

Detection of Aneuploid Human Sperm by Fluorescence In Situ Hybridization: Evidence for a Donor Difference in Frequency of Sperm Disomic for Chromosomes 1 and Y

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Summary

Fluorescence in situ hybridization with repetitive-sequence DNA probes was used to detect human sperm disomic for chromosomes 1 and Y in three healthy men. Data on these same men had been obtained previously, using the human-sperm/hamster-egg cytogenetic technique, providing a cytogenetic reference for validating sperm hybridization measurements. Air-dried smears were prepared from semen samples and treated with DTT and lithium diiodosalicylate to expand sperm chromatin. Hybridization with fluorescently tagged DNA probes for chromosomes 1 (pUC177) or Y (pY3.4) yielded average frequencies of sperm with two fluorescent domains of $14.2 \pm 2.4/10,000$ and $5.6 \pm 1.6/10,000$ sperm, respectively. These frequencies did not differ statistically from frequencies of hyperploidy observed for these chromosomes with the hamster technique. In addition, frequencies of disomic sperm from one donor were elevated ~ 2.5 -fold above those of other donors, for both chromosomes 1 ($P = .045$) and Y ($P = .01$), consistent with a trend found with the hamster technique. We conclude that fluorescence in situ hybridization to sperm chromosomes provides a valid and promising measure of the frequency of disomic human sperm.

Introduction

Aneuploidy is one of the most common and serious chromosomal abnormalities recognized in man. It is responsible for a large portion of human morbidity and mortality, including infertility, pregnancy loss, infant death, congenital malformations, mental retardation, and behavioral abnormalities (Hook 1985; Epstein 1986; Hecht and Hecht 1987). Most of the human aneuploidy load is considered to be of germ-cell origin arising from errors in maternal and paternal meiotic chromosomal segregation (Bond and Chandley 1983; Bond 1987). The ability to identify and screen human sperm for aneuploidy would lead to understanding of

the factors causing this chromosomal abnormality as well as possible prevention strategies.

Over the past 25 years, various histochemical and physical methods have been proposed for detecting sperm carrying chromosomal abnormalities. Barlow and Vosa (1970) reported that staining with quinacrine or quinacrine mustard yielded a domain of bright fluorescence in human sperm chromatin (also known as "F body" and "Y body") believed to represent the distal end of the long arm of the Y chromosome. Sperm with two fluorescent bodies were, therefore, thought to be sperm disomic for the Y chromosome. Kapp et al. (1979) found an elevated frequency of sperm with two F bodies in men occupationally exposed to dibromochloropropane. However, quinacrine staining (1) typically yielded underestimates of the 50% expected frequency for the Y chromosome in mature sperm (Barlow and Vosa 1970; Wyrobek et al. 1983) and (2) overestimated the frequency of sperm with two fluorescent bodies when compared with results from both nuclear mass measurements (Sumner and Robinson 1976) and the human-sperm/hamster-egg cytogenetic technique

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(Wyrobek et al. 1984; Brandriff and Gordon 1990; Martin and Rademaker 1990). Other histochemical techniques for detecting chromosomal abnormalities in sperm, such as staining secondary constriction regions of chromosomes 1 and 9 (Bobrow et al. 1972; Pearson 1972), had similar limitations. Flow-cytometric methods based on DNA content of sperm proved to be effective for distinguishing between Y- and X-bearing sperm in mice and other species (Meistrich et al. 1979; Pinkel et al. 1982; Johnson et al. 1989) but were inefficient for detecting small subpopulations of chromosomally abnormal sperm induced by testicular X-irradiation in mice (Pinkel et al. 1983).

To date, the human-sperm/hamster-egg cytogenetic technique (hamster technique) developed by Rudak et al. (1978) and now in use in several laboratories (e.g., see Brandriff and Gordon 1990; Martin and Rademaker 1990) is the only established method for the direct analysis of human sperm chromosomes. It is the "gold standard" against which any new sperm cytogenetic method must be evaluated. The hamster technique has provided normative data for numerical and structural abnormalities involving all chromosomes (Brandriff and Gordon 1990; Martin and Rademaker 1990). In addition, this method has been used to characterize disjunctive patterns in translocation carriers (Brandriff et al. 1986a; Martin et al. 1990), effects of age (Martin and Rademaker 1987), and effects of radiation and drug therapy on the frequencies of chromosomal abnormalities in human sperm (Martin et al. 1986; Jenderny and Roehrborn 1987; Brandriff et al. 1988; Genesca et al. 1990). However, the large effort required to obtain sperm metaphases has hampered widespread application and generalized utility of this technique in exposed populations.

In situ hybridization with chromosome-specific DNA probes has proved useful for detecting chromosome abnormalities in lymphocytes (Eastmond and Pinkel 1990) and offers a new approach for detecting chromosomally abnormal sperm. However, previous attempts to apply hybridization to ejaculated and testicular sperm and sperm nuclei, using DNA probes specific for chromosomes 1 and Y, were not completely satisfactory (Joseph et al. 1984; Guttenbach and Schmid 1990, 1991; Pieters et al. 1990; Coonen et al. 1991). Although hybridizations using the Y chromosome identified Y-bearing sperm with the expected frequency of ~50%, frequencies of sperm with two fluorescent domains far exceeded what would be expected when compared with aneuploidy data obtained either

by using the hamster technique or from live births. This was also the case for hybridizations using probes for chromosome 1. In addition, there has been no consensus among authors regarding the necessity of any decondensing pretreatment of sperm. Thus, lack of objective internal standards, variable hybridization efficiencies, and high baseline frequencies of cells with two fluorescent domains raised questions about the validity of these hybridization-based procedures for detecting disomy in human sperm.

Previously, we developed a method to expand the chromatin of sperm nuclei in suspension (Wyrobek et al. 1990), which resulted in high hybridization efficiency with repetitive-sequence probes. In the current study, we modified this method to use air-dried smears, which greatly simplified the pretreatment procedure. The purpose of the current study was to evaluate the method of fluorescence in situ hybridization for the detection of sperm aneuploidy, using the hamster technique as a reference measurement. Semen was obtained from 3 of 24 subjects for whom sperm cytogenetic data had been previously obtained by the hamster technique, and ~57,000 sperm cells were analyzed by fluorescence in situ hybridization. We found that the hamster technique and hybridization method agreed very closely. Both methods detected differential frequencies of sperm disomic for chromosomes 1 and Y and pointed to one donor with increased disomy frequencies for these chromosomes.

Material and Methods

Semen Samples

Study participants were from a larger cohort of anonymous healthy volunteers enrolled in an ongoing semen donor program at Lawrence Livermore National Laboratory. The three donors selected for this study had previous sperm cytogenetic analyses performed using the hamster technique, as summarized by Brandriff and Gordon (1990). Two to six semen samples from each of the three donors were provided in clean plastic containers (Baxter Healthcare Corporation, Valencia, CA). Semen was liquified at room temperature, mixed by gentle pipetting, aliquoted into 2-ml freezing vials, and frozen for as long as 15 mo at -20°C before being analyzed. No effort was made to utilize conditions that maintain viability. Donors had normal ranges of sperm shape abnormalities, and sperm concentrations ranged from $\sim 80 \times 10^6/\text{ml}$ to $140 \times 10^6/\text{ml}$.

Preparation of Sperm for In Situ Hybridization

Semen samples were thawed at room temperature, after which aliquots of $\sim 7 \mu\text{l}$ were spread into thin smears on clean glass microscope slides and allowed to air-dry. Smears were stored at room temperature for at least 24 h (and as long as 12 wk) prior to sperm decondensation. Decondensation was accomplished using the method of Wyrobek et al. (1990), with the following modification: Smears were incubated in 10 mM DTT (Sigma Chemical, St. Louis) in Coplin jars on ice for 30 min, followed by incubation at room temperature in 4 mM lithium diiodosalicylate (LIS; Sigma Chemical) for 90 min. Slides were allowed to air-dry and then were taken immediately into the hybridization procedure or stored under nitrogen at -20°C until hybridized.

Probe Preparation and Fluorescence In Situ Hybridization

Two probes were used: pY3.4, derived from a 3.4-kb fragment of a highly repeated sequence on the distal arm of Yq (Smith et al. 1987), and pUC177, a 1.77-kb cloned *EcoRI* fragment of human satellite III DNA specific to chromosome 1 (Cooke and Hindley 1979). Probes were labeled with biotin, using nick-translation. Hybridizations were performed as described by Wyrobek et al. (1990), with the following modifications: 20 ng of chromosome-specific repetitive probe was used per slide; slides were air-dried after the ethanol dehydration series; posthybridization washes were done twice for 10 min each at 45°C in 50% formamide, $2 \times \text{SSC}$, pH 7.0 (SSC = 0.15 M NaCl, 0.015 M sodium citrate), followed by two 15-min room-temperature washes in PN buffer (0.1 M NaH_2PO_4 , 0.1 M Na_2HPO_4 , pH 8.0, 0.1% Nonidet P-40 [Sigma Chemical]).

Scoring and Analysis of Hybridized Sperm

A Zeiss photofluorescent microscope III with Zeiss filter combination 487709, which allowed simultaneous visualization of red (chromatin) and green (fluorescent domain), was used to identify and score the proportion of sperm with zero, one, or two fluorescent domains. A sperm cell was scored as having two fluorescent domains if it contained two well-delineated fluorescent spots separated by $\sim 1/2$ domain diameter or more (fig. 1). At least 5,000 sperm were scored from each semen sample for each probe. To protect against bias in scoring, scorers were blinded with respect to identity of the specimen. Tests for statistical signifi-

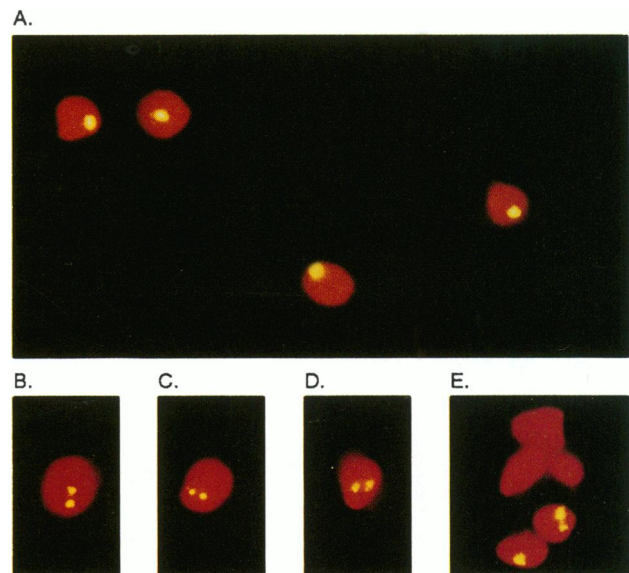


Figure 1 Fluorescence in situ hybridization of human sperm on air-dried smears, using probes for chromosome-specific repetitive DNA sequences. A, Field of sperm cells hybridized with FITC-labeled pUC177 and counterstained with propidium iodide. Each cell contains a single fluorescent domain. B and C, Sperm cells hybridized with pUC177, demonstrating two fluorescent domains. D, Sperm cell hybridized with pY3.4, demonstrating two fluorescent domains. E, Field of sperm cells hybridized with pY3.4, demonstrating cells with zero, one, or two fluorescent domains.

cance were done, using Cochran's test for equal proportions (Snedecor and Cochran 1967).

Results

Pretreatment of sperm and fluorescence in situ hybridization using probes pUC177 or pY3.4 resulted in reliable detection of sperm carrying zero, one, or two compact hybridization domains, as illustrated in figure 1. Four semen samples from one donor (donor A) were selected to investigate sources of variation in scoring: three specimens were provided at ~ 1 -mo intervals, and the fourth was provided at ~ 1 year prior to the other three (table 1). The overall percent of cells showing one hybridization signal, using probe pY3.4, did not differ from the expected 50% ($P = .7$). Also, there was no significant sample-to-sample variation in frequency of cells with either one ($P = .99$) or two fluorescent domains ($P = .99$). These experiments indicate that (1) specimens stored frozen as long as 1 year were hybridized reliably and (2) there was no statistically significant within-donor variation over that time period.

Table 1

Sample-to-Sample Variation in Frequencies of Sperm with Fluorescent Domains for Donor A Using Probe pY3.4

COLLECTION DATE OF SEMEN SAMPLE	NO. OF SPERM SCORED	NO. OF FLUORESCENT DOMAINS PER SPERM CELL			FREQUENCY OF VARIANT SPERM ^a (per 10,000 sperm)
		Zero	One	Two	
April 24, 1990	5,934	2,836	3,098	3	5.0
May 9, 1991	5,521	2,755	2,766	2	3.6
June 4, 1991	5,199	2,635	2,563	2	3.8
July 3, 1991	7,064	3,525	3,539	3	4.2
Overall	23,718	11,751	11,966	10	4.2

^a A variant sperm was a propidium iodide-positive nucleus carrying two distinct fluorescent domains separated by a distance of $1/2$ domain diameter or more.

Three donors (donors A, I, and Z) were then evaluated with respect to the frequencies of sperm carrying zero, one, or two fluorescent domains, using probes pUC177 or pY3.4 (table 2). Two semen samples were

analyzed from each donor. No statistical difference was found within any pair of samples, and thus the totals for each pair are presented in table 2. The overall frequency of sperm with at least one fluorescent domain when

Table 2

Sperm Hybridized Using DNA Probes Specific for Chromosome I (pUC177) or Chromosome Y (pY3.4)

DONOR AND PROBE	NO. OF SPERM SCORED ^a	NO. OF FLUORESCENT DOMAINS PER CELL			FREQUENCY OF VARIANT SPERM (per 10,000 sperm) ^b
		Zero	One	Two	
A:					
pUC177	11,795	240	11,543 (97.8%) ^c	12	10.2
pY3.4	11,249	5,620	5,625 (50.0%) ^d	4	3.6
I:					
pUC177	10,197	257	9,929 (97.4%)	11	10.8
pY3.4	10,077	4,935	5,139 (50.9%)	3	3.0
Z:					
pUC177	10,195	292	9,881 (96.9%)	22	21.5 ^e
pY3.4	10,530	5,270	5,249 (49.8%)	11	10.4 ^f

^a Two ejaculates per donor were prepared and scored independently.

^b A variant sperm was a propidium iodide-positive nucleus carrying two distinct fluorescent domains separated by a distance of $1/2$ domain diameter or more.

^c Percent of total sperm scored carrying one fluorescent domain when using probe pUC177; same for donors I and Z.

^d Percent of total sperm scored carrying one fluorescent domain when using probe pY3.4; same for donors I and Z.

^e Using Cochran's Test for Equal Proportions, significantly different from frequency obtained with the same probe for donors A and I, at $P = .045$.

^f Using Cochran's Test for Equal Proportions, significantly different from frequency obtained with the same probe for donors A and I, at $P = .011$.

hybridized using pUC177 was $97.5\% \pm 0.2\%$, a hybridization efficiency consistent with that generally found when hybridizing this probe to lymphocytes (Eastmond and Pinkel 1990). The overall frequency of sperm with at least one fluorescent domain, using pY3.4, was $50.3\% \pm 0.4\%$, which is not significantly different from the expected 50%.

The frequencies of cells with two fluorescent domains (per 10,000 cells) for donors A, I, and Z were 10.2, 10.8, and 21.5, respectively, using pUC177, and were 3.6, 3.0, and 10.4, respectively, using pY3.4 (table 2). For each donor the frequencies of variant cells were higher for chromosome 1 than for Y.

A consistent difference in frequencies of cells with two fluorescent domains was observed among these three donors (table 2). Using probe pY3.4, donor Z showed frequencies of cells with two fluorescent domains ~ 2 times higher than those in either donor A or donor I ($P = .045$). Similarly, using probe pUC177, donor Z had baseline frequencies ~ 3 times higher than those of donor A and ~ 3.5 times higher than those of donor I ($P = .011$). The frequencies of variant cells for donors A and I did not differ significantly from each other (table 2).

To evaluate the validity of the sperm hybridization method for detecting disomic sperm, the chromosomal and donor differences observed with the hybridization method were compared with frequencies of aneuploid sperm obtained by the hamster technique (tables 3 and 4). Table 3 compares two data sets, by donor. The hybridization data are the frequency of sperm with two domains, using probes for chromosomes 1 and Y, respectively. Two sets of hamster data are presented: (1) the frequencies of sperm metaphases hyperhaploid for any chromosome and (2) the frequency of metaphases with either gain or loss for any chromosome. With the hamster technique, the baseline frequencies of hyperploid or hypoploid sperm produced by donors A and I were different neither from each other nor from the group average of the 24 men surveyed. For donor Z, the frequency of hyperploid cells alone, which is considered to be a more conservative measure of sperm aneuploidy, was not significantly elevated; however, the sum of hyperploid plus hypoploid cells was significantly elevated ($P < .01$). Therefore, data from both systems support the conclusion that donor Z is elevated in the frequency of aneuploid cells when compared with donors A and I.

Table 4 compares the results of the sperm hybridization method and hamster technique, by chromosome.

Table 3

Comparison, by Donor, of Aneuploidy Frequencies Obtained by the Human-Sperm/Hamster-Egg Cytogenetic Technique (Hamster Technique) and Fluorescence In Situ Hybridization of Sperm (Sperm Hybridization Method) Using Repetitive-Sequence DNA Probes Specific for Chromosomes 1 and Y

DONOR	HAMSTER TECHNIQUE ^a		SPERM HYBRIDIZATION METHOD: FREQUENCY OF VARIANT SPERM FOR CHROMOSOMES ^b (%)	
	Frequency of Hyperploid Metaphases (%)	Frequency of Hyper- plus Hypoploid Metaphases (%)	1	Y
A6	1.3	.102	.036
I5	1.5	.108	.030
Z	1.2	3.8 ^c	.215 ^d	.104 ^d

NOTE.—All data are based on the following numbers of cells or metaphases analyzed: for donor A, 11,795 sperm for chromosome 1 hybridization, 11,249 sperm for chromosome Y hybridization, and 793 sperm metaphases with the hamster technique; for donor I, 10,197 sperm for chromosome 1 hybridization, 10,077 sperm for chromosome Y hybridization, and 603 sperm metaphases with the hamster technique; and, for donor Z, 10,195 sperm for chromosome 1 hybridization, 10,530 sperm for chromosome Y hybridization, and 339 sperm metaphases with the hamster technique.

^a Data from all chromosomes are combined. Data are from Brandriff et al. (1986b) and are updated with data from Brandriff and Gordon (1990).

^b Data are from table 2. A variant sperm was a propidium iodide-positive nucleus carrying two distinct fluorescent domains separated by a distance of $1/2$ domain diameter or more.

^c Differs from A and I, at $P < .01$.

^d Differs from A and I, at $P < .05$.

Too few aneuploid sperm metaphases were obtained for each donor by the hamster technique to permit a chromosome-by-chromosome comparison, so grouped data from three men analyzed by the sperm hybridization method are compared with grouped data for 24 men analyzed by the hamster technique. There was no statistical difference between frequencies observed with the hamster technique and the sperm hybridization method for chromosome 1 ($P = .6$) or for chromosome Y ($P = .5$). The preponderance of chromosome 1 aneuploidy was observed by both systems.

As a further test of the validity of the sperm hybridization method, table 4 compares sperm data with frequency data for chromosome 1 trisomy and XYY aneu-

Table 4

Comparison, by Chromosome, of Aneuploidy Frequencies Obtained by Human-Sperm/Hamster-Egg Cytogenetic Technique (Hamster Technique), by Fluorescence In Situ Hybridization of Sperm (Sperm Hybridization Method) with Chromosome-specific DNA Probes, and in Live Births

CHROMOSOMAL DISOMY	ANEUPLOIDY FREQUENCY (per 10,000 sperm)		
	Hamster Technique ^a	Sperm Hybridization Method	Live Births ^b
1-1	16.7	14.0 ^c	~0
Y-Y	3.3	5.7 ^d	5.4

^a Based on a survey of 5,998 sperm metaphases from 24 men, as reported by Brandriff and Gordon (1990).

^b Prospective study of 34,910 newborns, using the frequency of XYY offspring over the total number of male and female offspring surveyed (Nielsen and Wohler 1991).

^c Based on 32,187 sperm (obtained from 3 of 24 men evaluated by Brandriff and Gordon [1990]) hybridized with a DNA probe specific for chromosome 1.

^d Based on 31,856 sperm (obtained from 3 of 24 men evaluated by Brandriff and Gordon [1990]) hybridized with a DNA probe specific for chromosome Y.

ploidy in human live births. Chromosome 1 aneuploidy has never been observed at birth, although it has been reported in an eight-cell embryo (Watt et al. 1987) and with the hamster technique. This suggests that fertilization with a sperm disomic for chromosome 1 results in early fetal loss with no direct impact on aneuploidy at birth. However, the frequencies of sperm disomic for the Y chromosome, by both the sperm hybridization method and the hamster technique, were not significantly different from the rate of XYY aneuploidy reported in live births. This indicates a strong concordance between the frequency of Y chromosomal aneuploidy in sperm and at birth, with little evidence for negative selection during development.

Discussion

The present study successfully applied the technique of fluorescence in situ hybridization to sperm air-dried onto glass slides. Validity of the sperm hybridization method for measuring aneuploidy frequencies in sperm was evaluated by selecting donors for whom sperm chromosomes had been previously analyzed for chromosomal abnormalities via the hamster technique.

Among three donors (donors A, I, and Z) and for two chromosomes (1 and Y), the frequency of sperm with two fluorescent domains found by fluorescence in situ hybridization did not differ statistically from the frequency of hyperhaploid cells obtained with the hamster technique. This strong concordance supports the interpretation that sperm cells carrying two fluorescent domains are disomic for the specific chromosome being probed.

A donor difference was evident with the sperm hybridization method and hamster technique. The hamster technique showed an increased frequency of aneuploid cells for donor Z (when hypo- plus hyperhaploid cells are summed) and suggested a trend toward an increased frequency, when only hyperhaploid cells for this donor are considered. By sperm hybridization, donor Z showed elevated baseline frequencies of disomic sperm, using probes for both chromosomes 1 and Y, when compared with donors A and I. Donors A and I did not differ from each other in baseline frequencies of disomy for either chromosome, by either the sperm hybridization method or the hamster technique. Although the finding of a statistical difference in donor baseline aneuploidy frequency by using the hybridization method is based on only three men, we report it because it is consistent with the trend observed in the hamster technique. Concordance in findings from the two methodologies supports the hypothesis that donor differences in baseline frequencies of aneuploidy do exist for the two chromosomes studied. However, further work must be done to determine whether similar differences among these donors occur for other chromosomes and the variance in aneuploidy frequencies among a larger group of normal healthy men.

Both the sperm hybridization method and the hamster technique found an elevated frequency of chromosome 1 aneuploidy compared with chromosome Y. However, one must be cautious in trying to extrapolate these findings to other chromosomes. With the hamster technique, Brandriff and Gordon (1990) found chromosome 1 disomy elevated compared with other autosomes, suggesting that chromosome 1 was unusual. Hybridizations with DNA probes specific for other autosomes are needed to search for other possible differences among chromosomes.

Reliability of the sperm hybridization method and the utility of using frozen semen specimens aged greater than 1 year was demonstrated when four different semen specimens from one donor were hybridized at different times and showed no significant difference in

baseline frequency of sperm cells with two fluorescent domains. These results also suggest a within-donor stability in aneuploidy frequency, over at least 1 year's time.

Experience in this laboratory suggests that pretreatment to decondense sperm chromatin is necessary for successful hybridization. Using repetitive-sequence probes, sperm cells swollen 1.5 to 1.7 times their original size hybridized efficiently with well-localized fluorescence domains, while cells decondensed greater than this gave diffuse hybridization signals with poor resolution of signals. Cells less swollen did not label reliably.

There are limitations when using single-probe hybridizations, related to false negatives and false positives for numerical aneuploidy. First, it cannot be established whether absence of a hybridization signal reflects hypohaploidy or failure of that particular cell to hybridize. Second, using a Y probe, a single signal does not allow discrimination between cells hypoploid for the Y chromosome versus cells carrying an X chromosome. Third, since only a relatively small region of a chromosome is marked when using a single probe, a complete translocation of this region onto another chromosome would not be discernible. Fourth, a single probe does not allow discrimination between $2n$ or diploid cells versus cells that are disomic for the chromosome being probed. Fifth, using a single probe, it is not possible to distinguish between sperm that carry two copies of an entire chromosome versus sperm that have a break within the repeated sequence so that the two parts are separated spatially. The limitations of the single-probe method are presently being addressed with hybridizations using multiple repetitive-sequence probes (Wyrobek et al. 1992*b*) and with whole chromosome-painting probes (Wyrobek et al. 1992*a*).

Fluorescence in situ hybridization to sperm on smears, using chromosome-specific repetitive probes, provides a powerful supplement to the conventional human-sperm/hamster-egg cytogenetic technique. The conventional hamster technique has the advantage of being able to detect both numerical and structural aneuploidy for all chromosomes per sperm metaphase. Although the sperm hybridization method is limited, at present, to detection of numerical aneuploidy in specific chromosomes probed, it has the advantage of screening large numbers of sperm with comparatively minimal effort and time. Ten thousand cells can be readily scored from a single semen sample in less than 2 d. Using power calculations, we estimate that counting 10,000 cells would allow detection of approximately a

doubling in baseline frequency of disomic cells at 80% power and alpha of .05. Assuming random effects across chromosomes, the hybridization methodology offers great promise in investigations of induced aneuploidy effects. The ability to analyze thousands of sperm easily and quickly, as well as the utility of a system that utilizes air-dried smears, makes this an advantageous procedure for monitoring men exposed to potential germinal mutagens as a result of environmental, lifestyle, or occupational exposures.

The ability to identify individuals with elevated frequencies of disomic sperm allows the possibility of screening men with various constitutive chromosomal abnormalities, as well as men having difficulties fathering healthy children. Men carrying elevated frequencies of disomic sperm are expected to be at increased genetic risk of fathering children carrying chromosomal aneuploidies.

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