THE BROOD PATTERN OF MITOMYCIN-C-INDUCED TRANSLOCATIONS IN *DROSOPHILA MELANOGASTER* MALES: THE EFFECT OF TIME

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ABSTRACT

Translocations between the two large autosomes were scored in the progeny of males that had been fed mitomycin-C. The frequencies were the same in spermatogonia sampled after 12 and 25 days and in spermatozoa stored for the same periods in untreated females.

MITOMYCIN-C (MMC) produces a very unusual brood pattern in both males and females (MUKHERJEE 1965; SCHEWE, SUZUKI and ERASMUS 1971). Sex-linked recessive lethals were found in all germ-cell stages, but in both sexes their frequency was lower in gonia than in postmeiotic stages. The frequency of translocations showed the opposite trend. In both sexes, no increase over controls was found in the first three broods of two days each; after this, the translocation frequency in both sexes increased, at first slightly and then steeply, reaching a peak in the last brood, sampled 12 to 14 days after treatment. Similar results were obtained for dominant *versus* recessive lethals in Habrobracon (SMITH 1969).

Many hypotheses can be put forward to account for these contrasting brood patterns of point mutations and chromosome rearrangements, but they are not easily put to the test. We have, instead, examined the effect of a very simple variable: passage of time. It is well known that chromosome breakage by alkylating agents is subject to a "storage effect," *i.e.*, a striking increase in frequency when treated spermatozoa are stored in the seminal receptacles of untreated females (reviewed in LEE 1976). The length of the storage period required for a noticeable effect varies between mutagens; in general, it appears to be longer for monofunctional than for bifunctional alkylating agents. On the contrary, there is no storage effect on point mutations. We wanted to see whether the high frequency of translocations in gonia was due simply to the long interval between treatment and the sampling of germ cells that had been treated as gonia. To this end, we compared the frequencies of translocations between the two large autosomes in treated spermatozoa that had been stored for 12 or 25 days, and in spermatogonia that had been sampled at the same times.

MATERIALS AND METHODS

Three-day-old males of an OrK stock were treated by feeding them 1.5 mM mitomycin-C in 1% sucrose solution for two days according to the technique of LEWIS and BACHER (1968). Treatment was carried out at 20° in the dark because of the known deleterious effect of light on mitomycin-C. After treatment, the males were left one day without females in order to prevent mitomycin-C from being carried over into the females at fertilization. On the second day after treatment, the males were mated for the first time; this is called Day one in our results. On the following day, males and females were separated; the females were allowed to lay eggs for another two days. In order to secure regular utilization of germ cells, the males were given fresh virgin females at regular intervals: the brood pattern, in days, starting with the second day after treatment, was 1–2–2–2–2–2–3–3–3–4. In the experiments, only two broods were used in addition to the first: that produced on Days 12 to 15 and that produced on Days 25 to 27, representing, respectively, treated late and early spermatogonia. In the storage series, the inseminated females were kept on protein-free food until Day 12 or 25.

Males from the same treatment were divided into two lots. In one, for the detection of sex-linked lethals, the males were mated to Muller-5 females. In the other, for the detection of translocations, the males were mated to *bw*;*st* (brown; scarlet) females. These markers are on the two large autosomes (*bw* on *2*, *st* on *3*), and translocations between these chromosomes are detected as pseudo-linkage between *bw* and *st*. The crosses of the treated males were done in bottles at a ratio of 2 females per male.

RESULTS

Three experiments were performed. In two of them (I and II), tests were carried out on (a) mature spermatozoa, sampled on Day 1, (b) mature spermatozoa, stored for 12 days in untreated females, (c) mature spermatozoa, stored for 25 days in untreated females, and (d) spermatogonia, sampled on days 12 to 15.

For a comparison with (c), a further experiment (III) was carried out in which spermatogonia were tested on Days 25 to 27 after treatment. The results are shown in Table 1.

TABLE 1

The frequencies of sex-linked lethals (s-l-l) and 2–3 translocations (tr) in mature stored and unstored spermatozoa and in late and early spermatogonia

Expt.	Method	Day	Treated stage	s-l-l		tr		
				n	%	*n*	tr	%
I(a)		1	spermatozoa	200	5.0	600	0	0
II(a)			spermatozoa	200	6.0	600	0	0
I(d)	broods	12–15	spermatogonia	200	3.5	150	1	0.7
II(d)	broods	12–15	spermatogonia	400	2.5	600	5	0.8
I(b)	storage	12	spermatozoa			600	2	0.3
II(b)	storage	12	spermatozoa			600	1	0.2
III(e)	broods	25–27	spermatogonia	400	2.0	560	7	1.2
I(c)	storage	25	spermatozoa	200	6.0	175	2	1.1
II(c)	storage	25	spermatozoa	175	5.7	375	5	1.2

DISCUSSION

Sex-linked lethals

Germ cell stages (a,d,e): Lethals were found in all tested broods. This agrees with the more complete brood pattern obtained by MUKHERJEE (1965). Thus, MMC penetrates into cells of all stages and produces damage to DNA. In mature spermatozoa (a), sex-linked lethal frequency was from two to three times as high as in spermatogonia. This differs from the data of MUKHERJEE (1965), who found a very low frequency of lethals on the first day, with a peak on the third. The discrepancy may be due to the fact that our Day 1 corresponds to his Day 2.

Storage effect (a,c): The frequency of sex-linked lethals did not change during storage for 25 days at 25°. This confirms previous results obtained with various alkylating agents (LEE 1976).

Translocations

Germ cell stage (a,d,e): No translocations were found in 1200 spermatozoa, while 400 spermatozoa sampled on the same day yielded between 5 and 6% sex-linked lethals. There were six translocations in 750 late spermatogonia, in which the frequency of lethals had dropped to about 3%, and there were seven translocations in 560 early spermatogonia, in which lethal frequency had dropped to 2%. This agrees with the brood pattern obtained by SCHEWE, SUZUKI and ERASMUS (1971) for translocations between the *X* and the *Y* chromosomes; they found the first indication of induced translocations between Days 8 and 10, followed by a steep rise between Days 10 and 14, when they were sampled for the last time.

Storage effect (a,b,c): Treated spermatozoa that had been stored in untreated females for 12 days carried three translocations in 1200 tested gametes, as compared with none in 1200 before storage. Statistically, the increase is not significant, but the fact that it occurred in both experiments is suggestive. Moreover, the negative result for unstored spermatozoa is reinforced by the data of SCHEWE, SUZUKI and ERASMUS (1971) who found no increase in the frequency of *X-Y* or *Y-Y* translocations among nearly 14,000 spermatozoa, after a treatment which produced almost 3% translocations in spermatogonia.

After 25 days of storage, the result of our experiments was unambiguous. Using Yates' correction for small numbers, the χ^2 for the difference between stored and unstored spermatozoa is about 13 ($P < 0.001$). The frequency of translocations after 25 days of storage was the same as that in spermatogonia sampled at the same time. After only 12 days of storage, the frequency of translocations in simultaneously sampled spermatogonia was higher than that in stored spermatozoa. If this is not due to chance, it may indicate a steep increase in translocation frequency during Days 13 and 14, which were included in the spermatogonial sample, but not in that of stored spermatozoon.

General conclusions

In agreement with previous results, our incomplete brood patterns for sex-linked lethals and translocations show opposite trends. While sex-linked lethal

frequency dropped from mature sperm to spermatogonia, translocation frequency was zero in mature sperm, very low in late spermatogonia and fairly high in early ones. This shows that one of the explanations offered for the high translocation frequency in gonial cells, namely the amplification of a single initial event during the gonial divisions (SCHEWE, SUZUKI and ERASMUS 1971) cannot be correct. If it were, the frequency of lethals should be equally increased by cluster formation. Actually, this explanation is invalid on general grounds: if "events" are multiplied by cell divisions, so are "nonevents," in this case the number of spermatogonia without a translocation, and in the overall result the two will compensate each other. The only difference that cluster formation makes to the evaluation of the data lies in the standard error, which increases with cluster size; a formula for its calculation in dependence on cluster size has been given by MULLER (1952). For the special case of translocations, however, there exists the possibility that the nonevents will be multiplied more than the events, since translocations may be eliminated through mitotic or meiotic segregation. If this were the case in our experiments, the frequency of induced translocations may have been somewhat higher in spermatogonia than in stored spermatozoa, although the frequencies of recovered translocations were the same.

The differences between the brood patterns for MMC-induced sex-linked lethals on the one hand and translocations on the other must rest on differences in the mechanisms by which these effects are produced. Both require primary effects on DNA, followed by secondary steps. In rearrangement formation, the primary effect consists of lesions that lead to chromosome breakage, and this has to be followed by rejoining of broken ends into new configurations of the karyotype. Secondary steps are also involved in the production of point mutations; but not much is known about them in eukaryotes. If, as seems likely, repair enzymes play a role, they will be different from the one that allows reunion between broken chromosome ends.

There are many differences between spermatozoa and spermatogonia in metabolism, enzyme content, degree of condensation of the chromosomes, the position of the chromosomes in regard to each other, and so on. Hence, it is not difficult to imagine that conditions in spermatogonia favor those secondary steps that result in the formation of rearrangements, while conditions in spermatozoa do not, and that an opposite trend exists for point mutations.

There is, however, one difference between the production of point mutations and rearrangements that occurs at or near the level of primary damage: their response to storage. The frequency of point mutations remains unaffected by storage, while that of rearrangements increases strikingly. This is due, not to an effect on the secondary steps of contact and rejoining of broken chromosome ends, but to a manifold increase in the frequency of lesions that, after fertilization, open into chromosome breaks and can be rejoined by enzymatic repair in the zygote. Chromosome breaks that do not rejoin result in dominant lethality, and this, too, is increased by storage of Drosophila sperm treated with ethyl methanesulphonate or ethylene imine (ŠRAM 1970a) and, in late broods of Habrobracon males treated with mitomycin-C (SMITH 1969). It is well known

that chromosome breaks in Drosophila sperm, produced by whatever agent, do neither restitute nor rejoin before fertilization. The nature of these "pre-breaks" and the primary lesions preceding them is still unknown. The slow loss of alkylations in stored sperm, probably by hydrolysis (JANCA, LEE and AARON 1978) may play a role in the accumulation of pre-breaks. Whatever their nature, our data show that MMC can produce the requisite primary effects in spermatozoa, and that, given sufficient storage time, these can develop into pre-breaks and, subsequently, into breaks and rearrangements. The length of the required storage period is similar to that of monofunctional alkylating agents (ŠRAM 1970b) and suggests that MMC might act as such an agent when producing chromosome breakage (VIG 1977).

While it is tempting to conclude from our results that the passage of time alone is responsible for the peculiar brood pattern of mitomycin-C-induced translocations, we cannot exclude the possibility of chance coincidence between the effect of storage and of continued breeding in these particular experiments. This may be tested, and we intend to do it in the near future. We base ourselves on the fact that it is the pre-breakage lesions and not the completed translocations that are subject to a storage effect. If conditions in spermatogonia favored the secondary steps leading to translocations, then these would be completed already in the spermatogonial cells and should no longer respond to storage in spermatozoa. If, on the other hand, primary lesions in spermatogonia required the same time as those in spermatozoa to mature into pre-breaks, then they should still respond to further storage in spermatozoa. If the latter alternative should turn out to be correct, one would have to imagine that the primary chromosome damage can persist through more than one cell cycle. A number of observations on Drosophila (SLIZYNSKA 1957; MUÑOZ and BARNETT 1977), mice (GENEROSO *et al.* 1977) and human lymphocytes (ISHII and BENDER 1978) suggest that this is true.

Our results bear similarity to those obtained with the monofunctional alkylating agent (diethylsulphate, DES). This substance is highly mutagenic in Drosophila (RAPOPORT 1947; PELECANOS and AIDERSON 1964), but so far has failed to yield translocations in spermatozoa and larval spermatogonia (PELECANOS 1966). Positive results have, however, been obtained after prolonged storage (MUÑOZ and BARNETT 1977). When spermatozoa were treated with a dose that yielded 27% sex-linked lethals, no translocations were found in immediately utilized spermatozoa nor in sperm that had been stored for two weeks. When, however, storage was continued from the 16th to the 20th day, the frequency of translocations rose from 2% to nearly 6%. It would be interesting to see whether this substance, too, appears to produce translocations exclusively in spermatogonia in a conventional brood pattern analysis. It is true that PELECANOS (1966) found no translocations in treated larval spermatogonia. This suggests either that these cells are not susceptible to the treatment (in which case they should also fail to yield sex-linked lethals) or that the period between treatment and sperm utilization, presumable ten to 12 days, was too short for the primary lesions to develop into pre-breaks.

Finally, we want to draw attention to the bearing of our results on a problem that has first been pointed out by SLIZYNSKA (1969). It is usually assumed that chemicals that produce mutations only after one or more mitoses must require DNA synthesis for their action. This has, for example, been assumed for chemicals that in plant cells produce only chromatid breaks even when acting on the undivided chromosomes in G1 (KIHLMAN 1966; KIHLMAN, NATARAJAN and ANDERSON 1978; EVANS and SCOTT 1969). Similar conclusions have been drawn for chemicals that produce delayed chromosome breakage in mice (BREWEN and PAYNE 1976). SLIZYNSKA (1969) pointed out that in most systems it is very difficult to distinguish between passage of time with and without concomitant cell divisions, and she suggested that some of the observed delayed effects might not be attributable to a requirement for DNA synthesis. Very recently, unfortunately after her death, this prediction has been verified in at least one case. GICHNER and VELEMINSKY (1977) found that DES applied to barley seeds in the G1 phase produced almost exclusively chromatid aberrations and very few chromosome aberrations (193:4). When, however, the treated seeds were stored for one to four weeks under conditions that did not allow DNA replication, the vast majority of aberrations were of the chromosome type (4052 out of 4059). Thus, during storage, chromatid aberrations (which in Drosophila would not have been spotted as translocations) were transformed into chromosome aberrations. GENEROSO *et al.* (1977) have tentatively drawn a similar conclusion for delayed chromosome breakage by TEM in mice. In these cases, too, it appears that passage of time, rather than mitosis, is required for the formation of chromosome breakage.

Our results suggest caution in the interpretation of brood patterns for chromosome breakage. Differences in sensitivity between mature sperm and spermatogonia or spermatocytes need to be validated by allowing the spermatozoa a storage period that extends to the time when the younger germ cell stages are sampled.

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