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Original Article

Failure of differentiation of the nuclear-perinuclear skeletal complex in the round-headed human spermatozoa

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ABSTRACT Acrosomeless round-headed spermatozoa from three men were studied under electron microscopy and indirect immunofluorescence microscopy using the anti-calicin antibody that recognizes a basic protein of the sperm perinuclear theca (Longo *et al.*, 1987). Electron microscopy revealed the existence of anomalies of the nuclear envelope, the nuclear matrix underlying the nuclear envelope, and the perinuclear layer. The absence of sperm labeling with the anti-calicin antibody confirmed that the formation of the perinuclear theca was impaired. Data obtained from both mature spermatozoa and ejaculated spermatids suggest that i) round-headed sperm head anomalies result from a failure of differentiation of the sperm-specific skeletal complex related to the nucleus, and ii) the acrosome spreading over the nucleus, the nuclear elongation and the post-acrosomal sheath formation are dependent on such nuclear-perinuclear differentiations. In contrast, chromatin condensation, cytokinesis and some events of the acrosomal shaping appear not to depend on those nuclear-related differentiations. The possible processes allowing the maintenance of the sperm head structures and their subsequent morphogenesis are discussed.

KEY WORDS: *calicin, cytoskeleton, cytomatrix, nuclear envelope, nuclear matrix, human spermatozoa*

Introduction

Round-headed human spermatozoa are characterized in electron microscopy by a failure of nuclear elongation and an absence of acrosome (Schirren *et al.*, 1971; Holstein *et al.*, 1973). They are also devoid of the post-acrosomal sheath (Anton-Lamprecht *et al.*, 1976) and nuclear ring (Baccetti *et al.*, 1977). In addition, the chromatin may be coarse-granular (Kullander and Rausing, 1975) and coiled flagella have been observed (Bisson *et al.*, 1974; Pedersen and Rebbe, 1974; Tyler *et al.*, 1985). Whereas round-headed spermatozoa fail to fuse with the oolemma (Weissenberg *et al.*, 1982), they are capable of nuclear decondensation following stimulation by the ooplasm (Syms *et al.*, 1984; Lanzendorf *et al.*, 1988).

The biochemical analysis of round-headed human spermatozoa has confirmed the lack of acrosomal constituents (Castellani *et al.*, 1978; Lalonde *et al.*, 1988; Florke-Gerloff *et al.*, 1985; Jeyendran *et al.*, 1985; Baccetti *et al.*, 1988) and the variability of chromatin condensation (Baccetti *et al.*, 1977). Anomalies of the distribution of the lectin binding sites have also been suggested (Wollina *et al.*, 1989). The presence of acrosomal structures being subsequently eliminated with the residual body has been reported in spermatids from men with round-headed sperm (Holstein *et al.*, 1973; Florke-Gerloff *et al.*, 1985). On the other hand, the microtubular manchette may be absent (Baccetti *et al.*, 1977).

These data have suggested that the nucleus (Nistal *et al.*, 1978), the acrosome (Schirren *et al.*, 1971; Holstein *et al.*, 1973; Baccetti *et al.*, 1977), or the Golgi apparatus (Castellani *et al.*, 1978) is responsible for the observed anomalies. In addition, the observation of this sperm dysgenesis in brothers suggests a genetical disorder (Kullander and Rausing, 1975; Nistal and Paniagua, 1978). However, the morphogenetic mechanism(s) and molecular factor(s) possibly responsible for the formation of acrosomeless round-headed spermatozoa remain unknown.

The sperm nuclear modeling involves nucleoproteins (Fawcett *et al.*, 1971), the microtubular manchette (Zamboni *et al.*, 1971; Courtens and Loir, 1981; Russell *et al.*, 1983) and the perinuclear material (Courtens *et al.*, 1976). The perinuclear material is also known to participate in the acrosome anchorage on the nucleus (Fawcett, 1975) and constitutes the perinuclear theca (Bellvé and O'Brien, 1983), whose posterior region is termed the calyx (Longo *et al.*, 1987). A sperm-specific protein localized to the calyx (named

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calicin) has recently been identified in the bull (Longo *et al.*, 1987) and the human (Paranko *et al.*, 1988). Moreover, it has been suspected that calicin plays a role in the morphogenesis of the spermatid head (Longo *et al.*, 1987).

The aim of this study was to investigate whether acrosomeless round-headed spermatozoa may result from perinuclear-nuclear anomalies. The spermatozoa from 3 men with this type of dysgenesis have, therefore, been examined in electron microscopy for their nuclear and perinuclear substructure and in indirect immunofluorescence microscopy for their calicin status. Ultrastructural data suggested that a complex of nuclear and perinuclear matrix elements (involved in the cohesion of the head components) is implicated in the observed anomalies. Disturbances of the nuclear-related skeletons in those cases were supported at the molecular level on the basis of immunocytochemical data showing an absence of labeling with the anti-calicin antibody.

Results

In light microscopy, investigated spermatozoa were characterized by round-shaped heads with no discernible acrosome (Table 1).

Round-headed sperm ultrastructure

In electron microscopy, the studied spermatozoa exhibited the already known main characteristics of round-headed spermatozoa (Table 2), *i.e.* round nucleus, absence of the acrosome and post-acrosomal sheath (Figs. 1A-C). Additional subcellular disturbances were observed:

Overall head architecture

In most studied cells, the plasma membrane was close to the nuclear apical pole (Figs. 1A, B). However, 4% (case 1) to 19% (cases 2 and 3) of the sperm cells exhibited a nucleus totally surrounded by the spermatid cytoplasm which had not been eliminated and which contained the flagellum (Fig. 1C). In addition, 5-8% of the sperm sections showed acrosome-like structures far from the nucleus, exhibiting typical thicknesses but an atypical shape (Figs. 1B-E). They were delimited by an outer and inner membrane and contained a matrix with the same density as the normal acrosome (Figs. 1D, E). The implantation fossa was normal with a typical basal plate facing the connecting piece (Figs. 2 A, B).

TABLE 1

SEMEN PARAMETERS OF THE THREE ROUND-HEADED SPERM SAMPLES AS EXAMINED IN LIGHT MICROSCOPY

Semen samples	1	2	3
Semen Volume (ml)	5	4	2.5
Sperm count (10^6/ml)	45	73	28
Progressive motility (%)	50	25	15
Non-progressive motility (%)	5	5	5
Viability (%)	68	62	74
Round-headed sperm (%)	98	92	94
Acrosomeless sperm (%)	100	100	98
Coiled flagella (%)	14	30	24

TABLE 2

ULTRASTRUCTURAL CHARACTERISTICS OF THE ROUND-HEADED AND CONTROL SPERMATOZOA
(values are in %)

CONTROL SPERM	CASES WITH ROUND HEADED SPERMATOZOA				
	1	2	1	2	3
Normal Heads	23	23	0	0	0
Nucleus					
irregularly shaped	33	40	0	0	0
poorly elongated	23	10	0	0	0
round shaped	10	0	100	100	100
coarse granular chromatin	27	23	43	53	30
large nuclear vacuole	54	73	44	45	31
Acrosome					
malformed	47	27	0	0	0
absent	0	3	100	100	100
acrosomal-like structures	0	0	5	8	6
Post-acrosomal sheath					
incomplete	20	27	0	0	0
absent	10	10	100	100	100
Nuclear ring					
absent	7	3	100	100	100
Flagella					
coiled	3	3	4	19	19
absence of axonemal doublets	24	30	34	42	49

Substructure of the sperm head components

a) Nucleus

The chromatin (Table 2) exhibited either normal (47% to 70% of the sperm heads: cases 2 and 3 respectively) or slightly incomplete condensation (coarse granular aspect) (Fig. 1C). A voluminous round-shaped vacuole was present in the nuclear center in 31% (case 3) to 45% (case 2) of the heads (Fig. 1C).

The nuclear envelope displayed numerous anomalies except in the implantation fossa where it assumed a typical appearance (Fig. 2A). Elsewhere, the nuclear envelope never had the sperm-specific ultrastructural features (Table 3). Indeed, contrary to the morphologically normal spermatozoon (Figs. 3A, B), the nuclear envelope of the round-headed spermatozoa exhibited a space between the nuclear membranes as between the inner membrane and the chromatin (Figs. 2D, E) and 3 main atypical aspects randomly distributed among spermatozoa: i) multilayered envelope with or without nuclear pores (Figs. 1A and 2B); ii) envelope with the 2 membranes widely separated (Figs. 1B and 2B, C) and iii) unilayered envelope with or without pores (Figs. 2D, E). Occasionally, the portion of multilayered nuclear envelope assumed an aspect of annulate lamellae (Fig. 2B). The nuclear envelope may also be absent except in the implantation fossa region (Table 3). In such cases, envelope-like elements were observed in the cytoplasm far from the nucleus (Fig. 1C). Strands emerging from the chromatin mass could be observed (Figs. 2D, F) and ribosome-like particles were sometimes present on the outer nuclear membrane (Fig. 2E).

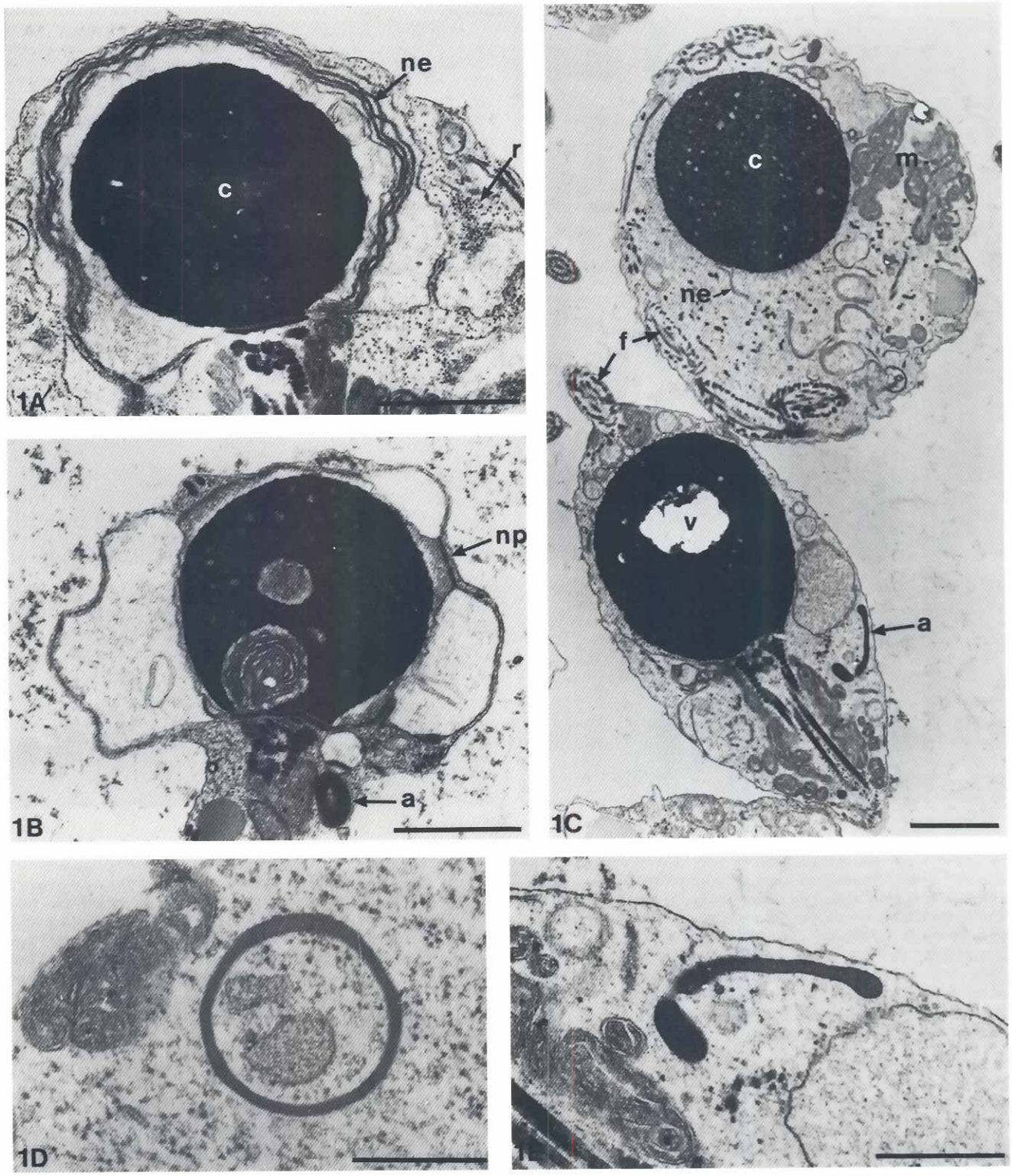

Fig. 1. Main ultrastructural characteristics of round-headed spermatozoa. (A) *A multilayered nuclear envelope (ne) separated by a space from the round-shaped chromatin mass (c). r= clusters of ribosome-like particles. Bar= 1 μm.* **(B)** *The nuclear envelope comprises areas containing nuclear pores (NP) and areas where the nuclear membranes are widely separated. a= acrosome-like structure. Bar= 1 μm.* **(C)** *The nuclear envelope is lacking and a coiled flagellum (f) observed within the residual cytoplasm. The nucleus exhibits either poorly condensed chromatin (c) or a large round vacuole (v). a=acrosomal elements; ne=nuclear envelope-like components; m=clusters of mitochondria. Bar=1 μm.* **(D)** *A ring-like acrosomal element in a residual cytoplasm exhibiting regions whose thicknesses are typical of either the principal (700 nm) or equatorial (350 nm) part of the normal acrosome. Bar=0.2 μm.* **(E)** *High magnification of the acrosomal elements of the lower spermatozoon of Fig. 1 C. The shorter element appears as thick as the principal part and the longer one as thick as the equatorial part of the normal acrosome. Bar=0.5 μm.*

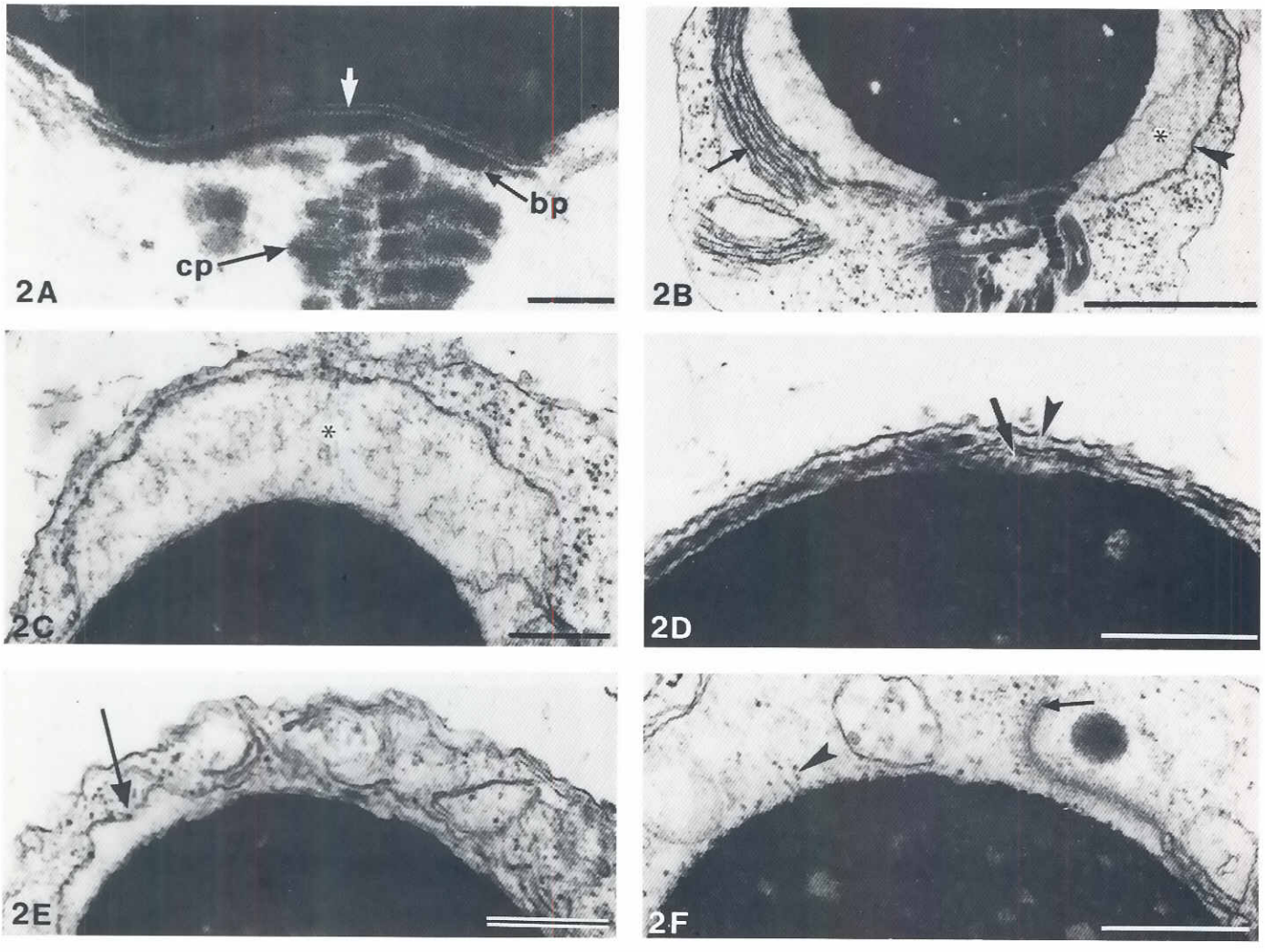

Fig. 2. Ultrastuctural characteristics of the nuclear envelope of round-headed spermatozoa. (A) *In the implantation fossa region, the nuclear envelope exhibits normal ultrastructural features. No nuclear pores can be seen. Periodically disposed cross-links (arrow) are observed between the two membranes. The envelope is closely apposed to the chromatin and a basal plate (bp) is discerned on the outer face of the nuclear envelope. cp=connecting piece. Bar=0.2 μm.* **(B)** *The nuclear envelope regions adjacent to the implantation fossa. The left hand side region consists of a multilayered envelope with well-discernible pores (arrow). The right hand side region comprises two membranes separated by a wide space (star). Ribosome-like particles are present on the outer membrane (arrowhead). Bar=1 μm.* **(C)** *The nuclear envelope (apical region) has split off giving rise to a gap between the two membranes (star). Bar=0.5 μm.* **(D)** *The nuclear envelope (apical region) exhibits well-discernible nuclear pores. Thin cross-links are present between the nuclear envelope and the plasma membrane (arrowhead) as in the space underlying the nuclear envelope (arrow). Bar=0.5 μm.* **(E)** *The space between the nuclear envelope (apical region) and the plasma membrane exhibits membranous elements. Arrow=ribosome-like particles. Bar=0.5 μm.* **(F)** *The nuclear envelope ends abruptly at the equatorial region level and exhibits striations between the membranes (arrow) which look like those of the implantation fossa (Fig. 2 A). Thin strands emerge from the chromatin mass (arrowhead). Bar=0.5 μm.*

b) Perinuclear cytoplasmic layer

In the implantation fossa region, the perinuclear layer contained the typical structures, *i.e.* the basal plate on the outer face of the nuclear envelope and cross-bridges linking the basal plate to the connecting piece (Fig. 2A). In contrast, the other areas of the perinuclear layer did not exhibit the sperm-specific substructures which, in the normal human spermatozoon, consist of cross-links of 7-9 nm in diameter interconnecting the nucleus and the adjacent structures (Figs. 3A, B). In that respect, it is worth noting that the rare acrosomeless spermatozoa from the control samples displayed typical perinuclear cross-bridges (Fig. 3C). In round-headed spermatozoa only thinner elements were observed (Figs. 2D and 3D) assuming the appearance of the bridges normally present in the periacrosomal layer (Fig. 3A).

Nuclear envelope-plasmalemma association in a ring-like fashion was rarely encountered and always incomplete (*i.e.* discerned on only one nuclear side) (Fig. 3D). Surprisingly, in some round-headed spermatozoa, successive nuclear envelope-plasma membrane associations could be observed (Fig. 3E). Finally, in 1% of the head sections, an undefined dense plate was observed between

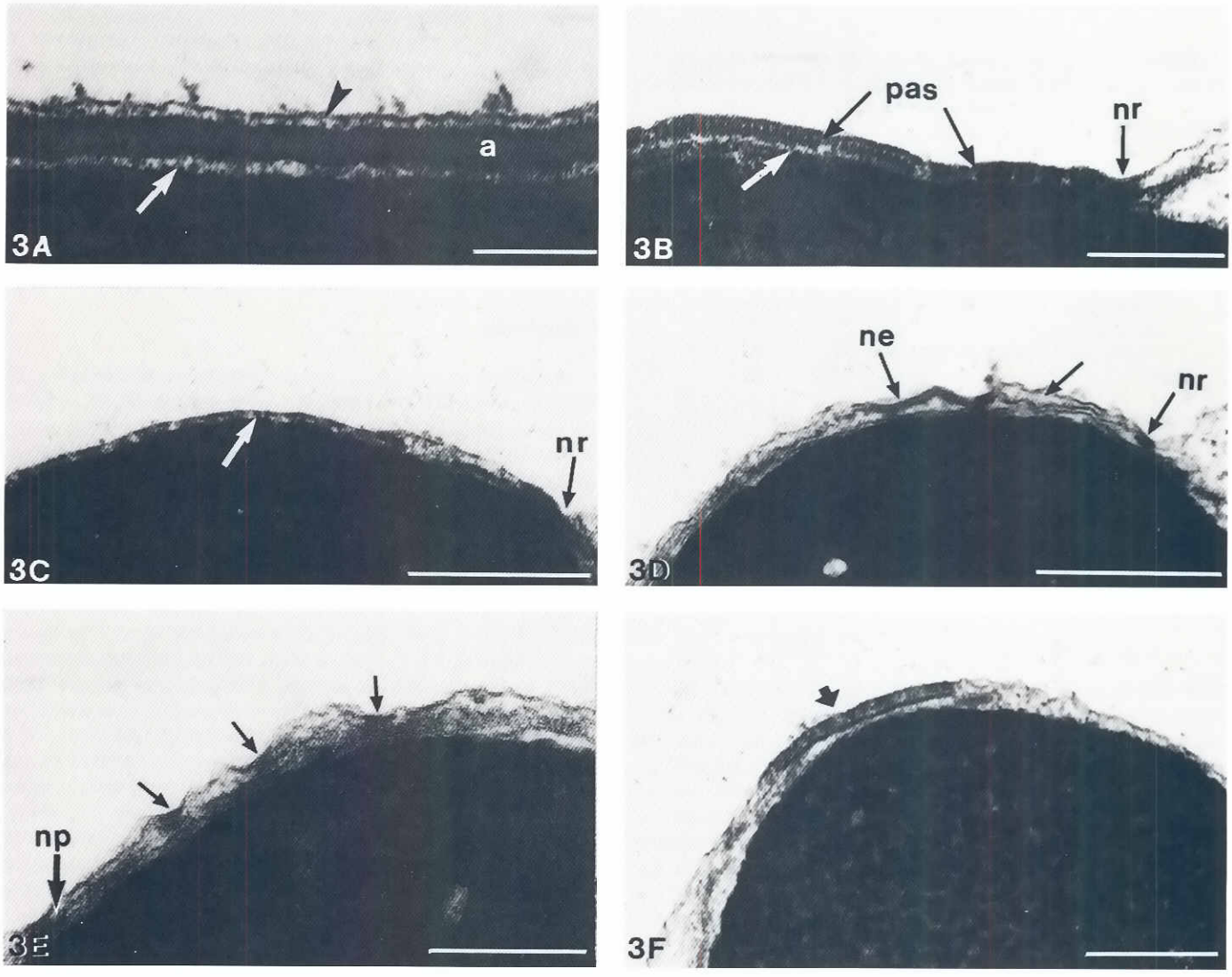

Fig. 3. Ultrastructural characteristics of the sperm perinuclear layer (**A-C:** *control semen;* **D-F**: *round-headed spermatozoa*). **(A)** *In the normal sperm head both the perinuclear (arrow) and periacrosomal (arrowhead) layers of the cytomatrix exhibit cross-links periodically disposed. The nuclear membranes are in close apposition as are the inner nuclear membrane and the chromatin. a=acrosome. Bar=0.2 μm.* **(B)** *The calyx region of a normal human sperm head containing the post-acrosomal sheath (pas). Cross-links are present between the pas and the nuclear envelope (arrow). nr=nuclear ring. Bar=0.2 μm.* **(C)** *An occasional control spermatozoon without acrosome. Perinuclear cross-links are seen between the nuclear envelope and the plasma membrane (arrow). nr=nuclear ring. Bar=1 μm.* **(D)** *The calyx region of a round-headed spermatozoon. The nuclear envelope (ne) is malformed and separated from the chromatin mass. The perinuclear layer (arrow) exhibits thin elements of the cytomatrix but not the typical cross-links. nr=nuclear ring. Bar=0.5 μm.* **(E)** *Abnormal presence of a dense material (arrows) associating the nuclear envelope and the plasma membrane in regions containing nuclear pores (np). Bar=0.5 μm.* **(F)** *A rare round sperm head exhibiting an undefined dense plate in the subplasmalemmal layer (arrow). Bar=0.5 μm.*

the plasma membrane and the nuclear envelope close to the anterior nuclear region (Fig. 3F).

Cytoplasmic remnants

The cell components present within the cytoplasm (Figs. 1A, C) were typical of those observed in the normal human spermatid (see Holstein and Roosen-Runge, 1981). In addition, clusters of ribosome-like granules were found in the vicinity of the nucleus (Fig. 1A). Similar dense granules were present throughout the cytomatrix (Figs. 1D and 2B, C).

Flagellar ultrastructure

A normal flagellar ultrastructure was observed in 51% [case 3] to 66% [case 1] of the sections, with the remaining sections exhibiting an absence of axonemal doublets (Table 2). The incidence of coiled flagella (Fig. 1C) ranged from 4% [case 1] to 19% [cases 2 and 3].

Ejaculated spermatids

Some spermatids were encountered in the semen samples of the three cases studied (Figs. 4A-C) as in the control semen (Figs. 4D, E). The spermatids of the round-headed sperm cases were frequently at step 5 of spermiogenesis according to Holstein and Roosen-Runge's classification (1981). They exhibited normal transitory components (Fig. 4A). Spermatids with a microtubular manchette were also encountered (Fig. 4B). Spermatids at steps 2-3 showed an acrosomal vesicle loosely associated with the nuclear

TABLE 3

ULTRASTRUCTURAL CHARACTERISTICS OF THE NUCLEAR ENVELOPE OF THE STUDIED ROUND-HEADED SPERMATOZOA
(Values are in %)

CASE	1	2	3
NUCLEAR ENVELOPE REGIONS			
Anterior			
Normal	0	0	0
Undifferentiated:			
continuous	77	63	14
discontinuous	10	10	45
widely separated membranes	10	23	31
Absent	13	27	41
Adjacent to the implantation fossa			
Undifferentiated and redundant	83	67	34
Undifferentiated and discontinuous	7	13	52
Widely separated membranes	7	3	28
Absent	10	20	14
Implantation fossa			
Normal	93	100	93
Absent	7	0	7

membrane by cytomatrix elements (Fig. 4C). Those acrosomal vesicles failed to extend over the nucleus. However, they tended to form a cap causing disruption of the associated cytomatrix elements in the concave region. The typical perinuclear material (Figs. 4D, E) normally present between the acrosomal cap and the nucleus was not formed. In contrast,typical cross-links of the cytomatrix extended between the acrosomal vesicle and the plasma membrane. Finally, close association of the nuclear envelope leaflets and dense material in the nuclear matrix layer underlying the nuclear envelope (Figs. 4D, E) were not observed in round-headed spermatids (Fig.4C).

Ultrastructural characteristics of the control spermatozoa

Only few control spermatozoa showed a round nucleus, an absence of acrosome and/or post-acrosomal sheath (Table 2). However, part of control spermatozoa exhibited an incomplete chromatin condensation [case 1: 27%; case 2: 23%] and a large, more or less elongated nuclear vacuole [case 1: 54%; case 2: 73%]. The nuclear envelope and perinuclear cytoplasmic layer displayed normal ultrastructural characteristics in all head regions (Figs. 3A, B). At the flagellar level, axonemal doublets may be lacking [case 1: 24%; case 2: 30%]. The percentage of coiled flagella, however, was low (3%).

Indirect immunofluorescence microscopy

In the two control samples treated with Triton X-100, the post-acrosomal region was brightly stained with the anti-calicin antibody in 78-81% of the spermatozoa. Labeling extended throughout the whole calyx region and was peripheral to the nucleus, as seen in grazing views (Fig. 5B). Treatment with acetone instead of Triton X-100 resulted in a similar although less intense labeling. No such staining was observed in the sperm heads of the three cases studied, whatever the treatment used (Fig. 5D). Only a small rod-like fluorescence, located at the head apex, was observed in 3% of the spermatozoa of case 2 only (Fig. 5D). In addition, both control and round-headed spermatozoa exhibited a weak fluorescence of the flagella (especially in their proximal part) and of the residual cytoplasm (Figs. 5B, D).

Controls performed by substituting the anti-calicin antibody by PBS showed no staining of the calyx region, while the flagellar proximal regions were again faintly stained in both normal and round-headed spermatozoa (Fig. 5F). The calyx region of both control and studied samples incubated with irrelevant guinea pig antibodies was not stained either (data not shown).

Discussion

In addition to the already known ultrastructural anomalies, this study revealed that round-headed spermatozoa exhibit extensive nuclear and perinuclear anomalies and fail to be stained by an anti-calicin antibody known to recognize a protein of the perinuclear theca in normal spermatozoa.

Nuclear and perinuclear differentiation events

During normal spermiogenesis, the nuclear envelope membranes become closer and denser and the nuclear pores are no longer discernible (Franklin 1968; De Kretser, 1969; Sandoz, 1970). A dense layer appears in the peripheral layer of the nuclear matrix (Fawcett, 1975; Afzelius *et al.*, 1982). The perinuclear theca forms by accumulation of periodic material (Nicander and Bane, 1966; Zamboni *et al.*, 1971) and the nuclear apical pole temporarily flattens (Burgos and Fawcett, 1955; Dooher and Bennett, 1973).

According to our results, all these differentiations failed to occur in round-headed spermatozoa and the nuclear pores were randomly distributed. In contrast, the chromatin appeared able to condense, suggesting that the substitution of histones by protamines (for review, see Grimes, 1986) was not affected. Moreover, the cytokinesis was apparently not impaired since the nucleus had reached the cell border, and the flagellum gained its site of attachment to the nucleus. The acrosome differentiated (even though its shape was atypical) and its matrix was normally filled by proacrosin, as observed in indirect immunofluorescence microscopy using an anti-proacrosin monoclonal antibody (data not shown). Finally, the flagellar structures were considered unaffected since their percentage of anomalies did not differ from that usually encountered in the human (David and Escalier, 1988). Our data, therefore, suggest that the anomalies of round-headed spermatozoa mainly affect a structural complex comprising the peripheral nuclear matrix, the nuclear envelope, and the perinuclear theca.

Structural and biochemical relationships between the nuclear matrix and the perinuclear theca have already been demonstrated in the mouse (Bellvé and O'Brien, 1983) and bull spermatozoon (Courtens *et al.*, 1980). Moreover, the perinuclear material has been observed to be closely associated with both the nuclear envelope and acrosome (Rahi *et al.*, 1983; Olson and Winfrey, 1985). In the human spermatozoon, the perinuclear material forms cross-links contributing to cell cohesion (Escalier, 1984). Such a function of the matrix elements in the maintenance of the head components might explain several of the nuclear and acrosomal round-headed sperm anomalies. Suprisingly, in those spermatozoa the differentiation of the nucleus-related matrix linkages did occur in the region of the implantation fossa. This observation suggests

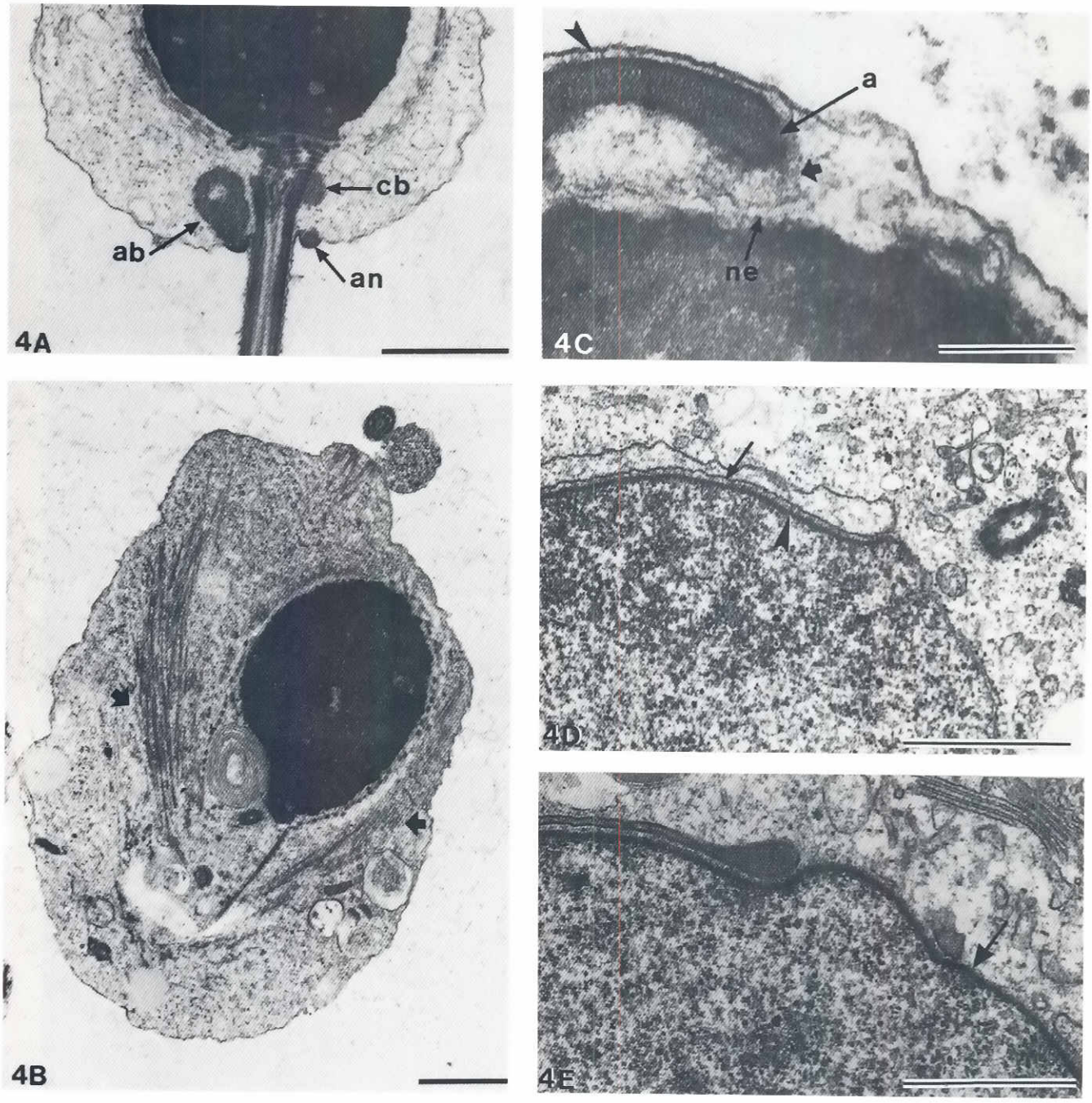

Fig. 4. Ultrastructural characteristics of ejaculated spermatids (**A-C:** round-headed spermatids; **D-E:** spermatids from control semens). **(A)** *Spermatid showing the centriole-associated body (cb), an incomplete annular body (ab) and the annulus in formation (an). Bar=1 μm.* **(B)** *A round nucleus surrounded by the microtubules of the manchette (arrows). Bar=1 μm.* **(C)** *A short curved acrosomal cap (a) in the vicinity of a round nucleus. The cytoplasmic elements between the acrosomal cap and the malformed nuclear envelope (ne) are present at the acrosomal border (arrow) but are disrupted in the acrosome concave region. Typical cross-links are present in the periacrosomal space (arrowhead). Bar=0.1 μm.* **(D)** *A control spermatid at step 2. Perinuclear material (arrow) and the nuclear differentiating structures of the nuclear envelope and lamina (arrowhead) are present in the region where the acrosome lies on the nucleus. Bar=0.5 μm.* **(E)** *In this control spermatid, the acrosomal cap is malformed and fails to continue to spread over the nucleus. However, the nuclear differentiations extend to nuclear lateral faces (arrow). Bar=1 μm.*

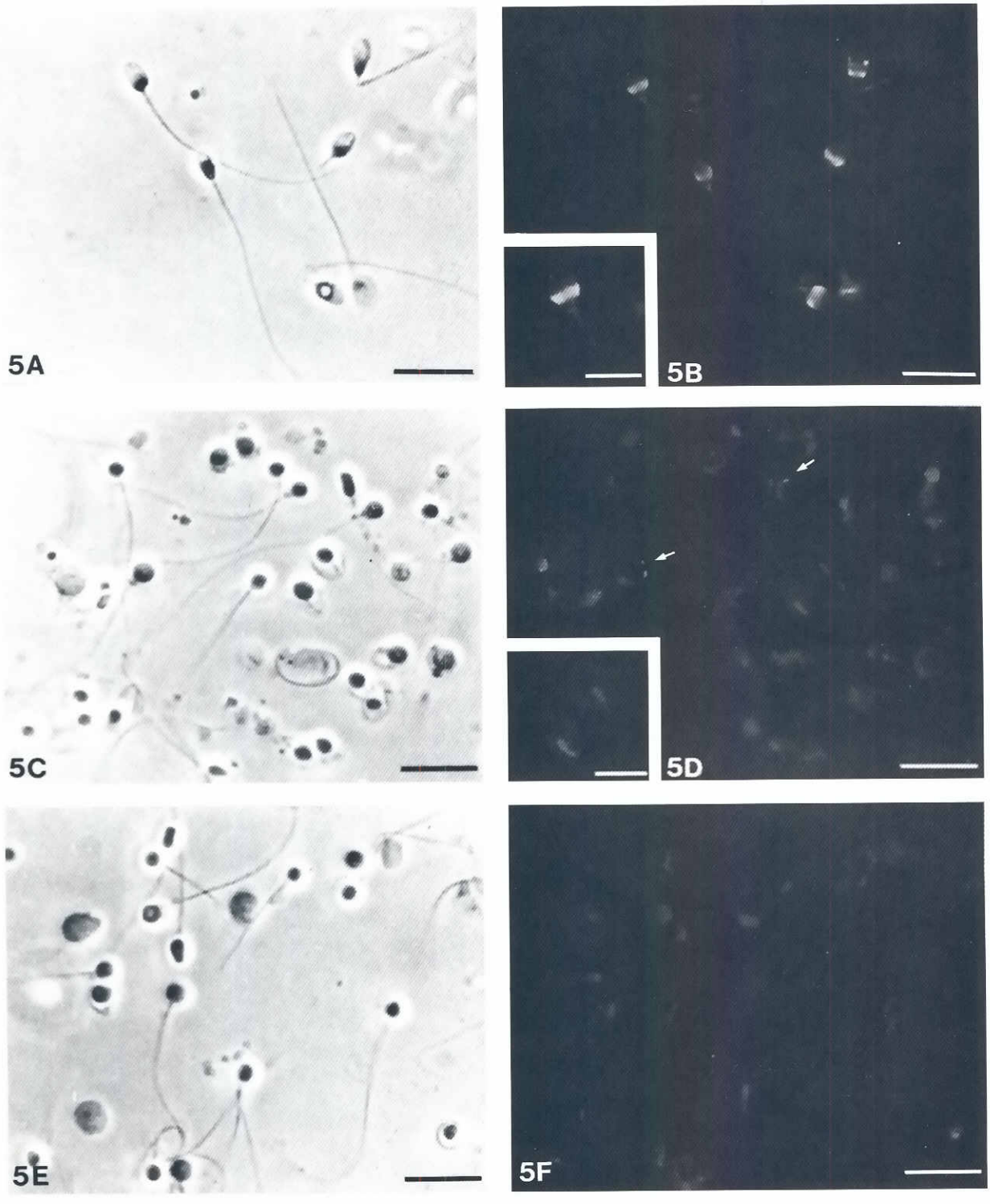

Fig. 5. Calicin status of the sperm studied. *Paired phase contrast* **(A, C, E)** *and immunofluorescence micrographs* **(B, D, F)** *of the same cells.* **(A, B)** *Normal spermatozoa stained with the anti-calicin antibody. Bar=10 μm. Insert: higher magnification of a sperm head. Bar= 5 μm.* **(C, D)** *Round-headed spermatozoa (case 2) are unstained with the anti-calicin antibody except for rare spermatozoa that exhibit a rod-like fluorescence at the apex of the head (arrowheads). Bar=10 μm. Insert: higher magnification of two round-headed spermatozoa. Bar=5 μm.* **(E, F)** *Control round-headed spermatozoa (case 2) for which the anti-calicin antibody was substituted by PBS. Bar= 10 μm.*

that the sperm nucleus-related skeletons are under, at least, two distinct morphogenetic controls depending on the nuclear regions. As assumed in an earlier study, the perinuclear linkages may be composed of distinct cytoskeletal proteins as suggested by differences in their ultrastructural characteristics (Escalier, 1984).

Disorders of the nuleus-related proteins

Basic proteins have been found to accumulate during spermiogenesis under the newly formed sub-acrosomal space and to extend into the post-acrosomal region concomitantly with the acrosome elongation (Nicander and Bane, 1966; Courtens *et al.*, 1976; Dadoune and Alfonsi, 1986). Longo *et al.* (1987) have recently identified two types of those basic proteins in the bull: the Mr 60,000 calicin and the Mr 56-74,000 multiple-band polypeptides (MBPs). The authors have assumed that those cytoskeletal proteins contribute to the formation of the perinuclear theca as an architectural element involved in the shape changes and the intimate association of the nucleus with the acrosome.

Labeling of normal human spermatozoa by means of the anti-calicin antibody has been found to extend over the whole post-acrosomal region in a belt-like fashion (Paranko *et al.*, 1988; this study). In contrast, data of the present study indicate that the anti-calicin antibody does not bind to round-headed spermatozoa. The fact that the ejaculated spermatids were also unlabeled leads to exclude the possibility that calicin is synthetized during spermiogenesis and subsequently eliminated within the cytoplasm, as is the case for the acrosomal constituents. Nevertheless, 3% of the case 2 sperm cells only were found to exhibit a small calicin labeling close to the nucleus (Fig. 5), a result that remains to be elucidated.

Therefore, in round-headed spermatozoa, at least one protein constitutive of the perinuclear theca (*i.e.* calicin) is either absent or present in a form that is not recognized by the corresponding antibody. This finding strongly supports the above hypothesis that the nuclear and perinuclear frameworks are predominantly implicated in the anomalies observed. However, the absence of calicin may not be primarily responsible for impaired maintenance of the sperm head components and the consecutive observed anomalies. Indeed, contrary to the MBPs, calicin is apparently no longer present in the subacrosomal space in late spermatids (Longo *et al.*, 1987). The absence of calicin in round-headed spermatoza may, therefore, also reflect disturbances of other structural or regulatory factors. To clarify this point, one should ascertain that calicin is not synthetized (and, thus, not present in an immature form), and determine whether other cyto- and/or karyoskeletal proteins are involved.

Among these proteins, lamins would be the ones most likely implicated due to their roles in reconstituting the nuclear envelope after cell division and interconnecting the pore complexes (see reviews in Franke *et al.*, 1981; Krohne *et al.*, 1989). However, somatic cell type-lamins seem to be absent (or present in very low amounts) in spermatids (Longo *et al.*, 1987) and spermatozoa (Schatten *et al.*, 1985). Their homologues, which may be sperm-specific (Pruslin and Rodman, 1983), and proteins of the sperm nuclear matrix have been identified only in non-human species (see Bellvé and O'Brien, 1983 for review).

Sperm morphogenesis controls

A failure of nuclear elongation was observed in round-headed spermatozoa although a microtubular manchette was found in ejaculated spermatids (Fig. 4B). Since the microtubules of the manchette are normally associated with the perinuclear material (Courtens and Loir, 1981), the absence of such an association in the cases studied might explain why the manchette failed to play its role. This, therefore, supports the notion that the nuclear envelope and/or matrix may contribute to nuclear morphogenesis (Myles and Hepler, 1982).

Despite the absence of the perinuclear material and spreading over the nucleus (Fig. 4C), the acrosomal formations of the studied mature spermatozoa exhibited many of the structural characteristics of the normal acrosome (Fig. 1). This suggests that the structural elements of the acrosome itself are involved in the acrosome flattening (including the formation of domains with different thicknesses) as in the acrosomal curvature processes. The typical acrosomal shape may, therefore, result from the involvement of both the acrosome and the perinuclear theca. Acrosomal caps loosely associated with the spermatid nucleus as observed in our cases have been found to degenerate in other studies on round-headed spermatozoa (Baccetti *et al.*, 1977; Castellani *et al.*, 1978; Nistal and Paniagua, 1978).

Degeneration of proacrosomal vesicles lying on the spermatid nuclear envelope has also been observed in acrosomeless mouse spermatozoa in spite of normal nuclear differentiation (Sotomayor and Handel, 1986). In procarbazine-treated rats, several small acrosomal vesicles have been observed to spread over the nucleus and nuclear changes occur even in regions that normally should have been covered by an acrosomal cap (Russell *et al.*, 1983). These data suggest that: i) the stimulus signal for nuclear differentiation emanates from the attachment of the proacrosomal granule; ii) the continuation of the process and maintenance of nuclear envelope modifications may reportedly not depend on acrosome assembly, and iii) acrosome spreading is induced by changes first occurring in the nuclear membrane (Russell *et al.*, 1983; Sotomayor and Handel, 1986). Our observations (Figs. 4C-E) support these notions in the human.

During spermiogenesis, the acrosome and the nucleus may, therefore, produce reciprocal inducing processes allowing their subsequent differentiation. This implies acrosomal and nuclear ability to deliver – but also to respond – to such inductions. In this respect, anomalies of distinct components of the round-headed spermatozoa may result from their interaction (relational pleiotropy). In addition, assembly of the structural complex comprising the nuclear envelope, the peripheral nuclear matrix and the perinuclear theca may either also require their interaction or the same gene product (mosaic pleiotropy).

As for the macronuclear human spermatozoa (Escalier, 1983, 1985), our study on round-headed human spermatozoa shows that, while sperm morphogenesis is seriously affected at early steps of spermiogenesis, the genetic program for further sperm differentiation is not arrested. Our data also suggest the existence in the human of separate controls of cytokinesis and nuclear differentiation on the one hand, and of nuclear morphogenesis and chromatin condensation on the other. The same assumption has already been made following experimentations in other species (Courtens *et al.*, 1980; Risley *et al.*, 1982). The presence of several sperm defects induced by mutation concomitant with the occurrence of independent sperm morphogenetic events has also been reported in invertebrates (Ward *et al.*, 1981).

In conclusion, our data suggest the existence of interconnected nuclear-perinuclear frameworks in the human sperm head which

would fail to differentiate in round-headed spermatozoa. The absence of sperm staining with the anti-calicin antibody supports this hypothesis. However, it has not been possible to determine whether the absence of calicin is at the origin of this dysgenesis or simply reflects other anomalies involved in the differentiation of this complex of holding structures. Nevertheless, data suggest that the molecular disturbance primarily implicated in the structural anomalies of round-headed spermatozoa affects a sperm-specific factor related to the sperm-head matrices. Gene disorder(s) at this level may, therefore, exist in the human. Further investigations on the molecular constitution of those sperm-head constituents should make it possible to pin-point the factor involved in this dysgenesis.

Materials and Methods

Semen samples

Three cases of men with acrosomeless round-headed spermatozoa as described earlier (Schirren *et al.*, 1971) had been detected in the course of routine semen analysis for male infertility. Semen samples were collected after 3 days of sexual abstinence. After semen liquefaction at 37° C for 30 min, semen parameters were evaluated (Table 1). Cell viability was estimated by the nigrosin/eosin staining method (WHO, 1987). The semen from two healthy donors considered as normal according to standard criteria (Jouannet and Feneux, 1987) were used as controls.

Morphological analysis

Light microscopy

A drop of each semen sample was smeared onto slides, air-dried and stained according to the Shorr's technique (WHO, 1987). The morphological characteristics of 100 spermatozoa from each semen (Table 1) were classified taking into account associated anomalies of the different sperm regions (David *et al.*, 1975).

Electron microscopy

A 1.5 - 2 ml aliquot from each semen was fixed with two volumes of 2.5% v/v glutaraldehyde in 0.1 M phosphate buffer (450 mOsM). After 1 h fixation at 4° C, cells were centrifuged at 800 g for 10 min, washed for 15 min with 0.1 M phosphate buffer containing 4% w/v sucrose, centrifuged again at 800 g for 10 min, and embedded in 2% agar. Post-fixation was for 1 h in 1% w/v osmic acid in 0.1 M phosphate buffer with 4% w/v sucrose. After dehydration in ascending concentrations of ethanol, small pieces of agar containing spermatozoa were embedded in araldite. Sections were cut on a Reichert OmU3 ultra-microtome, stained with uranyl acetate (4% in 70% ethanol, 20 min) and lead citrate (10 min) and examined under a Siemens Elmiskop CT 150 transmission electron microscope.

Ultrastructural quantitative analysis

Two different grids were observed per sample. Thirty longitudinal sections passing through the basal plate were examined for sperm head morphology. Head sections exhibiting plasma membrane disruption or more extended damage were excluded. The presence of coiled flagella and axonemal anomalies having been previously reported in round-headed spermatozoa (Pedersen and Rebbe, 1974), 50 transverse flagellar sections were investigated for such anomalies.

Indirect immunofluoresence microscopy

Immunolabeling of normal and round-headed spermatozoa was performed on either fresh semen samples or spermatozoa stored in liquid nitrogen until use. Samples were washed twice with phosphate buffered saline (PBS, pH 7.3) and sperm concentration was adjusted to 1 x 10^6 spermatozoa/ml with PBS. Ten microliters of each diluted sample were placed onto slides and air-dried. Spermatozoa were then permeabilized for 5 min with either 0.5% Triton X-100 in PBS (see Paranko *et al.*, 1988) or cold acetone, washed twice in PBS and incubated for 15 min in PBS containing 1% bovine serum albumin (PBS/BSA). Primary antibody was the guinea pig anti-calicin antibody obtained from the calyx fraction of bull sperm heads (a gift from Dr G. Krohne). Spermatozoa were incubated for 30 min with the anti-calicin antibody diluted 1:75 in PBS/BSA. After extensive washing in PBS, they were incubated for another 30 min with a goat anti-guinea pig immunoglobulin G antibody conjugated to fluorescein-isothiocyanate (GPG/F(ab')2/FITC) (Jackson Research Laboratories, USA) diluted 1:30 in PBS. After washing in PBS, cells were counterstained with 0.02% Evans blue and mounted in Citifluor (glycerol/PBS, Citifluor Ldt, London, UK).

For controls the anti-calicin antibody was substituted by i) PBS; or ii) a guinea pig antibody known to recognize the acrosomal region of the human spermatozoon; or iii) a guinea pig antibody that recognizes the human sperm mid-piece. Slides were examined with an Olympus BH-2 microscope fitted with epi-illumination and FITC filters. The fluorescence photographs were taken at x1250 magnification (45 sec exposure) and with T-Max film (400 ASA, Kodak, France).

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