Generation of Flagella by Cultured Mouse Spermatids

GEORGE L. GERTON and CLARKE F. MILLETTE
Department of Anatomy and Laboratory of Human Reproduction and Reproductive Biology, Harvard Medical School, Boston, Massachusetts 02115

ABSTRACT During the short-term culturing of mouse spermatogenic cells, flagella were generated by round spermatids previously lacking tails. Unseparated germ cells were obtained by enzymatic treatments and round spermatids (>90% pure) were purified by unit gravity sedimentation. As determined by Nomarski or phase-contrast microscopy, no cells had flagella immediately after isolation; flagella were first clearly detected after 6½ h of culture in Eagle's minimal essential medium containing 10% fetal bovine serum and 6 mM lactate. After 24 h, ~20% of round spermatids had formed flagella. Multinucleated round spermatids often formed multiple flagella, the number never exceeding the number of nuclei per symplast. Round spermatids were the only spermatogenic cells capable of tail formation. Flagella elongation was blocked by 1 μM demecolcine, an inhibitor of tubulin polymerization. Indirect immunofluorescence localized tubulin in the flagella. As seen by scanning electron microscopy, flagella developed as early as 2 h after culture and continued to elongate over the next 20 h, reaching lengths of at least 19 μm. Transmission electron microscopy demonstrated that flagella formed in culture resembled flagella from Golgi-phase round spermatids in situ; the flagella consisted of "9+2" axonemes lacking other accessory structures such as outer dense fibers and the fibrous sheath. As determined by acridine orange staining of the developing acrosomes, all spermatids that formed flagella in culture were Golgi-phase spermatids. By these criteria, the structures are indeed true flagella, corresponding in appearance to what others have described for early mammalian spermatid flagella in situ. We believe this is the first substantiated report of limited in vitro differentiation by isolated mammalian spermatids.

Spermatogenesis represents a complex system of cellular differentiation involving mitotic stem cell proliferation, meiosis, and subsequent remodeling of haploid spermatids to produce mature spermatozoa (4, 20). These modifications include formation of the acrosome, condensation and shaping of the nucleus, development and dissolution of the microtubular manchette surrounding the condensing nucleus, and the generation of a motile flagellar apparatus consisting of a "9+2" axoneme and associated accessory structures (48).

In vitro systems for the study of mammalian spermatogenesis have been severely limited in the past since even organ cultures of testis fail to allow differentiation of male germ cells beyond the pachytene stage of the first meiotic prophase (54). Recent studies indicate that alternate in vitro culture systems may facilitate more detailed analysis of early spermatogenic cell development (28, 47, 58), but the differentiation of haploid spermatids in these experiments has not yet been documented. Although it is possible to obtain highly purified populations of mammalian cells at particular stages of spermatogenesis (6, 7, 51), isolated male germ cells do not undergo extended differentiation in vitro using any culture techniques reported to date. These cells may, however, be maintained in vitro for short-term studies designed to investigate a variety of metabolic events (10, 27, 33, 56). During experiments intended to study the biosynthesis of plasma membrane glycoproteins present in purified populations of mouse pachytene spermatocytes and round spermatids (24), we noted that some round spermatids elaborate flagella-like structures in vitro. Flagellar elongation by cultured mammalian spermatids has not previously been reported. Here we demonstrate using light microscopy, immunofluorescence, ultrastructural analysis, and inhibitor experiments that the observed structures are indeed true flagella, corresponding in appearance to early mammalian spermatid flagella described in situ (29–31, 59, 60).

MATERIALS AND METHODS

Animals and Cell Preparation: Adult CD-1 mice aged 60–120 d obtained from Charles River Laboratories (Wilmington, MA) or adult

TAC:(SW)fBR mice of the same age from Taconic Farms, Inc. (Germantown, NY) were used for these studies. Similar results were obtained with mice of either strain.

Testicular cells were isolated by the procedures of Romrell et al. (51) and of Bellvé and co-workers (6, 7). Testes were removed, decapsulated, and immediately placed into sterile enriched Krebs-Ringer bicarbonate buffer (EKRB).[1] With the exception of the cell separation step, all subsequent procedures utilized sterile technique. Seminiferous cell suspensions were prepared using sequential incubations in 0.5 mg/ml collagenase and 0.5 mg/ml trypsin as described previously (6, 7). Purified populations (>90% pure) of pachytene spermatocytes and round spermatids were obtained by unit gravity sedimentation in linear 2–4% wt/vol gradients of BSA (Fraction V, US Biochemical Corp., Cleveland, OH). Logistically it was not possible to perform these cell separations in a sterile environment. To minimize microbial contamination we autoclaved all glassware and tubing before use and sterilized the BSA solutions before use (Millipore IVAA0103F prefilter followed by SVGS01015 sterilizing filter; Millipore Co., Bedford, MA).

Cell Culture: Seminiferous cell suspensions, purified pachytene spermatocytes, or purified round spermatids were washed twice in sterile EKRB prior to cell culture. Cells (2×10^7 per 25-mm^2 culture flask) were incubated in 5–10 ml of Eagle's minimal essential medium supplemented with 10% dialyzed fetal bovine serum (Gibco Laboratories, Grand Island, NY), 6 mM sodium lactate, 1 mM sodium pyruvate, 2 mM glutamine, 100 μg/ml penicillin, and 100 mcg/ml streptomycin. For some experiments the medium also contained 1 μM demecolcine (Sigma Chemical Co., St. Louis, MO), a colchicine analog. Cultures were incubated at 33°C with 5% CO_2 in humidified air. Since adult mouse spermatogenic cells do not adhere to plastic substrata, cells were harvested at the indicated times by gentle agitation of the culture flask. Cell viability assays were performed using trypan blue in saline.

Light Microscopy and Immunofluorescence: Cell morphology and purity were assayed by phase-contrast or Nomarski differential interference contrast microscopy (51). Immunofluorescence procedures were adapted from Byers et al. (12). Briefly, spermatogenic cells were attached to polylysine-coated coverslips. After being washed in EKRB, the cells were fixed for 15 min at room temperature in 0.1 M PIPES, pH 6.8, containing 2.0 mM EGTA, 20 mM $MgCl_2$, 2.0% paraformaldehyde, and 0.1% glutaraldehyde. After extensive rinsing in 0.15 M Tris-HCl, pH 7.4, the cells were washed in PBS and then incubated for 2 min in 0.2% Triton X-100 in PBS. The cells were subsequently washed in PBS before sequential treatment with primary and secondary antisera. Primary sera were from a nonimmune rabbit and a rabbit immunized with purified tubulin. The antitubulin serum was kindly provided by Dr. K. Fujiwara, Department of Anatomy, Harvard Medical School and has been characterized extensively (22). Secondary antibody was fluorescein-conjugated Fab fraction of goat anti-rabbit IgG (Cappel Laboratories, Inc., Cochranville, PA). Epifluorescence was visualized with a Zeiss photomicroscope III and photographed via automatic exposure of Kodak Tri-X film using an ASA of 1600.

Scanning Electron Microscopy: Cells incubated for various times were removed from culture, attached to polylysine-coated coverslips, and fixed for 30 min at room temperature in 2.0% glutaraldehyde in 0.1 M sodium phosphate, pH 7.2–7.4. After being washed in the phosphate buffer the cells were postfixed for 30 min at 0°C in 0.5% osmium tetroxide in 0.1 M sodium phosphate, pH 6.0. After a subsequent rinse with distilled water, samples were dehydrated in a graded series of ethanols and critical-point dried using a Polaron chamber. Dried cells were coated with gold (Hummer, Technics Inc., Alexandria, VA) and observed with a JSM-35 scanning microscope.

Transmission Electron Microscopy: After culturing, the cells were washed twice in EKRB. Pellets from the second wash were fixed for 30 min at room temperature in 2.0% glutaraldehyde in 0.1 M sodium phosphate, pH 7.4, and postfixed with 1.0% osmium tetroxide in 0.1 M sodium phosphate, pH 7.4, for 1 h, also at room temperature. Samples were dehydrated in a graded series of ethanols and embedded in Epon-Araldite. Thin sections were cut and stained with aqueous uranyl acetate and lead citrate according to standard methods. Sections were studied using 60 kV on a Philips EM300 instrument.

Acridine Orange Staining: Vital staining of developing spermatid acrosomes was performed with acridine orange using a protocol modified from Bishop and Smiles (8) and Clark et al. (13). In these studies 1×10^7 unseparated germ cells were pelleted at 1,000 *g* for 15 s in a Fisher microfuge (Fisher Scientific Co., Pittsburgh, PA) and resuspended in 0.5 ml of EKRB containing 10 μg/ml acridine orange (Sigma Chemical Co.). Cells were incubated for 15 s at room temperature before they were repelleted and washed twice in fresh EKRB. Following the last wash, cells were resuspended in 0.5 ml EKRB and observed using epifluorescence microscopy. Acridine orange stains nuclei green, while acrosomes and lysosomes stain red. Photomicrography of spermatid acrosomes with black and white film was facilitated by elimination of the green fluorescence using the Zeiss #48-77-14 rhodamine filter combination. Photographs were taken on Kodak Tri-X film at ASA 1600.

[1] *Abbreviations used in this paper:* EKRB, enriched Krebs-Ringer bicarbonate buffer.

RESULTS

Isolated Mouse Round Spermatids Produce Flagella in Culture

Round spermatids produce flagella in vivo as early as steps 1 and 2 of spermiogenesis as determined by ultrastructural examination (30, 31). However, when enzymatic and mechanical forces were used to dissociate testicular cells in the present study, all flagella were lost from early round spermatids. The appearance of round spermatids immediately after isolation is shown in Fig. 1, while Table I quantitates the lack of flagellar structures on these cells. Initial viabilities of the isolated seminiferous cells were always in excess of 95% (Table I). Furthermore, other studies conducted by this laboratory have demonstrated that these cells actively synthesize proteins during short-term culture, suggesting that they remain physiologically competent (24, 38).

After seminiferous cell mixtures (unseparated pachytene spermatocytes, round spermatids, and residual body cytoplasts) or purified round spermatids were cultured overnight, approximately one-fifth of the round spermatids elaborated flagella-like structures (Table I). These structures were distinguished by nonrandom flexing movements and a uniform

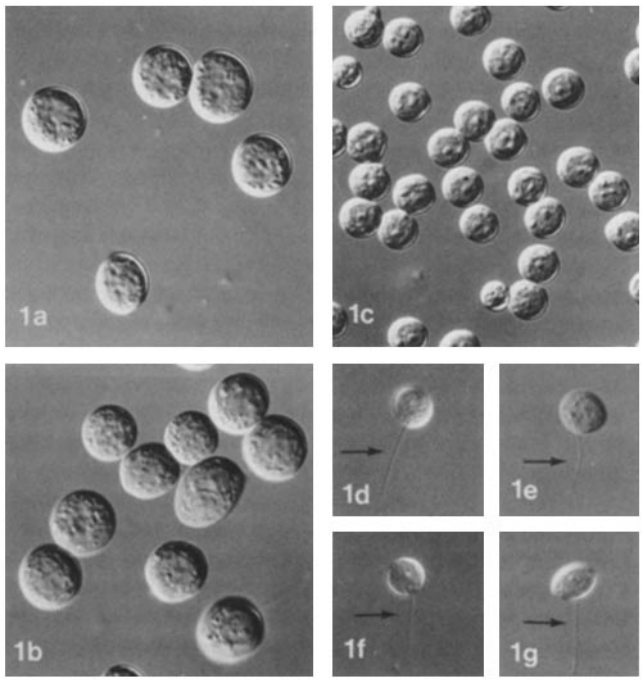


FIGURE 1 Purified pachytene spermatocytes and round spermatids observed using Nomarski differential interference microscopy. (*a*) Purified spermatocytes before short-term in vitro culture. Cells are generally spherical and uniform in appearance. (*b*) Spermatocytes after 17.5 h in vitro culture. Cellular morphology is unchanged. (*c*) Purified round spermatids before short-term culture. These cells are of uniform size and are considerably smaller than pachytene spermatocytes. Note the prominent nucleolus characteristic of early spermatids. (*d–f*). Representative examples of cultured spermatids which have elaborated flagella after 17.5 h in vitro. Individual mononucleated spermatids invariably exhibit a single flagellum. × 320.

TABLE I
*Inhibition of Flagellar Formation**

	No culturing	24-h culture, no demecolcine	24-h culture in 1 μM demecolcine
	%	%	
Round spermatids with tails	1.0 ± 0.7	19.0 ± 4.1	0.3 ± 0.1
Round spermatids without tails	99.0 ± 0.7	81.0 ± 4.1	99.7 ± 0.1
Viability	97.4 ± 0.9	94.2 ± 2.1	91.3 ± 2.3

* Each value represents the average ± standard deviation of triplicate experiments. Over 500 round spermatids in a population of cultured seminiferous cells were counted for each experiment.

thickness along their length (Fig. 1). At any given time of in vitro culture, all flagella detected were of similar length. Multinucleated round spermatids often formed multiple flagella and the number of flagella in any single symplast never exceeded the number of nuclei in that same cell. Flagella were never detected on any pachytene spermatocytes (Fig. 1), condensing spermatids, or residual bodies when mixed populations of seminiferous cells were examined. It should also be noted that the generation of flagella by isolated populations of purified round spermatids indicates that this process is independent of other cellular influences of the seminiferous epithelium such as might be provided in situ by Sertoli cells.

Flagellar Formation Is Inhibited by Demecolcine

Flagellar formation should be blocked by inhibitors of microtubule polymerization since the major constituent of sperm tail axonemes is tubulin. Accordingly, demecolcine, a colchicine analog, was tested at concentrations from 1 to 100 μM for its ability to block the elongation of the flagella-like structures developed by cultured round spermatids. As shown in Table I, 1 μM demecolcine, a concentration known to be inhibitory in other systems of microtubule assembly (53), completely blocked the formation of flagella by isolated mouse spermatids. Demecolcine had no noticeable effect on cell viability as measured by trypan blue dye exclusion assays. As assayed by light microscopy, no other morphological alterations in cell size, shape, or behavior were detected in the presence of the drug.

Tubulin Is Present in Flagella of Cultured Spermatids

The inhibitor data implicated the involvement of tubulin in structural elements of the newly produced flagella. Using indirect immunofluorescence, antitubulin serum labeled the flagella as well as the cytoplasmic cytoskeletal elements of round spermatids cultured for 20 h (Fig. 2, *c–f*). All flagella formed by spermatids in culture were stained by this antibody. Other germ cell types present in the mixed seminiferous cell cultures, including pachytene spermatocytes, revealed only staining of cytoplasmic microtubules. Nonimmune serum controls yielded negligible, diffuse staining of mouse spermatogenic cells. There was no detectable staining of the tail regions of cultured round spermatids (arrow, Fig. 2, *a* and *b*).

Flagella Develop Early in Culture

To visualize more effectively the early stages of flagellar elongation, we cultured cells for various lengths of time and subsequently processed them for scanning electron microscopy. All cells prior to culture were generally spherical with smooth surfaces, devoid of microvilli, and exhibited occasional surface blebs (Fig. 3, *a*, *c*, and *d*). Although the origin of these blebs has not yet been determined precisely, we believe that they represent the remnants of the extensive cytoplasmic bridges which normally interconnect developing germ cells within the seminiferous epithelium (16).

Elongating flagella first became readily apparent after only 2 h of culture and continued to increase in length during at least the next 20 h of observation (Fig. 3). By 22 h of culture, tails were about 19 μm long. Each tail had an intact plasma membrane and a relatively uniform thickness of ~0.2 μm along its length. As previously mentioned, multinucleated round spermatids exhibited multiple flagella. The elongation of the individual flagella in symplasts occurred at approximately the same rate (Fig. 4), as might be expected since the symplasts derive from developing cells at the same stage of spermatogenesis in vivo (16). There was no evidence of any significant invagination of the plasma membrane surrounding the region of the cell surface from which the flagella extruded.

Flagella from Cultured Spermatids Resemble Early Flagella In Situ

The flagellum of a mature mammalian spermatozoon is a complex structure composed of the plasma membrane, the axoneme, and additional accessory structures which include the outer dense fibers, the fibrous sheath, and the mitochondrial gyre (21). Flagella elaborated by cultured mouse round spermatids were examined by transmission electron microscopy and were found to be composed only of the normal "9 +2" axoneme surrounded by intact plasma membrane (Fig. 5). No accessory structures were detected.

Attempts were also made to correlate flagellar growth with the stage of spermiogenic differentiation. It was not possible to obtain statistically significant numbers of appropriate images allowing direct visualization of both an elongating flagellum and the developing acrosome. However, particular attention was concentrated upon acrosomal morphology since the early stages of spermiogenesis are defined by the morphological development and intracellular positioning of this organelle (43). All sections that did allow correlation of a flagellum and acrosomal differentiation showed that tail elongation occurred in early round spermatids only, before the evident formation of an acrosomal cap covering the anterior portions of the spermatid nucleus. Spermatids of this type are classed as step 1 or 2 cells in the "Golgi phase" of spermiogenesis.

In addition, Tang et al. (57) have correlated the morphological appearance and positioning of other cytoplasmic organelles, particularly the chromatoid body and the multivesicular body, with acrosomal development during rat spermiogenesis. Using these criteria, all appropriate thin sections again showed that only spermatids in steps 1 or 2 of spermiogenesis had elaborated flagella in vitro (Fig. 5).

Spermatids That Produce Flagella in Culture Have Golgi-phase Acrosomes

The round spermatid population consists of cells from steps 1 through 7 or 8 of spermiogenesis as defined by Oakberg (43) for the mouse. The steps of spermiogenesis are identified morphologically on the basis of acrosomal development, nu-

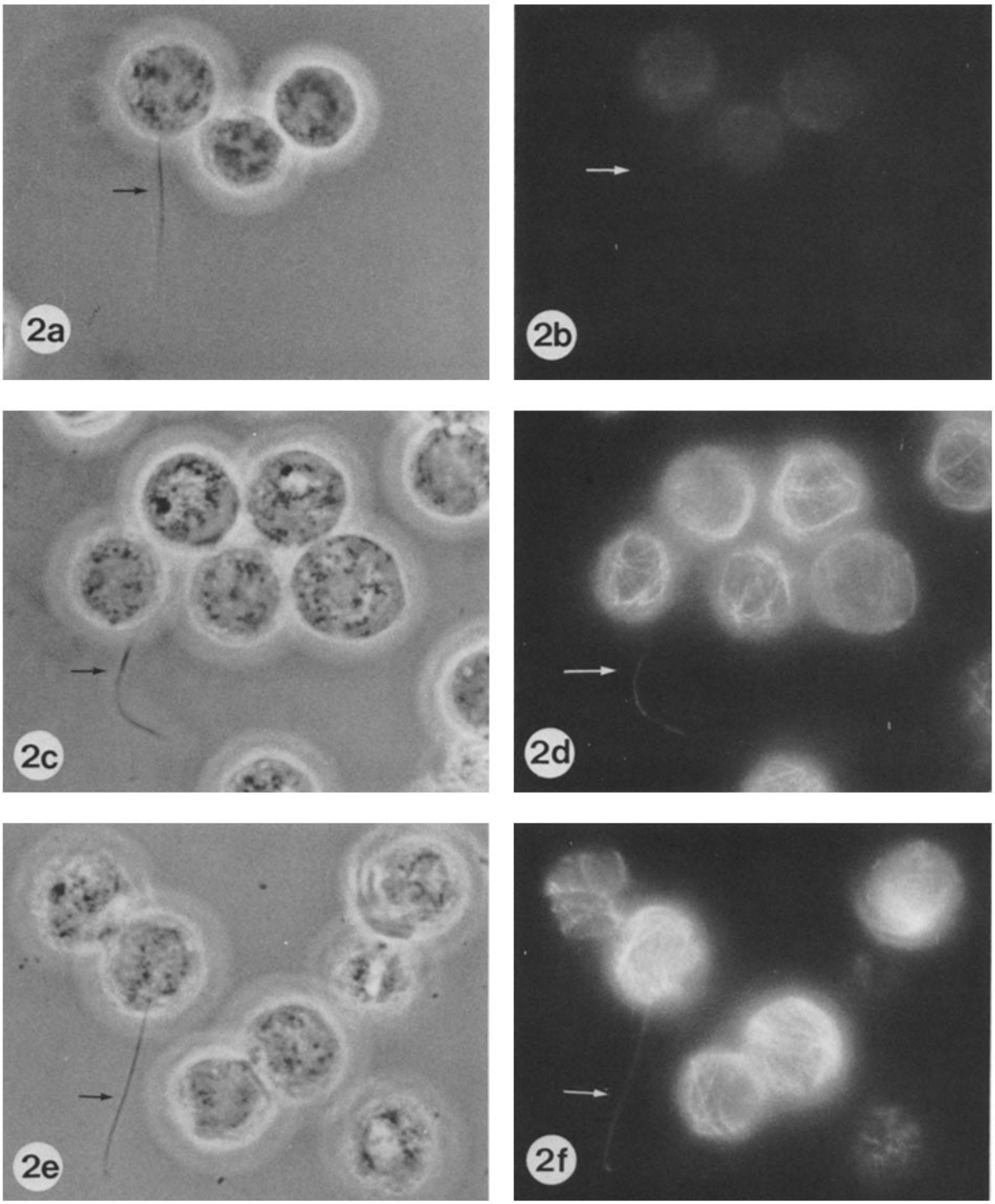


FIGURE 2 Immunofluorescent localization of tubulin in flagella generated by cultured round spermatids. (*a* and *b*) Control experiment using nonimmune rabbit serum. Diffuse, weak staining of cytoplasm is noted, but the flagellum (arrow) is completely negative. (*c–f*) Two representative fields of cells stained with antitubulin antibody. In addition to labeling of the cytoplasmic microtubular network, the flagella (arrows) are stained intensely. All cells have been fixed and stained after 20 h of in vitro culture. × 1500.

clear condensation, and nuclear shape. Early round spermatids are classified as "Golgi-phase" cells, if the developing acrosome has not yet attached to the nucleus, or as "cap-phase" cells, if the acrosome has begun to spread posteriorly over the nuclear surface. Therefore, vital staining of acrosomes using acridine orange was employed to distinguish between Golgi-phase and cap-phase spermatids.

Direct comparisons using acridine orange fluorescence and phase-contrast microscopy revealed that without exception all cultured mouse spermatids that exhibited flagella were Golgi-phase cells (Fig. 6). No cap-phase spermatids identified using this procedure exhibited flagella after any culture interval examined. In addition, in two separate experiments quantitating Golgi-phase spermatids, 32 and 34% of these cells

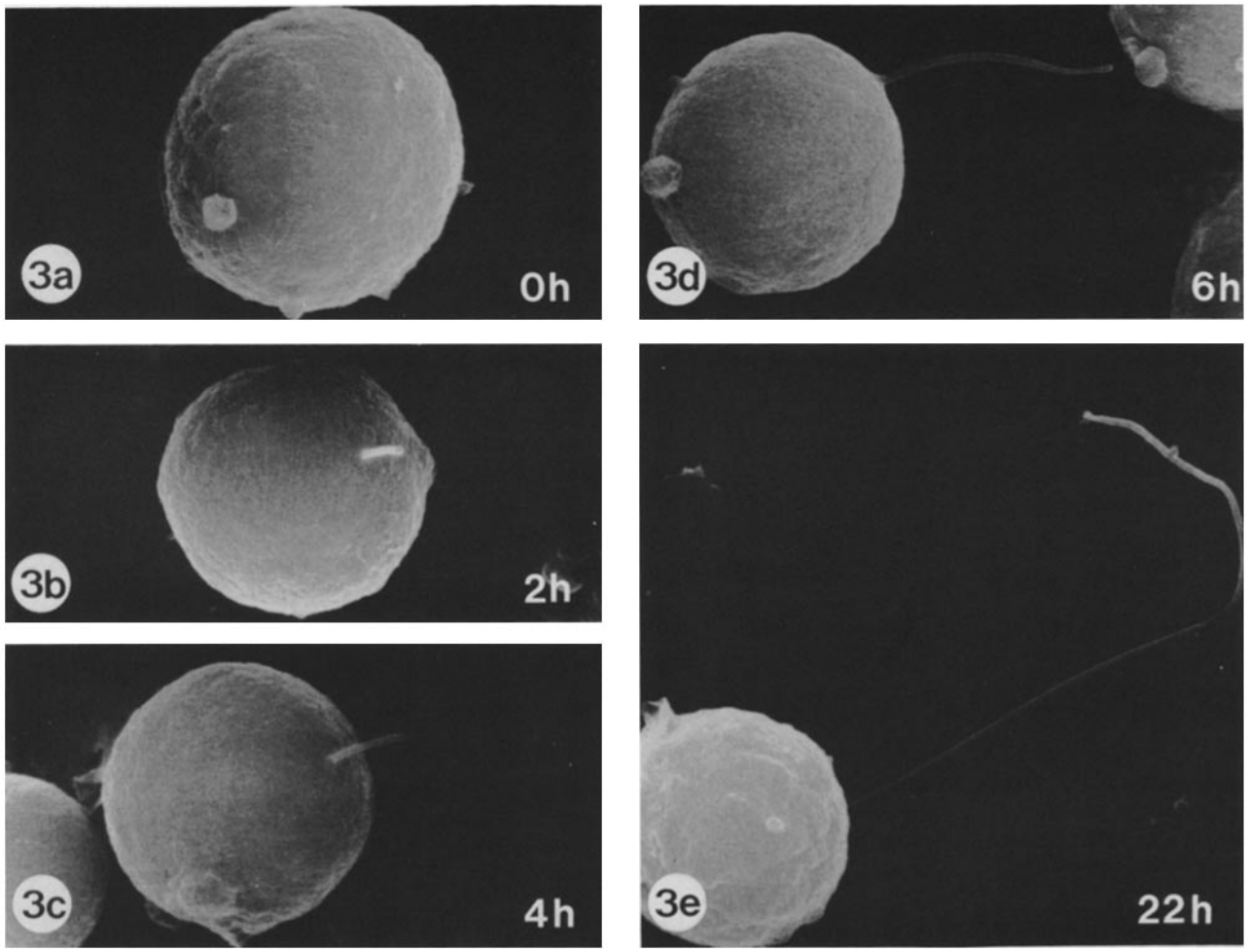


FIGURE 3 Scanning microscopic views of flagella after various times of culture. (a) Cells before culture are spherical and exhibit only an occasional surface bleb. No microvilli or other projections are observed. (*b*–e) Representative round spermatids after limited culture incubation. Single flagella are noted as early as 2 h after isolation and the flagella lengthen as the in vitro culture proceeds. Note that there is no significant invagination of the plasma membrane at the base of the developing sperm tail. × 5,360.

formed flagella after 24 h in vitro. These data indicate that only spermatids early in spermiogenesis are able to elongate flagella under the culture conditions tested, a finding in excellent agreement with the ultrastructural results presented in Fig. 5.

DISCUSSION

The data presented here indicate that isolated populations of mammalian round spermatids are able to undergo limited differentiation in vitro, as defined by the generation of flagella. Previous substantiated reports detailing the in vitro differentiation of haploid spermatids have concerned nonmammalian organisms, particularly *Xenopus laevis* (50) and newt (1). Primary spermatocytes isolated from these species proceed through both meiotic reductions to yield spermatids. The spermatids continue differentiation at least until the development of flagella. For example, Abe (1) reported that after 1 mo of incubation, newt spermatids had developed from spermatocytes and had not undergone nuclear condensation, but 80% of these cells had developed motile flagella ~500 μm long. Evidence of complete spermatozoon maturation in vitro has not yet been obtained in either *X. laevis* or the newt.

In contrast, studies of mammalian spermatogenesis in culture have been less successful. Occasional early reports of limited spermatogenesis in vitro (3, 32) were not investigated further as it became apparent that even testicular organ cultures failed to support the later stages of spermatogenic differentiation (54). Recently, however, the availability of isolated cell populations from the mammalian testis has stimulated renewed interest in the biochemistry of spermatogenesis. As a result, numerous investigators have developed relatively simple procedures for the short-term culture of pachytene spermatocytes and round spermatids from both the rat and the mouse (10, 33). Experiments reported to date concern primarily intracellular metabolic events such as protein synthesis (10), protein glycosylation (27), and lactate utilization (39).

The culture conditions used in the present study are similar to those employed in previous biochemical studies, but flagellar elongation in these earlier experiments was not reported. Flagella formed in vitro may have been previously overlooked for several reasons. First, our initial observations were conducted using live mounts under Nomarski differential interference optics, conditions that facilitate the detection of the flagella. We have found it difficult to quantitate flagellar formation using routine histological stains, such as the Romanowsky derivatives or hematoxylin and eosin, most often applied. Furthermore, few of the previous studies reported

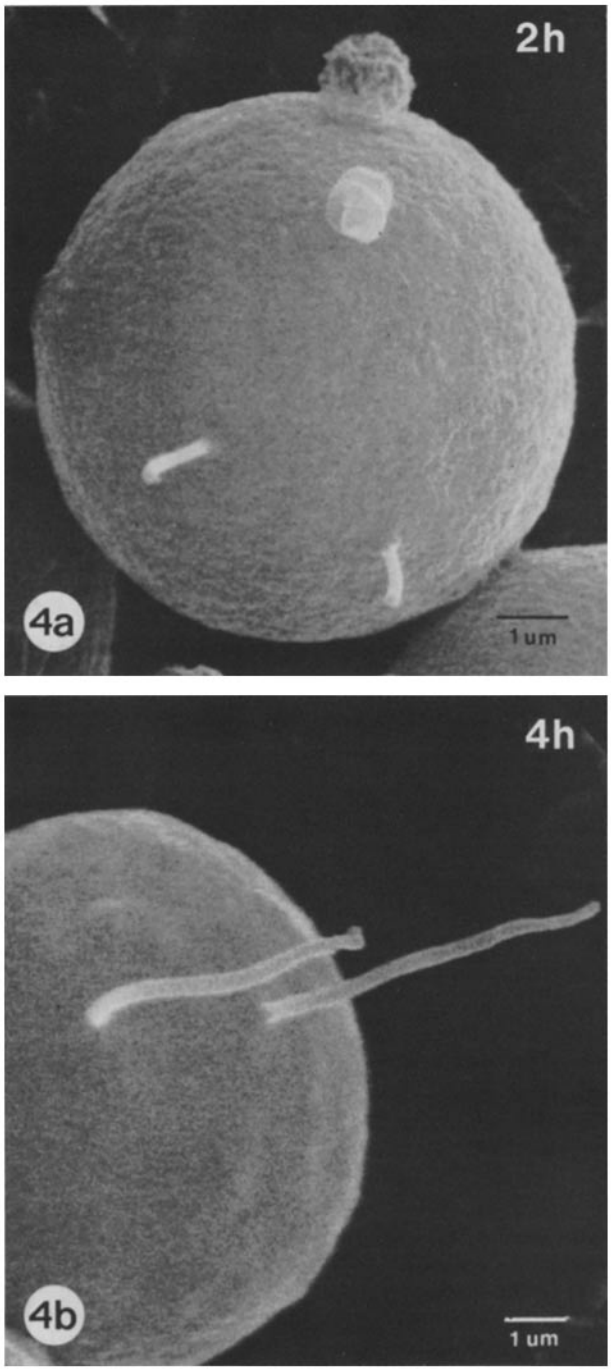


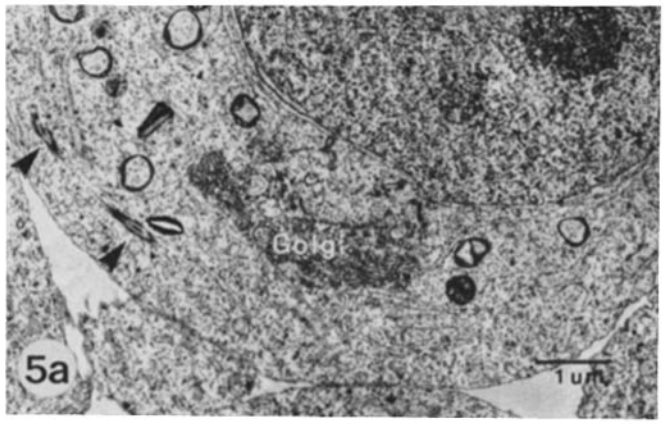
FIGURE 4 Scanning microscopic views of multiple flagella elaborated by round spermatid symplasts. Phase-contrast and interference microscopy indicate that the number of flagella in any single symplast never exceeds the number of nuclei present. Note that all flagella in any single symplast are of approximately equal length. (*a*) × 9,000. (*b*) × 8,000.

morphological examinations of any type, with the exception of Nakamura et al. (42) and Boitani et al. (10) who presented ultrastructural data demonstrating the integrity of cultured rat spermatids. Second, although scanning electron microscopy indicates that flagellar elongation is initiated within the first few hours of culture, we have noted that elongating tails are not readily visible using normal light microscopic procedures until ~6–8 h of incubation. Most of the earlier reports maintained mammalian spermatogenic cells in vitro for <8 h. Third, only recently has it become apparent that the cellular metabolism of spermatids isolated from the rat is greatly stimulated by the inclusion of exogenous lactate in the culture medium (33, 39, 41). Our culture conditions included 6 mM lactate and in addition, the spermatogenic cells were originally isolated using EKRB, instead of PBS. Romrell et al. (51) have shown that EKRB better supports the metabolic activity of mouse spermatogenic cells as evidenced by oxygen consumption measurements. It is possible that these improved culture conditions facilitate spermatid differentiation. The culture medium is certainly not optimal, however, since it does not approximate the unusually high osmolarity, low protein content, and altered Na^+/K^+ ionic composition of the seminiferous fluids normally surrounding developing spermatids in situ (52).

Morphologically, the flagella generated in vitro appear physiologically normal. Recently, Walt (59, 60) has fragmented living rat seminiferous tubules and examined flagellated spermatids with the light microscope. The flagellated round spermatids described in these studies appear morphologically identical to those from our cultures. Irons and Clermont (30, 31) have completed a detailed ultrastructural analysis of flagellar formation during spermiogenesis in the rat. The developing sperm tail is first noted in step 1 spermatids as the axoneme begins to extend from the cytoplasmic centriolar diplosome. During step 2 of spermiogenesis the centrioles migrate from the cell periphery to a position adjacent to the nuclear envelope, causing an invagination of the plasma membrane. Accessory flagellar structures such as the outer dense fibers and fibrous sheath are not readily apparent in situ until the later "acrosomal" and "maturation" phases of spermatogenesis. In our cultures, we have observed only "9 + 2" axonemal structures, devoid of accessory fibers. The ultrastructural results indicate, therefore, that all spermatids elaborating tails are Golgi-phase cells, no later than step 3. Although acridine orange staining does not provide sufficient resolution to distinguish step 1 cells from step 2 cells, the scanning microscopic observations reveal an apparent lack of invaginated plasma membrane, suggesting that perhaps only step 1 Golgi-phase spermatids generate flagella.

We have not observed flagellar growth by cultured spermatids in the cap phase of acrosomal maturation. The reasons for this restricted tail elongation response are not clear. Pre-

FIGURE 5 Ultrastructural examination of cultured round spermatids. (*a*) Cell exhibiting cytoplasmic flagellum in oblique section (arrowheads). The extensive Golgi apparatus and the midcytoplasmic positioning of mitochondria indicate that this cell is an early spermatid. (*b*) Round spermatid exhibiting a developing flagellum (arrowhead). Note that the basal centriole is subjacent to the plasma membrane, that the multivesicular body (*MVB*) is in close proximity, and that the developing acrosome (*Ac*) contains an acrosomal granule with an associated Golgi complex. These morphological features indicate that this cell is also early in spermiogenesis, probably step 1 (60). (*c*) View of a basal centriole in cross section, illustrating the "9+0" triplet structure. The chromatoid body (*CB*) position, the proximity of the centrioles to the plasma membrane, and the lack of surface membrane invagination indicate again that this cell is a step 1 spermatid. (*d–f*) Additional sections demonstrating that only early Golgi-phase spermatids elaborate flagella in vitro. (*g*) Cross section of a flagellum elaborated in vitro. The normal "9+2" axonemal unit is evident. Note that no accessory structures, such as the outer dense fibers, are seen in this or any of the other micrographs. All cells were fixed and sectioned after 48 h in vitro culture. (*a*) × 10,000; (*b*) × 15,000; (*c*) × 26,600; (*d*) × 14,500; (*e*) × 14,700; (*f*) × 13,000; (*g*) × 60,000.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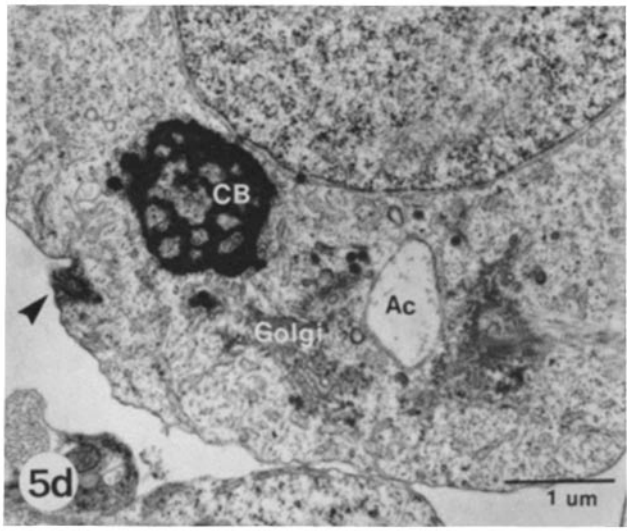
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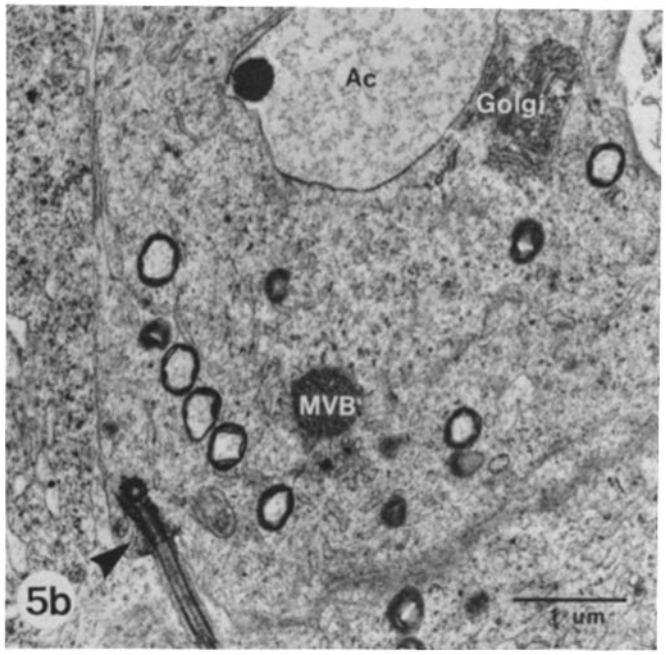
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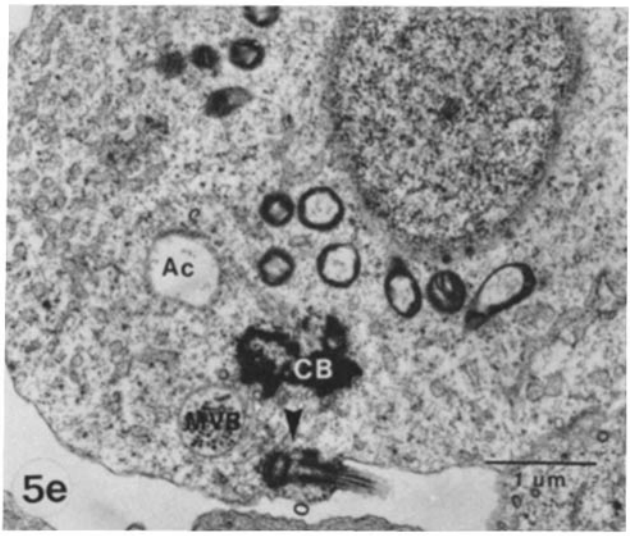
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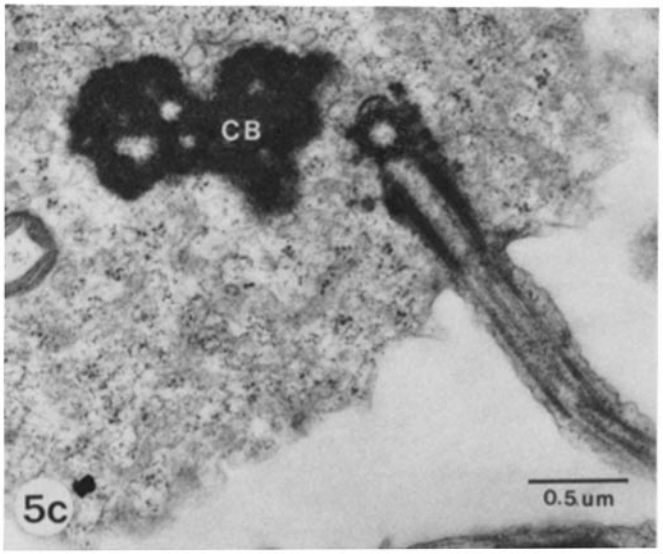
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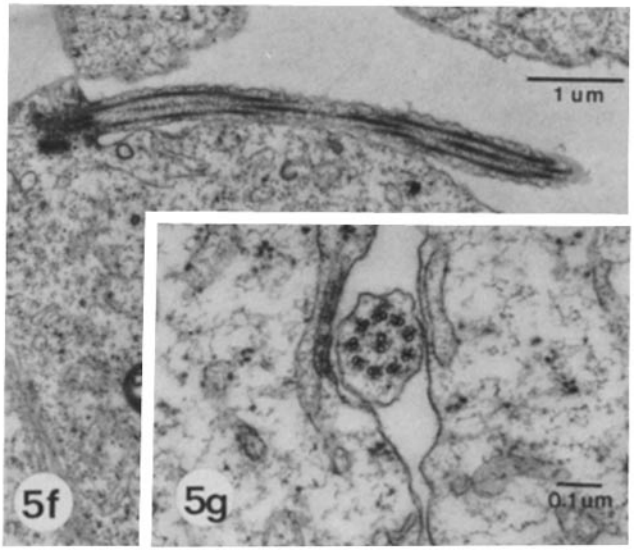
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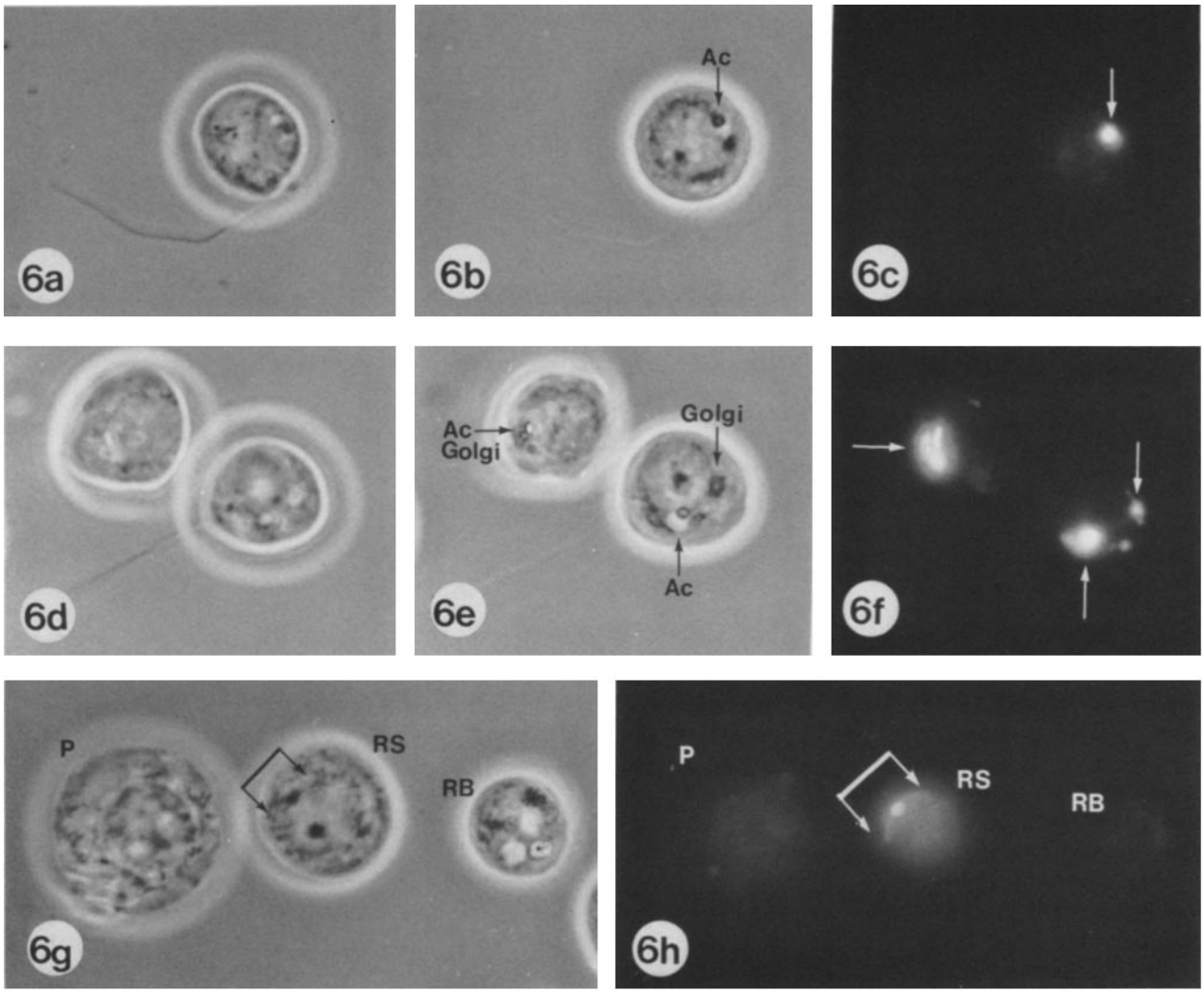

FIGURE 6 Cultured spermatogenic cells labeled with acridine orange. (*a–c*) Flagellated round spermatid viewed in different focal planes with phase contrast to allow observation of the flagellum as well as the developing acrosomal vesicle containing a prominent acrosomal granule (*Ac*). Using acridine orange the acrosome labels strongly (*c*). This cell is in the "Golgi phase" of spermiogenesis. (*d–f*) Another representative field showing Golgi-phase spermatids in culture. The developing acrosomes (*Ac*) and regions presumably representing the Golgi complex are visible in the phase-contrast micrographs. Acridine orange (*f*) stains these intracellular regions intensely. Note that not all Golgi-phase spermatids develop flagella in culture. (*g* and *h*) "Cap-phase" spermatids are easily distinguished from the earlier Golgi-phase cells using both phase contrast and acridine orange. The acrosomal cap in the round spermatid (*RS*) is indicated by the bracket. Note that neither the pachytene spermatocyte (*P*) nor the residual body (*RB*) is labeled by acridine orange in an intense or localized manner. No cap-phase spermatids elongated flagella under the conditions used in these studies. All cells were stained with acridine orange after 19 h of in vitro culture. × 1,328.

sumably, all of the round spermatids in our cultured preparations originally had flagella in situ which were lost during the enzymatic and mechanical procedures used in the dissociation of the testis and seminiferous tubule. It is not known at present what portion of the original axoneme remains within the spermatid cytoplasm following enzymatic and mechanical trauma, but the centriolar pair is probably intact since it is situated immediately adjacent to the nucleus by step 2 of spermiogenesis (29). Cultured cap-phase spermatids should, therefore, possess a nucleation site for flagellar regeneration.

The fact that these cells do not regenerate flagella may simply reflect inadequate culture conditions, but it seems more probable that our results reflect biochemical differences between spermatids at various stages of maturation. Few biochemical parameters of cellular metabolism have been measured during mammalian spermatogenesis. However, the available data demonstrate that extensive alterations in biosynthetic activity do occur and that the changes may often be correlated with particular stages of spermiogenesis. Protamines, for example, are not synthesized until late in spermatid differentiation (steps 12–15), after extensive nuclear condensation has occurred (5, 26, 34, 40, 49). Structural components of the developing spermatozoon are also synthesized de novo by mammalian spermatids (2, 44, 46), but many polypeptides of spermatozoa result from earlier biosynthetic events in diploid pachytene spermatocytes (45). Synthesis of LDH-C_4, the testis-specific isozyme of lactate dehydrogenase, is initiated during pachytene and is not completed until late in spermiogenesis (37). It is conceivable, therefore, that cap-phase sper-

matids cannot regenerate a flagellum due to a depletion of cytoplasmic tubulin stores, concomitant with an inability to synthesize additional tubulin monomers de novo. Golgi-phase spermatids would presumably lose less tubulin upon deflagellation owing to the shortened length of developed axoneme and would therefore have sufficient tubulin pools remaining for polymerization. Many other explanations are, of course, also feasible. Golgi-phase cells could differ from later cap-phase spermatids in their ability to synthesize tubulin. Some evidence of haploid gene expression by mammalian spermatids has been obtained (15, 17–19), but it is not yet proven that the translation of specific proteins during spermiogenesis does not derive from messenger RNAs originally transcribed by the diploid spermatocyte genome (25, 55, 56).

The biosynthesis of tubulin in cultures of isolated mammalian spermatids has not yet been investigated. Consequently we do not know whether tubulin synthesis is depressed in the presence of colchicine as has been found in other systems (14). It is interesting to note, however, that when protein synthesis is blocked by cycloheximide, meiosis of newt spermatocytes is blocked at the second division, yet these cells produce flagella nonetheless (35). These results suggest that tubulin synthesis during early spermiogenesis may not be required for flagella formation.

Recent studies suggest that various cell and organ culture systems may be used to analyze biochemical parameters of mammalian spermatogenesis (28, 49, 58), particularly the molecular events during spermatogonial proliferation and during meiotic prophase. Our results suggest that although complete maturation of mouse spermatids cannot yet be achieved in vitro, some of the molecular parameters determining early spermiogenesis may now be identified and characterized. Other in vitro systems for the analysis of flagellar development have in general been limited to nonmammalian organisms such as *Chlamydomonas*, *Euglena*, or the sea urchin (9, 11, 23, 36). It will be of interest to apply the investigative methods used in these and similar studies for the direct biochemical analysis of mouse spermatid tail growth.

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