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EMBRYONIC AND POST-EMBRYONIC LETHALS INDUCED BY DIETHYL SULFATE IN MATURE SPERM OF *DROSOPHILA MELANOGASTER*

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Summary

It has recently been reported that, in *Drosophila melanogaster*, when sperm treated with diethyl sulfate was stored in the females, II–III translocations were detected as from the 6th day after the treatment, though none was recovered without storage. Chromosome breaks being currently considered the main cause of dominant lethality and the embryonic period lasting about one day at 25°C, it was thought of interest to study the ability of DES to induce this type of damage with and without storage. It was found that the treatment increased embryonic lethality (measured as frequency of unhatched eggs) and post-embryonic lethality (measured as frequency of larval and pupal death) over the control values. The frequency of embryonic lethals after storage in the females for 6 days was similar to that shown by the unstored samples. In contrast with this, the yield of post-embryonic lethality was markedly raised by that storage time. It is suggested that: (1) lesions are induced as “pre-breaks”, and storage and cell divisions are instrumental in their opening; (2) potential breaks can undergo DNA replication and cell division as such and become open in different cell cycles, impairing embryonic and post-embryonic development; (3) chromosome breaks induced by DES seem to behave in a way similar to those induced by other mono- and poly-functional alkylating agents; and (4) when the potential ability of chemical compounds to induce chromosome breaks is assessed, post-embryonic lethality can be used as a simple one-generation preliminary test, to establish delayed effects.

Introduction

It has recently been reported that in *Drosophila melanogaster*, when sperm treated with diethyl sulfate (DES) is stored in untreated females, II–III trans-

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locations are recovered with a frequency that increases with storage time [13]. With the higher concentration used (0.75%), translocations were detected as from the 6th day after treatment. In line with previous findings [14], no translocation was observed without storage, and it was then suggested that DES induces potential breaks that in time open.

Since chromosome breaks are considered to be the main cause of dominant lethality and at 25°C the embryonic period lasts about a day, it was considered of interest to assess the ability of DES to induce this type of damage with and without storage.

This paper reports results on embryonic lethality (measured as frequency of unhatched eggs) and post-embryonic lethality (measured as frequency of larval and pupal death) after treatment of *Drosophila* sperm with DES.

Materials and methods

Diethyl sulfate (DES) ($C_2H_5)_2SO_4$, mol. wt. 154.19 (British Drug Houses Ltd.) dissolved in ethanol, was made up into 0.5 and 0.75% solutions in 5% glucose. It was administered to 7-day-old wild-type *Samarkand* males by means of saturated filter paper attached to the walls of 180-ml bottles (150 males/bottle). Immediately after treatment the males were mated to *Oregon K* virgin females, 4 to 6 days old.

The 0.5% solution was used to study embryonic lethality (unstored) with flies exposed to the chemical for 2.5 h (Series I) and 3 h (Series II). The 0.75% solution was used for embryonic and post-embryonic lethal tests (with and without storage). The duration of treatments was: Series I, 2.5 h; Series II, 2.5 h (1-h interval in regular food), 1 h (total time of treatment 3.5 h). For all the controls the males were fed on an ethanol-5% glucose solution for 3 h.

To study the effect of storage on the treated sperm, the inseminated females were kept at 17°C for 6 days, with regular food (the deposition of eggs is slightly reduced by this storage technique). After this period, the females were again placed in oviposition chambers and the eggs laid were scored. All experiments were carried out at 25°C unless otherwise indicated.

The frequencies of embryonic and post-embryonic death were corrected for control values by using the formula:

$$R = \frac{Pt - Pc}{1 - Pc}$$

(Pt , proportion in the treated sample; Pc , proportion in the control), adapted from Abbot's correction [1,21]. To estimate the significance of the differences, a test based on a Z parameter was used

$$Z = \frac{R1 - R2}{\sqrt{\text{var. } R1 + \text{var. } R2}}$$

where the variance of R was calculated from the following expression [17]:

$$\text{var. } R = \left(\frac{1}{1 - Pc} \right)^2 \text{var. } Pt + \frac{(1 - Pt)^2}{(1 - Pc)^4} \text{var. } Pc$$

Embryonic lethality

For these experiments the males were individually mated with the females in empty vials, and after one observed mating (this ensures the exclusive sampling of sperm that at the time of treatment was mature and motile), the males were discarded and the females transferred to oviposition chambers for 24 h. A medium of agar, sugar, yeast, molasses and acetic acid was used that encouraged oviposition and was dark enough to allow the eggs to be easily distinguished.

The eggs were counted after a 24-h oviposition period. The unhatched eggs were scored 24 h later and again after an additional 24-h period to ensure the detection of late hatching. All vials with 100% unhatched eggs were discarded.

Post-embryonic lethality

These experiments were carried out to determine the number of individuals that go through embryogenesis successfully but do not reach the adult stage (larval and pupal death). The oviposition chambers in which hatched and unhatched eggs had been scored, both from stored and unstored sperm, were incubated until emergence of the adults, that were counted daily until exhaustion. To prevent distortion of the data [29], care was taken to avoid overcrowding.

Results

(a) Embryonic lethality

The results of the embryonic lethal tests after treatment of mature sperm with DES are shown in Table 1. It is evident that this compound was highly efficient in increasing embryonic death when treated males were mated with untreated females. This increase, measured as frequency of unhatched eggs, was dose-dependent.

In Series I (2.5 h of treatment) the frequency of embryonic death with 0.75% DES was 2.2 times as high as it was with a 0.5% solution. In Series II,

TABLE 1
EMBRYONIC LETHALITY INDUCED BY TREATING MATURE SPERM WITH DES

	$\frac{\text{Unhatched eggs}}{\text{Total eggs}} \times 100^a$	
	DES 0.5%	DES 0.75%
Control	5.32 (136/1991)	5.25 (193/1962)
Series I	24.52 (593/2423)	54.79 (862/1508)
Series II	31.23 (630/1801)	73.51 (498/660)

Statistical significance: in all cases $\alpha < 6 \times 10^{-5}$

^a% in the treated series corrected as indicated in Materials and methods. Duration of treatment: DES 0.5%: Series I, 2.5 h; Series II, 3 h; DES 0.75%: Series I, 2.5 h; Series II, 2.5 h + 1 h (1-h interval); Control: 3 h.

with DES 0.75% (3.5 h of treatment) the frequency was 2.3 times as high as it was with DES 0.5% (3 h of treatment). With DES 0.5% the frequency of embryonic death in Series II was 1.3 times as high as in Series I; with a concentration of 0.75% the difference was also 1.3 times greater.

To study further the action of DES 0.75% the treated sperm was stored for 6 days in untreated females. The first part of Table 2 shows that the frequency of embryonic death remained unchanged after this storage period. The frequency of embryonic lethals in Series II was 1.4 times as high as in Series I.

(b) Post-embryonic lethality

The study of post-embryonic death (lethals that act in the larval or pupal stages) was carried out both with and without storage of the treated sperm in the females. Post-embryonic lethals, as can be seen in the second part of Table 2, also increased significantly over the controls in both series after treatment with a 0.75% solution. But, in contrast with the results shown by embryonic lethals, the yield of post-embryonic lethality in the unstored sample was not affected by the length of exposure to the chemical. Analysis of the data obtained after storing the treated sperm for 6 days led to the following conclusions. (1) The frequencies in the treated series were significantly higher than in the control; (2) the frequency of lethals in Series II (3.5 h treatment) was significantly higher than that in Series I (2.5 h treatment); and (3) all the frequencies obtained were significantly higher than those shown by the unstored samples.

TABLE 2

EFFECT OF STORAGE ON THE FREQUENCIES OF EMBRYONIC AND POST-EMBRYONIC LETHALITY INDUCED BY TREATING MATURE SPERM WITH 0.75% DES

	Embryonic lethality			Post-embryonic lethality		
	Unhatched eggs			Unhatched eggs — adults		
	Total eggs			Hatched eggs		
	Control (C)	Series I (SI)	Series II (SII)	Control (C)	Series I (SI)	Series II (SII)
Non-stored (A)	5.25 (103/1962)	54.79 (862/1508)	73.51 (498/660)	2.42 (45/1859)	11.96 (91/646)	11.85 (26/186)
Stored for 6 days (B)	4.55 (39/857)	52.07 (595/1089)	74.49 (379/501)	5.38 (44/818)	20.84 (124/494)	30.04 (72/210)
Statistical significance (only values of $\alpha \leq 0.05$ shown)						
(A) vs. (B)	—	—	—	<0.001	<0.001	<6 × 10 ⁻⁵
(SI) vs. (SII)			<6 × 10 ⁻⁷			—
(SI) vs. (SII)			<6 × 10 ⁻⁷			<0.03
In all cases: (C) vs. (SI) and (SII) $\alpha < 6 \times 10^{-7}$						

Duration of treatments: Series I: 2.5 h; Series II: 2.5 h + 1 h (1-h interval); Control: 3 h.

^a % in the treated series corrected as indicated in Materials and methods.

Despite the differences shown by the controls, which are presently under study, the differences found between stored and non-stored sperm in the treated series are real, since all results have been corrected for control values as indicated in Materials and Methods.

Discussion

From the data presented here, it is evident that DES, in addition to being a powerful mutagenic agent in *Drosophila* [18,19,15,3,16,14], is highly efficient in increasing embryonic death when treated males are mated to untreated females. Since chromosome breaks are currently considered to be the main cause of dominant lethality, the obvious explanation for the high frequency of embryonic lethality observed would be that chromosome breaks were induced. However, this would raise a seeming discrepancy with the lack of II—III translocations in unstored DES-treated *Drosophila* sperm [14,13].

Therefore, a critical evaluation of other causes that might account for the observed increase is called for before the high frequency of embryonic death reported here is imputed to chromosome breakage. It is conceivable that, after treatment of sperm with a mutagen, damage leading to dominant lethality other than chromosome breaks could also be induced: (1) point mutations; (2) physiological alterations of the sperm; and (3) injuries to structures other than chromosomes or genes.

The possibility that point mutations with a dominant-lethal effect could have contributed substantially to the high frequency of embryonic death seems remote. In a thorough analysis of segmental aneuploidy in *Drosophila*, Lindsley, Sandler et al. [12] reached the conclusion that in the entire genome of this organism there is only one triplo lethal locus that is located in the third chromosome, and that a few loci must be present in two doses for the survival of the fly. This implies that only a few dominant lethal mutations result from a change in the dosage of a gene. The production of dominant phenotypes would be restricted to changes that result in uncontrolled gene function or the production of an abnormal gene product.

In connection with the second point it should be mentioned that Sankaranarayanan [21] found that, after nitrogen treatment to males, physiological damage causes the rapid exhaustion of usable sperm in the females, leading to a steep increase in the proportion of unhatched eggs in successive egg-laying periods, spuriously increasing the frequency of embryonic death. This possibility cannot be completely ruled out after treatment of sperm with DES. Yet, in that case a still higher frequency of unhatched eggs should have been observed after storage (the deposition of eggs is diminished but not inhibited by the storage technique used). The observation that the frequency of embryonic lethals did not increase over that found without storage suggests that physiological damage, if inflicted on the sperm, was not the only cause of the effect observed.

It has been postulated that, after treatment of *Drosophila* sperm, damage to structures essential for normal cleavage might lead to dominant lethality [8]. Owing to the high toxicity of DES to adult flies, this possibility could not be excluded without further analysis of the action of this drug. To this end, the

frequency of post-embryonic death (lethals that act at the larval and pupal stages) was determined since the non-chromosomal alterations already mentioned would not be expected seriously to affect post-embryonic death.

The increase in post-embryonic death found was taken as an indication that chromosome breaks were actually induced, bearing in mind that the probability of inducing point mutations with a dominant-lethal effect is very low. Moreover, a still higher frequency of these lethals was obtained after storage, and it is known that, in *Drosophila*, storage acts only upon breakage events [23,9,10, 6,26,20,28,2,7].

Based on the data already discussed, the embryonic lethals induced by DES can also be ascribed to chromosome breakage despite the lack of increase after storing for 6 days. It is worth noting that, with ethyleneimine, Štátník [27] observed a storage effect on dominant lethals measured as unhatched eggs only after 6 days, and with ethylmethane sulfonate this effect became really evident after that period. The same result could probably be reached by storing DES-treated sperm for more than 6 days.

The evidence given above, together with the absence of II-III translocations without storage [14,13], strongly suggests that the breaks induced are mainly potential and that the time that elapsed between their induction and the union of pronuclei after fertilization does not suffice to allow for their opening and the exchange of fragments. The opening of breaks should therefore take place mostly beyond the first cleavage divisions.

The fixation of breaks during cleavage divisions would lead to the formation of mosaics some of which would be eliminated as embryonic lethals, and others, though successfully passing through embryogenesis, would impair larval or pupal development. Since II-III translocations were not detected before 6 days of storage, only a few of those embryonic lethals found with that storage period could have been the consequence of asymmetrical chromosome exchanges of the complete type. The term "mosaic" is used in reference to breaks that open during cleavage divisions and "complete" to those that open during pronucleolus formation or before, and not in connection with damage to one or two DNA strands.

The lack of storage effect for embryonic lethals (as well as for II-III translocations) could lead to the assumption that DES induces actual breaks that cannot establish the necessary exchange conditions, or that potential breaks open simultaneously during embryogenesis, independently of the time that elapsed after treatment. However, the increase of post-embryonic lethals with storage suggests that new lesions become effective, in addition to those leading to embryonic and post-embryonic lethality without storage. These latent breaks opening later during embryogenesis (or during post-embryonic stages) might not prevent the hatching of eggs, but would adversely affect the normal development of larvae and pupae. This implies that chromosomes with pre-breakage lesions induced by DES can undergo DNA replication and the breaks are realized in subsequent cell cycles. With ethyl methanesulfonate it has recently been reported that some of the alkylations induced in the DNA of sperm cells are retained throughout cleavage divisions [11].

There is an apparent discrepancy between the long storage time needed for translocations to be detected and the high embryonic lethality observed with

out storage. (At 25°C the embryonic stage lasts about one day.) This could be explained on the assumption that storage time and cellular divisions are both instrumental in the opening of potential breaks. While sperm remain in the storage organs of the female, time would be the decisive factor, but after fertilization, when cleavage divisions start, the latter would play a preponderant role, despite the short time that elapsed, operating through chromosome movements and/or DNA replication. Potential breaks which surpass these difficulties, and become fixed later on may contribute to post-embryonic lethality.

It could be argued that the embryonic lethals scored without storage result only from the breaks that become effective immediately after treatment and not from those that open during the short embryonic period. These breaks, though insufficient in number to give rise to a sizeable frequency of translocations, could lead to dominant lethality. Against this view would be the observation that, after treatment of mature sperm with ionizing radiations (broken ends are readily available for exchange), translocations are detected with doses that induce lower frequencies of embryonic lethals. (For a recent review see Sankaranarayanan and Sobels [22].)

Our interpretation of the data fits the hypothesis put forward by Auerbach [4,5] that alkylating agents would induce potential breaks that remain latent for a period that may extend over many cell cycles, and with Slyzinska's work that provided the cytological evidence of the nature of the storage effect at chromosomal level [24,25]. The fact that Slyzinska used triethylene melamine and ethyleneimine suggests that DES shares the characteristic features of those alkylating agents in their chromosome-breaking ability.

It can be concluded that the data presented here, together with those reported earlier for translocations [13], are consistent in showing that: (1) DES is efficient in breaking *Drosophila* chromosomes; (2) lesions are induced mainly as "pre-breaks", and storage and/or cell division are instrumental in their opening; (3) potential breaks can undergo DNA replication and cell divisions as such and become open in different cell cycles, impairing embryonic and post-embryonic development; (4) chromosome breaks induced by DES seem to behave in a similar way to those induced by other mono- and poly-functional alkylating agents; (5) when the potential ability of chemical compounds to induce chromosome breaks is assessed, it is important to establish delayed effects. To this end, post-embryonic lethality can be used as a simple one-generation preliminary test.

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