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Cyclophosphamide in the Male Rat: New Pattern of Anomalies in the Third Generation

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Several abnormalities, such as postnatal deaths and behavioral impairments, have been previously reported in the progeny of male rats exposed to the cytostatic drug cyclophosphamide 60 days prior to mating. The anomalies were transmitted to the second generation (F2). The present results concern the third generation. Two experimental groups have been studied: a hybrid group, resulting from crosses between control subjects and either experimental F2 males or females, and a nonhybrid group, obtained by mating experimental F2 subjects together. Significant abnormalities were found in all experimental groups, whether the F2 subjects were male or female. F2 females had smaller litters whether they were mated with control or experimental males. Body weight was significantly increased in both hybrid and nonhybrid males. Increased postnatal mortality and learning deficit were also observed in the hybrid group. Such complex phenotypic changes confirm that frequent mutations probably have been inherited from the treated males but also suggest that genetic rearrangements have occurred from one generation to the next.

Key words: cyclophosphamide, male rat, spermatogonia, progeny, physical and behavioral changes, genotoxicity.

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The improved results in cancer therapy have given rise to concern about the effects of cytostatic treatments on reproductive function. Besides the possibility of irreversible sterility (Byrne et al, 1987), little is known about the risk of new mutations in the progeny of men recovering fertility after such treatments. Epidemiologic surveys have focused mainly on malformations and the results are sometimes conflicting (Li and Jaffe, 1974; Senturia et al, 1985). In fact, most of our knowledge in this field is limited to data from animal studies.

Cyclophosphamide is a widely used cytostatic drug whose mutagenic properties are well documented (Mohn and Ellenberger, 1976, Mirkes, 1985). A postmeiotic exposure of male rat gametes to this drug can induce severe abnormalities in the offspring, such as embryonic deaths and obvious malformations (Trasler et al, 1985), but also more subtle behavioral anomalies (Adams et al, 1981, 1982, 1984; Fabricant et al, 1983). In our previous works, we

have observed increased postnatal lethality and behavioral impairments in rat offspring after spermatogonial exposure to cyclophosphamide, the effects being more severe when the time interval between treatment and mating was shortened (Auroux and Dulioust, 1985, Auroux et al, 1986). Moreover, a neurochemical factor involved in memory, hippocampal choline acetyltransferase, was also modified (Auroux et al, 1987). The disorders appeared to be transmitted to the next generation, thus supporting the hypothesis that mutations had been induced (Auroux et al, 1988). In the present work, we investigate further the consequences of such genetic changes by comparing the third generation from treated males, a hybrid group obtained by mating control and experimental subjects, and a control third generation.

Materials and Methods

First and Second Generations

A detailed description of the methods and the results concerning the first and the second generation has been published previously (Auroux et al, 1986, 1988). A summary is given below.

Wistar male rats were mated with untreated females 60 days after the end of a treatment with either cyclophosphamide alone (10 mg/kg intraperitoneally daily for 15 days, n = 15), or with a combination of cyclophosphamide (same regimen) and vinblastine (ip, 250 μg/kg on the 1st day and 150 μg/kg on the 8th day, n = 6). The parameters studied were pregnancy rate and, in the offspring, litter size, sex ratio, frequency of gross external malformations and, during the first 4 months, mortality rate and body weight. At adulthood, behavioral tests were performed, studying spontaneous activity (open field) and learning ability (conditioned avoidance response). Finally, several months later, neurochemical assays were performed in the male subjects. They focused on an enzyme, hippocampal choline acetyltransferase, involved in memory processes (Jaffard et al, 1979, Squire, 1981).

The first generation from treated males showed an increased mortality rate in the postnatal period. Later. spontaneous activity was decreased and learning ability was impaired in two ways. First, the percentage of conditioned animals (success rate) was lower than in the control group. Second, the conditioned males showed a higher frequency of failures before conditioning (success mode) than the conditioned controls. No difference was observed between the animals descended from males treated with either cyclophosphamide alone or with cyclophosphamide plus vinblastine. The neurochemical assays revealed a decreased hippocampal choline acetyltransferase activity.

A second generation was produced by random matings within each group, control and experimental, the latter including offspring from both groups of treated males (cyclophosphamide and cyclophosphamide plus vinblastine). The same parameters as in the first generation were evaluated and a similar pattern of anomalies was found: increased postnatal mortality rate, behavioral abnormalities, and decreased hippocampal choline acetyltransferase activity. Additionally, it was shown that natural inheritance of behavioral traits or foster mother influences could not explain the observed effects. Subtle differences between the first and second generation were observed.

Third Generation

When 7 months old, second generation subjects from the treated rats (F2T) and from the controls (F2C) were randomly selected and mated as follows: F2C male/F2C female; F2C male/F2T female; F2T male/F2C female; F2T male/F2T female.

In this way, four groups were obtained in the third generation, one control group and three experimental groups, consisting of one nonhybrid group obtained exclusively from F2T subjects, and two hybrid groups obtained from crosses between F2T and control subjects (the results from the two hybrid groups were pooled because no differences were observed).

Identity of the couples and corresponding progeny was coded until the experiment was completed. Once the code was broken, the exact genealogy of each subject was established. The cages were randomly distributed in the animal house. The general conditions of breeding and the detection of the pregnancies corresponded to the methods previously described (Auroux et al, 1986).

At the end of gestation, the pregnant females were examined twice daily and newborn pups were counted. The births took place over 6 days. On the 3rd day after birth, the litters were weighed and the pups were carefully inspected for sex determination and detection of gross external malformations. Dead or missing pups were recorded.

Between 3 and 5 days of age, each litter was culled to four randomly selected animals, two males and two females. In three litters, where such numbers were not available, a sex ratio (number of males/total) of 3/4 or 1/4 was accepted. In one litter made up of four females, two females were replaced by two males from another litter. At the same time, to control for postnatal maternal factors, each litter was given to a randomly chosen foster mother from one of the three groups. When 4 weeks old, the young were weaned and males and females were separated and housed six or seven per cage. Throughout the experiment, the cages were randomly distributed in the animal house. The animals were weighed again at 17 and 39 weeks of age.

Behavioral testing started at 18 weeks of age. Each subject was submitted to the two tests used in our previous experiments: learning ability in an automated shuttle box, and spontaneous activity in open field (Auroux et al, 1986). Learning ability was assessed within a period of 11 days, between 10 A.M. and 3 P.M. Each day, randomly chosen male and female subjects from the different groups were tested alternately. The parameters evaluated for all the animals were: 1) Success rate—percentage of animals showing a conditioned response (six consecutive successful attempts). 2) Total stimulation time—the total duration of the 50 attempts of the test.

TABLE 1. Fertility of the Parents

	Controls	Hybrids		Nonhybrids
Parental origin (♂× ♀)	$F_2C \times F_2C$	$F_2C \times F_2T$	$F_2T \times F_2C$	$F_2T \times F_2T$
Pregnancy rate*	21/30 70%	8/15 53,3%	11/15 73,3%	23/30 76,7%
Litter size ± SD†	9,5 ± 2,7[a]	6,75 ± 1,16[b]	11,09 ± 1,38[c]	8,3 ± 2,6[d]

*$X^2 = 2.69$, df = 3, NS.

†a-b, c-d, a-c, b-d : variances are significantly different.
Wilcoxon's tests—a-b , c-d (maternal factor) : $p < 0.01$.
a-c , b-d (paternal factor) : $p > 0.05$.

Successful subjects only were evaluated for the success mode: percentage of failures up to and including conditioning.

Spontaneous activity was evaluated 40 days after learning ability, using an Opto-Varimex Colombus device. The modalities of the test were the same as in the second generation (Auroux et al, 1988). The following data were collected: total distance covered by the animal, resting time, number of stereotypic movements (e.g. grooming behavior), number of rearings.

Finally, hippocampal choline acetyltransferase activity was determined in the males at 10 months of age using the same procedure as in the previous generations (Auroux et al, 1987) Twenty males in each group were studied.

Statistical Analysis. Preliminary analysis compared the two groups of hybrid offspring. Since no difference was observed, results from these groups were pooled, and comparisons were made between the controls and either the nonhybrid or the hybrid subjects. Whenever possible, the effect of paternal (control or experimental) and maternal (idem) origin was studied in a two-way analysis of variance. Quantitative results were compared using analysis of variance and *t*-test, or using Wilcoxon's test when the variances were significantly different. Qualitative results were compared using the chi-square test.

Results

Fertility of the Parents

Pregnancy rates and litter sizes for the different matings are presented in Table 1. Pregnancy rates were not different, but significant variations were observed in litter size. The two-way analysis of variance seemed to show opposite maternal and paternal effects; the experimental mothers were associated with reduced litter sizes, and the experimental fathers with increased litter sizes. However, the validity of this analysis was impaired by significantly different variances. Wilcoxon's tests confirmed the maternal but not the paternal effect ($P < 0.01$).

General Characteristics in the Third Generation.

The general characteristics of the third generation are summarized in Table 2. No difference was observed between the hybrid groups for any of the parameters. It was verified that the random choice of foster mothers ensured a good balance of different maternal conditions. No changes were observed in sex ratio. We found no external malformations. However, both nonhybrids (F3T) and pooled hybrids (F3H) showed significant abnormalities when compared with the controls (F3C).

Hybrid Group (F3H). The mortality rate after cross-fostering was significantly increased ($P < 0.05$). Clustered deaths in two litters were excluded. Males and females seemed equally affected ($X^2 = 0.48$, NS). The mortality rate was unrelated to the origin of either the parents or the foster mother. Increased body weight was observed in the male subjects at 39 weeks of age. Their mean weight at that age was intermediate between the control and nonhybrid means. High weight was not related to the origin of the foster mother. Female weights were not different from control values.

Non-Hybrid Group (F3T). Body weight was significantly increased in the males at 17 weeks of age (about 7%, $P < 0.01$). The difference between this group and the controls increased with age and reached about 10% of the mean weight of the controls at 39 weeks ($P < 0.001$), far exceeding the physiologic increase at that age. Again, no foster mother effect was found. Females were not affected.

The results of the two-way analysis of variance for the male weights are presented in Table 3. Only maternal effects were significant at 17 weeks of age. At 39 weeks of age, both experimental fathers and mothers seemed to be associated with increased weights, though only the paternal effect was significant. While these variances differed significantly, the paternal effect remained significant in one separate Wilcoxon's test ($P < 0.04$). As discussed below, such parental effects were not linked to higher body weights in F2 parents.

TABLE 2. General Characteristics

		Controls (a)	Hybrids (b)	Nonhybrids (c)	Tests
Sex ratio (♂/♀) (4th day)		73/80	96/87*	88/84	a-b : X^2 = 0.75 NS a-c : X^2 = 0.39 NS
Malformations		0	0	0	—
Deaths	0-4 days	9/192 4.7%	7/160 4.4%	11/183 6%	a-b : X^2 = 0.01 NS a-c : X^2 = 0.32 NS
	5-30 days	4/76 5.3%	10/60 16.7%*	6/88 6.8%	a-b : X^2 = 4.7, $p < 0.05$ a-c : X^2 = 0.17 NS
Weight	3 days (♂ + ♀)	7.40 ± 0.80	7.38 ± 0.88*	7.86 ± 1.14	a-b : t_{37} = 0.07 NS a-c : t_{41} = 1.47 NS
	♂ 17 weeks	424.2 ± 43.2	428.7 ± 36.3*	457.2 ± 42.7	a-b : t_{60} = 0.44 NS a-c : t_{75} = 3.36, $p < 0.001$
	♀	256.6 ± 20.6	259.8 ± 19.2*	266.9 ± 22.7	a-b, a-c : NS
	♂ 39 weeks	506.5 ± 53.0	533.5 ± 34.4*	556.3 ± 64.1	a-b : Wilcoxon's test $p <$ 0.04†
					a-c : t = 3.78, $p < 0.001$
	♀	293.1 ± 34.4	294.7 ± 27.0*	295.7 ± 29.0	a-b, a-c : NS

*No significant difference between the hybrid subgroups (see also Table 3).
†Variances are significantly different.

Behavioral Data

Learning Ability. The data are presented in Table 4. No difference was found between the hybrid groups. No foster mother effect was observed. The results were unrelated to the learning performance (successful or not) of the parents. The hybrid males showed a significant increase in total stimulation time ($P < 0.01$), and their success rate was low, although not significantly different from that of the controls. The success mode was not modified. In males, the two-way analysis of variance revealed a significant interaction between the paternal and maternal factors ($P < 0.01$). The females were not different from the controls.

The nonhybrid animals, whether male or female, did not differ from the controls, whatever the parameter.

Spontaneous Activity. No difference in spontaneous activity were observed, whatever the parameter or the mode of analysis (Table 5).

Neurochemical Data

No difference was found, either between the hybrid subgroups or between experimental and control groups: (μmoles acetylcholine/g.prot./h)F3C: 31.52 ±2.22; F3H: 32.49 ± 2.15, F3T: 32.75 ± 1.61.

Discussion

Several findings emerged from this experiment. F2T females had smaller litters whether they were

TABLE 3. Male Weights—17 and 39 Weeks

	Controls (a)	Hybrids (b)	Hybrids (c)	Nonhybrids (d)	Tests
Parents (♂ × ♀)	$F_2C \times F_2C$	$F_2C \times F_2T$	$F_2T \times F_2C$	$F_2T \times F_2T$	
17 weeks	424.2 ± 43.2	431.8 ± 35.5	426.4 ± 38.0	457.2 ± 42.7	Two-way analysis of variance Interaction : NS Maternal factor (a-b , c-d) : p = 0.046 Paternal factor (a-c , b-d) : p = 0.15
39 weeks*	506.5 ± 53.0	526.8 ± 32.3	537.9 ± 36.1	556.3 ± 64.1	Two-way analysis of variance Interaction : NS Maternal factor (a-b , c-d) : p = 0.127 Paternal factor (a-c , b-d) : p = 0.017 Wilcoxon's test : a-c : $p < 0.04$ a-b, c-d, b-d : NS

*Variances are significantly different.

TABLE 4. Learning Ability

		Controls (a)	Hybrids* (b)	Nonhybrids (c)	Tests
Total stimulation time†	♂	1747 ± 581	2189 ± 700	1879 ± 569	a-b : t_{60} = 2.71, $p < 0.01$‡ a-c : t_{75} = 1, NS
	♀	1854 ± 601	1767 ± 341	1742 ± 514	a-b, a-c : NS
Success rate	♂	24/36 66.7%	11/26 42.3%	22/41 53.7%	a-b : X^2 = 3.64, NS a-c : X^2 = 1.34, NS
	♀	16/3 45.7%	15/24 62.5%	23/40 57.5%	a-b, a-c : NS
Success mode	♂	61.06 ± 11.02	63.70 ± 9.98	62.45 ± 12.02	a-b, a-c : NS
	♀	60.35 ± 11.03	64.87 ± 8.98	61.14 ± 12.83	a-b, a-c : NS

*No difference between the hybrid subgroups.
†Arbitrary units.
‡The two-way analysis of variance showed a significant interaction between parental factors ($p < 0.01$).

mated with F2T or control males. Both males and females from hybrid matings, but not from F2T × F2T matings, had an increased risk of postnatal mortality. Male offspring of F2T parents in any mating regimen (F2T × F2T or F2T × control) weighed more than controls, a trait inherited from both F2T fathers and mothers. A learning impairment was observed in hybrid (F3H) males but not in nonhybrids (F3T). Therefore, abnormalities were found in both hybrid and nonhybrid subjects, providing new evidence that cyclophosphamide-induced mutations are inherited from the treated males and raising the question of the frequency of such mutations. The differences between hybrids and nonhybrids, and the changes between the second and third generations emphasize the complexity of the underlying processes. In addition, as in our previous experiments, differences were observed between males and females.

Anomalies Observed in the Two Experimental Groups

Reduced Litter Size. The relationship between the second generation experimental males and increased litter sizes needs further verification. However, it is clear that the experimental females produced smaller litters than the control females. Maternal genotype is known to influence size, and induced genetic changes might have resulted in decreased fertility or in disorders during pregnancy in these animals (Wilmut et al, 1986). Litter size may also be reduced by consanguinity but this does not explain the difference between control and experimental females, since the same effect was observed whatever the origin of the male. The defects might also concern the embryos themselves. Embryonic lethality was probably not sex-linked, because the sex ratio was not modified. Chromosomal rearrangements may lead to embryonic death. However, transmission of induced chromosomal anomalies

TABLE 5a. Spontaneous Activity—Males

	Controls	Hybrids*	Nonhybrids	Tests
Distance (cm)	355 ± 270	385 ± 233	372 ± 272	F^2_{100} = 0.13 NS
Time resting (sec)	97.2 ± 36.4	92.3 ± 36.1	95.5 ± 37.9	F^2_{100} = 0.23 NS
Stereotypic movements (N_0)	108.1 ± 37.4	114.7 ± 39	108.7 ± 37.1	F^2_{100} = 0.34 NS
Vertical movements (N_0)	9.3 ± 7.6	11.3 ± 8.2	11.5 ± 11.4	F^2_{100} = 0.66 NS

TABLE 5b. Spontaneous Activity—Females

	Controls	Hybrids*	Nonhybrids	Tests
Distance (cm)	511 ± 271	508 ± 272	416 ± 273	F^2_{96} = 1.06 NS
Time resting (sec)	74.7 ± 32.6	75.7 ± 34.7	85.7 ± 36.6	F^2_{96} = 0.84 NS
Stereotypic movements (N_0)	123.7 ± 32.6	123.4 ± 33.9	115 ± 37.7	F^2_{96} = 0.56 NS
Vertical movements (N_0)	14.4 ± 8.7	14.5 ± 9.9	10.8 ± 7.6	F^2_{96} = 1.48 NS

*No difference between the hybrid subgroups.

from spermatogonia appear to be rare. We failed to observe major changes in preimplantation embryos obtained after a paternal spermatogonial exposure to cyclophosphamide (Auroux et al., in press). Dominant gene mutations represent another possibility, since they may have more severe effects when they are maternally transmitted as, for example, the T locus mutations in the mouse (McGrath and Solter, 1984). Finally, such asymmetric paternal and maternal effects on embryonic viability might also result from incompatibility between the embryos and some uterine factors (Rossant et al, 1983) or between certain genomic and cytoplasmic factors as observed in mice between the DDK strain and other strains (Mann, 1986).

Increased Body Weight. This anomaly was unexpected. Age-related or technical biases have been excluded, as well as foster mother effects. The mean weight of the controls corresponded to the breeder's values. Food consumption was not increased. There was no difference between the hybrid subgroups. Moreover, the weight increase, affecting both experimental groups, occurred earlier in the nonhybrids. Furthermore, at 39 weeks of age, the difference between nonhybrids and controls reached nearly twice the difference between hybrids and controls. Therefore, the anomaly appeared to result from equivalent and cumulative paternal and maternal effects.

Parental weights were verified but F2T parents did not weigh more than control parents, although this point cannot definitely be ruled out because, in the second generation, body weight was recorded up to the 13th week only (this problem suggests the importance of long term investigations in such experiments). Moreover, while body weights either at 17 or at 39 weeks were significantly related to the mean weight of the parents in male and female controls and in all experimental females ($r > 0.6$, $p < 0.02$), such correlations did not exist at 17 weeks in both hybrid and nonhybrid males ($r < 0.32$, NS). Therefore, it seems difficult to explain the increased weight by natural heritability after a spontaneous shift, which itself hardly could have occurred within only three generations and without selection for high or low weight. Consanguinity effects also seem irrelevant: only three males born of two sibling matings were kept in the experiment. On the other hand, an increased weight has been reported in the progeny of irradiated male mice (Ramel, 1983). In the present case, if we suppose that induced mutations were involved, the apparent discrepancy between the second and third generation suggests that particular mutated genes and genetic backgrounds might have been selected through genetic rearrangements.

Other Anomalies in the Hybrid Group: Postnatal Deaths and Learning Impairment

In addition to abnormal weight, a higher postnatal mortality rate and, in the males, learning impairment, were also observed in the hybrids. As in our previous experiments, the precise cause of death remained unknown. Maternal factors cannot be excluded, although the deaths did not predominantly concern the litters born of (or fostered by) experimental females. As regards learning, although the success rate itself was not significantly reduced, the difference with the controls (about 25%) was close to the significant differences observed in the previous generations. We therefore cannot conclude that learning ability was really less impaired in this generation. However, a decrease in learning deficit might correspond to a lack of neurochemical changes. In other respects, the learning deficit was not related to higher weight.

In our studies, the association of increased postnatal mortality and learning impairments has been observed in three successive generations from the treated males. In the first and in the hybrid third generation, only one of the parents was an experimental subject, suggesting dominant mutations, where penetrance and expressivity would be variable. Such mutations would probably show other phenotypic effects, but nervous function might be particularly susceptible to mutations as it seems clear from the high number of different mRNAs found in the brain (Van Ness et al, 1979) that numerous genes are expressed in nervous tissue. Therefore, it is not surprising to have repeatedly observed behavioral abnormalities, in association with other disorders.

Frequency of Induced Mutations

In the present experiment, as in the previous generations, the quantitative differences observed did not result from major anomalies in few subjects, but rather from slight deviations in numerous animals. Even if every mutation showed pleiotropic effects, a high frequency of induced mutations is suggested. Such an interpretation may seem unusual but, interestingly, several similar experiments in

drosophila have come to the same conclusion: studying polygenic traits in a quantitative way yields mutation rates that are much higher than those calculated from punctual mutations. Mutations inducing clear-cut phenotypic effects might therefore represent a minority of the changes possible in a complex organism (Ramel, 1983). Actually, this conclusion agrees with current knowledge about the subtlety of gene expression and protein function. Evidence for such a hypothesis might come from detecting minor cytogenetic and/or molecular changes at the individual level.

Differences Between the Two Experimental Groups

Contrary to the results concerning weight, which was increased in both experimental groups in a consistent manner, some discrepancies were observed in postnatal mortality and learning impairment, which affected the hybrid but not, apparently, the nonhybrid animals. Such differences need further investigation but raise interesting problems.

The absence of increased mortality or learning deficit in the nonhybrid (F3T) group cannot be explained in terms of a high percentage of animals free from any induced mutation. First, the increased weights would have to be explained. Second, no anomaly should have been found in the hybrid (F3H) group, since the parents were randomly chosen. Third, such an explanation would imply a low frequency of induced mutations, which seems to conflict with the other results.

Actually, the main difference between hybrid and nonhybrid subjects was that the latter could have inherited cyclophosphamide-induced mutations (but not necessarily the same ones) from both of their parents. If we suppose that, indeed, such an event has frequently occurred, we might hypothesize that interactions between mutations corrected some of their consequences. Several examples of mutations that simultaneously increase some functions while reducing others have been reported in drosophila: some of them can thus suppress the effects of other mutations and restore a normal phenotype (Scott, 1987). Similar processes might be involved in our observations.

Sex Differences

Once again, obvious differences were observed between sexes. Only the increased postnatal mortality affected males and females equally. The experimental females, whether hybrid or not, had no weight anomaly. No learning impairment was found in the hybrid females. Such differences have been observed several times in our previous works (Auroux, 1983, Auroux and Dulioust, 1985; Auroux et al, 1986, 1988). Each time, the males exhibited more severe troubles than the females. Here, similar alterations were found in the progeny of experimental females mated with control males and in the progeny of experimental males mated with control females. Therefore, at least in this experiment, the differences between the sexes might reflect a higher sensitivity of the male organism to the effects of certain mutations rather than more frequent or particular mutations in the males.

Conclusions

Significant abnormalities have been observed over three generations in the progeny of cyclophosphamide-treated male rats. In the second and third generation, all experimental groups were affected. Cerebral biochemical changes were also oberved in the first and second generations (Auroux et al, 1987). Our results support the hypothesis that alkylating drugs can induce transmissible mutations in the spermatogonia. This suggests the possibility of a long lasting genetic risk after exposure of the male to mutagenic agents. Furthermore, it seems that mutation rates based on qualitative criteria such as malformations or other clear-cut phenotypic changes probably underestimate the overall frequency of genetic changes in complex organisms. In fact, mutations inducing viable anomalies might be frequent. Screening for functional disorders might then become a useful tool in mutagenesis studies. The relevance of such experimental results to human situations is still very difficult to evaluate. However, these findings, which agree with those of other authors (Adams et al, 1981, 1984, Fabricant et al, 1983), would seem to justify more extensive studies in man.

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