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PNA on human sperm: a new approach for *in situ* aneuploidy estimation

Franck Pellestor*,[1], Brigitte Andréo[1], Krihan Taneja[2] and Brett Williams[2]

[1]CNRS-UPR 1142, F-34396 Montpellier, France; [2]Applied Biosystems, Bedford, MA, USA

Peptide nucleic acids (PNAs) are a relatively new class of synthetic DNA mimics based on a peptide-like backbone. Since their introduction, PNA probes have become established as an efficient variation on the standard FISH procedure for chromosomal identification. In this report we have experimented with centromeric PNA probes on human sperm preparations. Both NaOH and DTT sperm decondensation procedures have been tested and comparative estimates of disomies X, Y and 1 have been performed in sperm from two donors using PNA, FISH and PRINS techniques. Similar results were obtained with the three methods, demonstrating the efficiency of PNA probes in the analysis of human sperm. The fast kinetics, stability and high specificity of PNA probes make PNA-based methodologies very valuable for *in situ* cytogenetic investigations.
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Introduction

Direct chromosomal analysis of spermatozoa is an essential approach for the investigation of the occurrence and etiology of chromosomal abnormalities in humans under a wide variety of clinical conditions. To date, numerous chromosomal analyses on human sperm have been performed using fluorescence *in situ* hybridization (FISH) (for a review, see Downie *et al*[1] and Egozcue *et al*[2]). These reports demonstrated the efficiency of the *in situ* labelling procedure on male gametes, but also pointed out the limitations of FISH on this biological material, which are essentially linked to the size of the probes and the reliability of the associated sperm decondensation treatments.[2,3]

The primed *in situ* (PRINS) reaction has offered an alternative approach for the direct chromosome analysis of human spermatozoa. Several studies have demonstrated that PRINS labelling was just as efficient as FISH on human sperm and exhibited higher specificity.[4–6] The great

potential of this method based on the PCR principles, lies in the small size and the high specificity of the selected primers.

Peptide nucleic acid (PNA) probes provide a new and interesting alternative to FISH and PRINS. PNAs are synthetic mimics of DNA in which the deoxyribose phosphate backbone supporting the nucleic acid bases is replaced by a noncharged peptide backbone conferring greater stability and affinity to PNA probes than DNA probes. The efficiency of PNA probes has been demonstrated in the studies of telomere and centromere repeat sequences[7,8] and more recently for the specific *in situ* identification of human chromosomes.[9] All of these reports have demonstrated the superiority of PNA probes over conventional repeat DNA probes in both signal intensity and sequence discrimination. However, no PNA probes have been yet produced for the detection of unique DNA sequences, such as telomere- and locus-specific sequences, for which efficient FISH probes are available. Consequently, we conducted a study to test PNA probes on human sperm preparations. In the present work, we report on the elaboration of an efficient multicolor PNA protocol for sperm nuclei analysis and the results of the first estimate of sperm disomy using this new type of probe.

*Correspondence: Dr. F Pellestor, CNRS-UPR 1142, IGH, 141, rue de la Cardonille, F-34396 Montpellier cedex 5, France. Tel: 33 (0)4 99 61 99 12; Fax: 33 (0)4 99 61 99 01; E-mail: Franck.Pellestor@igh.cnrs.fr

Materials and methods

PNA probes

PNA probes specific for chromosomes X, Y and 1 were kindly provided by Applied Biosystems (formerly Boston Probes Inc.). Each probe consists of a mixture of several short synthetic sequences (18–22 base units) specific for the centromeric tandem repeat sequence of the targeted chromosomes. The probe specific for chromosome X was labelled with fluorescein. The chromosome Y-specific probe was labelled with rhodamine and the chromosome 1-specific probe was labelled in blue with diethylamino-coumarin. The PNA probes were supplied ready to use in hybridization buffer.

Slide preparation

Metaphase chromosomes were freshly prepared from peripheral blood lymphocytes by standard cytogenetic methods, fixed in methanol: glacial acetic acid (3:1) and spread on cleaned slides. Slides were passed through an ethanol series (70, 90, and 100%), 2 min each step, and air-dried. Slides were stored at room temperature and aged for at least 4 h at room temperature before proceeding with the hybridization steps. They were denatured by immersion in 70% formamide, $2 \times SSC$ at $72^{\circ}C$ for 3 min and then dehydrated in a series of ice-cold ethanol washes (70, 90 and 100%) before being allowed to air-dry.

Sperm samples were obtained from two healthy males with normal peripheral blood karyotypes. Each ejaculate was collected in a sterile container and kept at room temperature for 30 min. After liquefaction, an aliquot of the specimen was used for semen analysis. The rest of the sample was washed twice in 1xPBS by centrifugation (five min at 2000 rpm) and fixed for one hour in fresh fixative (3:1 methanol:glacial acetic acid) at $4^{\circ}C$. The sperm suspension was then dropped onto clean microscope slides and air-dried. Slides were aged for 1 day at room temperature. At this step, two pretreatments were tested and sperm preparation slides from each ejaculate were divided into two groups. For the first group of slides, the simultaneous decondensation–denaturation NaOH protocol was used by immerging sperm slides in a 0.5 M NaOH solution at room temperature for 4 min, followed by an ethanol series (70, 90 and 100%) and air-drying. In the second group, a decondensing treatment based on dethio-threithol (DTT) was applied, consisting of the immersion of slides in a 1 M DTT solution for 30 min at $4^{\circ}C$, followed by two washes in $2 \times SSC$ (2 min), an ethanol series and air-drying. The slides were then denatured in 70% forma-mide, $2 \times SSC$ at $72^{\circ}C$ for 3 min, dehydrated in a series of ice-cold ethanol washes (70, 90 and 100%) and air-dried.

Hybridization and posthybridization washes

Probe aliquots of $5 \mu l$ were mixed and denatured for 6 min in a water bath preheated to $72^{\circ}C$. The probe solution was applied to the preparation slide, covered with a 22×22 mm coverslip and put in a humidified hybridization chamber 5–60 min at room temperature.

At the end of the hybridization, the coverslips were removed by washing of slides in $1 \times PBS$, 0.1% Tween-20 for 2 min. The slides were then transferred to a $58^{\circ}C$ pre-warmed $1 \times PBS$, 0.1% Tween-20 for 20 min and rinsed in $2 \times SSC$, 0.1% Tween-20 for 1 min. The excess fluid was drained from the slides, which were then mounted in Vectashield antifade solution (Vector laboratories, Burlingame, CA, USA) containing a mixture of propidium iodide $(0.01 \mu g/ml)$ and DAPI $(0.3 \mu g/ml)$.

Disomy estimate: control, microscopic analysis, scoring criteria and statistical analysis

FISH and PRINS procedures were used as controls. Thus, in parallel with the PNA hybridizations, both FISH and PRINS estimates of disomy rates for chromosomes X, Y and 1 were performed on sperm preparations from the same two donors. FISH assays were performed with the Vysis satellite probes CEPX, CEPY and CEP1 (Vysis, Downers Grove, IL, USA) directly labelled with the Spectrum Aqua™, Spectrum Green™ and Spectrum Orange™, respectively. The FISH procedure was performed after DTT pre-treatment of sperm slides, according to the protocol recommended by Vysis, with an overnight hybridization at $37^{\circ}C$. The PRINS assays were performed using NaOH pretreatment of sperm slides and primers specific for chromosomes X, Y and 1, as previously described.[5,10] Their sequences and optimal technical conditions have been reported elsewhere.[11]

Two independent observers analyzed the slides, using a Leitz DMRB epifluorescence microscope (Leica SA, Rueil-Malmaison, France) equipped with appropriate excitation and emission filters. Each observer scored a minimum of 5000 sperm nuclei per slide. The haploid sperm nuclei displayed spots of different colors corresponding to each labelled chromosome. Nuclei were considered disomic when they displayed two signals similar in size and intensity, separated by at least the diameter of one fluorescent spot.[12]

The χ^2 test was used to compare the disomy rates for the three chromosomes tested in each sperm sample, to compare this data between the two subjects and to test the variability of disomy values obtained with each of the three types of procedures (PNA, PRINS and FISH). For each donor, the homogeneity of the disomy rates, according to the labelling techniques used, was also tested by the Student's *t*-test. A value of $P < 0.05$ was considered to be significant.

Results

The specificity and the labelling intensity of the PNA probes were first tested on metaphases and interphase nuclei using single- and two- or three-color PNA hybridizations. Examples of labelling on peripheral blood
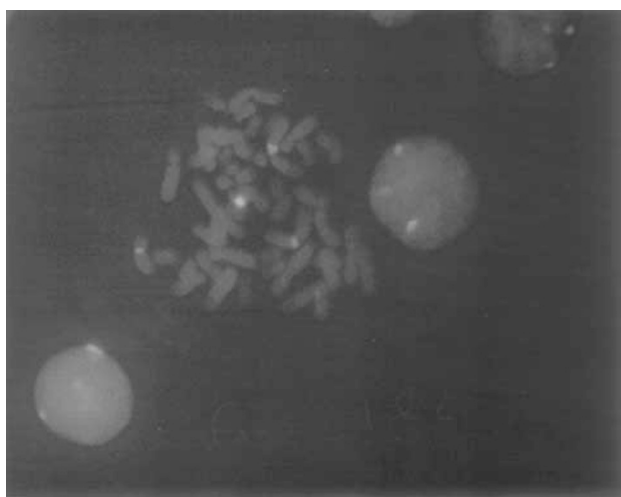
Figure 1 Three-color PNA labelling on human metaphase and interphase nuclei. Chromosome 1 is labelled in blue, chromosome X in red and chromosome Y in yellow.

lymphocytes are given in Figure 1. For each probe, resulting signals were specific and easily scorable. In double and triple hybridization experiments, the signals remained well defined without crosshybridization and significant background. The hybridization efficiency, estimated on 100 metaphases and nuclei, reached 100% in single-color experiments and ranged from 97 to 100% in three-color PNA experiments. In order to assess the limits of PNA hybridization, several durations of hybridization time (from 5 to 60 min) were tested. Satisfactory results, that is, well-distinguishable spots, were obtained in both metaphases and interphase nuclei following 20–60 min hybridization. The shortest hybridization did not allow enough time to obtain an efficient labelling of the targeted chromosomes.

On sperm preparation, the quality of the labelling was similar with the two decondensation pretreatments tested. As illustrated in Figure 2a, well-defined and bright signals were obtained on sperm nuclei without background, thus allowing an easy and reliable scoring of the targeted chromosomes. Several hybridization durations were tested on sperm preparations in order to define the minimal time of hybridization. With both DTT and NaOH pretretaments, the limit of *in situ* labelling was reached with a 25 min hybridization time.

According to these data, standard hybridization conditions on human sperm were fixed at 45 min with both NaOH and DTT pretreatments. The size of the fluorescent spot obtained was then similar to the size of the FISH signals, or the PRINS signals shown in Figure 2b.

Table 1 summarizes the results of the PNA estimates of disomy rates for chromosomes X, Y and 1 in parallel with the estimates drawn from FISH and PRINS assays. In the PNA experiments, the ratio of X- and Y-bearing spermatozoa was 1:1 as expected. The frequencies were 0.14 and
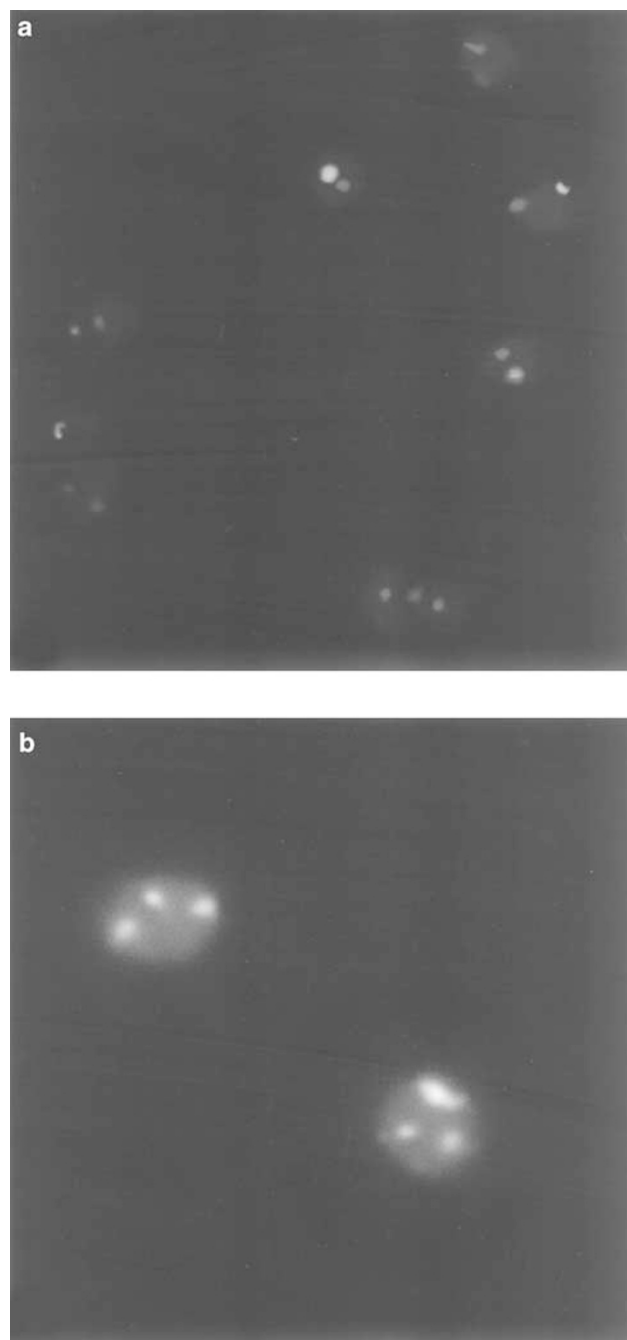

Figure 2 (**a**) Specific labelling of chromosome 1 (blue), X (red) and Y (yellow) on human sperm nuclei using PNA technique. (**b**) Detection of chromosome 1 (orange), chromosome X (red) and chromosome Y (green) on human sperm nuclei using PRINS method.

0.08% for XX disomy, 0.10 and 0.10% for YY disomy, 0.12 and 0.10% for XY disomy, and 0.13 and 0.18% for chromosome 1 disomy, in donors 1 and 2, respectively. The overall incidence of sex chromosome disomy (i.e. XX+YY+XY disomy rates) was 0.36% for donor 1 and

Table 1 Results of sex ratio and disomy estimates for gonosomes and chromosome 1 using PNA, PRINS and FISH techniques on sperm preparations from two normal men

| Methodology | Donor | No. of screened nuclei | Sex ratio | | Overall sex chromosome disomy (%) | Disomy | | | |
			X	Y	XX+YY+XY	XX	YY	XY	II
PNA	1	10 008	4981 (49.77)	4990 (49.86)	0.36	16 (0.14)	10 (0.10)	12 (0.12)	13 (0.13)
	2	10 036	5010 (49.92)	4998 (49.80)	0.28	8 (0.08)	10 (0.10)	10 (0.10)	18 (0.18)
PRINS	1	10 040	4999 (49.79)	4992 (49.72)	0.39	9 (0.09)	13 (0.13)	17 (0.17)	10 (0.10)
	2	10 005	4988 (49.85)	4981 (49.78)	0.36	12 (0.12)	14 (0.14)	10 (0.10)	23 (0.23)
FISH	1	10 010	4987 (49.82)	5023 (50.17)	0.40	11 (0.11)	10 (0.10)	19 (0.19)	13 (0.13)
	2	10 013	5009 (50.03)	5004 (49.97)	0.32	9 (0.09)	10 (0.10)	13 (0.13)	21 (0.21)

0.28% for donor 2. Similar values were found in FISH and PRINS experiments (Table 1). These results are in agreement with the data previously reported in the literature.

No significant difference ($P > 0.05$) was observed between the disomy rates estimated by either PNA, FISH or PRINS for a same chromosome. The three approaches displayed no significant interindividual differences in gonosome disomy rates ($P > 0.2$) and chromosome 1 disomy rates ($P > 0.3$). For each patient, the comparative analysis of the PNA, FISH and PRINS data indicated no significant variation ($0.020 < t < 0.386$; $P > 0.5$). With the three procedures, a significant statistical difference was found between the overall sex chromosomes disomy rate and the chromosome 1 disomy rate in sperm from donor 1 ($P < 0.01$), but not in sperm from donor 2 ($P > 0.05$).

Discussion

The PNA probes present multiple advantages for the *in situ* detection of nucleic acid sequences. Several studies have already reported their successful use on metaphases and interphase nuclei for chromosomal identification.[13,9] However, the adaptation of this new class of probes to human spermatozoa constituted a new step in the development of PNA methodology and a very interesting challenge because of the particularities of human sperm nucleus in terms of genomic compaction and accessibility of DNA sequences. On human sperm, two chromosomal labelling techniques have been successfully adapted, that is, the FISH and the PRINS methods.[4,14,15] They constitute two well-distinct approaches for *in situ* chromosomal identification, and each presents advantages and disadvantages. Owing to its relative simplicity and the commercial availability of numerous probes, FISH has become the standard technique for sperm analysis, despite the lack of specificity of some alphoid probes.[2] The PRINS reaction requires a thermocycler and sequential reactions, but provides a more specific and faster labelling procedure for the repeat DNA sequences.[3,16] When applied to human

sperm preparation, both techniques need to be associated with a sperm nucleus decondensing treatment in order to made the targeted DNA region accessible. The quality of the fluorescent signals, and consequently the reliability of the sperm analysis results are directly related to the efficiency of the *in situ* decondensation.[17,18] Pretreatment to decondense sperm chromatin is also indispensable with PNA probes. No PNA labelling of sperm nuclei is obtained without decondensation pretreatment. We have tested the two decondensation procedures that give efficient and reproducible results with FISH and PRINS reactions, that is, the DTT and the NaOH treatments. The two procedures yielded satisfactory results with PNA and gave similar kinetics for the labelling reaction. The hybridization timing of PNA probes appeared to be considerably shortened in comparison with FISH reaction on sperm, which requires an overnight hybridization in order to be efficiently completed. The fast hybridization kinetics of PNA is more similar to the kinetics of PRINS reaction on which sperm chromosomal labelling can be performed in less than 20 min.[10] The similarity between PNA and PRINS might be essentially because of the small size of both PNA oligoprobes (18–20 bp) and PRINS primers, which do not exceed 30 bases in length. Reduced labelling timing (around 3 h) has also been reported in some sperm FISH studies, but using short satellite oligonucleotide probes.[19,20] This data points out the importance of the probe size, and the use of a decondensation procedure in sperm labelling assays. Similar performance of PNA and PRINS methods has already been reported for the *in situ* detection and sizing of telomeric repeat sequences.[21] Both techniques presented comparable features in terms of specificity, staining intensity and efficiency, but PRINS always displayed faster turnaround reaction time. This could reflect the fact that PRINS is an 'active' reaction involving an ultrafast biochemical reaction of primer extension catalyzed by a *Taq* polymerase. In the case of PNA reaction, the rapidity of the labelling is because of the neutral backbone of the PNA molecules, which allows for PNA/DNA binding to occur more rapidly and more tightly

than DNA/DNA binding. Moreover, PNA probes can bind to DNA under low ionic strength conditions that disfavor reannealing of complimentary genomic strands. This advantage is particularly important for in situ hybridization experiments that target repetitive sequences, because both the length and the repetitive nature of the target sequences will affectively favor renaturation over hybridization with labelled probes. The high affinity of PNA probes to DNA constitutes an important feature for chromosomal analysis. Previous studies have demonstrated that PNA probes could discriminate between two centromeric DNA repeats that differ by only a single base pair.[8,13] Identical results were obtained with PRINS primers[22] and oligonucleotide probes,[19] whereas standard FISH probes are unable to discriminate sequences with a single base resolution. The study of chromosomal polymorphism could benefit from the discriminating power of PNA. On sperm, this could be useful for the in situ distinction of autosomal nondisjunction occurring at meiosis I and meiosis II, when satellite polymorphisms exist between two homologous chromosomes.[20]

The PNA probes constitute new and valuable tools for in situ chromosomal investigation. They can advantageously be used to complement FISH and PRINS techniques and one can predict that the PNA technology is going to make significant progress during the next years. The successful testing of PNA probes on human sperm has proven that these reagents could be used on difficult biological material. Given their main features, that is, stability, specificity, rapidity, the PNA probes also have a great potential for clinical applications, in particular when chromosomal identification must be performed on limited amounts of material and in a limited time period such as in preimplantation chromosomal diagnosis.

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