DNA CONTENT AND MORPHOLOGICAL PROPERTIES OF PRESUMABLY ANEUPLOID SPERMATOOA OF TOBACCO MOUSE HYBRIDS

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(Received 12th July 1971, accepted 27th September 1971)

Summary. A sharp reduction in fertility of the F_1 hybrids between the tobacco mouse (*M. poschiavinus*) and the laboratory mouse can be attributed to irregular segregation of trivalents in meiotic anaphase I. In male meiosis, more than 50% of aneuploid secondary spermatocytes are produced. The cytophotometric measurements of the relative Feulgen-DNA content of cytologically mature, morphologically normal spermatozoa revealed a significantly greater variation in F_1 males as compared to *M. poschiavinus* and the laboratory mouse. This difference is regarded as being due to the presence of a considerable fraction of aneuploid spermatozoa. Obviously, they are derived from the aneuploid precursor cells and have developed normally in spite of their abnormal number of chromosomes. However, the frequency of malformed spermatozoa was also higher in F_1 males than in the parental strains. The additional analysis of the number of spermatozoa, their motility and the respective proportions of living or dead spermatozoa and of spermatozoa with normal or abnormal acrosomal caps did not show differences between F_1 males and the parental strains. The head area of F_1 spermatozoa was markedly reduced.

INTRODUCTION

It has been established that reproductive failure due to fetal aneuploidy resulting from irregular meiotic segregation in gametogenesis is an important feature in man (Carr, 1970) as well as in other mammalian species (Ford, 1970). Qualitative and quantitative analyses of this phenomenon were provided by an investigation of the factors responsible for the sharp reduction of fertility in F_1 hybrids of the tobacco mouse, *M. poschiavinus*, and the laboratory mouse (Gropp, Tettenborn & von Lehmann, 1970). The F_1 hybrids are heterozygous for seven Robertsonian fusion metacentrics, and, therefore, they display seven trivalents in meiosis I. These peculiar cytological conditions enhance irregular segregation in meiotic anaphase I, thereby producing considerable gametic aneuploidy (Tettenborn & Gropp, 1970) and, consequently, a great proportion

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of chromosomally unbalanced, mostly lethal zygotes (Gropp, Giers, Ford, Clegg & Evans, in preparation). There is, therefore, evidence that aneuploid spermatogenic or oogenic precursor cells develop to mature gametes which are, at least in part, capable of fertilization.

The observations presented in this report extend and supplement earlier studies on regular versus irregular meiotic segregation in F_1 hybrid males as compared to the tobacco and laboratory mouse (Tettenborn & Gropp, 1970). The main objective of this study, however, was to measure the DNA content in cytologically mature epididymal spermatozoa. In addition, the morphology and motility of the spermatozoa were investigated.

The results of these studies and their comparative analysis should answer the question of whether or not the majority of aneuploid secondary spermatocytes, as revealed by abnormal metaphase II counts, develop to functioning epididymal spermatozoa.

MATERIALS AND METHODS

Animals

For the morphological and functional analyses and for DNA measurements of epididymal spermatozoa, twelve male tobacco mice (*M. poschiavinus*) from our laboratory stock, twenty-seven males of a laboratory mouse strain ('NMRI' Hannover) and thirty-two male (*M. poschiavinus* ♂ × NMRI ♀) F_1 hybrids were used. Meiotic preparations of three additional tobacco mouse males, four NMRI males and three F_1 males were made to supplement the meiotic metaphase II data presented by Tettenborn & Gropp (1970). All animals were between 10 and 16 weeks old.

The tobacco mouse has a diploid chromosomal set of 26 (Pl. 1, Fig. 1) with 7 pairs of fusion metacentrics (Nombre fondamental (N.F.) = 40). In F_1 hybrids, the somatic cells invariably show 33 chromosomes (Pl. 1, Fig. 1) including 7 heterozygous metacentrics (N.F. = 40).

Meiotic preparations

The occurrence of abnormal chromosomal segregation in meiotic anaphase I can be detected by the numerical evaluation of metaphase II figures. A 0.3-ml vol. of colchicine (0.5%) was injected intraperitoneally 3 hr before the animals were killed. Air-dried preparations (Evans, Breckon & Ford, 1964) were stained with acetic orcein. They yielded sufficient numbers of M II figures for the score of 100 plates per animal. The total number of chromosome arms (N.F./2) as well as the number of chromosomes (n) and metacentrics were recorded for each metaphase II plate. However, according to the procedure applied in a previous study (Tettenborn & Gropp, 1970), only the number of chromosome arms was used for determining metaphase II euploidy (N.F./2 = 20) or aneuploidy (N.F./2 > or < 20).

Assays for DNA content of spermatozoa

Spermatozoa were obtained from the vas deferens immediately after killing the animals. Smear preparations of spermatozoa from individual F_1 males were made simultaneously on separate areas of the same slide together with smears of

PLATE 1

FIG. 1. Somatic karyotype of a male tobacco mouse (top), *M. poschiavinus*, and of a male F₁ hybrid (bottom). The metacentric chromosomes are designated by the symbols T1 to 7 Bnr as proposed by Gropp, Tettenborn & Léonard (1970).

PLATE 2

Figs. 1 to 6. (1) Normal unstained (living) spermatozoon in eosin-nigrosin. (2) and (3) Eosin-stained (dead) spermatozoa with normal (2) and with abnormal (3) acrosomal caps. (4-6) Malformed spermatozoa: unstained (4 and 6) or eosin-stained (5); with double tail (5, 6) or giant head (6). $\times 2350$.

either tobacco or (NMRI) laboratory mouse spermatozoa. In this manner, a series of smears (Series I; Table 2) of spermatozoa from each of nine F₁ hybrids were arranged on the same slide with a smear from one of nine NMRI males. Similarly, in another series (Series II; see Table 2), corresponding smears of five F₁ and five tobacco mouse males were made. The fresh, wet smears were fixed (1 hr) in ethanol:formalin, 9:1, and then kept until hydrolysis (the same or the following day) in 96% ethanol. Immediately before hydrolysis (11 min in N-HCl at $60 \pm 1^\circ \text{C}$), the smears were rinsed in water for 20 min. Feulgen staining (Schiff solution, 1 hr) was carried out simultaneously and in one working procedure for all the slides of each series. As a control procedure, hydrolysis was omitted in one slide of each series. These slides were negative.

Feulgen-DNA measurements. Using a Barr & Stroud Integrating Microdensitometer, Type G.N., for cytophotometric measurements, at least fifty or more morphologically normal spermatozoa per mouse were selected. The conditions under which the measurements were carried out were: lamp 9 V; filter 550 nm; objective $\times 100$; aperture 1.30; ocular size $\times 10$; extinction level 0.75; absorption range 5.

Sperm counts

The number and density of the spermatozoa obtained from the vas deferens of nine NMRI and F₁ males were determined in two counts for each mouse in a Fuchs-Rosenthal chamber.

Morphological criteria of spermatozoa and assays for viability and motility

Fresh smears of spermatozoa were treated with eosin-nigrosin stain (Hancock, 1951; Beatty & Sharma, 1960) for the differentiation of viable (unstained) and dead (eosin-stained) spermatozoa. The observations of this staining method and some additional criteria were recorded for evaluation of:

- (a) proportion of living (unstained) spermatozoa (Pl. 2, Fig. 1);
- (b) proportion of spermatozoa with a 'normal' acrosomal cap (Pl. 2, Fig. 2);
- (c) proportion of malformed spermatozoa: slight or heavy (Pl. 2, Figs. 3 and 4) malformations of the sperm head, giant heads (Pl. 2, Fig. 6) duplication of the tail (Pl. 2, Figs. 5 and 6);
- (d) size of living 'normal' spermatozoa as measured by the area occupied by the head. Planimetric measurements were taken from photographs (negatives) projected on a screen.

Nine NMRI, seven tobacco mouse and nine F₁ males were used for these studies, with the exception of the estimation of living spermatozoa for which only six F₁ males were available. The respective counts refer to 200 spermatozoa per animal for the number of living spermatozoa and of spermatozoa with a normal acrosomal cap, and to 300 cells for the estimation of malformed spermatozoa. For the planimetric measurements of the head area, ten spermatozoa were randomly selected from each of the same animals.

The motility of the spermatozoa was determined according to the criteria established by Emmens (1947). The following two suspension media were

TABLE I
DISTRIBUTION OF EUPLOID ($N.F./2 = 20$) AND ANEUPLOID ($N.F./2 < \text{OR} > 20$) METAPHASE II (M II) FIGURES IN MALE NMRI LABORATORY MICE, *M. poschiavinus* AND F_1 HYBRIDS

N.F./2	NMRI Laboratory mouse†						Total	<i>M. poschiavinus</i> †						Total	<i>F₁</i> hybrids†						Total	NMRI	% M.p.	<i>F₁</i>
	399	359	356	362	a*	b*		393	404	408	a*	b*	366		367	402	a*	b*						
<17					1		1						1	1	1	9	2	14	0.16				1.9	
17						1	1						2	3	2	8	2	17	0.17				2.3	
18					2						1		3	2	4	19	11	44	1.2				5.8	
19					4		16				5		30	18	24	17	62	29	150	2.6			19.9	
20	1	2	2	2	4		578	7	4	9	5	126	53	40	53	148	55	349	95.2				46.2	
21	2	2	2	2	96		3	92	95	90	93		14	19	21	48	22	124	0.5				16.4	
22	97	96	96	1	1	1		1	1	1			7	4	2	20	8	41					5.4	
23													1	1		8	2	12					1.6	
>23					1											1	1	4	0.16				0.5	
							607											755						

* Values from Tettenborn & Gropp (1970).

† Statistical analysis: $m \times n$ table; χ^2 test.

(a) No significant differences of the distribution of M II plates within the individual groups of NMRI, *M. poschiavinus* (*M.p.*) and F_1 hybrids ($P \geq 0.05$).

(b) No significant difference between NMRI and *M. poschiavinus* ($P > 0.05$).

(c) Significant differences between F_1 hybrids and NMRI or *M. poschiavinus*, respectively ($P < 0.001$).

used: (a) 3% glucose, 0.4% NaCl, (b) Ringer solution (0.9% NaCl; 0.042% KCl).

RESULTS

Segregation of chromosomes in first meiotic anaphase (euploidy/aneuploidy of spermatocytes II)

In addition to the end-to-end associated XY bivalents, diakinesis figures of meiosis I invariably showed 19 and 12 autosomal bivalents in NMRI and *M. poschiavinus* respectively, and 7 chain trivalents together with 5 bivalents in the F₁ hybrids (Gropp, Tettenborn & von Lehmann, 1970).

TEXT-FIG. 1. Frequency diagram showing the distribution of euploid (N.F./2 = 20) and aneuploid metaphase II counts in spermatocytes of NMRI and *M. poschiavinus* (a) and F₁ hybrids (b).

Counts of chromosome arms (N.F./2) were made in a total of 607 M II plates of six NMRI males, in 533 plates of five tobacco mouse males and in 755 plates of five F₁ hybrid males. Data from a former investigation (Tettenborn & Gropp, 1970; see Table 1) are included in these numbers, which are a composite of additional data collected by a study of more animals. Table 1 shows the distribution of the counts of the euploid (N.F./2 = 20) and the hypo- or hypermodal (N.F./2 < and > 20) M II plates. Their relative percentile proportions are further represented in Text-fig. 1a and b. In the parental strains, NMRI and *M. poschiavinus*, the frequency of the euploid M II plates considerably exceeded

90% (95.2 and 93.1%, respectively). There was a low peak of only 46.2% euploid M II plates in the F_1 males and the presence of a considerably broader dispersion of values. In all three groups, the variation from the euploid value ($N.F./2 = 20$) was not completely symmetrical. There was an excess of hypomodal counts which may have resulted from artificial losses of chromosomes due to the spreading procedure.

The mathematical analysis of the distribution of M II counts did not reveal statistically significant differences of the individual specimens within each of the three groups (NMRI, *M. poschiavinus* and F_1). There was also no significant

TEXT-FIG. 2. Histograms representing the statistical variation of the Feulgen-DNA content as revealed by two comparative measurements of spermatozoa from NMRI compared to F_1 (a and b; see Table 2, Series I) and from *M. poschiavinus* compared to F_1 (c and d; see Table 2, Series II).

difference between the NMRI- and *M. poschiavinus*-males ($P > 0.05$). However, the differences of the distribution of the M II counts in F_1 males were statistically significant in comparison to NMRI and *M. poschiavinus* ($P < 0.001$) (see Table 1).

Feulgen-DNA measurements of spermatozoa

The histograms of Text-fig. 2a to d show the distribution of the Feulgen-DNA values of the spermatozoa of F_1 hybrids as compared to spermatozoa of the

parental NMRI laboratory mouse strain and *M. poschiavinus*. Lower peaks and a definitely broader variation of the Feulgen-DNA values characterize F_1 males. This is documented by the comparative measurements of F_1 and NMRI (Table 2, Series I; Text-fig. 2a and b) and of F_1 and *M. poschiavinus* as well (Table 2, Series II; Text-fig. 2c and d).

Detailed data of the absorption values as measured in the individual animals are represented in Table 2 (Series I of comparative measurements of nine F_1 and nine NMRI; Series II comprising five F_1 and five *M. poschiavinus*). Since the absorption measurements of F_1 versus NMRI or *M. poschiavinus* were carried out on spermatozoa fixed, hydrolysed and stained on the same slide,

TABLE 2
FEULGEN-DNA ABSORPTION VALUES OF MOUSE SPERMATOZOA: SERIES I, COMPARATIVE MEASUREMENTS OF F_1 AND NMRI; SERIES II, COMPARATIVE MEASUREMENTS OF F_1 AND *M. poschiavinus*

Preparation no.	Spermatozoa n	AU $\pm$ S.D.	Spermatozoa n	AU $\pm$ S.D.	F	P _F
SERIES I						
	F_1 hybrids		NMRI laboratory mouse			
1	51	15.20 1.37	50	15.68 1.23	1.24	$P > 0.10$
2	62	16.63 1.60	60	15.77 0.99	2.59	$P < 0.005$
3	54	16.11 1.55	51	16.40 1.19	1.69	$P < 0.05$
4	52	15.18 1.41	55	16.02 1.23	1.32	$P > 0.10$
5	51	16.21 1.38	50	15.59 1.07	1.68	$P < 0.05$
6	50	15.12 1.68	50	16.69 1.03	2.67	$P < 0.005$
7	50	16.37 1.40	50	15.35 1.06	1.76	$P < 0.05$
8	55	14.63 1.33	54	16.72 1.06	1.58	$P < 0.05$
9	54	15.94 1.54	60	16.44 0.98	2.47	$P < 0.005$
Total	479	15.71 1.47	480	16.07 1.09		
SERIES II						
	F_1 hybrids		<i>M. poschiavinus</i>			
I	65	17.23 1.64	56	16.93 1.18	1.92	$P < 0.01$
II	56	17.40 1.64	53	16.74 1.14	2.07	$P < 0.01$
III	60	17.42 1.51	66	16.75 1.11	1.85	$P < 0.01$
IV	60	17.07 1.53	62	17.22 1.20	1.63	$P < 0.05$
V	54	17.39 1.59	70	17.10 1.10	2.09	$P < 0.005$
Total	295	17.30 1.58	307	16.95 1.15		

Mean Feulgen-DNA absorption values expressed in absorption units (AU) $\pm$ S.D.

identical conditions for the comparative numbers were guaranteed. While the absorption measurements of the individual males within all three groups of animals were statistically homogeneous, there were distinct and statistically significant differences (P_F) between the variances of the DNA values in most of the F_1 males in comparison to spermatozoa of NMRI or *M. poschiavinus* measured on the same slide (Table 2). The variances of the Feulgen-DNA values were, however, statistically not significant in the case of the comparative measurements of two slides of Series I (Preparation nos. 1 and 4; F_1 against NMRI), even though the standard deviation was greater in the F_1 than in the NMRI preparations.

Sperm counts

The pooled counts of the density of the spermatozoa of nine NMRI and F₁ hybrid males gave the following mean values:

NMRI	757,800 per mm ³ ± 416,000 S.D.
F ₁ hybrids	523,800 per mm ³ ± 318,000 S.D.

The difference of 234,000 per mm³ between both mean values is statistically not significant ($P_t > 0.10$).

Viability and morphology of spermatozoa

The proportion of living spermatozoa (not stained in the eosin-nigrosin test; Pl. 2, Fig. 1, but also 4 and 6) was 21.4% in F₁ males and 19.4% or 27.2% in NMRI or *M. poschiavinus*, respectively. The differences are statistically not significant. Similarly, the number of 79.1% spermatozoa with a normal acrosomal cap (Pl. 2, Fig. 2) in F₁ males did not differ significantly from 81.3% in NMRI and 82.1% in *M. poschiavinus*.

The mean percentage of abnormal or malformed spermatozoa (Pl. 2, Figs. 5 to 6) was 9.6% in counts from nine F₁ males. This frequency is significantly higher than the respective comparative value of 2.7% in nine NMRI or of 1.7% in seven *M. poschiavinus* males ($P < 0.001$).

The results of the planimetric measurements of the heads of 'normal' and unstained (living) spermatozoa are shown in Text-fig. 3. The peaks of the variation curves of NMRI and *M. poschiavinus* are both distinctly higher than the peak for F₁ spermatozoa. The distribution is unimodal in the three groups. The mean measurements of 22.74 µm² in F₁ males is significantly lower ($P < 0.01$) than the mean values of 24.18 µm² for NMRI and of 23.25 µm² for *M. poschiavinus*.

Evaluation of the motility of the spermatozoa

The motility of spermatozoa, of NMRI and F₁ hybrid males, suspended in glucose solution as well as in Ringer solution was assessed at hourly intervals for 27 hr. The curves shown in Text-fig. 4 represent the mean values of nine animals of both groups as expressed in grades of the motility index according to Emmens (1947). There were no significant differences between the spermatozoa of the NMRI and of the F₁ hybrid males.

DISCUSSION

The chromosome counts of meiotic metaphase II plates made in the course of this study confirmed similar observations presented in a previous paper (Tettenborn & Gropp, 1970). The combined results emphasize the prevalence of regular anaphase I disjunction and of euploid M II figures in the parental strains, on one hand, and the importance of structural heterozygosity in the (*M. poschiavinus* × NMRI) F₁ males in inducing meiotic non-disjunction on the other hand. In fact, less than 50% of all M II plates in the F₁ animals were euploid while the proportion of euploid M II figures was 95.2% in NMRI and 93.1% in *M. poschiavinus*.

The cytophotometric assay on Feulgen-stained smears was used for the estimation of DNA because this method gives sufficiently accurate information

TEXT-FIG. 3. Frequency distribution of planimetric measurements of the heads of spermatozoa of NMRI, *M. poschiavinus* and F₁ hybrid males.

TEXT-FIG. 4. Motility index of NMRI (○) and F₁ hybrid (■) spermatozoa (mean values from nine males of each group) at different time intervals following suspension in glucose medium or Ringer.

about the comparative DNA content per cell, despite some objections which are discussed below. As a main result of the DNA evaluations of epididymal spermatozoa, it was shown that lower peaks and a broader variation of the DNA values characterize the (*M. poschiavinus* × NMRI) F_1 hybrids in contrast to NMRI and *M. poschiavinus*. The differences are statistically significant. Possible sources of error inherent to comparative DNA quantification in Feulgen-stained nuclei seem to be reduced to a minimum due to the selection of animals of the same age (Bouters, Esnault, Salisbury & Ortavant, 1967), held under similar dietary condition (Ely & Ross, 1951), and without any influence of extreme temperature like cold shocks (La Cour, Deeley & Chayen, 1956; Leeman, 1959; Viola, 1964). Furthermore, influences exerted by variable length of storage of the slides or by a variation of the time of staining (Hauschteck-Jungen, 1970a, b) could almost be excluded by the simultaneous application of all hydrolysing and staining procedures and by restricting the comparative measurements to cells spread on the same slide.

Since it was shown that the vast majority of the M II plates in NMRI and *M. poschiavinus* have a euploid chromosomal set, it is most probable that the DNA histograms of the spermatozoa from these two strains reflect the condition of a preponderantly or even almost pure euploid population, provided that euploid secondary spermatocytes do not produce aneuploid spermatozoa at a considerable rate. Though unexplained, slight but significant differences of the absolute DNA content of the spermatozoa of different individuals have been observed in man (Sandritter & Grosser, 1964), and in boars (Leidl & Stolla, 1969), the assumption of the constancy of the Feulgen DNA content of spermatozoa of different euploid mouse strains is stressed by the study of Godowicz & Krzanowska (1966), who determined (also by a chemical method) the absolute DNA content per spermatozoon to be about 4.5×10^{-9} mg.

Therefore, the conclusion may be drawn that the greater variation of the DNA content of (NMRI × *M. poschiavinus*) F_1 spermatozoa is due to the marked karyotypical heterogeneity in the presence of an important fraction of aneuploid spermatozoa. This variation seems to parallel the distribution of euploid and aneuploid M II counts in the precursor cells of the mature spermatozoa. It is, therefore, probable that a considerable proportion, if not a majority, of aneuploid (hypomodal as well as hypermodal) secondary spermatocytes develop into cytologically mature spermatozoa of normal shape and appearance, since only such spermatozoa had been selected for the DNA measurements. It is not possible, however, to determine the exact proportion of euploid ($n = 20$) and aneuploid ($n < \text{or} > 20$) spermatozoa of the F_1 males by comparing their respective DNA histograms with those of NMRI and *M. poschiavinus* males. A massive loss or degeneration of the aneuploid precursor cells in the course of their maturation in F_1 males can almost be ruled out by the absence of a statistically significant difference of the sperm counts in NMRI and F_1 males. In the F_1 animals, a rise of the frequency of morphologically abnormal spermatozoa (9.6%) was observed. However, their number did not seem to be sufficiently great to warrant special DNA measurements of statistical relevance. The assumption may nevertheless be correct that the chromosomal imbalance of aneuploid precursor cells is at least one of the factors responsible for the

increase of the frequency of malformed spermatozoa. Yet the importance of further peristatic or genetic factors cannot be excluded. Among others, gene imbalance could act also in the chromosomally euploid, but variably recombined, heterozygote genome of the F_1 spermatozoa. Thus, according to Braden (1959) and Beatty & Sharma (1960), sperm morphology and the frequency of sperm abnormalities are determined genetically or may be controlled by a factor located on the Y chromosome (Krzanowska, 1966, 1969). In both parental strains (NMRI and *M. poschiavinus*), the percentage of sperm abnormalities was very small and was least in *M. poschiavinus* (1.7%) which contributed the Y chromosome to the F_1 males. This observation is in contrast to the conclusions drawn by Krzanowska (1969) from her findings in crosses of the KE and CBA strains.

Without doubt, a genetic factor inherent to the recombined heterozygote haploid genome of the (NMRI $\times$ *M. poschiavinus*) F_1 spermatozoa is responsible for the variation of the head area of the F_1 spermatozoa in comparison to both NMRI and *M. poschiavinus*. Yet, the fact that the mean area occupied by the head of F_1 spermatozoa is lower than in the parental strains is noteworthy compared to observations of heterosis in sperm heads in outbred strains or in crossings between inbred strains of mice reported by Beatty & Sharma (1960), Sharma (1960) or Godowicz & Krzanowska (1966).

The variation in the DNA content, the frequency of malformations, and the area of the head are the only morphological or functional criteria of the spermatozoa which differ in the F_1 animals and the parental strains. No significant variation exists for the respective proportions of living spermatozoa in the eosin-nigrosin test or for spermatozoa with a normal acrosomal cap as well as for the motility of the spermatozoa. It can therefore be assumed that the presence of a fraction of aneuploid spermatozoa in F_1 animals does not affect the statistical variation of the latter properties in the different groups of animals investigated.

Our results show that aneuploid precursor cells in spermatogenesis may develop at a considerable rate to cytologically mature aneuploid spermatozoa. They eventually constitute a fraction of the total number of morphologically and functionally 'normal' spermatozoa, even though some aneuploid precursor cells may degenerate or produce malformed spermatozoa. These observations may provide better means for a comprehensive analysis of gametogenesis as a link in the sequential relationship of abnormal meiotic segregation, zygotic aneuploidy and reproductive failure.

ACKNOWLEDGMENTS

This study was supported by a grant (No. Gr 71/28) of the Deutsche Forschungsgemeinschaft, Bad Godesberg. For their introduction in the use of the Barr & Stroud integrating microdensitometer and for advice, the authors are indebted to Dr H. G. Schwarzacher and Dr F. Pera, Anatomisches Institut der Universität, Bonn.

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