Human Reproduction Vol.21, No.7 pp. 1749–1758, 2006 doi:10.1093/humrep/del051
Advance Access publication February 23, 2006.

Chromosome constitution and apoptosis of immature germ cells present in sperm of two 47,XYY infertile males

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BACKGROUND: In order to assess sperm alterations observed in some XYY males, we analysed the chromosome constitution as well as apoptosis expression in germ cells from two oligozoospermic males with high count of immature germ cells in their semen. METHODS: Sex chromosome number and distribution were assessed at pachytene stage by fluorescence *in situ* hybridization (FISH). Immature germ cells and spermatozoa were examined by FISH and TdT (terminal deoxynucleotidyl transferase)-mediated dUDP nick-end (TUNEL) assay, combined with immunocytochemistry using the proacrosin-specific monoclonal antibody (mAb 4D4). RESULTS: For patients 1 and 2, two Y chromosomes were present in respectively 60.0 and 39.6% of pachytenes. The three sex chromosomes were always in close proximity and partially or totally condensed in a sex body. XYY spermatocytes I escape the pachytene checkpoint and achieve meiosis. Nevertheless, nuclear division and/or cytokinesis were often impaired during meiosis leading to diploid (mainly 47,XYY cells) and tetraploid (94,XXYYYY) meiocytes. The presence of binucleated (23,Y)(24,XY) immature germ cells resulting from cytokinesis failure agree with a preferential segregation of the two Y chromosomes during meiosis I. In addition, 69.6% (patient 1) and 53.12% (patient 2) of post-reductional round germ cells were XY. However, high level of apoptotic round germ cells (94.9% for patient 1 and 93.3% for patient 2) was detected and may explain the moderate increase of hyperhaploid XY spermatozoa. Segregation errors also occurred in the XY cell line responsible for disomic 18 and X, as well as 46,XY diploid spermatozoa. CONCLUSIONS: Our data are in agreement with the persistence of the extra Y chromosome during meiosis in XYY oligozoospermic males responsible for spermatogenesis impairment and a probable elimination via apoptosis of most XYY germ cells not solely during but also after meiosis.

Key words: apoptosis/chromosome anomalies/immature germ cells/meiotic disorders/XYY males

Introduction

In the general male population, 47,XYY and 47,XXY karyotypes are the most common chromosome anomalies (Hook and Hamerton, 1977; Hecht and Hecht, 1987). XYY males are generally fertile and there is no evidence of transmission of the extra Y chromosome to their progeny (Melnyk *et al*., 1969; Chandley, 1985). It has been suggested that fertile XYY males generally loose the extra Y chromosome prior to meiosis (Gabriel-Robez *et al*., 1996). In contrast, recent meiotic studies performed in infertile XYY males revealed that severe oligozoospermia is often associated to the persistence of the extra Y chromosome in a significant number of spermatocytes I at pachytene stage, from 30% (Speed *et al*., 1991) to 100% (Solari and Rey Valzacchi, 1997). Different pairing configurations of sex chromosomes have been described at pachytene stage in XYY males after synaptonemal complex analysis by electron microscopy. In humans, a majority of XYY pachytenes show a YY bivalent and an X univalent (YY+X) (Berthelsen *et al*., 1981; Speed *et al*., 1991; Solari and Rey Valzacchi, 1997). However, other meiotic configurations are also observed such as XY bivalent and Y univalent (XY+Y) (Speed *et al*., 1991; Gabriel-Robez *et al*., 1996), three independent univalents (X+Y+Y) (Speed *et al*., 1991; Solari and Rey Valzacchi, 1997) and an XYY trivalent (XYY) (Gabriel-Robez *et al*., 1996; Solari and Rey Valzacchi, 1997). Furthermore, studies using fluorescence *in situ* hybridization (FISH) have also indicated the presence of four possible pairing configurations for the sex chromosomes in XYY males (Blanco *et al*., 1997, 2001; Rives *et al*., 2005).

In patients with high rates of XYY pachytenes, abnormal spermatocytes I are then rapidly eliminated (Blanco *et al*., 2001; Rives *et al*., 2005). A major decrease of the proportion of XYY cells between the pachytene (30%) and the metaphase I (MI; 5%) stages has also been reported in an XYY male (Speed *et al*., 1991). Furthermore, in previous studies, the extra Y chromosome was never or rarely observed at MI (for review Berthelsen *et al*., 1981). An exception was the case of Hulten and Pearson (1971) presenting 45% of MI spermatocytes with two Y chromosomes.

In the light of these data, it appears that despite a major cell degeneration, some XYY spermatocytes are able to survive to MI. Furthermore, XYY trivalent appears to be the only configuration in XYY male compatible with the progress of meiosis by saturation of pairing sites preventing to trigger the pachytene checkpoint (Miklos, 1974; Rodriguez and Burgoyne, 2000; Roeder and Bailis, 2000). The outcome of these XYY germ cells along the meiotic process is unknown. The normal meiosis of some XYY germ cells could explain, in part, the moderately increased frequency of sex chromosome anomalies in spermatozoa generally observed in XYY males (for review Blanco *et al*., 2001; Rives *et al*., 2003).

Analysis of immature germ cells sometimes present in semen samples of XYY oligozoospermic patients can be helpful in order to answer this question when testicular germ cells are not accessible by testis biopsy. Previous works have shown that most immature ejaculated germ cells have an XYY or XY sex chromosome constitution. However, the distinction between the different types of germ cells and somatic cells was not always possible (Martini *et al*., 1996; Blanco *et al*., 1997).

In the present study, semen samples from two oligozoospermic XYY patients with high count of immature germ cells were explored. Immunocytochemistry using the proacrosin monoclonal antibody mAb 4D4 was combined (i) with multicolour FISH for assessment of germ cell chromosome constitution (Rives *et al*., 2005) and (ii) with terminal deoxynucleotidyl transferase)-mediated dUDP nick-end (TUNEL) assay for DNA fragmentation evaluation. The results provide a better understanding of meiotic disorders and maturation arrest affecting most XYY cells that have escaped the pachytene checkpoint. Furthermore, we have demonstrated how the extra Y chromosome can segregate and consequently how aneuploid spermatozoa proceeding from XYY cell line can also be produced.

Materials and methods

Patients

The two XYY patients were respectively 34 and 30 years old and consulted for primary infertility. Cytogenetic analysis of peripheral lymphocytes revealed a 47,XYY karyotype in all metaphases studied. Two semen analyses in the space of three months demonstrated an oligozoospermia with a mean sperm count of 0.8×10^6/ml (2.3×10^6/ejaculate) for patient 1 and of 1.7×10^6/ml (11.6×10^6/ejaculate) for patient 2. Round cell counts were 5.5×10^6/ml (16.5×10^6/ejaculate) for patient 1 and 0.9×10^6/ml (6.1×10^6/ejaculate) for patient 2. No leukocytes were detected when using peroxidase test in semen samples. The spermocytogram confirmed that most of these round cells were immature germ cells. Ten healthy probands of proven fertility, aged between 28 and 40 years, were included in the study as a control group for FISH and TUNEL assays of spermatozoa. Semen analysis demonstrated normal parameters according to the World Health Organization (WHO, 1999) standards. All participants gave their informed consent to participate in the study.

Pachytene cell analysis by FISH

Nuclei preparation

Semen samples were washed by centrifugation at 300 *g* for 10 min with IVF medium (Medicult®, Lyon, France). Then, the pellets were submitted to an hypotonic shock in a 0.88% KCl solution for 5 h, before fixation in methanol:acetic acid (3:1). Cells were re-suspended and spread onto glass slides stored at –20°C until use (Superfrost-Plus, Menzel-Gläser®, Menzel GmbH & Co, Braunschweig, Germany).

Probes

The different probes used during FISH procedure are indicated in Table I. Pachytenes were first analysed by three colour FISH using (i) alpha satellite centromeric probes for chromosomes 18 (CEP 18, Spectrum Orange™) and X (CEP X, Spectrum Green™); and (ii) satellite III probes for chromosomes Y (CEP Y satellite III, Spectrum Orange™, Spectrum Green™; Vysis®, Abbott, Rungis, France). Fluorescent signals were red for chromosome 18, green for chromosome X and yellow for chromosome Y. The yellow colour was obtained by mixing an equal volume of chromosome Y probes labelled with Spectrum Orange™ and Spectrum Green™. In order to evaluate more precisely sex chromosome number and distribution at pachytene stage, an other combination of probes was also chosen using alphasatellite centromeric probe for chromosome Y (CEP Y, Spectrum Orange™, Vysis®, Abbott) and a whole chromosome paint probe for chromosome X (WCP X, Spectrum Green™, Vysis®, Abbott).

Fluorescence in situ hybridization procedure and scoring

Germ cell nuclei and DNA probes were simultaneously denatured at 73°C on heated plate (Hybaid Omnigene®, Hybaid, Ashford, UK) and hybridized overnight at 37°C. After revelation, cell nuclei were counterstained with 4,6-diamidino-2-phenylindole (DAPI; Vysis®).

The slides were screened using a ×100 objective in an epifluorescence microscope (DMRD®, Leica, Germany) equipped with a triple band-pass filter [fluorescein isothiocyanate (FITC)/Rhodamine/DAPI]. Although sex chromosome pairing can only be conclusively demonstrated by synaptonemal complex analysis (Solari and Rey Valzacchi, 1997), pachytene cells were classified taking into account the distribution of hybridization signals when a Y alphasatellite centromeric probe and a whole chromosome paint probe for chromosome X were used as follows: (i) a sex vesicle (SV) with an XY chromosome constitution (XY) when one signal for the X and one signal for the Y chromosome in close contact were seen (Figure 1A); (ii) three sex chromosomes in close proximity with SV (XYY) when FISH showed three signals in close contact with condensed sex chromosomes (Figure 1D); and (iii) pachytene with an XY chromosome constitution but no sex vesicle (X+Y) when two separated signals, one for the X and one for the Y chromosomes, were detected.

Chi-square test was performed to compare the frequency of pachytenes with an XYY sex chromosome constitution between the two 47,XYY males. A value of $P < 0.05$ was considered to be significant.

Table I. DNA probes used during fluorescence *in situ* hybridization procedure according to the different categories of germ cells analysed

Cells	Probes
Pachytenes[a]	X WCP+Yαsat CEP Xαsat CEP+Y satIII CEP+18αsat CEP
Macronuclear and Interphase germ cells (mono or binucleated)	mAb 4D4 Anti-proacrosin+Xαsat CEP+Y satIII CEP+18αsat CEP
Spermatozoa	Xαsat CEP+Y satIII CEP+18αsat CEP

[a]Obtained after hypotonic treatment.
WCP, whole chromosome paint probe; CEP, chromosome enumeration probe; Sat, satellite.

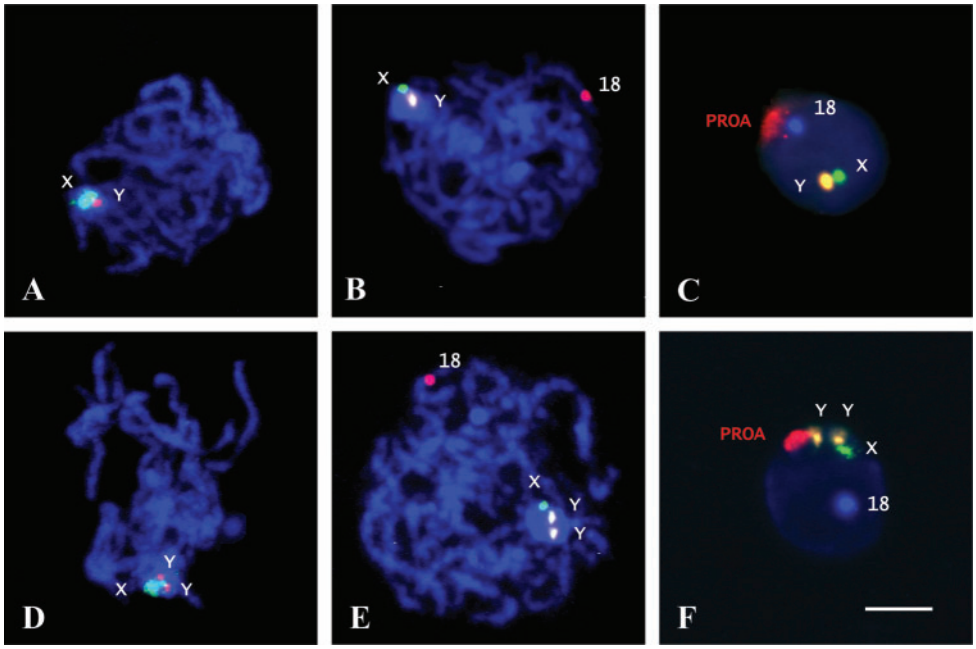

Figure 1. Multicolour FISH on pachytenes obtained after hypotonic shock of immature round germ cells present in semen samples of two XYY males. (**A**, **D**) Pachytenes showing sex chromosomes in close proximity, with one signal for the chromosome X (whole chromosome paint probe in green) and one (**A**) or two (**D**) signals for the chromosome(s) Y (satellite III probe in red). (**B**, **E**) Pachytenes with a single fluorescent signal due to synapsed centromeres for the chromosomes 18 (alphasatellite centromeric probe in red) and showing sex chromosomes in close proximity, with one signal for the chromosome X (alphasatellite centromeric probe in green) and one (**B**) or two (**E**) signals for the chromosome(s) Y (satellite III probe in yellow). (**C**, **F**) Pachytenes labelled with mAb 4D4 anti-proacrosin antibody and multicolour FISH showing a single signal for chromosomes 18 (alphasatellite centromeric probe in red) and X (alphasatellite centromeric probe in green), and one (**C**) or two (**F**) signals for the chromosome(s) Y (satellite III probe in yellow). All figure parts are printed at the same magnification (×1000). Bar = 10 µm.

Interphase germ cells analysis by FISH and proacrosin labelling

Nuclei preparation, FISH and proacrosin labelling procedure

Cells present in ejaculates were washed in phosphate-buffered saline (PBS) (0.01 M phosphate buffer, 0.0027 M KCl, 0.137 M NaCl), pre-fixed at –20°C and spread onto dry slides (Polysine, Superfrost-Plus, Menzel-Gläser®, Menzel GmbH & Co, Braunschweig, Germany).

A three-colour FISH was coupled with the immunodetection of proacrosin. Alpha satellite centromeric probes for chromosome 18 (CEP 18, Spectrum Aqua™, Vysis®) and X (CEP X, Spectrum Green™, Vysis®) as well as a satellite III probe for chromosome Y (CEP Y satellite III, Spectrum Orange™, Spectrum Green™, Vysis®, Abbott) were used. The mouse anti-human proacrosin-specific 4D4 monoclonal antibody (mAb 4D4) was a generous gift from Dr Denise Escalier (University of Paris, France). Signals were respectively blue, green and yellow for chromosomes 18, X and Y, and red for proacrosin.

Proacrosin immuno-labelling was first performed: slides were incubated at room temperature with mAb 4D4 antibody (dilution 1:100) revealed by a biotinylated anti-mouse antibody (Anti-mouse Affinity Isolated Biotin Conjugated, Sylenus®, Hauthorn, Australia). The detection was performed with Avidin Texas Red (detect B3, STAR*FISH, Cambio®, Adgenix, Voisins le Bretonneux, France). Labelled germ cells and DNA probes were simultaneously denatured at 73°C and hybridized overnight at 37°C. Cell nuclei were counterstained with 4,6-diamidino-2-phenylindole (DAPI, Vysis®).

Microscope evaluation

After detection, fluorescent signals were captured at ×1000 magnification with a digital imaging system (Mac Probe® version 3.3; Perceptive Scientific International, Chester, England).

Only germ cells presenting simultaneously hybridization signals and 4D4 immuno-staining were analysed. Diffuse fluorescent signals and overlapping nuclei were classified as ambiguous and were not included in the count. Post-meiotic germ cells with one, two or four blue spots (chromosome 18) were respectively considered as haploid, diploid and tetraploid. In haploid spermatids with a normal size (diameter <10 µm), cells with one signal for the X chromosome and one signal for the Y were classified as hyperhaploid XY. 4D4-positive cells with two nuclei were considered as the result of incomplete or absent cytokinesis following the first or second meiotic nuclear division. For these cells, cytokinesis impairment was considered to occur: (i) during the first meiotic division when the two nuclei had a different sex chromosome constitution; and (ii) during the second meiotic division when the two nuclei had a similar sex chromosome constitution.

Chi-square tests were performed to compare the frequency of each hybridization pattern between the two 47,XYY males. A value of $P < 0.05$ was considered to be significant.

Spermatozoa analysis by FISH

A three-colour FISH was performed according to a previously published protocol (Rives *et al*., 2003). Alphasatellite centromeric probes specific for chromosome 18 (CEP 18, Spectrum Orange™) and X (CEP X, Spectrum Green™) and a satellite III centromeric probe for chromosome Y (CEP Y sat III, Spectrum Orange™, Spectrum Green™, Vysis®) were used.

The slides were screened using a ×100 objective in an epifluorecence microscope (DMRD®, Leica, Reuil-Malmaison, France). An FITC/Rhodamine/DAPI triple band-pass filter set was used for the count and a single band-pass filter set (FITC) to differentiate a spermatozoon from somatic or immature germ cells. Green, red and yellow signals detected chromosomes X, 18 and Y respectively. Two

fluorescent spots of comparable size and intensity, separated by at least one spot diameter, were considered as two copies of the corresponding chromosome. Spermatozoa with diffuse fluorescent signals and overlapping nuclei were classified as ambiguous and were not included in the count. When the sperm count was sufficient, at least, 10 000 sperm nuclei were scored per chromosome.

Chi-square tests were performed to compare the frequency of each hybridization pattern obtained from 47,XYY males and controls. A value of $P < 0.05$ was considered to be significant. In the control group, the values are noted as mean ± SE.

Detection of DNA fragmentation

Fixed smears of cells present in semen samples were processed for TUNEL using a Cell Death Detection Kit (Roche Molecular Biology®, Penzberg, Germany). This method was combined with proacrosin immunodetection allowing immature germ cells analysis. After the completion of the proacrosin staining procedure described above, TUNEL labelling was carried out according to the manufacturer's instructions.

TUNEL-positive nuclei were green due to the addition of FITC-labelled dUTP to the exposed ends of multiple DNA fragments resulting from the apoptosis-induced internucleosomal DNA breakage. A counterstaining with 4,6-diamidino-2-phenylindole (DAPI, Roche Molecular Biology®) was used to visualize in blue TUNEL-negative nuclei. Acrosomal bodies labelled with anti-proacrosin monoclonal antibody were red.

Therefore, 4D4-positive cells were classified in four categories as follows: (i) large round or slightly elongated germ cells (diameter >10 μm) with one nucleus considered as spermatocytes I or macronuclear spermatids; (ii) round or slightly elongated germ cells (diameter <10 μm) with one nucleus considered as spermatids (Sa, Sb, Sc); (iii) round or slightly elongated germ cells with two nuclei, and (iv) late elongated spermatid (Sd) or spermatozoa with one nucleus.

Chi-square tests were carried out to compare the frequency of TUNEL-positive spermatozoa between XYY males and controls, and $P < 0.05$ was considered to be significant.

Results

FISH analysis of pachytenes

A total of 117 pachytenes for patient 1 and 44 pachytenes for patient 2 were first analysed after hypotonic shock with alphasatellite centromeric probes for chromosomes X and 18 and satellite III probes for chromosome Y (Figure 1B and E). A single red fluorescent signal corresponding to synapsed centromeres was always observed for chromosome 18. Due to the size of the chromosome Y probe, distinction between two Y chromosomes in close proximity was not always possible. Furthermore, the use of a centromeric probe for the chromosome X was not sufficient to evaluate the real distance between the chromosomes X and Y. Consequently, the number and distribution of sex chromosomes at pachytene stage were determined with a Y alphasatellite centromeric probe and an X whole chromosome paint probe (Figure 1A and D). Therefore, 100 pachytenes for patient 1 and 53 for patient 2 were explored (Table II). For patient 1, the frequency of XYY pachytenes (60.0%) was higher compared with the frequency of XY pachytenes (40.0%). For patient 2, an inversion of these frequencies was observed. In addition, the proportion of XYY cells at pachytene stage was significantly increased in patient 1 compared with patient 2 ($P < 0.05$). No MI or metaphase II (MII) figures were found.

Table II. Sex chromosome distribution at pachytene stage assessed by fluorescence *in situ* hybridization with a whole chromosome paint probe for the chromosome X and an alphasatellite centromeric probe for the chromosome Y

Sex chromosome distribution and association	Patient 1 [*n* (*f* %)]	Patient 2 [*n* (*f* %)]
XYY		
One X and two Y chromosomes in close proximity with a sex vesicle X chromosome partially or totally condensed	60 (60.0)	21 (39.6)
XY		
One X and one Y chromosomes in close proximity with a sex vesicle	40 (40.0)	29 (54.7)
X+Y		
One X and one Y chromosomes independent without sex vesicle	0 (0.0)	3 (5.7)
Total	100	53

n, Number of nuclei; *f*, frequency.

FISH analysis of anti-proacrosin-positive immature germ cells

During spermatogenesis, proacrosin appears in the Golgi complex as early as the mid-pachytene stage. Therefore, its detection allows the identification of immature germ cells initiating meiosis. Moreover, the number, position and shape of anti-proacrosin-labelled bodies change during spermatogenesis and are consequently useful for the identification of germ cells maturation stages (Escalier *et al*., 1991).

At late pachytene and spermatocyte II stages as well as during meiotic divisions, germ cells present two 4D4-labelled bodies due to the partition of the Golgi complex. In 4D4-positive cells, the most frequently encountered are primary spermatocytes (diameter >10 μm) and spermatids (diameter <10 μm). They present a single body labelled with anti-proacrosin and they can, normally, be distinguished according to their respective nuclear size (Mendoza *et al*., 1996). Nevertheless, due to the presence of macronuclear spermatids in XYY semen samples, this distinction was not always possible in the present study. Hybridization patterns obtained for pachytenes after hypotonic shock were helpful to eliminate the macronuclear cells from the final count that could be primary spermatocytes (with one signal for the chromosome 18 and one signal for the chromosome X in close proximity with one or two signals for the Y; Figure 1C and F). Other 4D4-positive cells were classified as normal (diameter <10 μm) or macronuclear (diameter >10 μm) spermatids.

A total of 653 mAb 4D4-positive cells for patient 1 and 343 for patient 2 were scored (Table III). Cells unlabelled with anti-proacrosin could be somatic or premeiotic germ cells. They were not taken into account. The identification of macronuclear germ cells was uncertain. They could be either 24,XY or 25,XYY giant hyperhaploid spermatids or pachytenes. Therefore, they were also not taken into account and pachytene configurations were determined only after hypotonic shock.

Illustrations of some mAb 4D4-positive cells encountered are shown in Figure 2. Amongst interphase germ cells with one nucleus (Table III), the proportion of spermatids with a normal

Table III. Sex chromosome constitution and ploidy determined by three-colour fluorescence *in situ* hybridization on immature germ cells labelled with 4D4 anti-proacrosin monoclonal antibody

Chromosome constitution		Patient 1			Patient 2		
		n	*f* (%)	*f′* (%)	*n*	*f* (%)	*f′* (%)
XY or XYY Pachytenes (or macronuclear haploid spermatids)*							
Total (T1)		175	100.0		30	100.0	
Interphase germ cells (one nucleus)							
Haploid <10 μm	23,Y	30	7.0[a]	22.2[a′]	71	23.4[a]	40.1[a′]
	23,X	8	1.9[b]	5.9[b′]	8	2.6[b]	4.5[b′]
	24,YY	3	0.7	2.2	4	1.3	2.3
	24,XY	94	22.0[c]	69.6[c′]	94	30.9[c]	53.1[c′]
	Total (t)	135	31.5[d]	100.0	177	58.2[d]	100.0
Diploid	46,YY	2	0.5	1.1	7	2.3	6.9
	46,XY	16	3.7[e]	8.6[e′]	22	7.2[e]	21.8[e′]
	47,XYY	160	37.4[f]	86.5[f′]	62	20.4[f]	61.4[f′]
	48,XXYY	7	1.6[g]	3.8[g′]	10	3.3[g]	9.9[g′]
	Total (t′)	185	43.2[h]	100.0	101	33.2[h]	100.0
Tetraploid	94,XXYYYY	71	16.6[i]	100.0	9	3.0[i]	100.0
Others**		37	8.6[j]		17	5.6[j]	
Total (T2)		428	100.0		304	100.0	
Germ cells with incomplete cytokinesis (two nuclei)							
Haploid ×2	(23,Y)2	4	8.0		0		
	(23,X)(23,Y)	2	4.0		0		
	(24,XY)(23,Y)	19	38.0[k]		9	100.0[k]	
	(24,XY)2	7	14.0		0		
Diploid ×2	(47,XYY)2	18	36.0		0		
Total (T3)		50	100.0		9	100.0	
Total		653			343		

a–d–f–f′–I, $P < 0.0001$.
a′–k, $P < 0.001$.
c–c′–e′–h, $P < 0.01$.
e–g′, $P < 0.05$.
b–b′–g–j, Not significant.
n, Number of nuclei; *f*, *n*/T1 or *n*/T2 or *n*/T3 (%) ; *f′*, *n*/t or *n*/t′ (%).
*Macronuclear germ cells (>10 μm) with one signal for X and 18 chromosomes and one or two signals for Y chromosome.
**Different interphase germ cells (one nucleus) with a chromosome constitution very rarely observed in the two patients, representing less than 1% of proacrosin positive cells for the two patients. They mostly correspond to tetra or diploid cells with various non-distinguishable signals.

chromosome constitution (23,X or 23,Y) was significantly lower in patient 1 compared with patient 2 (8.9 versus 26.0% respectively, $P < 0.0001$). Sex ratios (X/Y) were 0.27 and 0.11, for patients 1 and 2 respectively, with an over-representation of 23,Y spermatids. These values differ significantly from the expected 1:1 sex ratio ($P < 0.001$). Most of mononucleated interphase germ cells had an abnormal chromosome constitution with a cumulative frequency of 91% for patient 1 and 74% for patient 2. The most frequent chromosome constitutions observed were 47,XYY for patient 1 (37.4%) and 24,XY (30.9%) for patient 2. The incidence of hyperhaploid XY spermatids was significantly lower for patient 1 (22.0 versus 30.9%, $P < 0.01$). A majority of diploid 4D4-positive cells were 47,XYY (86.5% for patient 1 and 61.4% for patient 2, $P < 0.0001$). Most of these cells were macronuclear (diameter >10 μm) (62.5 and 61.3% respectively for patients 1 and 2). Tetraploid cells were more frequently observed in patient 1 (16.6 versus 3.0%, $P < 0.0001$). Furthermore, 4D4-positive labelled bodies with a morphologically disturbed shape (large, irregular or with a strongly labelled cap) accounted for (i) 83.1 and 33.3% of tetraploid cells; (ii) 88.1 and 31.7% of diploid cells, as well as (iii) 81.5 and 44.1% of haploid cells for patients 1 and 2 respectively.

A total of 50 (patient 1) and 9 (patient 2) binucleated cells were explored. For patients 1 and 2, (24,XY)(23,Y) cells originated from XYY spermatocytes I that had completed the first meiotic nuclear division with an absence of cytokinesis. For patient 1: (i) (24,X)(23,Y) cells originated from XY spermatocyte I; and (ii) (47,XYY)×2, (24,XY)×2 and (23,Y)×2 cells proceeded respectively from 47,XYY, 24,XY and 23,Y spermatocytes II. Cells with cytokinesis failure carried one large (92%) or two opposite small (8%) 4D4-labelled bodies.

Secondary spermatocytes (10 μm diameter cells with two 4D4-positive bodies) were rarely detected in XYY semen samples. For patient 1, one 47,XYY, and for patient 2, one 47,XYY and one 24,XY spermatocytes II were observed.

FISH sperm analysis

A total number of 100 795 sperm nuclei were scored for control group, 2971 and 21 424 for patients 1 and 2 respectively. Summaries of the results obtained are shown in Table IV. The ratio of X- to Y-bearing spermatozoa did not significantly differ from 1:1 for patient 1 (0.997) but was decreased for patient 2 (0.942, $P < 0.0001$). For the two XYY males, the incidence of hyperhaploid XY, disomic Y and 18 as well as diploid sperm nuclei was significantly increased when compared with controls.

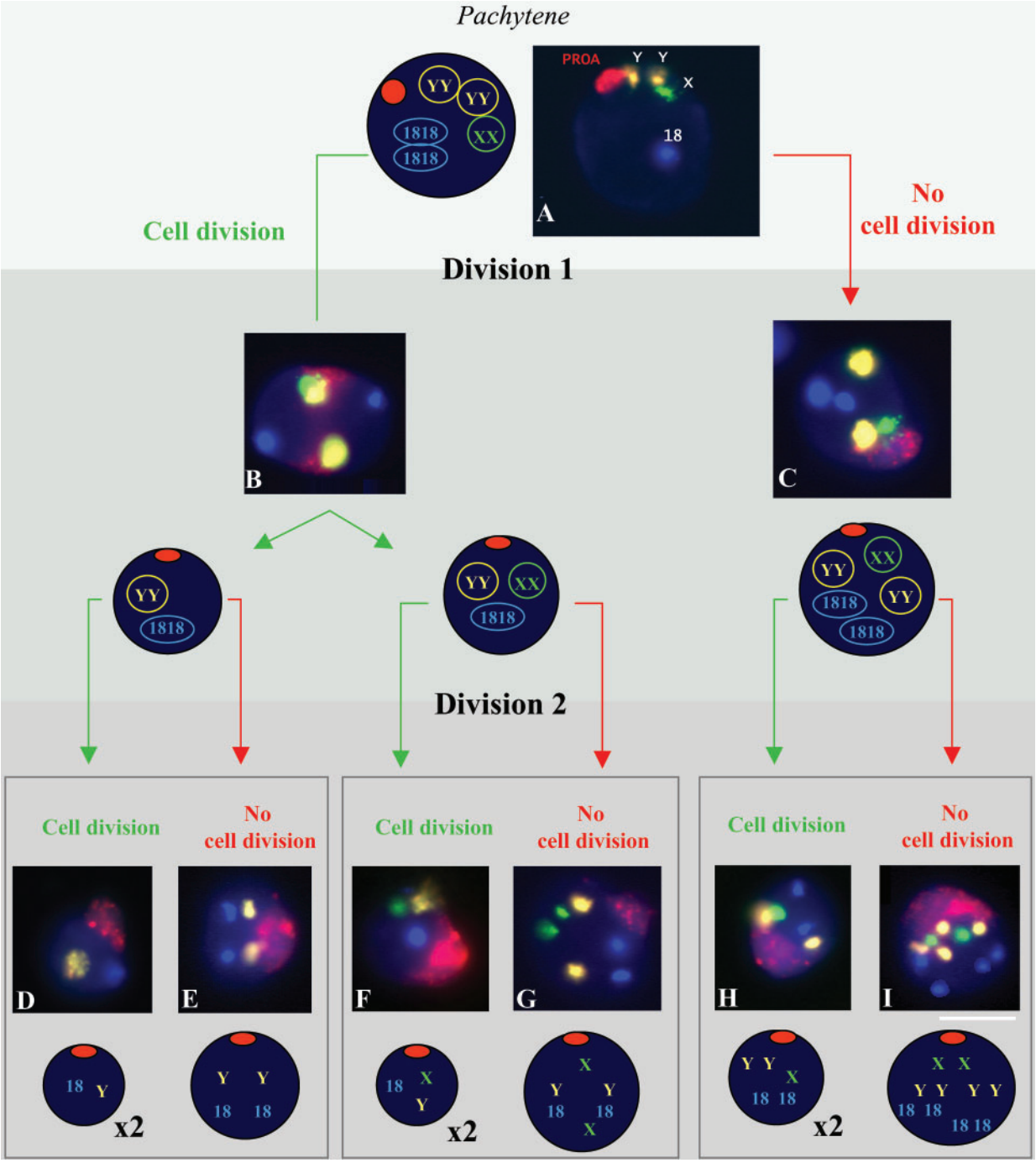

Figure 2. Progression of XYY cells through meiosis. Pachytenes with an XYY trivalent configuration can enter into meiosis but homologous chromosomes segregation (division 1) and sister chromatids separation (division 2) are not always followed by nuclear and cytoplasmic divisions. Red arrows indicate an absence of nuclear division and cytokinesis after chromosomes separation. Green arrows indicate that complete nuclear and cytoplasmic divisions occur. (**A**) XYY pachytene. (**B**) Incomplete cytokinesis during first meiotic division. (**C**) 47,XYY macronuclear hyperdiploid germ cell that probably corresponds to a spermatocyte II with unpartitioned Golgi complex (a single mAb 4D4-labelled body). (**D**) 23,Y spermatid. (**E**) 46,YY diploid spermatid. (**F**) 24,XY hyperhaploid spermatid. (**G**) 48,XXYY hyperdiploid spermatid. (**H**) 47,XYY hyperdiploid spermatid. (**I**) 94,XXYYYY macronuclear hypertetraploid spermatid. All figure parts are printed at the same magnification (×1000). Bar = 10 µm.

TUNEL analysis of anti-proacrosin-positive germ cells

For both XYY patients, very high levels of DNA fragmentation were observed in 4D4-positive immature germ cells (Figure 3, Table V). The frequencies of apoptotic sperm nuclei were increased in XYY males when compared to controls (14.7% for patient 1, and 15.6% for patient 2 versus 4.02 ± 0.41% in controls).

Discussion

In this study, we showed how some XYY germ cells that have escaped the pachytene and spindle-assembly checkpoints can progress through meiosis.

It is well established that the pachytene checkpoint prevents progression of germ cells through the meiotic prophase I, when

Table IV. Incidence of chromosome anomalies in ejaculated spermatozoa from two XYY patients and 10 control subjects assessed by three-colour fluorescence *in situ* hybridization with alphasatellite centromeric probes for chromosomes X, Y and 18

Presumed karyotype	Patient 1 percentage of sperm	Patient 2 percentage of sperm	Control group percentage of sperm
23,X	48.56[a]	47.66[a′]	49.70 ± 0.16[a″]
23,Y	48.69[b]	50.59[b′]	49.77 ± 0.12[b″]
24,XY	0.60[c]	0.22[c′]	0.14 ± 0.01[c″]
24,XX	0.13[d]	0.1[d′]	0.07 ± 0.01[d″]
24,YY	0.20[e]	0.17[e′]	0.08 ± 0.01[e″]
46,XY	0.23[f]	0.14[f′]	0.03 ± 0.005[f″]
46,XX	0.30[g]	0.06[g′]	0.006 ± 0.002[g″]
46,YY	0.36[h]	0.03[h′]	0.008 ± 0.004[h″]
24,X/Y+18	0.09[i]	0.24[i′]	0.033 ± 0.004[i″]

a′a″–cc″–e′e″–ff″–f′f″–gg″–g′g″–hh″–i′i″, $P < 0.0001$.
b′b″–c′c″, $P < 0.01$.
ee″–h′h′–ii″, $P < 0.05$.
aa′–bb″–dd″–d′d″, Not significant.

recombination and chromosome synapsis are incomplete (Roeder and Bailis, 2000). In the special case of the sex bivalent in males, the pseudoautosomal region (PAR) asynapsis triggers the checkpoint. Consequently, in XYY males, it is possible that only pachytene spermatocytes that have achieved full PAR synapsis in a trivalent configuration are compatible with the progress of meiosis (Miklos, 1974; Rodriguez and Burgoyne, 2000, 2001; Blanco *et al*., 2001; Rives *et al*., 2003). In the present study, all XYY pachytenes analysed by FISH had three signals for sex chromosomes in close proximity and therefore a probable XYY trivalent configuration (Table II). In fact, other sex chromosome configurations—one or three univalents—were not observed. Spermatocytes I with such chromosome configurations have probably been produced but rapidly eliminated at the testicular level. Our two patients present simultaneously pachytenes in probable XYY trivalent configuration and post-reductional germ cells of the XYY line. These results are in accordance with the hypothesis that in XYY males, only pachytenes with three synapsed sex chromosomes can achieve meiosis (Rodriguez and Burgoyne, 2000, 2001). Furthermore, in our patients, sex chromosomes were always partially or totally condensed (Figure 1D). Sex chromosome condensation in the sex body has been considered as a morphological manifestation of meiotic sex chromosome inactivation (MSCI) (for reviews, see McKee and Handel, 1993; Solari, 1994). In normal meiosis, MSCI may allow to mask the non-synapsed regions of sex chromosomes, in order to avoid the activation of pachytene checkpoint (Jablonka and Lamb, 1988; Odorisio *et al*., 1998). Therefore, not only the association of the three sex chromosomes but also their condensation and the formation of a 'XYY-body' may be essential to allow XYY spermatocytes I to circumvent meiotic arrest (Rodriguez and Burgoyne, 2001; Rives *et al*., 2005).

XYY spermatocytes escaping the pachytene stage lead to a heterogeneous population of immature germ cells, according to our FISH analysis of proacrosin positive germ cells (Figure 2). Spermatogonia and somatic cells do not express proacrosin and are not labelled with 4D4 antibody. Consequently macronuclear cells with a presumed 47,XYY constitution and labelled

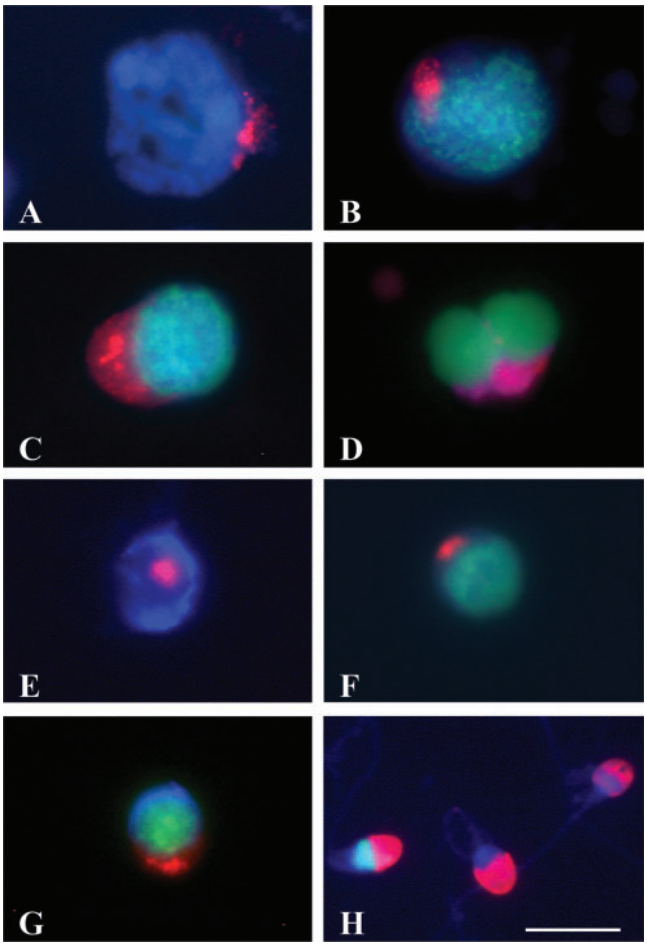

Figure 3. Apoptotic (green and diffuse fluorescent signal in the nucleus due to TUNEL reaction) and healthy (blue florescent signal due to DAPI counterstain) immature germ cells from XYY patients. Immunofluorescence with the germline marker 4D4 antibody gives a red signal in the acrosomal body. (**A**) Healthy pachytene. (**B**) Apoptotic pachytene (or giant round spermatid). (**C**) Apoptotic giant spermatid at the acrosomal step. (**D**) Apoptotic cell with incomplete cytokinesis. (**E**) Healthy round spermatid. (**F**) Apoptotic round spermatid. (**G**) Apoptotic elongating spermatid. (**E**) One apoptotic and two healthy spermatozoa. All figure parts are printed at the same magnification (×1000). Bar = 10 μm.

with 4D4 antibody were considered as germ cells that had, at least, performed the separation of homologous chromosomes during meiosis I, with an impairment of nuclear and cytoplasmic divisions. Furthermore, the presence, in patient 1, of immature germ cells presenting two 47,XYY nuclei indicate that some of these macronuclear cells may enter and complete the second meiotic division. Thus, smallest 47,XYY immature germ cells (diameter <10 μm) (Figure 2H) may originate from 47,XYY macronuclear germ cells able to achieve the second meiotic division and correspond certainly to spermatids. 47,XYY macronuclear cells were possibly equivalent to spermatocytes II, insofar as homologous chromosomes have been separated. Nevertheless, two 4D4-labelled bodies were very rarely seen in these cells. At spermatocyte I and II stages, proacrosin is normally found in the Golgi complex (Escalier *et al*., 1991). Consequently, the presence of a single 4D4-labelled body generally observed in 47,XYY macronuclear

Table V. Occurrence of apoptosis DNA fragmentation amongst germ cells labelled with 4D4 anti-proacrosin monoclonal antibody from XYY patients

		Germ cells with DNA fragmentation					
		Patient 1			Patient 2		
Nucleus features	Possible identification	*n*	*t*	*f* (%)	*n′*	*t′*	*f′* (%)
Large and round or slightly elongated (diameter>10 μm)	Spermatocyte I or macronuclear spermatid	93	104	89.4	174	192	90.6
Round or slightly elongated (diameter<10 μm)	Spermatid (Sa, Sb, Sc)	163	168	97	82	3	98.8
Binucleated cells	Incomplete cytokinesis after meiotic division I or II	40	40	100	10	10	100.0
Elongated with a flagellum	Late elongated spermatid (Sd) or spermatozoon	28	191	14.7	183	1173	15.6
Total		296	312	94.9	266	285	93.3

n, Number of apoptotic cells for patient 1; *t*, total number of cells explored for patient 1; *f*, *n*/*t* (%); *n′*, number of apoptotic cells for patient 2; *t′*, total number of cells explored for patient 2; *f′* : *n′*/*t′* (%).

cells and also in figures of incomplete cytokinesis (with only one signal for the two nuclei) certainly traduces a failure of Golgi partition and displacement (Figure 3D). Nuclear and cytoplasmic division impairments, Golgi partition failure as well as irregular size and shape of 4D4-labelled body observed in the present study are comparable to anomalies previously described in meiotic division deficiency (MDD) syndromes (Escalier, 2002). In MDD cells, a blockage of organelle displacement at the pachytene stage disables the assembly of a bipolar meiotic spindle. MDD spermatocytes escape the pachytene and spindle-assembly checkpoints, but anaphase is incomplete and telophase absent. This process gives rise to macronuclear spermatids as observed in our XYY males (Escalier, 2002) or tetraploid spermatozoa (Escalier, 1983; Pieters *et al*., 1998).

Tetraploid cells with a 92,XXYY chromosome constitution have already been observed in semen samples of XYY males (Han *et al*., 1994; Chevret *et al*., 1997). However, tetraploid spermatids with four Y chromosomes (94,XXYYYY presumed karyotype) were, to our knowledge, for the first time described in the present study (Figure 2I). They can only proceed from XYY spermatocytes I that have undergone homologous chromosome and sister chromatid separations with an absence of nuclear and cytoplasmic divisions leading first to a 47,XYY spermatocyte II and then to a 94,XXYYYY spermatid.

However, meiotic disorders did not affect all the XYY germ cell line. Even if 24,XY hyperhaploid spermatids can, *a priori*, proceed from non-disjunctions in the XY germ cell line during meiosis I, they can also originate from the XYY line achieving two normal meiotic divisions. In our patients, among haploid interphase germ cells (<10 μm, Table II), 24,XY hyperhaploid cells were the most frequently observed (69.6% for patient 1, 53.1% for patient 2; Figure 2F). The presence of (24,XY)(23,Y) binucleated germ cells with incomplete cytokinesis (Figure 2B) indicate that in XYY spermatocytes I, homologous chromosome segregation can occur and be followed by nuclear separation. In the situation of a normal cytokinesis, (24,XY)(23,Y) germ cells may lead to 23,Y and 24,XY spermatocytes II that can undergo the meiosis II with (complete meiosis) or without (incomplete meiosis) nuclear and cytoplasmic divisions. In our two patients (i) complete meiosis II produced 23,Y haploid (Figure 2D) and 24,XY hyperhaploid (Figure 2F) spermatids resulting respectively from 23,Y and 24,XY spermatocytes II and (ii) incomplete meiosis II produced 46,YY diploid (Figure 2E) and 48,XXYY hyperdiploid (Figure 2G) spermatids resulting respectively from 23,Y and 24,XY spermatocytes II.

Binucleated germ cells resulting from abnormal cytokinesis during meiosis I can help to improve understanding of sex chromosome segregation in the XYY line. For patients 1 and 2, (24,XY)(23,Y) cells obtained after a nuclear division with separation of the two Y chromosomes represented respectively 90.5 and 100.0% of cells with incomplete first meiotic cytokinesis. In contrast, (24,YY)(23,X) germ cells, without segregation of the two Y chromosomes, were never observed. Furthermore, amongst haploid spermatids, 24,YY (2.2%) and 23,X (5.1%) cells were very exceptional compared to 24,XY (60.3%) and 23,Y (32.4%) spermatids. The cumulative frequencies of 24,YY+23,X spermatids (7.3%) and 24,XY+23,Y (92.7%) spermatids differ significantly ($P < 0.00001$) from the expected distribution (respectively 1/3 and 2/3) resulting from a random segregation of the three sex chromosomes during division I. These results are in agreement with a preferential separation and segregation of the Y chromosomes when the first meiotic division occurred normally in the XYY cell line (Solari and Rey Valzacchi, 1997).

In the light of these data, we can consider that increased frequencies of 23,Y (patient 2), 24,XY (patient 1 and 2), 24,YY (patient 1 and 2) and 46,YY (patient 1 and 2) spermatozoa may, for a part, result from the completion of spermatogenesis by some XYY germ cells. Moreover, elevated rates of disomic 18, diploid XX and XY sperm nuclei also suggest meiotic errors in the XY germ cell line, probably induced by a compromised environment (Mroz *et al*., 1998). Consequently, we can presume that aneuploid post-meiotic cells originate from both XYY and XY cell line as previously suggested for other XYY patients (Mercier *et al*., 1996; Chevret *et al*., 1997; Rives *et al*., 2005).

The over-representation of XYY germ cells and their subsequent elimination result in low sperm counts. In particular, the

Table VI. Relation between the sperm count and the frequency of XYY pachytenes in XYY males

	Sperm count ($\times 10^6$/ml)	XYY pachytenes (%)	Sex chromosome distribution in XYY pachytenes (*n*)			
			XYY	YY+X	XY+Y	X+Y+Y
Gabriel-Robez *et al*., 1996[a]	Normal	0				
Speed *et al*.,1991[a]	5.0	30		13	1	2
Blanco *et al*., 2001[b]	Oligozoospermia	58	20	13		
Blanco *et al*., 1997[b]	3.0	61		20		
Rives *et al*., 2005[b]	2.0	64	13	28	6	30
Gabriel-Robez *et al*., 1996[a]	<1.0	83	1	2	2	
Solari and Valzacchi, 1997[a]	0.0006	100	7	64		3

n, Number of pachytenes observed.
[a]Sex chromosome pairing configuration determined after electron microscopy studies of synaptonemal complexes.
[b]Sex chromosome distribution determined by fluorescence *in situ* hybridization using probes for chromosomes X and Y.

proportion of XYY pachytenes present in testis is negatively correlated with the concentration of spermatozoa in ejaculates (Table VI). This tendency was verified between our two XYY males: patient 1 presented higher percentage of XYY pachytenes (60 versus 40%) and lower sperm count (0.75 versus 1.7×10^6/ml) compared with patient 2. Results of DNA fragmentation analysis were in accordance with previous studies showing that in XYY men, most abnormal cells that have escaped the pachytene checkpoint are then continuously eliminated along spermatogenesis (Solari and Rey Valzacchi, 1997; Blanco *et al*., 2001; Rives *et al*., 2005). Thus, increased incidence of aneuploid and diploid spermatozoa remained moderate despite the high rate of immature germ cells with chromosomal abnormalities. In particular, 47,XYY diploid and 94,XXYYYY tetraploid chromosome constitutions, largely represented in proacrosin positive round cells and proceeding from XYY cell line, were not observed in mature spermatozoa. We can presume that most of these cells never ended spermiogenesis and were eliminated via apoptosis in our two XYY males. The presence of spermatozoa with fragmented DNA also agrees with a probable elimination of aneuploid spermatozoa via apoptosis, as suggested previously in infertile males with a normal or abnormal karyotype (Schmid *et al*., 2003; Baccetti *et al*., 2005). However, a direct evaluation of aneuploidy in apoptotic spermatozoa was not performed, as in previous studies.

In conclusion, our results revealed that in oligozoospermic XYY males, XYY germ cells initiating meiosis generally had compromised spermatogenesis due to nuclear and cytoplasmic division failure during meiosis I and/or II. These cells were eliminated via apoptosis and never led to aneuploid spermatozoa. An exception may be the case of 46,YY spermatozoa that could proceed from XYY cells with a normal first meiotic division but an abnormal second meiotic division. For few XYY cells, meiosis occurred normally with a preferential segregation of the two Y chromosomes during division I, resulting in the production of 23,Y and 24,XY spermatids. Most of these cells were eliminated via apoptosis but some of them probably gave rise to 23,Y and 24,XY spermatozoa. To a lesser extent, the increased frequency of 24,YY spermatozoa could be due, in part, to a normal meiotic process of XYY germ cells without segregation of the two Y chromosomes. The other sperm chromosome abnormalities could only be explained by segregation errors in XY germ cells. Our data are in agreement with the persistence of the extra Y chromosome during meiosis in XYY oligozoospermic males responsible for spermatogenesis impairment and a probable elimination via apoptosis of most XYY germ cells not solely during but also after meiosis.

Acknowledgements

The authors thank Richard Medeiros, Rouen University Hospital Medical Editor, for his valuable editorial assistance.

Authors' confirmation

The authors confirm that all experiments performed in this study comply with the current French legislation.

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Submitted on December 21, 2005; resubmitted on January 26, 2006; accepted on January 27, 2006