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# Prepubertal Expansion of Dark and Pale Type A Spermatogonia in the Rhesus Monkey (*Macaca mulatta*) Results from Proliferation During Infantile and Juvenile Development in a Relatively Gonadotropin Independent Manner[1]

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## ABSTRACT

**The purpose of the present study was to determine whether dark and pale type A spermatogonia (Ad and Ap, respectively) are mitotically active during prepubertal development and whether proliferation of these germ cells during this protracted phase of primate development occurs predominantly during infancy before gonadotropin secretion is arrested. Four neonate (1–2 days of age), four infant (4–5 mo of age), and four juvenile (14–17 mo of age) rhesus monkeys (*Macaca mulatta*) were castrated 2 h after receiving an i.v. bolus of 5-bromo2′-deoxy-uridine (BrdU, 33 mg/kg body weight). Tissue was fixed in Bouin solution, and 5-μm paraffin sections were cut. Using periodic acid-Schiff reagent/Gill hematoxylin staining, the number per testis of Ad and Ap spermatogonia were determined. BrdU S-phase-labeled nuclei were identified using immunofluorescence. Conservative criteria were employed for classifying cell types, and this resulted in a fraction of A spermatogonia remaining unclassified. Ad, Ap, and the unclassified A spermatogonia each showed an approximately 4-fold increase over the 5-mo period from birth to infancy, and a similar increase was observed over the 10-mo period between infancy and the juvenile stage of development. Both Ad and Ap (and unclassified A spermatogonia) exhibited robust and similar S-phase labeling at the three stages of development. We conclude that the prepubertal expansion of Ad and Ap spermatogonia is achieved by mitotic proliferation that is relatively gonadotropin independent. This conclusion raises the question of the nature of the signal that arrests the cell cycle of Ad in adult testis.**

*developmental biology, follicle-stimulating hormone, luteinizing hormone, male reproductive tract, testis*

## INTRODUCTION

Undifferentiated or type A spermatogonia in the testis of the adult monkey have generally been classified into two categories on the basis of nuclear staining patterns produced with hematoxylin [1]. The first category, called dark type A (Ad) spermatogonia, are mitotically quiescent in the post-pubertal testis [2, 3] and have been traditionally viewed as reserve stem cells [1, 4, 5]. The second category, termed pale type A (Ap) spermatogonia, are mitotically active and classically have been considered to be renewing stem cells [1, 4, 5]. A third category of type A spermatogonia with an intermediate nuclear staining pattern, referred to as transitional type A (At) spermatogonia, has also been described [3, 5, 6].

Puberty in the male rhesus monkey occurs at 3–4 yr of age [7], and in the adult testis there are approximately 500 million type A spermatogonia, which compares to a value of less than 2 million in the testis at 2–3 wk of age [8]. The postnatal ontogeny of Ad spermatogonia is of particular interest for two reasons. First, some or all of these cells are likely to constitute the spermatogonial stem cell of the primate testis. Second, although this category of type A spermatogonia divide only rarely in the adult testis, their number increases several-hundred-fold from birth to adulthood.

The purpose of the present study was 1) to determine whether type A spermatogonia, and in particular Ad spermatogonia, are mitotically active during prepubertal development, and 2) to examine the extent to which proliferation of type A spermatogonia during this prolonged phase of primate development is dependent on gonadotropin stimulation. Accordingly, in vivo S-phase labeling of type A spermatogonia with 5-bromo2'-deoxy-uridine (BrdU) was studied during infancy (birth until 6 mo of age) when gonadotropin secretion is elevated, and during juvenile development when LH and FSH release is markedly attenuated [7].

## MATERIALS AND METHODS

### Experimental Design and Tissue Preparation

The results reported here were derived from the same testes that had been used in an analogous study of the ontogeny of the Sertoli cell [9]. All monkeys were maintained in accordance with NIH Guidelines for the Care and Use of experimental animals, and the experimental procedures were approved by the University of Pittsburgh Institutional Animal Care and Use Committee. Neonate (1–2 days of age), infant (4–5 mo of age), and juvenile (14–17 mo of age) rhesus monkeys (*Macaca mulatta*) were castrated (n = 4/group) 2 h after receiving an i.v. bolus injection of BrdU (Sigma Chemical Co.; 33 mg/kg body weight as a 2% solution in PBS). One testes from each animal was weighed and bisected perpendicular to the long axis. Tissue from this testis was fixed overnight in Bouin solution and subsequently stored in 70% ethanol until embedding in paraffin.

### Morphometric Analysis

Morphometric analyses were performed as previously described [9]. Five 5-μm sections taken at 50-μm intervals were stained with periodic acid-Schiff reagent (PAS)/Gill hematoxylin. Volume fraction of the seminiferous cord was determined by the point counting method and used to calculate the total volume of seminiferous cord per testis. The total length of the seminiferous cord per testis was calculated using the cross-sectional area, determined by measuring the diameter of 20 seminiferous cords. Spermatogonia nuclei were counted from circular profiles of seminiferous cord cross sections. These numbers were then corrected using Abercrombie formula. Total number of spermatogonia per testis was derived by multiplying the corrected average number of cells per

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cross section by the total length of seminiferous cord and dividing by the thickness of the histological section.

### *Immunocytochemistry*

Three immunocytochemical methods were employed in an attempt to reveal the nuclear BrdU label:

Method 1: An anti-BrdU monoclonal antibody and biotinylated horse anti-mouse antibody were used as the primary and secondary antibody, respectively, as previously described for the analogous study of the Sertoli cell [9]. The avidin-horseradish peroxidase complex was used to amplify the signal, which was visualized with 3,3'-diaminobenzidine (DAB). Sections were then counterstained using PAS/Gill hematoxylin. The BrdU-labeled nuclei were dark brown.

Method 2: This was similar to method 1, except that a fluorescent (Cy-3) conjugated goat anti-horse antibody (1:200 dilution in 50 mM PBS/0.05% Triton; Jackson Laboratories) was used instead of the avidin-horseradish peroxidase complex. Sections were counterstained using PAS/Gill hematoxylin or hematoxylin only. Bright yellow BrdU-labeled nuclei were detected using a fluorescent microscope.

Method 3: This method was a modification of that recently described by Ehmcke et al. [10]. After removing paraffin and rehydrating, the sections were treated with 1 M HCl for 15 min, washed with distilled water, and stained with Mayer hematoxylin (Sigma) for 3 min. The sections were then treated with 0.1% trypsin (Sigma) in Tris-buffered saline (TBS) for 10 min and sequentially washed with distilled water and TBS. Sections were then incubated in TBS containing 5% normal goat serum and 1% BSA (both reagents from Sigma) for 30 min. Sections were then incubated with an anti-BrdU mouse IgG (clone BU-33; Sigma or Biomeda) diluted 1:50 in TBS + 0.1% BSA, at 4°C overnight. After washing in TBS, sections were incubated with a goat anti-mouse biotinylated antibody (Sigma) at a 1:100 dilution in TBS + 0.1% BSA for 1 h. After washing with TBS, sections were incubated in streptavidin-conjugated AlexaFluor 488 (Molecular Probe) at a 1:100 dilution in TBS + 0.1% BSA for 60 min. After additional washing in TBS, sections were mounted with VectaShield Mounting Medium (Vector Laboratories). Bright yellow-green BrdU-labeled nuclei were detected using a fluorescent microscope.

### *Labeling Index*

Labeling indices were determined on 5-μm sections in which BrdU had been visualized using AlexaFluor 488 (method 3). Corresponding bright-field and fluorescent micrographs were systematically taken along the x and y axes of the microscope stage over the entire area of two sections. Total and labeled cell numbers were determined from the bright-field and fluorescent micrographs, respectively. A total of 60–90 micrographs per animal (130–703 type A spermatogonia per animal) were analyzed. Labeling index is expressed as a percent of labeled cells divided by total cell number.

### *Numerical Analysis*

Data are expressed as mean ± SD. The significance of differences in means was determined using one-way repeated-measures ANOVA followed by the Neuman-Keuls test for multiple comparisons. $P \leq 0.05$ was considered significant.

## RESULTS

### *Morphometry of the Testes*

Testis weight increased significantly with age (Table 1). This increase was associated with corresponding changes in the seminiferous cord length and its total volume (Table 1). Volume fraction of the cord increased from the neonatal to juvenile stage of development, although the increase between infant and juvenile was not significant. Cord diameter was similar at the three stages of development.

TABLE 1. Testis weight and seminiferous cord parameters (mean ± SD) of neonate, infant, and juvenile rhesus monkey (N = 4 per group).

| Parameter | Neonate[a] | Infant[a] | Juvenile |
|---|---|---|---|
| Testis weight (g) | 0.09 ± 0.02[b] | 0.19 ± 0.03[c] | 0.26 ± 0.06[d] |
| Volume fraction of cord (%) | 28 ± 4[b] | 41 ± 6[c] | 45 ± 6[c] |
| Volume of cord ($cm^3$) | 0.03 ± 0.01[b] | 0.08 ± 0.02[c] | 0.12 ± 0.04[d] |
| Diameter of cord (μm) | 48 ± 3[b] | 45 ± 2[b] | 43 ± 2[b] |
| Length of Cord (m) | 14.1 ± 4.1[b] | 50.8 ± 17.7[c] | 79.8 ± 22.8[d] |

[a] Data for neonate and infant were previously reported [9].
[b–d] Values with different letters are significantly different from each other; $P \leq 0.05$.

The histology of the testis at all three stages of prepubertal development was similar [9]. Immature Sertoli cells and type A spermatogonia were observed in the seminiferous cords, together with an occasional B-like spermatogonia. Based on nuclear size and staining pattern revealed by PAS/Gill hematoxylin, type A spermatogonia were placed into one of four categories (Fig. 1).

The first were cells with small, spherical and darkly stained nucleus with dense and homogeneous chromatin. Two or more hemispherically shaped nucleoli attached to the inner side of the thin nuclear envelope were usually seen. The nuclear envelope was generally separated from the chromatin by a clear zone. The average nuclear diameter was approximately 6.4 μm. These cells were unequivocally type Ad spermatogonia.

The cells in the second category contained a relatively large ovoid and lightly stained nucleus, with coarsely granular chromatin. The nuclear envelope was distinct, and a clear zone was not seen. Heterochromatin associated with the nuclear envelope was evident. The average nuclear diameter was approximately 8.4 μm, although nuclear and nucleolar size varied broadly. These cells were unequivocally type Ap spermatogonia.

The cells in the third category contained an oblong nucleus with coarse chromatin. The nuclear envelope was discernable, but a clear zone was not seen. Occasionally, clear vesicles were found inside the nucleus. Heterochromatin associated with the nuclear envelope was evident. The average nuclear diameter was approximately 6.9 μm. These cells were also classified as type Ap spermatogonia. For quantitative purposes, the two types of Ap spermatogonia, Ap large (l) and Ap small (s), respectively, were analyzed separately.

The fourth category of cell shared some morphological similarities to type Ad, that is, a thin nuclear envelope

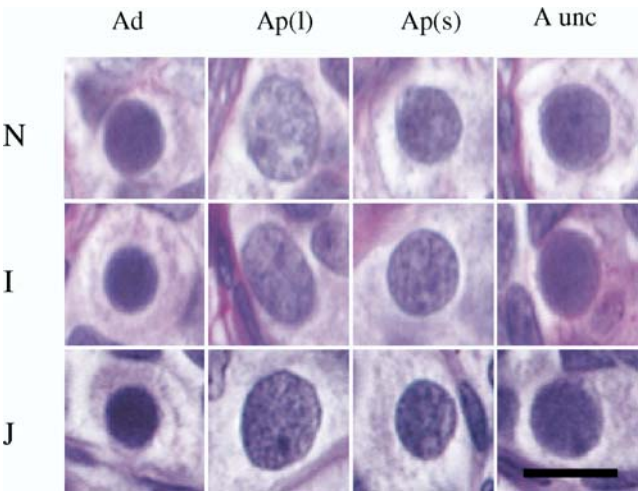


FIG. 1. Photomicrographs obtained from PAS/ hematoxylin stained sections from testes of neonate (N), infantile (I), and juvenile (J) rhesus monkeys illustrating the morphological characteristics of A spermatogonia observed at these three stages of prepubertal development. Approximately 75% of undifferentiated germ cells were unambiguously classified as dark type A (Ad) or pale type A (Ap) spermatogonia: left and two center panels, respectively. Ap spermatogonia were further subdivided into large (l) and small (s). A spermatogonia that could not be unequivocally identified remained unclassified (A unc) and are shown in the right panels. Bar = 10 μm.

TABLE 2. Nuclear-diameter (μm; mean ± SD) of type Ad, Ap(l), Ap(s), and unclassified A spermatogonia (A unc) of neonate, infant, and juvenile monkeys (N = 4 per group).

| Spermatogonia type | Neonate | Infant | Juvenile |
|---|---|---|---|
| Ad | 6.61 ± 0.40[a] | 6.35 ± 0.23[a] | 5.85 ± 0.03[b] |
| Ap(l) | 8.53 ± 0.29[a] | 8.18 ± 0.36[a] | 8.40 ± 0.15[a] |
| Ap(s) | 7.17 ± 0.27[a] | 7.02 ± 0.11[b] | 6.70 ± 0.27[b] |
| A unc | 7.64 ± 0.37[a] | 7.44 ± 0.07[a] | 7.44 ± 0.09[a] |

[a,b] Values with different letters are significantly different from each other; $P \leq 0.05$.

TABLE 3. Mean number (± SD; million) per testis of Ad, Ap(l), Ap(s), and unclassified A (A unc) spermatogonia in neonate, infant, and juvenile rhesus monkey (N = 4 per group).

| Spermatogonia type | Neonate | Infant | Juvenile |
|---|---|---|---|
| Ad | 0.47 ± 0.21[a] | 1.95 ± 1.80[a] | 5.79 ± 1.77[b] |
| Ap(l) | 0.47 ± 0.13[a] | 1.75 ± 1.86[a] | 5.05 ± 2.33[b] |
| Ap(s) | 0.13 ± 0.10[a] | 0.59 ± 0.61[a] | 3.06 ± 1.51[b] |
| A unc | 0.19 ± 0.08[a] | 0.80 ± 0.79[a] | 4.53 ± 2.33[b] |

[a,b] Values with different letters are significantly different from each other; $P \leq 0.05$.

unassociated with heterochromatin and a clear zone. In contrast to type Ad, however, nuclear size was larger, and the chromatin was less dense and less homogeneously distributed. The nuclear and nucleolar size varied broadly, with an average nuclear diameter of approximately 7.5 μm (Table 2). These A spermatogonia could not be unambiguously assigned to either Ad or Ap and therefore remained unclassified.

Although A spermatogonia were usually located on the basement membrane, these cells were occasionally found in the central area of the cord separated from the basement membrane (Fig. 2). The morphological features of these centrally located cells were identical to those situated on the basement membrane and therefore were included in the quantitative analysis. These centrally located spermatogonia were observed with the same frequency in neonate and juvenile subjects (<1–3 per 100 cells). However, only one (3 per 100 cells) out of four infant testes showed this phenomenon.

### *Total Undifferentiated Spermatogonial Number per Testis*

At birth, the number of Ad, Ap(l), Ap(s), and unclassified A spermatogonia was 0.47 ± 0.11, 0.47 ± 0.06, 0.13 ± 0.05, and 0.19 ± 0.04 ($\times 10^6$), respectively. All four populations showed an approximately 4-fold increase over the 5-mo period from birth to infancy, and a 3- to 6-fold increase was observed over the 10-mo period from infancy to the mid-juvenile stage (Table 3 and Fig. 3).

In the neonate and infant, the ratio of Ad:Ap(l):Ap(s): unclassified A was approximately 1:1:0.4:0.4. In the juvenile, the ratio was 1:1:0.5:0.8.

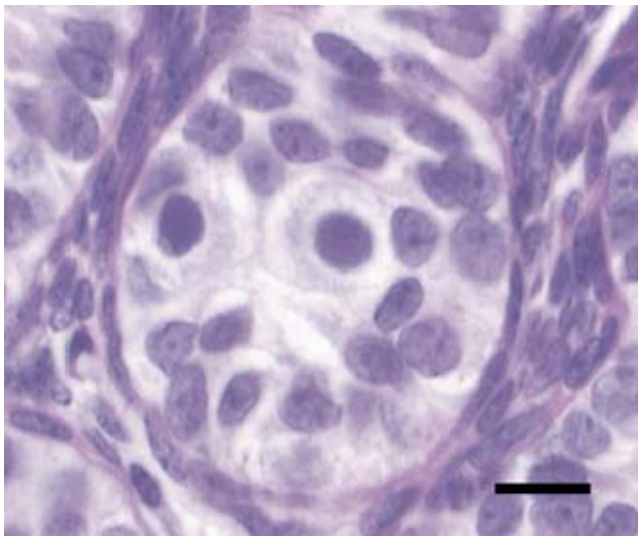

FIG. 2. Photomicrograph obtained from a PAS/hematoxylin-stained section from testis of an infant rhesus monkey illustrating an A spermatogonia centrally located within the seminiferous cord. Bar = 20 μm.

### *BrdU Labeling*

When DAB was used to visualize BrdU-labeled nuclei (method 1), the intensity of the DAB reaction frequently masked the hematoxylin counterstain, making it very difficult to classify cell types. This was especially difficult in the case of type Ad and the unclassified A spermatogonia.

Although substitution of the fluorescent label (Cy-3) for DAB (method 2) facilitated cell identification, in this case, the hematoxylin counterstain led to a reduction in the intensity of the fluorescent signal, making it difficult to identify BrdU-labeled nuclei. Nevertheless, labeled Ad, Ap, and the unclassified A spermatogonia were observed with this method (Fig. 4).

Reduction in the intensity of the fluorescent label was eliminated in method 3 by counterstaining sections before visualization of BrdU. This procedure, however, led to a reduction in the morphological integrity of the sections. Most notably, spermatogonia nuclei were enlarged and appeared less dense (Fig. 5). These changes were associated with a redistribution of cells within the four categories. As might be expected, the major redistribution occurred within Ad and the unclassified A spermatogonia. The average ratio of Ad:unclassified A spermatogonia across the three developmental stages as revealed by PAS/H staining was 2.1:1, and this compared to a corresponding ratio of 0.4:1 when BrdU was visualized with method 3. Comparable ratios for Ap(l):Ap(s)

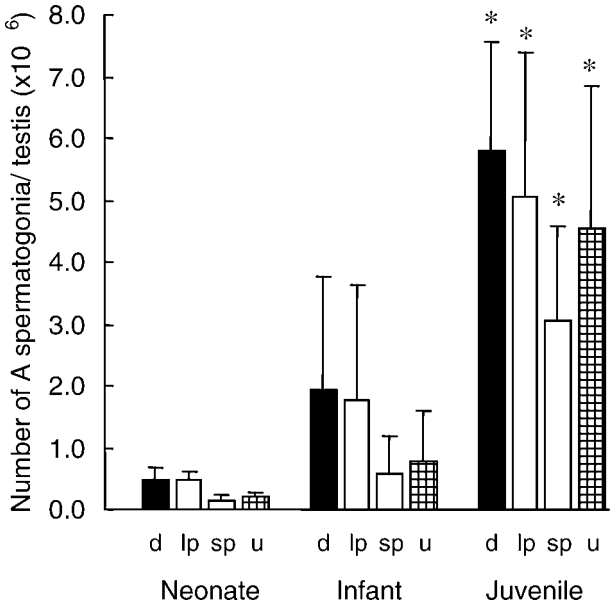


FIG. 3. Increase in the number per testis (mean ± SD) of Ad (d), large and small Ap (pl and ps, respectively), and unclassified A (u) spermatogonia from birth (neonate) through infancy (infant, 4–5 mo of age) until 14–17 mo of age (juvenile). *Indicates significantly different from other stages of development.

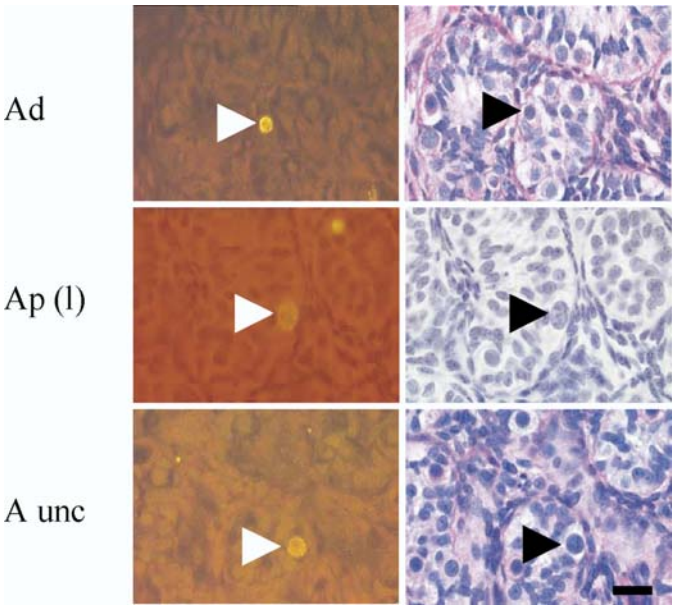


FIG. 4. Photomicrographs of S-phase-labeled Ad, large Ap, Ap(l), and unclassified A (A unc) spermatogonia revealed by method 2 using a Cy-3 conjugated secondary antibody (left panels) before counterstaining with PAS/H or H (right panels). Bar = 20 μm.

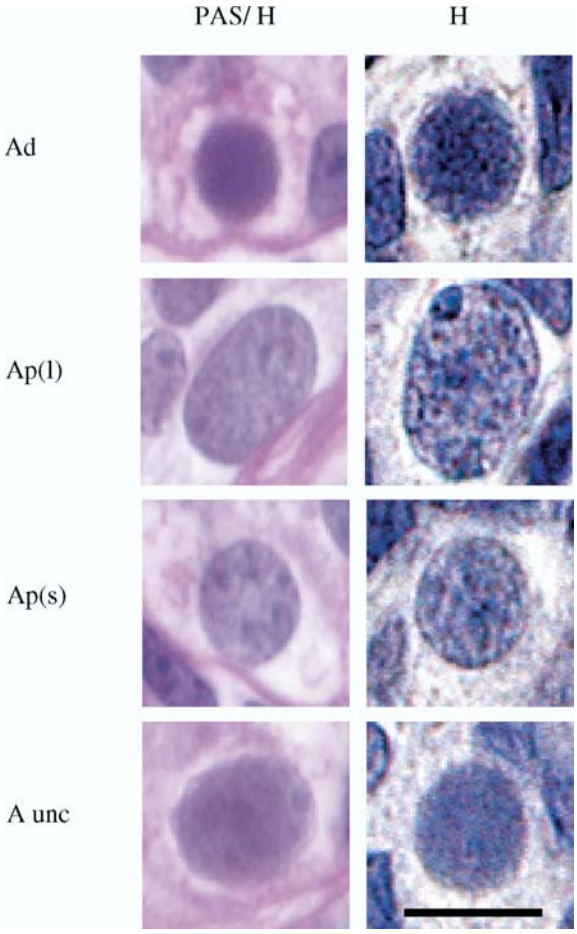


FIG. 5. Photomicrographs comparing the morphology of Ad, large and small Ap (l and s, respectively), and unclassified A (A unc) spermatogonia observed in sections stained with PAS/H (left) and in sections stained with H and subsequently subjected to immunocytochemistry using method 3 that employs a trypsin treatment before incubation with primary antibody (right). Bar = 10 μm.

were 2.7:1 and 3:7:1, respectively. Regardless, labeled Ad continued to be observed, as were Ap(l) and the unclassified A spermatogonia (Fig. 6). For these reasons, we avoided drawing quantitative inferences from the labeling indices.

### *BrdU Labeling Indices*

Because of the superiority of method 3 in revealing BrdU-labeled cells, and in particular those with dense chromatin, this method was used for determining the labeling index of type A spermatogonia. The labeling index of Ad, Ap(l), and the unclassified A spermatogonia were not significantly different at the three developmental stages (Fig. 7). The labeling indices of Ad in the neonate, infant, and juvenile were 14.1%, 15.1%, and 22.4%, respectively, while those for Ap(l) were 4.5%, 9.6%, and 4.4%, respectively, and the unclassified A spermatogonia were 14.0%, 6.7%, and 12.1%, respectively. No labeled Ap(s) were observed, although a total of 539 of this cell type were counted from all animals.

Overall, the labeling indices of all type A spermatogonia in the neonate, infant, and juvenile were indistinguishable—8.5, 8.9, and 9.1, respectively.

### *Additional Evidence of Mitosis*

On occasion, Ad and the unclassified A spermatogonia, but not Ap, were observed in apparent incomplete cytokinesis (Fig. 8). Also, chains of Ad and Ap(l) were occasionally noticed, both in circular and in oblique cross sections of the cord (Fig. 8).

## DISCUSSION

As has been the case in studies of the testis of adult macaques by others [3, 5, 6, 11], we found it difficult to precisely place type A spermatogonia in the testes of prepubertal rhesus monkeys into one of the two categories originally described for this genus by Clermont and Leblond [1], namely, type A1 and A2 spermatogonia, later and currently recognized as type Ad and Ap spermatogonia [12], respectively.

The classification of type A spermatogonia in the present study into four categories was based on the following considerations. Most important, because Ad spermatogonia are mitotically quiescent in the adult [2, 4] and because a major aim of the present investigation was to determine whether proliferation underlies the marked expansion in the population of this undifferentiated spermatogonia during infancy and juvenile development, we were intentionally conservative in categorizing A spermatogonia as type Ad. The cell had to possess a small nucleus with dense and homogeneous chromatin and a nuclear envelope separated from the chromatin by a clear zone. On the other hand, type A spermatogonia with a nucleus containing coarse granular chromatin, a nuclear envelope associated with heterochromatin, and the absence of a clear zone were classified, by convention, as type Ap spermatogonia. Ap spermatogonia were further divided into Ap(s) and Ap(l) spermatogonia according primarily to relative nuclear size.

The unclassified A spermatogonia in the present study bore resemblance to Ad spermatogonia, but the larger nuclear size and the less dense chromatin excluded them from the Ad classification because, as stated previously, the assignment of cells to the Ad category needed to be unequivocal. In an earlier study of neonatal and juvenile monkeys by this laboratory [8] in which type A spermatogonia were classified according to the scheme of Clermont and Leblond [1], the ratios of type Ad to Ap spermatogonia in testis from 1- to 3-wk-old and 15- to 17-mo-old animals were 1.2:1 and 1.4:1, respectively. Approxi-

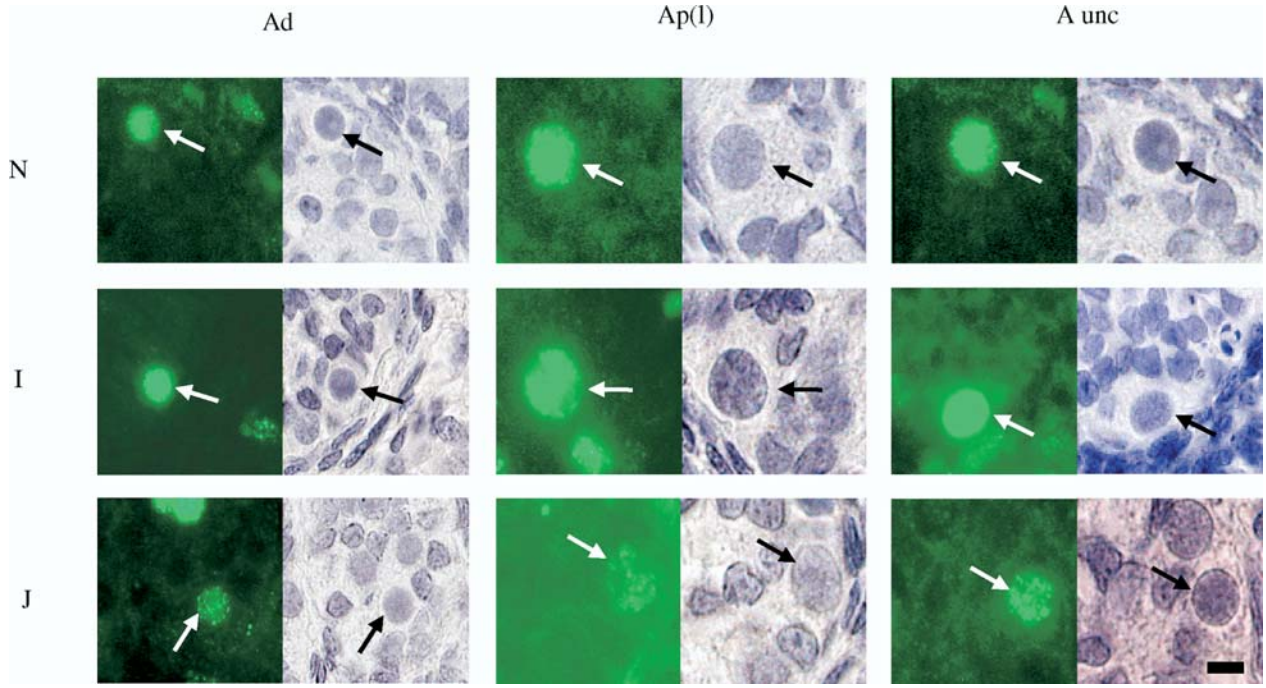


FIG. 6. Photomicrographs of S-phase-labeled Ad, large Ap (Ap[l]) and unclassified A (A unc) spermatogonia from testes of neonate (N), infant (I), and juvenile (J) rhesus monkeys. The left- and right-hand photomicrographs show fluorescent and bright-field, respectively. S-phase-labeled spermatogonia are indicated by the white arrow in the fluorescent fields. The corresponding cell in bright field is indicated by a black arrow. Bar = 10 μm.

mately equal numbers of Ad and Ap spermatogonia were earlier also reported for the infant cynomolgus monkey [5]. In the present study, the ratio of Ad to Ap spermatogonia was consistently less than 1:1 (0.7–0.8:1 depending on the developmental stage), and this difference would, by inference, suggest that the unclassified A spermatogonia would previously have been classified as Ad spermatogonia.

The same categories of A spermatogonia observed in the testis of the prepubertal monkey are also found in the adult (unpublished observations). Although an occasional type A spermatogonia was found in the center of the seminiferous cord of testes from neonates and the more mature groups studied, large spherical/ovoid germ cells with pale cytoplasm and nuclei containing lightly staining fine chromatin granules and one or more globular nucleoli, analogous to gonocytes reported in the newborn rodent testis [13–15], were not observed at any stage of development. In this regard, it is interesting to note that in an earlier investigation of testicular development in the rhesus monkey by van Wagenen and Simpson [16], A spermatogonia, but not gonocytes, were described in fetal and postnatal testis. A similar situation was reported for stillborn cynomolgus monkeys [3]. On the other hand, gonocytes have been described in fetal and newborn testes of humans [17–19].

In the present study, the number of Ad spermatogonia per testis in the neonate and infant were $0.47 \times 10^6$ and $1.95 \times 10^6$, respectively, and these values compared to a number of $1.71 \times 10^6$ in the testes of 7–18-day-old monkeys, reported earlier by this laboratory [8]. Corresponding values for Ad spermatogonia number for juveniles with comparable ages in the two studies was $5.79 \times 10^6$ and $15.43 \times 10^6$, respectively. As discussed previously, the relatively smaller numbers of Ad spermatogo-

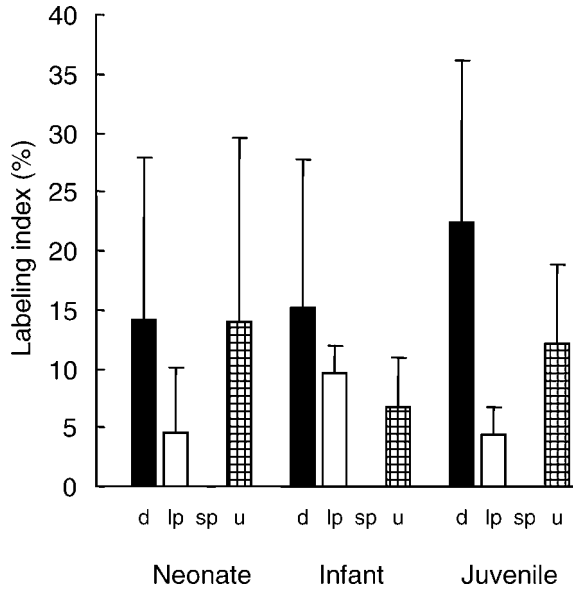


FIG. 7. Mean labeling index (±SD) of Ad (d), large Ap (lp), small Ap (sp), and unclassified A (u) spermatogonia in neonate (1–2 days of age), infant (4–5 mo of age), and juvenile (14–17 mo of age) rhesus monkeys. Significance differences were not observed. N = 4 for each age-group.

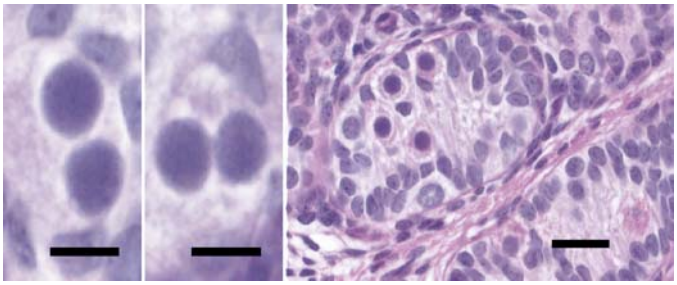

FIG. 8. Photomicrographs of PAS/H-stained sections from juvenile rhesus monkey testis showing Ad spermatogonia apparently captured at the time of fixation in a state of incomplete cytokinesis (two left panels) and an apparent chain of four Ad spermatogonia (right panel). Bars = 10 μm (left panels) and 20 μm (right panel).

nia in the present study is undoubtedly due to the very conservative criteria employed here to classify the germ cells.

On the other hand, in the case of Ap spermatogonia, agreement between the two studies is quite reasonable. In the present investigation, the number of these cells per testis in the neonate and infant were $0.61 \times 10^6$ and $2.35 \times 10^6$, respectively, and these values compared to that reported earlier of $1.44 \times 10^6$ in testes of 7–18-day-old monkeys [8]. Corresponding values for Ap spermatogonia number for juveniles in the present and earlier study was $8.12 \times 10^6$ and $10.79 \times 10^6$, respectively.

The number of Ad spermatogonia per testis increased 4-fold from birth until 5 mo of age and a further 3-fold over the next 10–13 mo to obtain a number of nearly 6 million in the juvenile. The fold increases in the other three types of A spermatogonia during the 5 mo of infancy (4-, 5-, and 4-fold; Ap[l], Ap[s] and the unclassified A spermatogonia, respectively) were similar to that of Ad spermatogonia. The fold increase in both types of Ap spermatogonia during the 10–13 mo spanning the infant-to-juvenile transition (3- and 5-fold; Ap[l] and Ap[s], respectively) was also similar to that for Ad spermatogonia during this phase of prepubertal developmental. In the case of the unclassified A spermatogonia, a 6-fold increase was observed from infancy to juvenile development.

Ad spermatogonia divide only rarely in the adult testis [2, 4], and therefore the finding that expansion of this population of A spermatogonia during prepubertal development was associated with robust S-phase BrdU labeling of this cell type in the prepubertal testes is particularly interesting. Moreover, robust labeling was also observed in those A spermatogonia that we were unable to categorically classify. It should be noted, however, that in previous studies of infant (2 mo of age) and juvenile cynomolgus monkey (12–18 mo) in which tritiated-thymidine was used either in vivo (intraperitonium or intratesticular injection) or in vitro to label cells in S-phase, a much lower labeling index ($<0.2\%$) of type Ad spermatogonia was reported [3, 5]. The reason for this discrepancy is unclear. Our observation in the rhesus monkey that type Ad spermatogonia are mitotically active during prepubertal development is consistent with the finding that two Ad nuclei were occasionally observed in cells that appeared to be fixed at the point of incomplete cytokinesis. Thus, it is reasonable to propose that the several hundred million Ad spermatogonia in the adult testis [8] is achieved by mitotic proliferation before the completion of puberty, a conclusion that raises the interesting question of the nature of the signals that arrest the cell cycle of Ad spermatogonia in the adult testis.

The finding that the progressive increase in the number of Ap spermatogonia during infancy and juvenile development was also associated with robust S-phase BrdU labeling of this cell type, which confirms earlier observations in the juvenile cynomolgus monkeys [3], was expected, as Ap spermatogonia are mitotically active in the adult [1, 3, 4]. That BrdU labeling of Ap spermatogonia in the present study was restricted, without exception, to the large subclass of this cell type, however, was remarkable. In the adult, Ap(l) are observed at those stages of the seminiferous epithelial cycle during which Ap spermatogonia divide [4, 10], while Ap(s) are found in the intervening stages. It is generally recognized that when a cell is approaching or entering S-phase, the nucleus becomes enlarged [20–22], suggesting that Ap spermatogonia classified as large are in S-phase or G2, while those classified as small are younger cells in G1. However the case may be, it must be concluded that Ap spermatogonia also increase their number during prepubertal development by mitotic proliferation. This conclusion, however, does not exclude the possibility that nonmitotic transformation of Ad to Ap also contributes to the expansion of the Ap population.

Gonadotropin secretion from birth until puberty in higher primates exhibits a characteristic pattern with elevated LH and FSH release during infancy and puberty, separated by a protracted hiatus in the secretory activity of pituitary gonadotrophs during juvenile development [7]. That the elevated levels of LH and FSH during infancy are biologically active is indicated by the findings that testicular hormone secretion (testosterone and inhibin B) is also elevated during this phase of development [23, 24]. Since in the present study monkeys were studied when LH and FSH secretion was elevated (infant) and arrested (juvenile), it is possible to relate the proliferative activity of A spermatogonia to circulating gonadotropin status. Interestingly, the labeling indices of all categories of A spermatogonia were comparable in newborn, infant, and juvenile testes. Thus, mitotic activity of A spermatogonia in the immature testis appears to be relatively gonadotropin independent. The recent finding of robust S-phase BrdU labeling of Ap spematogonia in adult monkeys rendered chronically hypogonadotropic by daily treatment with a GnRH receptor antagonist [25] suggests that gonadotropin-independent proliferation of this spermatogonial type is also a feature of the adult testis. The rate of increase in number of A spermatogonia during 10 mo of juvenile development (4–5 to 14–17 mo of age) when gonadotropin secretion is low, however, was less than that during the 4 to 5 mo of infancy (birth to 4–5 mo of age) when LH and FSH levels are elevated. Thus, it is possible that survival of A spermatogonia during the juvenile phase of development is compromised by the low levels of gonadotropin, perhaps because the rate of increase in Sertoli cell number and therefore germ cell niches is not optimal. In this regard, GnRH stimulation of endogenous gonadotropin secretion for 5–10 wk in the juvenile monkey leads to increases in the number of A spermatogonia [8]. However, labeling indices were not determined in this earlier study, and therefore an effect of gonadotropin stimulation on proliferation cannot be excluded.

The relatively gonadotropin-independent expansion of type A spermatogonia during infancy and juvenile development may be contrasted with that for the Sertoli cell, where the rate of increase in number of this somatic cell and its labeling index is markedly greater during infancy than during juvenile development [9] and therefore decline in parallel with the reduction in LH and FSH secretion during this phase of development. We had expected that proliferation of A spermatogonia would follow in the wake of an expanding population of Sertoli cells and that the ratio of spermatogonia to Sertoli cells across prepubertal development would therefore be similar. This, however, was not the case. The ratio of the germ cell to the somatic cell is notably greater during juvenile development, when Sertoli cell proliferation is retarded as a result of the hypogonadotropic state.

Interestingly, the ratio of A spermatogonia to Sertoli cells in the adult testis [8] is 4-fold higher than that observed in the juvenile. Although the relative time courses in the pubertal increases in A spermatogonia on the one hand and Sertoli cells on the other have not been systematically studied, we posit that expansion of the spermatogonial population during this phase of development lags that of the Sertoli cell. This notion is based on the relative changes in these cells types during 10 wk of precocious pubertal gonadotropin stimulation in the juvenile male [8]. In contrast to Sertoli cells, which attained 60% of their adult level after 10 wk of precocious gonadotropin stimulation, A spermatogonia attained only 15% of their adult number during this interval. Perhaps the best explanation for

this relationship is that the gonadotropin-driven pubertal expansion of the Sertoli cell population leads to an expansion of the number of niches for undifferentiated A spermatogonia that are subsequently occupied as constitutive proliferation of A spermatogonia continues.

As has been reported for the cynomolgus monkeys [3] and for humans [26], the occasional type B-like spermatogonia was observed in the present study. Since the generation of B spermatogonia in the adult is driven by gonadotropin secretion [27], and since the testis of the infant macaque is exposed to a hormonal milieu similar to that of the adult [7], the failure of the primate testis at this stage of development to produce differentiated germ cells is intriguing. The most probable explanation for this developmental block to spermatogenesis during infancy is that at this stage of development the Sertoli cell is immature. The absence of androgen receptor expression by the Sertoli cell of the infant testis [28] may underlie this immaturity.

In summary, the present study indicates that the adult complement of both Ad and Ap spermatogonia in the mature testis of the rhesus monkey, a representative higher primate, is produced, in part, by sustained mitotic proliferation during prepubertal development that is relatively gonadotropin independent. This is particularly interesting in the case of Ad spermatogonia because, in the adult testis, these cells are mitotically quiescent.

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