Quantitative fluorometry of abnormal mouse sperm nuclei

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Summary. An extensive quantitative analysis of deformed mouse spermatozoa was undertaken. Improvements over previous studies included the isolation and purification of sperm nuclei, a multifaceted analytical approach using several fluorochromes and the analysis of individual nuclei classified into shape categories.

Malformed sperm nuclei in BALB/c mice could not be distinguished from normal ones in terms of total and basic proteins, sulfhydryl and disulfide group concentration, DNA concentration and chromatin organization. The shape of sperm nuclei is therefore probably determined by the manner in which the internal biochemical components are assembled.

Keywords: quantitative fluorometry; sperm nuclei; mouse

Introduction

All species of mammal produce deformed spermatozoa during spermiogenesis. The proportion varies depending upon the species or the strains (Beatty & Sharma, 1960; Beatty, 1972; Wyrobek, 1979; Krzanowska, 1981), from 4% in SW mice (Wyrobek *et al.*, 1975; Pogany, 1987) to 99% in the *azh/azh* strain (Kot & Handel, 1987). The mechanisms responsible for nuclear deformation in these cells are not understood, but deformed spermatozoa are known to penetrate the zona pellucida of oocytes *in vitro* (Kot & Handel, 1987) and to fertilize eggs (Moore Smith, 1970; Krzanowska & Lorenc, 1983). Deformed spermatozoa may also contribute to reproductive dysfunctions and are suspected to cause habitual abortions (Giarola & Ballerio, 1962).

A large number of physical and chemical factors are known to temporarily increase the incidence of malformed spermatozoa (Wyrobek & Bruce, 1975; Wyrobek *et al.*, 1983; Pogany, 1987). Although means of selecting against these deformed spermatozoa within the female reproductive tract have been demonstrated (Krzanowska, 1974), embryos conceived during the time that the frequency of sperm shape abnormality is exacerbated may be at considerable genetic and/or teratogenic risks. Hence, it is important to determine the mechanism(s) behind the induction of head shape abnormalities.

The biochemical events responsible for the shape of spermatozoa have not yet been resolved although much is already known about the biochemistry of spermiogenesis. The process entails a complex series of reactions during which somatic histones are replaced by gametic protamines (Bode *et al.*, 1977; Willmitzer *et al.*, 1977). This intricate sequence of biochemical steps is coupled with the compaction of the haploid nucleus, which is probably the most important event in the final shaping of the spermatozoa. Perturbations in these events could therefore significantly alter the structural integrity of the sperm chromatin and influence the morphology of the cells.

A common approach to the analysis of sperm chromatin compaction involves the estimation of dye binding during their morphological maturation. Results have revealed a progressive reduction

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in dye binding reflecting the increased condensation of the chromatin (Ringertz *et al.*, 1970; Rodman *et al.*, 1979; Wagner & Minhas, 1982; Gledhill, 1985). The progressive oxidation of sulfhydryl groups in protamines (Bedford & Calvin, 1974; Saowaros & Panyim, 1979; Pellicieri *et al.*, 1983; Shalgi *et al.*, 1989; Balhorn *et al.*, 1991) has also been suggested to assist in this compaction, although the precise manner in which this is accomplished in maturing spermatozoa is unknown.

Other investigations of chromatin in abnormally shaped spermatozoa have focused on DNA content, chromatin structure or on both (Pinkel *et al.*, 1983; Evenson *et al.*, 1985, 1986). In most instances, these analyses involved large populations of spermatozoa without regard to their individual shape. In the few cases where single cells were analysed for the structural integrity of malformed chromatin (Gledhill *et al.*, 1977; Gledhill, 1985), no attempts were made to distinguish between types of abnormality.

In an attempt to identify biochemical defects that contribute to abnormal shaping in spermatozoa, we used biochemical and histochemical tests to analyse normal and abnormally shaped sperm nuclei individually. Three different fluorochromes were used to measure nuclear proteins, sulfhydryl groups and DNA. 7-Aminoactinomycin D and Acridine Orange were used to assess the integrity of sperm chromatin in deformed and normal nuclei.

Materials and Methods

Animals

BALB/c mice were purchased from Bantin & Kingman, Inc. (Fremont, CA) and from Simonsen Laboratories, Inc. (Gilroy, CA). They were kept individually in the animal facility of the Lawrence Livermore National Laboratory under a 12 h light : 12 h dark regimen and at a constant temperature of 22°C with free access to food (Certified Rodent Chow Checkers, Purina Mills, Inc., St Louis, MO) and water.

Isolation of sperm nuclei

Animals were killed with carbon dioxide and cervical dislocation. Cauda epididymides were removed, pooled in cold Tris-saline (TS) buffer (0.01 mol Tris l⁻¹ in 0.9% saline, pH 8.0), and their contents gently expressed with the blunt edge of a scalpel. After filtration through a fine silk filter (no. 11; Standard Swiss Silk Doufour; Abbe Engineering Co., Brooklyn, NY), they were sonicated to remove somatic cell contaminants and washed three times in 5 ml TS with intermittent centrifugation at 10 400 g for 4 min (Sorvall, Model RC2-B, HB-4 rotor; Norwalk, CT). Sperm nuclei were isolated (Balhorn *et al.*, 1977; Pogany *et al.*, 1981) in 4 ml TS to which 10 mg DTT (dithiothreitol; Boehringer-Mannheim, Indianapolis, IN) was added. After incubation on ice for 15 min, 1 ml of 5% MTAB stock (mixed alkyltrimethylammonium bromide; Sigma Chemical Co., St Louis, MO) in distilled water was added to a final concentration of 1% and the nuclear suspension was further incubated on ice for 60 min with frequent but gentle mixing. After centrifugation, the pellet was resuspended in 5 ml 1% MTAB and incubated for another 60 min at room temperature. The suspension was then centrifuged and the pellet containing pure nuclei dispersed in 10 ml TS by sonication.

The nuclear suspension was further diluted by transferring 2.5 ml of the suspension to 10 ml of TS. Plain microscope slides were cleaned in 70% ethanol and mounted into cytobuckets (International Equipment Company, no 1024; Needham Heights, MA). Aliquots of 0.25 ml were delivered to each of the three wells per slide and centrifuged at 350 g for 10 min (International Equipment Company, Model HN-SII). The slides were allowed to air dry overnight. They were subsequently rinsed with distilled water, air dried again and stored in a slide box for subsequent use. In general, fixation of the nuclei was omitted unless expressly required by the specific staining application.

Staining protocols

DAPI (4', 6-diamidino-2-phenylindole; Sigma Chemical Co., St Louis, MO) was used to determine the DNA content of sperm nuclei (Lin *et al.*, 1977). The dye was dissolved in distilled water to a stock concentration of 100 µg ml⁻¹. Just before use it was diluted to 0.5 µg ml⁻¹ in antifadant (Johnson & Noguiera Araujo, 1981). Drops of the stain were placed on slides and the edges of the coverslip sealed with fingernail polish. They were left to incubate in the dark for 1 h before quantitation. Nuclei were analysed at 365 nm (excitation) and 397 nm (emission).

Brilliant Sulfaflavine (Aldrich Chemical Co., Inc., Milwaukee, WI) was used to measure total and basic proteins (Leemann & Ruch, 1972). For total proteins, slides were first fixed in ethanol:glacial acetic acid (3:1) and stained at pH 2.8 in citric acid buffer (0.1 mol citric acid l⁻¹, 0.3 mol disodium phosphate l⁻¹) with sulfaflavine at 0.1% concentration for 30 min in the dark. Differentiation was obtained in buffer for 10 min. The slides were dehydrated, cleared in xylene, mounted in antifadant and analysed at 420 nm (excitation) and 520 nm (emission).

For the estimation of basic proteins with sulfaflavine, nuclei were fixed in buffered formalin (pH 7.0) for 10 min. The slides were then rinsed in water, and hydrolysed in 5% trichloroacetic acid at 60°C for 3 h. They were rinsed three times in 70% ethanol, twice in water (distilled), and once in TS. The slides were stained with Sulfaflavine at the same concentration as above for 30 min and differentiated in buffer. After dehydration and clearing, they were mounted in antifadant and analysed at the same wavelengths used for neutral proteins.

Free thiol groups were determined using the thiol-specific dye DACM (N-(7-dimethylamino-4-methylcoumarinyl)maleimide; Sigma Chemical Co., St Louis, MO) as reported by Pellicciari *et al.* (1983). A stock of 1 mg in 1 ml acetone was kept in the freezer and diluted to a final concentration of 10^{-5} mol l^{-1} in 0.04 mol PBS l^{-1} (phosphate-buffered saline, pH 7.0) just before use. Slides were washed in three changes in PBS and air dried. Slides were stained in DACM for 20 min in the dark. They were rinsed three times in PBS, allowed to air dry, and sealed with antifadant. Their analysis was performed at 405 nm (excitation) and 515 nm (emission).

Sperm chromatin integrity (Gill *et al.*, 1975) was assessed using the DNA intercalator 7-aminoactinomycin D (7-AMD; Calbiochem, San Diego, CA) as described by Balhorn *et al.* (1985). Slides of dried nuclei were stained for 16 h in phosphate buffer (pH 7.0) containing 5 μ mol 7-AMD l^{-1} . Brief rinsing in phosphate buffer was followed by air drying in the dark. Slides were immediately analysed at 546 nm (excitation) and 590 nm (emission).

Acridine orange (Polysciences, Inc., Warrington, PA) staining was used because of its known metachromatic property (Lerman, 1961; Darzynkiewicz *et al.*, 1975) which permits the discrimination between native (green fluorescence) and denatured DNA (red fluorescence). The technique assesses the susceptibility of DNA to acid depurination and denaturation when nuclei are exposed to acridine orange at a pH of 2.5–2.8. The one-step method of Tejada *et al.* (1984) was chosen because of its simplicity. Slides were fixed in fresh Carnoy's (3 parts methanol:1 part glacial acetic acid) for 2 h and air dried. A working solution of acridine orange was used by adding 10 ml of a distilled water stock (1 mg ml^{-1}) to a citric acid buffer (40 ml of 0.1 mol citric acid l^{-1} and 2.5 ml of 0.3 mol disodium phosphate l^{-1} , pH 2.55). Slides were stained for 5 min in the dark and briefly rinsed in distilled water. For the fluorometric analysis, slides were covered with water and the edges of the coverslip sealed with nail polish. The fluorescence of nuclei was also scored according to whether they stained green (native DNA) or red (denatured DNA). Acridine orange binding to nuclei was also quantitatively assessed at an emission of 490 nm and an excitation of 530 nm. All slides were analysed immediately after staining.

Image analysis

Fields of dispersed nuclei were located at $\times 100$ (Pan-NEOFLUAR, 1.30 aperture) using a Zeiss Axiophot microscope equipped with a COHU SIT Videcon camera (Model 5152; tube no 4804 HP2). A non-random approach was chosen to obtain representative images of all shapes, some of which were too rare to select in a random fashion. The images were focused onto a black and white TV screen and pipelined through a Trapix 5500/64 processing unit (Recognition Concept, Inc., Carson City, NV) at 512 $\times$ 512 pixel resolution. Image capture and analysis were executed by an ACUity program (version 6.2) specifically designed for object analysis (Young *et al.*, 1982). Optimal image segmentation parameters were selected for each stain to extract the best contour traces of the nuclei from the background. The digitized images were projected onto a colour monitor in pseudocolour video output and rastered to locate each object. When an object was encountered which conformed to the user-selected segmentation parameters, a contour trace was delineated around it. The various morphological types were classified as 'normal', 'hammer', 'acrosome', 'truncated', 'collapsed' and 'others' and given an identification number of 1 to 6 respectively. After the identification of each nucleus, the digitized images were saved in individual files until the end of the session when they were combined into a larger data file for subsequent statistical analyses. All results were expressed as total stain (brightness) per surface area of the nuclei.

Statistical analysis

Digitization and the subsequent morphometric analysis of each object by ACUity were driven by a DEC, MicroVAX II (Digital Equipment Corporation, Maynard, MA) computer and included a number of measurements. These have been described and defined by Young *et al.* (1982). Standard descriptive statistical analyses were performed as well as one-way analysis of variance (ANOVA) followed by the Scheffe multiple-range test of discrimination between the means.

Photographic records of the various nuclear shapes were obtained through a compound microscope. Intact sperm suspensions were first stained with 0.1 eosin Y for 60 min according to the method of Wyrobek & Bruce (1975), smeared onto clean slides, air dried, and covered with Permount. In the case of isolated nuclei, they were stained with Toluidine blue for 60 min and rinsed in distilled water. All photographs were taken with a Zeiss Ultraphot microscope at $\times 100$ magnification (Optovar = 1.60) and recorded on Panatomic X film. Negatives were developed in Microdol X for the recommended time and printed on multigrade RC paper.

Results

Approximately 60% of the sperm obtained from the cauda epididymides of BALB/c mice used in this study have abnormally shaped heads. These were classified into five categories of shape

anomalies in increasing order of severity (Fig. 1). Even though the categories are based primarily on the morphology of intact sperm, they apply equally well to isolated nuclei since the latter retain the shape of the cells from which they are derived (Fig. 1). This clearly indicates that the removal of the membranes with DTT and MTAB does not preferentially affect any one particular shape. The distribution of nuclear deformities is virtually identical to that of untreated, intact spermatozoa. The analysis of these nuclei was, therefore, considered representative of intact epididymal spermatozoa.

The mildest deformation, defined as 'acrosome', pertains to various degrees of blunting and bending of the tip of the head. Although isolated nuclei no longer possess acrosomes, the designation is retained for purposes of consistency. 'Truncated' sperm heads and nuclei are defined as having a tapered posterior aspect. Typically, these abnormalities include the deformation of the implantation fossa. A third class of deformations involves the ventral shift of the implantation fossa to the extent that the insertion of the mid-piece is characteristically perpendicular to the longitudinal axis of the head. These are designated 'hammer' types. The most severely deformed sperm and nuclei are classified as 'collapsed' and 'others'. Both are amorphic and, upon closer examination, reveal that they are thicker than the other types of deformity. The 'others' category includes nuclei that cannot easily be placed into any of the other morphological categories. Frequently, sperm heads are characterized by more than a single abnormality as seen in the 'truncated' example where the blunting of the acrosome is also evident. In these instances, heads are classified according to the most prominent aberration.

The most frequent shape anomaly in BALB/c mice is represented by 'acrosome' and 'truncated' types of deformity. In the former case, the proportion almost equals that of normal heads and nuclei. Most other shape aberrations are present less frequently, with the most severely distorted sperm heads and nuclei ('collapsed') being about twice as abundant as 'hammers' and 'others'.

A large number of slides were routinely prepared for analysis to minimize sample variation during the fluorometric measurements. The likelihood that these samples could deteriorate upon storage and, therefore, alter their quantitative fluorescence, was tested by performing the analysis at various time intervals up to 57 days. The results show that prolonged storage does not affect stainability and subsequent analyses (data not shown). Similarly, there is no evidence that the integrity of the chromatin is altered upon storage as tested with acridine orange staining during the same period (data not shown).

The image analysis capabilities of the program 'ACUity' were used to determine whether the application of the dyes affects the physical dimensions of the objects under consideration. Alterations in the overall size of the nuclei, as a result of staining, would significantly influence the quantitative comparisons between nuclei stained with different fluorochromes. When stained nuclei were compared, no change in size is observed except in the case of 7-AMD where a noticeable increase in surface area was noted (Table 1). Closer examination of the length and width parameters (routinely provided by ACUity) in 7-AMD-stained nuclei reveals that the increase is primarily due to nuclear elongation (42.11%). An increase in width is also observed but it is smaller (29.43%).

The distribution of the 5013 nuclei fluorometrically analysed in this investigation is shown (Table 2). As can be seen, the frequency of the various shapes analysed in this study deviates slightly from the normal distribution of abnormally shaped spermatozoa in BALB/c (Fig. 1). This deviation reflects the need to preferentially select certain abnormality types because of their low inherent frequency.

The results of the quantitative fluorometric analysis of normal and malformed sperm nuclei are shown in Fig. 2. For most of the fluorochromes, the range of brightness is very similar and extends from 30 (acridine orange) to 2000 (DAPI) fluorescence units. This latter stain stands out as a particularly sensitive dye. Small quantitative differences in brightness values can be seen between 'normal', 'acrosome', 'hammer' and 'truncated' shapes irrespective of the dyes used. Statistical analyses (ANOVA and Scheffe multiple-range test) show that these differences are not significant. However, the fluorescence of 'collapsed' and 'other' shapes is significantly different from normal

Fig. 1. Sperm head shape abnormalities in BALB/c mice; (a) various shape categories used to identify the malformations in intact spermatozoa; (b) the same shape abnormalities in sperm nuclei after the removal of the membranes. The retention of the distinctive shapes after denaturation demonstrates that the deformations are nuclear. The similarity in their frequency (percentages) further indicates that the chemical treatment used to remove the nuclear membrane has no effect on their shape. ($\times 910$).

Table 1. Effects of dye binding on the dimensions (pixels $\pm$ SD) of normal mouse sperm nuclei

Dyes	Surface area	Length	Width
Acridine orange	743.5 $\pm$ 89	44.9 $\pm$ 3.6	21.2 $\pm$ 2.3
<i>N</i> -(7-dimethylamino-4-methylcoumarinyl) maleimide (DACM)	881.2 $\pm$ 130	49.7 $\pm$ 5.0	22.9 $\pm$ 2.8
Brilliant sulfaflavine, pH 8.0	642.0 $\pm$ 101	40.2 $\pm$ 3.8	19.9 $\pm$ 2.1
Brilliant sulfaflavine, pH 2.8	812.7 $\pm$ 114	48.7 $\pm$ 4.7	20.9 $\pm$ 2.3
4',6'-Diamidino-2-diphenylindole (DAPI)	701.0 $\pm$ 79	43.6 $\pm$ 4.1	20.8 $\pm$ 2.1
Average	755.6	45.4	21.2
7-Aminoactinomycin D nuclei	1055.0 $\pm$ 137	64.5 $\pm$ 4.7	27.4 $\pm$ 2.6
% Increase:	39.6	42.1	29.4

Table 2. Distribution of types of nuclear shape

Dyes	Normal	Hammer	Acrosome	Truncated	Collapsed	Other	Total
Acridine orange	140	17	74	147	29	11	418
<i>N</i> -(7-dimethylamino-4-methylcoumarinyl) maleimide (DACM)	336	45	58	177	70	19	705
Brilliant sulfaflavine pH 8.0	432	63	136	237	59	18	945
Brilliant sulfaflavine pH 2.8	176	15	111	163	27	9	501
4',6'-Diamidino-2-diphenylindole (DAPI)	581	51	118	255	56	25	1086
7-Aminoactinomycin D (7-AMD)	429	65	263	492	85	24	1358
Totals:	2094	256	760	1471	326	106	5013
Frequency (%)	41.8	5.1	15.2	29.3	6.5	2.1	100

nuclei when stained with DAPI, brilliant sulfaflavine 8.0, and 7-AMD ($F > 2.381$; $P = 0.001$). The other stains (acridine orange, DACM, brilliant sulfaflavine 2.8) do not discriminate sufficiently between normal and these severely deformed nuclei to distinguish them.

Discussion

This study represents a comprehensive quantitative evaluation of sperm nuclear components. First, five different fluorochromes were used to quantify several critical components of sperm nuclei in contrast to previous investigations which were based on single parameter determinations. Second, six different types of sperm head morphology were recognized in an attempt to provide added delineation among them. The large number of individual sperm nuclei in each shape class provided sufficient statistical samples to generate meaningful comparisons between the various shapes. Lastly, the use of isolated and purified nuclei made it possible to dispense with concerns about the potential influence of membranes on the quantitative uptake of dyes.

Most deformed BALB/c sperm nuclei could not be distinguished from normal sperm nuclei in terms of total and basic proteins, sulfhydryl groups, DNA and chromatin organization as monitored by 7-AMD binding and acridine orange metachromasia. The only significant differences were those detected between the most deformed nuclei ('collapsed' and 'others') and normal ones. However, these differences, although statistically significant, may be artifacts. In addition to being highly distorted, these nuclei were consistently thicker. This increased thickness may have affected

Fig. 2. Quantitative fluorometry of mouse sperm nuclei using (a) *N*-(7-dimethyl amino-4-methylcoumarinyl) maleimide (DACM), (b) 4'6-diamidino-2-diphenyl indole (DAPI), (c) brilliant sulfaflavine pH 8.0, (d) brilliant sulfaflavine pH 2.8, (e) acridine orange and (f) 7-aminoactinomycin D (7-AMD). All values are expressed as stain per area and are plotted as arbitrary fluorescence units on the y axis. The numbers on the x axes represent the various nuclear shape categories (1: normal, 2: hammer, 3: acrosome, 4: truncated, 5: collapsed, 6: others). The total number of nuclei analysed in each shape category and for each dye is indicated within the graphs. The bars indicate the means and the lines represent the SEM; *significant differences from average of other shapes ($P < 0.05$).

both the proper focusing and the segmentation of the objects. It is, therefore, unclear whether the observed differences in staining for these two classes of nucleus are real or whether they arise from their peculiar shape.

The absence of a relationship between the binding of the structural probes (acridine orange and 7-AMD) and nuclear shapes contradicts a previous investigation in which binding of [^3H]actinomycin D was found to be higher in deformed bull spermatozoa than in their normal counterpart (Gledhill *et al.*, 1971). This was subsequently related to structural defects in the chromatin (Gledhill, 1972). However, the discrepancy may have resulted from determining dye binding on intact bull spermatozoa. It is therefore possible that the data from bulls may reflect only an altered

permeability of the membranes to the dye in deformed spermatozoa. Such a condition would not have been detected in our investigation since only amembranous nuclei were analysed. Similarly, it has been shown that spermatozoa from subfertile males are more susceptible to heat denaturation (Evenson *et al.*, 1980). However, the relationship between their individual shape and their propensity to denature under heat was not established.

Our determinations of potential differences between normal and abnormal sperm nuclei in terms of total and basic proteins are not shown. The harsh fixation and extraction treatments that preceded their quantitative assessments introduced uncertain experimental conditions that require further monitoring. Accordingly, additional experiments, using milder conditions, are planned for a more quantitative re-assessment of these parameters.

Staining nuclei with 7-AMD induced an increase in the size of the nuclei that may have been due to the prolonged exposure to either the dye or the buffer overnight (see Balhorn *et al.*, 1985). However, the preferential increase in length may be related to the observation that chromatin, inside sperm nuclei, is in a cholesteric configuration (Sipski & Wagner, 1977). A greater increase in length would, therefore, be anticipated, as a result of chromatin expansion, if the presumed cholesteric bundles were oriented along the longitudinal axis of the spermatozoa. The intercalation of 7-AMD between base pairs of the DNA in sperm chromatin could extend the length of these bundles.

Our analyses of protein, DNA and total sulphydryl groups in sperm nuclei support and complement previous reports in which the total protein contents of teratozoospermic cells was found to be identical to normal ones (Bustos-Obregon & Leiva, 1983). As demonstrated by de Boer *et al.* (1990), even aneuploid gametes could not be distinguished in terms of protamine content and cross-linking. Quantitative DNA analyses by Feulgen cytometry (Döring *et al.*, 1972) failed to discriminate abnormal sperm heads from normal ones. However, a UV absorption analysis of F₁ hybrids between *Mus musculus* and *Mus poschiavinus* showed a higher mean DNA content but the differences were not statistically significant (Stolla & Gropp, 1974). In a comprehensive analysis of the protein synthetic machinery of differentiating spermatozoa, Meistrich *et al.* (1988) showed that *azh/azh* mice possessed normal components and expression levels compared with a strain where the incidence of sperm shape abnormality is considerably lower. These authors concluded that the molecular entities themselves were biochemically unaffected but could be subjected to faulty assembly processes. They further suggested that microtubular elements in the manchette could be involved in the formation and deformation of spermatozoa. However, the participation of the manchette in the shaping of sperm nuclei has been strongly disputed by Fawcett *et al.* (1971) on the basis that there is insufficient diversity in its structure and distribution to account for the wide array of sperm shapes in animals. Accordingly, these latter authors propose that the final shaping of the sperm nucleus involves factors internal to the nucleus.

The similarity in the composition of nuclei from different shape classes suggests that the fundamental biochemical differentiation of the sperm nucleus antedates its formation during spermiogenesis. The forces that impose a morphological character on each sperm nucleus must, therefore, use components that are quantitatively similar in all. Accordingly, the formative events instrumental in shaping sperm nuclei must exhibit considerable morphological plasticity. This extends from sperm possessing minor acrosomal deformities to those having severely distorted nuclei ('others' and 'collapsed') and further suggests that it is the mode of packaging these similar components that is radically different in abnormal spermatozoa. A genetic component for this disturbance in packaging is strongly implied when one considers that the proportion of malformed spermatozoa in any strain of mice is constant from one generation to the other (Wyrobek, 1979).

A number of internal formative elements have already been implicated in the morphological development of spermatozoa, including the characterized stable DNA-protein complexes in the sperm nucleus of rams (Avramova & Tsanev, 1987). DNA-anchoring proteins, located at the implantation fossa of hamster spermatozoa, have also been characterized (Ward & Coffey, 1989). Residual histone components cannot be excluded either since they have been found in human

spermatozoa (Tanphaichitr *et al.*, 1978) and are also suspected to occur in mice (R. Balhorn, unpublished) albeit in smaller concentrations. Finally, the presence of a structural nuclear element similar to the nuclear matrix of somatic cells may need to be invoked to account for the shape of spermatozoa. Such a matrix element has recently been suggested for human sperm nuclei (Wyrobek *et al.*, 1990) and, if present, would have a significant impact on our understanding of normal and abnormal sperm maturation. The total absence of quantitative differences between malformed and normal sperm nuclei in BALB/c mice is consistent with the hypothesis that sperm nuclear deformation in this mouse strain may result from structural perturbations in a nuclear matrix.

Although the shape abnormalities analysed in BALB/c mice have an obvious genetic origin, it should be instructive to compare our results with deformed nuclei experimentally generated with shape-altering agents such as X-rays (Pogany, 1987).

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