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Constancy in Human Sperm DNA Content

(population heterogeneity/sex chromosomes/chromosomal translocation/fluorescent Feulgen stain/flow microfluorometer)

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ABSTRACT **The frequency distribution of DNA content of human sperm was measured in an automated flow microfluorometer. The flow method measures the DNA content by quantifying the amount of fluorescence emitted by the fluorescent Feulgen stained DNA of single sperm cells suspended in microdroplets. The variability in the mean value for the haploid amount of DNA in sperm from 15 randomly chosen donors was less than 1%. Statistical tests on the observed frequency distribution data indicated that each sperm population probably consists of two homogenous components present in almost equal proportions but differing in mean DNA content. The difference in their modal values for DNA is within the range of known values of DNA difference between the two sex chromosomes.**

Sperm of donors segregating balanced translocations, when compared to the random samples as a class, showed greater variability in the mean DNA content.

Sperm population in ejaculates may be regarded as a statistical sample of all meiotic products in the male (1, 2). With the availability of the rapid flow microfluorometer (3, 4) it may become possible to detect population heterogeneity by the direct examination of individual sperm cells as haploid segregants. The genetic information obtained through this procedure should be fruitful in interpreting certain cytogenetic observations associated with abnormal transmission ratios (5, 6). In addition one may now ask, what is the extent of variability in the segregation of DNA in meiosis compared to mitosis? Does somatic cell differentiation or replication intrinsically select for least variability in the DNA content (3), whereas meiosis (7) in contrast, purposefully provides mechanisms to achieve more variability in gametes?

In this report we describe the population heterogeneity of human sperm in the haploid DNA content, and the extent to which the variability could be determined by the segregation of specific chromosomes with structural rearrangements.

MATERIALS AND METHODS

Sperm Donors. Fifteen donors were chosen at random among those attending a family planning clinic (courtesy of Dr. E. T. Tyler, Tyler Clinic, Los Angeles) and among students (courtesy of Dr. V. J. Flynn, Scripps Hospital, La Jolla). The structural rearrangements of five subjects heterozygous for balanced translocations are described below:

Subjects:

B. S.; age: 19; translocation t(13, 14) (11p; 11q); semen vol: 1.2 ml; sperm count: 10^8/ml; unmarried; no children. (Courtesy of Dr. William Centerwall, Director, Division of Medical Genetics, Department of Pediatrics, Loma Linda School of Medicine, Riverside, Calif.).

J. G.; age: 41; translocation: t(21q; 14q); semen vol.: 0.5 ml.; sperm count: 5×10^5/ml.; married; one child with 46, XY t(21q; 14q) Down's syndrome.

R. P.; age: 41; translocation: t(1; 2)(321; 13q); semen vol.: 5 ml.; sperm count: 5×10^7/ml.; married; 3 children: 1 female t(1; 2) (32q; 13q); 1 male t(1; 2) (32q; 13q); 1 male with normal karyotype.

G. P.; age: 74; translocation: t(1; 2) (32q; 13q); semen vol.: 1.5 ml.; sperm count 4×10^7/ml; married; 3 sons: only 1, R. P., has had karyotype analysis. Other two sons are phenotypically normal.

R. S.; age: 69; translocation: t(14, 7) (22q; 36q); semen vol.: 6 ml.; sperm count: 10^8/ml.; married; 2 daughters: 1 daughter with normal karyotype and 1 with t(14; 7) (22q; 36q). Both daughters are phenotypically normal.

Fluorescent Feulgen Stain. Fresh ejaculated samples of semen were diluted 2-fold and washed three times on a cushion of 15% sucrose solution (15% sucrose w/v in phosphate-buffered saline with 0.1% glucose, pH 7.2–7.4) to remove contaminating cells and semen proteins from sperm. Washed sperm was fixed in 3.7% formalin overnight at 4°. Freshly drawn heparinized chicken blood was washed in balanced salt solution for erythrocytes and was formalin fixed in the same manner. Cells were washed to remove formalin and were hydrolyzed with 4 M HCl at room temperature for 20 min. Excess acid was removed by repeated wash. Cells were stained with acriflavine (staining solution: $K_2S_2O_5$, 500 mg;

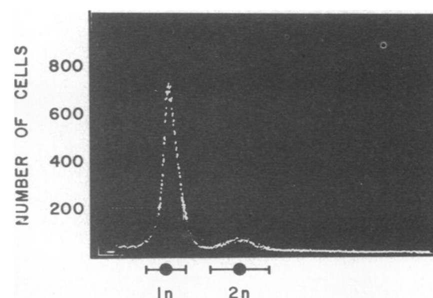

FIG. 1. Frequency distribution of Feulgen stained cells from one donor showing peaks at haploid (1n) and diploid (2n) regions; both ordinate (cell number) and abscissa (fluorescence intensity) are on the linear scale. The fluorescence intensity is measured in units of approximately 8 mV and the full scale is 8 V (1022 units).

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TABLE 1. *Normality tests by computing Fisher's k and g statistics**

Normality tests	If normal: Skewness = 0, Kurtosis = 3	If normal: Fisher's g statistics Both = 0	Variance thereof	If normal: X (<1.96)	If normal: Its probability 0.05
Skewness	0.267850	0.267882	0.000115	24.901571	<0.001
Kurtosis	3.704812	0.705083	0.000462	32.771871	<0.001

* See Fig. 2 for observed frequency distribution.

acriflavine, 30 mg; 0.5 M HCl, 10 ml; water, 100 ml) for 20 min and washed three times with cold acid–alcohol solution (1 ml of concentrated HCl in 100 ml of 70% ethanol) to remove excess unbound dye. The cells were then diluted in cold water (10^5 cells per ml) for fluorometric analysis. The staining procedure was obtained from Dr. H. A. Crissman of the Los Alamos Scientific Laboratory (3, 8).

Frequency Distribution of Cells Differing in DNA Content. The Salk Institute flow-system analyzer was used for determining frequency distributions of cell DNA content. The design of this instrument is based on the flow microfluorometer at the Los Alamos Scientific Laboratory. The instrumental factors that are potential contributors to dispersion have been described elsewhere (3). Variation in the intensity measurements (1%) was determined separately from the variation in cell staining (less than 1%). Stained chicken erythrocytes and human sperm from a particular donor were used as standards in all experiments. The amount of nuclear DNA in both cell types is about 3 pg (9). The data on frequency distributions were recorded directly on punched tapes for statistical analysis.

Statistical Analysis. Three major statistical procedures performed on the observed data are: (*a*) test for normality, (*b*) nonlinear regression analysis, and (*c*) variability analysis. (*a*) The test for normality consists of calculations for skewness, kurtosis, their k- and g-statistics (Fisher) (10), their variance, and finally, probabilities at which one is to accept or reject a null hypothesis that the observed data came from a normal parental population. The findings given in Table 1 clearly indicate that such a hypothesis can be confidently rejected. (*b*) The nonlinear regression analysis was done by the Gauss and the steepest descent methods (11) to decompose the observed data into two Gaussian curves. The modal values and the number of cells under each of the two Gaussian components (see Table 2) were computed by this analysis, under the constraint that the variances of the two populations are equal. This constraint was applied on the assumption that the variances of the X or Y chromosome-bearing sperms should be the same. (*c*) The variability analysis consists of calculation of mean, standard deviation, 95% confidence limits of the mean and standard deviation, and finally, ratio of standard deviation to mean.

RESULTS

The frequency distribution of Feulgen stained input cells as it is displayed in the window of the integrator, is shown in Fig. 1. The major fraction of input cells is recovered under the peak for haploid DNA (labeled 1n, in Fig. 1) and a variable number of cells, 0.1–4%, fall under a peak with twice as much fluorescence value (labeled 2n, in Fig. 1). The peak at 2n of fluorescence is made of exceptional sperm cells with diploid amounts of DNA (12) and contaminating epithelial cells which were not removed by the preparative procedure. The peak at 1n is considered for the purpose of this study. The cut off points for the haploid peak were arbitrarily determined to minimize the effects of the background and the contaminant cells in the diploid peak.

Two Gaussian components in the frequency distribution of acriflavine Feulgen stained sperm DNA

All sperm populations are inherently heterogeneous with respect to the sex chromosomes, which segregate asymmetrically into two different haploid populations. The heterogeneity should be detectable in the sperm DNA content because the sex chromosomes complemented with haploid amounts of autosomal DNA show a mean DNA difference of about 4% (12). Therefore, the haploid peak of the observed frequency curve of Fig. 1 should represent a bimodal distribu-

TABLE 2. *Nonlinear regression analysis: decomposition of frequency distribution data into two Gaussian components**

Analysis of variance for significance test: Source of variation	Sum of squares	D.F.	Mean squares	F
Regression	14348545.62149	5	2869709.12430	7223.16711
Error	87007.02739	219	397.29236	
Total	14435552.64890	224		
	Component 1	Component 2		
No. of cells	20011 (53.15%)	17633 (46.85%)		
DNA content:				
Normalized mean	102.18	97.82		
SD	10.03	10.03		

* Frequency distribution is shown in Fig. 2.

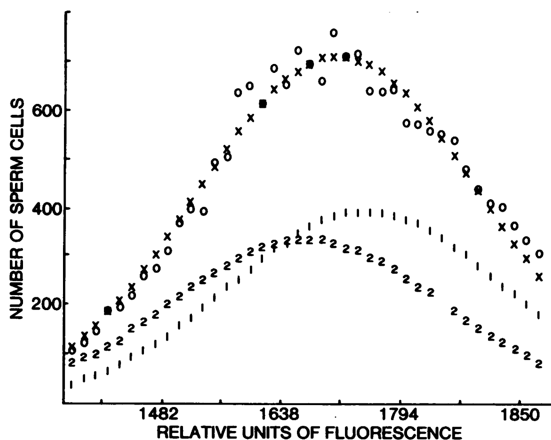

FIG. 2. Nonlinear regression analysis of the frequency distribution of sperm with respect to haploid amount of DNA. One of every two points in the sequence of the experimental data is presented for the clarity of drawing. The ordinate indicates number of cells, abscissa indicates fluorescence value in relative units (in mV). Both are on the linear scale. The experimental data are plotted (O) in combination with data of computed best fit (X). The two computed Gaussian components, 1 and 2 (see Table 2 for details) are derived by stipulating that the standard deviations of the two components be the same (see *text* for details).

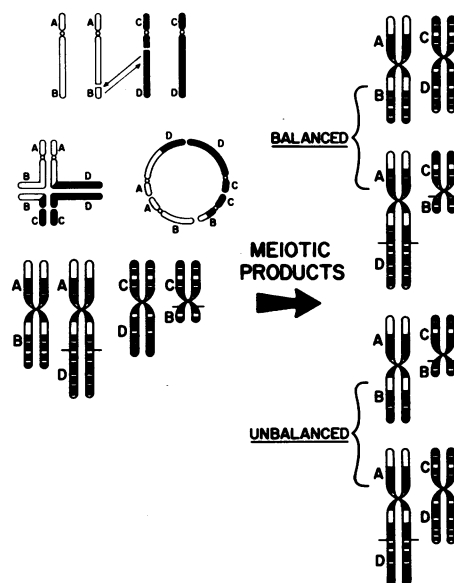

FIG. 3. Segregation of balanced translocation. The quadrivalent arrangements in meiosis are used to identify reciprocal exchange of segments between nonhomologous chromosomes; two of these arrangements, the paired and the ring form, are shown. The balanced segregants are reciprocal products of each other as are also the unbalanced ones. All four are products of normal disjunction and should be formed in equal proportions.

tion of two equal populations differing by 4% in the modal amounts of DNA. The bimodality is not observed in the experimental data, presumably because it is masked by the relatively high variance of the parental population. However, the statistical tests (see statistical analysis, *Materials and Methods*) on the observed data clearly indicate the heterogeneity of the population (probability is <0.001 that the population is homogeneous, see Table 1). The data were then processed by the computer to search for two homogenous components. We assumed that the sperm populations carrying the X or Y chromosome should have equal variance but would differ in mean. The assumption of equal variance was incorporated in the computer analysis to obtain the numerical values of two unknown variables, (a) the modal values, and (b) the number of cells, in each of the two components. The results of nonlinear regression analysis and the analysis of

TABLE 3. *Values of mean and standard deviation of human sperm DNA content (95% confidence limits)**

Sample no.	Lower limit	Mean	Upper limit	Lower limit	Standard deviation	Upper limit	Coefficient of variation
1	1596.3658	1597.8972	1599.4287	136.1215	136.6569	136.6722	.0855
2	1699.0270	1700.7621	1702.4973	169.0673	169.6743	169.6915	.0997
3	1562.1506	1563.7560	1565.3614	142.5598	143.1211	143.1372	.0915
4	1695.9463	1697.517	1699.088	153.6104	154.1599	154.1755	.0908
5	1680.3497	1681.9156	1683.4814	159.3933	159.941	159.9567	.0950
6	1718.5039	1720.0756	1721.6473	160.9937	161.5437	161.5592	.0939
7	1713.1952	1714.8114	1716.5475	184.515	185.1017	185.1181	.1079
8	1697.4667	1699.1687	1700.8708	187.2476	187.8433	187.860	.1105
9	1609.1649	1610.8530	1612.5411	168.0835	168.6741	168.6908	.1047
10	1662.0472	1663.9543	1665.8614	237.8216	238.4895	238.5081	.1433
11	1684.5849	1686.6128	1688.6406	263.4986	264.2089	264.2286	.1566
12	1655.5920	1657.7673	1659.9427	330.1769	330.9394	330.9603	.1996
13	1643.6965	1645.5312	1647.3658	250.4382	251.0810	251.0987	.1525
14	1745.4683	1747.3671	1749.2660	242.3500	243.0151	243.0335	.1390
15	1706.0299	1708.0199	1710.0099	236.3989	237.0958	237.1152	.1388
t(1;2) father	1941.153	1943.553	1945.953	335.138	335.979	336.002	.1728
t(1;2) son	1716.3393	1718.8916	1721.4439	418.4609	419.3557	419.3800	.2439
t(13,14)	2204.0840	2206.8101	2209.5361	326.9228	327.8774	327.9040	.1485
t(7,14)	1514.0693	1517.3098	1520.5503	297.8082	298.9414	298.9737	.1970
t(21q;14q)	1482.5275	1485.0890	1487.6505	154.9430	155.8361	155.8629	.1049
Chicken erythrocytes	1626.6389	1628.6544	1630.6699	126.7074	127.4104	127.4313	.0782

* At least 30,000 cells were used for each measurement. See Fig. 4 for scatter plot of these data.

variance for significance test ($P < 0.001$) are shown in Table 2. The two computed homogenous components are plotted as components 1 and 2 in Fig. 2 under the observed curve. The proportion of cells in each component is approximately equal, 53.15% for the heavier of the two components and 46.85% for the light component. Since the Y-bearing sperm is expected to have 4.23% less DNA, as found by optical density measurements of single cells under the light microscope (12), cells in component 2, which has 4.36% less modal amount of DNA, could presumably be the Y-bearing sperm (see Table 2). The proportion of sperm cells that stain specifically for Y chromosome is in the average 41.65% (range 37–47%, ref. 12) a value similar to the computed fraction of cells in component 2 (46.85%). The correspondence of the computed values in our experiment with the results of cytophotometric analysis by direct visualization of single cells under the microscope (12), in addition to the finding of the significance test of the variance analysis ($P < 0.001$ in Table 2), confirm the precision of the flow microfluorometer measurements and the statistical interpretation of the frequency distribution data.

Variability in haploid DNA in the segregation of structural rearrangements of specific chromosomes

The special properties of the segregation of balanced translocations are shown in Fig. 3. At meiosis the translocation chromosomes pair exactly with homologous segments of the normal members of the complement. For this to occur, the chromosomes enter a relationship forming different quadrivalent arrangements, two of which are shown in Fig. 3. The chromosomes may segregate in any fashion at meiosis, giving 16 possible genetic combinations (1, 7). The most common four products of disjunction of homologous centromeres, two balanced and two unbalanced, are derived as reciprocal products in equal proportions. The unbalanced gametes are deleted for certain genes at the expense of diploid number of others. The unbalanced pair containing twice the dose of segment B should have lost some DNA that in turn has been gained by the unbalanced pair with two doses of D. If all segregants matured into sperm, the two unbalanced gametes would fall on either extreme of the frequency distribution, giving rise to increased variance in DNA content without altering the modal value. The variance is also expected to increase due to nondisjunctive forms (not shown in Fig. 3) of segregation, if all such segregants mature into sperm. We have analyzed the effect of segregation of five such translocations on sperm DNA content (see sperm donors under *Materials and Methods* for the description of each translocation). The data of the mean and standard deviation obtained from the analysis of frequency distribution of sperm from 20 subjects are presented in Table 3 and as a scatter plot in Fig. 4.

The two distinct comparative features in the population characteristics of sperm from five carriers of translocations and 15 random samples are as follows: First, the standard deviations in DNA content of four out of five donors are higher than the average value for the 15 normal donors (see Fig. 4 and Table 3). This is consistent with the illustration given in Fig. 3. Genetically unbalanced gametes are expected to contribute toward increased standard deviation. The standard deviations for identical translocations (1; 2) carried by the father and the son are similar. The unexpected result was obtained from the carrier of (21; 14) translocation, the only oligospermic donor in our study (0.5% of average sperm

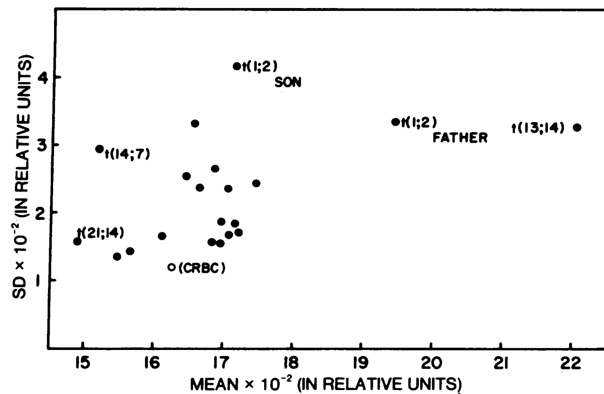

FIG. 4. Dispersion in the amount of human sperm DNA. In this scatter plot each point represents the relative values for the mean and standard deviation in the DNA content of the independent samples. Data from Table 3 are plotted; sperm DNA measurements (●), chicken erythrocyte DNA (CRBC) (O); translocations are indicated numerically (see sperm donors, *Materials and Methods*).

count). The lowest value for standard deviation was obtained in this case. It may either be due to an association of oligospermy with low standard deviation of haploid DNA, or to a specific effect of translocation involving chromosome 21. We are unable to determine whether the extent of standard deviation is uniquely determined by a given translocation until we have the opportunity to examine sperm from donors segregating a larger variety of translocations, as well as more examples of the kinds we have analyzed in this study.

The second important feature is that in the samples from carriers the mean value fluctuates rather unpredictably (see Fig. 4) over a wide range. For example, a relatively small standard error (SE = 13.05) in the mean (1673.06) DNA content of 15 random samples, which is within the range of errors caused by the instrumental and staining variations, demonstrates the remarkable constancy in the amount of sperm DNA for normal donors. In contrast, sperm from donors with structural rearrangements has on the average a mean DNA content of 1774.33 with a relatively high standard error (SE 134.05), about ten times higher than that of the random samples. The difference in the modal values of the two classes of donors can only be explained by assuming that the structural rearrangements cause an excess of aneuploid segregants that mature into sperm (13).

DISCUSSION

We have measured the population heterogeneity of human sperm with respect to the total DNA content. A relatively simple procedure that allows precision in fluorescent-DNA detection and rapid processing of large numbers of cells was used for this purpose. The standard errors in the measurement of sperm DNA in samples of a single donor collected at different times, and of 15 randomly chosen donors, were less than 1%, a value that is within the range of experimental errors. Thus, the extent of variability in the modal amount of sperm DNA was found to be less than 1%.

The close agreement of our computed data on the two homogenous components in the sperm population (see Table 2) with the values obtained by the direct cytophotometry of single cells visualized under the light microscope (12) probably attests to the validity of the computer analysis. However,

the validity needs direct testing by the application of cytogenetic techniques on sperm fractionated on the basis of DNA content. Procedures for sorting somatic cells on the basis of fluorescence are available (4) and could be adapted to sort sperm cells.

The coefficients of variation in the DNA content (see Table 3) were found to be consistently higher in sperm preparations (range 8.55%–24.39%) than in preparations of nucleated chicken erythrocytes (7.82%). The reported values of the coefficients of variation in the DNA content of somatic cells grown in tissue culture (3) are also lower than the values reported here for sperm cells. DNA constancy, despite variability in the chromosome number, has been reported for cultured mammalian cells in the above study (3). Perhaps greater variability in the amount of DNA is permitted in the maturation of sperm cells (13), whereas it is selected against in the differentiation and replication of somatic cells. The observation could also be interpreted to mean that the errors in DNA segregation occur more frequently in meiosis than mitosis.

The number of sperm donors heterozygous for chromosomal translocations was small; we need to examine a larger number of donors to establish if the positions on the scatter plot (see Fig. 4) are uniquely determined by specific chromosomes involved in translocations.

It has been estimated that approximately 5% of all gametes that effect a recognizable fertilization have a detectable chromosome aberration, and the rate of spontaneous occurrence of all new structural rearrangements of the autosomes is probably in the order of 2×10^{-3} per gamete per generation (14); the possibility of rapid detection of population heterogeneity of sperm might make the study of population cytogenetics more amenable to experimental analysis.

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