome reaction [Zao et al., 1985; Cummins and Yanagimachi, 1986; Tesarik et al., 1988], and this might accompany the swelling of the matrix seen prior to membrane fusion in the acrosome reaction as well as in other exocytotic events [Green, 1987b].

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