GENETICAL EFFECTS OF NITROGEN MUSTARD IN THE HOUSE MOUSE

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(With Plate 3)

Snell (1933, 1935) and Hertwig (1938, 1940) have shown that the F_1 of irradiated male mice contains a proportion of semi-sterile animals, which transmit their semi-sterility to about half their progeny. The cause for this heritable semi-sterility was thought to be the presence of X-ray-induced translocations, and its mechanism the lethal action of unbalanced chromosome complements on zygotes when either sperm or ovum contained a translocation. Embryological proof for zygotic lethality was obtained by Snell, Bodemann & Holländer (1934), Snell & Picken (1935) and Hertwig (1940). Cytological proof of the presence of translocations in semi-sterile lines was given by Koller (1944), who also established a relationship between the degree of semi-sterility and the type of translocation present in the line. Snell (1941, 1946) confirmed the translocation hypothesis genetically by means of linkage tests, and Slizynski (1952) demonstrated cytologically the presence of an interchange in one of these translocations.

Mustard gas and several nitrogen mustards resemble X-rays closely in their genetical effects. They have so far produced mutations in every organism which has been studied from this point of view. In mammals, the only case of a mutation which may have arisen through the action of nitrogen mustard is a new recessive in the mouse reported by Auerbach & Falconer (1949). Translocations in *Drosophila* have been produced with mustard gas and one nitrogen mustard (Auerbach & Robson 1947*a*, *b*; Kaufmann, Gay & Rothberg, 1949).

The experiments reported here were designed to test the effect of nitrogen mustard treatment of male mice in producing visible mutations and inherited semi-sterility. Part of the work (Exp. I) has already been reported briefly in connexion with the new mutant; the data are included in the present report. Exp. II was designed for the detection of translocations only, and Exp. III for that of both translocations and visible mutations.

Material and methods

The method of treatment was the same in all three experiments: it consisted in intraperitoneal injection of a solution of nitrogen mustard—methyl-*bis* (betachloroethyl) amine hydrochloride—into adult males. The solutions were made up with tap water and were buffered by the addition of small amounts of $NaHCO_3$. The doses used were: 0·08 mg. in 2 c.c. in Exp. I; 0·08 mg. in 1 c.c. in Exp. II; 0·06 mg. in 1·5 c.c. in Exp. III. With two exceptions, the treated males belonged to an inbred wild-type strain (Strong-CBA). The exceptions were two males in Exp. II, which were homozygous for Rex (**ReRe**) and were only moderately inbred. The untreated females to which the treated males were mated were **aa bb pp** (moderately inbred) in Exp. I, **aa bb cc** (inbred, Strong-A) in Exp. II, and wild-type (inbred, Strong-CBA) in Exp. III. In addition, in Exp. III there were three

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litters by mothers known to carry **a**, **b** and **c** in heterozygous condition, but no other mutant genes.

Genetically, the following tests were carried out:

(1) Inspection for dominant mutations in F_1. Where the females carried recessive marker genes (**a**, **b**, **c** or **p**), or where the treated males carried dominant markers not present in the females (**Re**), mutation to the recessive alleles at these loci, or chromosome loss or deletion covering any of the marked loci, would be revealed in the F_1. In view of the observation that in *Drosophila* mustard treatment often has a delayed effect resulting in mosaicism in F_1 (Auerbach, Robson & Carr, 1947), care was taken to examine the F_1 animals for the possible occurrence of mosaic spots.

(2) Breeding tests to detect recessive visible mutations. The method, namely, outcrossing of F_1 males and backcrosses of their daughters to them, has been described in an earlier communication (Auerbach & Falconer, 1949), and has been more fully evaluated in a separate paper (Falconer, 1949). The test litters were all kept till 3 weeks of age and were then carefully examined for external abnormalities. When any abnormality was found, proof of its genetical nature was sought by allowing the parents of the litter to continue breeding, and sometimes by additional breeding tests of the abnormal individuals. Autopsies were not made, nor was any attempt made to identify lethals acting before or soon after birth. The females used for these tests belonged to the inbred black C_{57} strain in Exp. I, and to the inbred wild-type CBA strain in Exp. III. In addition, some F_1 females of Exp. I were outcrossed and the progeny mated *inter se* as described in the previous papers. In Exp. II no tests for recessive visible mutations were carried out.

(3) Fertility tests of F_1 males to detect animals with transmissible semi-sterility. In Exps. I and III, F_1 males which had produced less than six young in their first outcross litter (see above (2)), were further tested by their second litter with the same female, and also by the addition of fresh females from outbred stocks, which had already proved their fertility. The F_1 males of Exp. II were tested only by outbred females. The females were examined daily for vaginal plugs, so that litters of zero size could be detected. Males which gave consistently small litters (less than six young) were kept for further genetical or cytological study.

For cytological investigations, testis material was used. The testes were removed immediately after killing, teased apart with stainless needles and transferred to a vial with acetocarmine. Squash preparations may be made after about half an hour; but fixation for several hours is preferable. Microscopical preparations were made from small pieces of the tubuli (about 1 mm. long). Cellophane squares were used instead of cover-slips, and the tissue was squashed by rolling test-tubes over the cellophane. The slides were then placed in a jar with tap water; this loosens the cellophane square so that it can be pulled off after about 10 min. Staining in hot fuchsin preceded by hydrolysis and followed by bleaching in SO_2-water was carried out as described previously (Slizynski, 1949, 1952). Camera lucida drawings were made at a magnification of 3600 × with an 80 ×, 1·32 n.a. oil-immersion objective and a 30 × eyepiece. For the drawing of details 6 × eyepieces were used. Microphotographs were made with the aid of a Cook, Troughton and Simms attachable camera, using a 45 ×, 0·95 n.a. fluorite oil-immersion objective, a 30 × eyepiece, and a Wratten filter no. 62 in front of the substage condenser.

Results

(1) *Effect of the treatment on the treated males themselves*

The toxic effect of the injections varied considerably, even between males of the same experiment. Thus, in Exp. I, two out of three injected males died within 3 days after treatment, while the third survived until killed between 2 and 3 months later. Similarly, in Exp. III, eight out of twelve injected males died between 4 and 9 days after injection, while the remaining four lived for about 2 months or were killed after 3 months. Possibly these variations in toxicity were in part due to slight variations in technique, in particular to the oozing out of drops of fluid from the injection site, which was observed in several cases.

Fertility of the treated males was not correlated with the length of their survival, except of course in those cases where sickness made the animals unwilling to copulate. In Exp. II all three injected males had to be killed on the sixth day following treatment, because they were manifestly ill; yet all of them had copulated and produced litters of at least average sizes (six, eight and nine young). In Exp. III, one male which died 4 days after treatment produced a litter of nine, while two others, which were killed after more than 3 months, had four and five young in their first litters.

As after X-ray treatment, an initial fertile period, yielding the 'Frühprobanden' (Hertwig, 1938), was succeeded in the survivors by a period of complete sterility, which in its turn was followed by a second fertile period, during which the 'Spätprobanden' were produced. In four males which in our experiments lived to produce 'Spätprobanden', the initial fertile period lasted from 0 to 8 days, and fertility was resumed after 34, 40, 40 and 41 days respectively, the times being reckoned from the day of treatment to the day of copulation. Litter size in the first fertile period ranged from 0 to 11, and in the second fertile period from 1 to 10, none of the males giving consistently small litters in either period.

(2) *Genetical results*

No dominant mutation or genetical loss at the marked loci was observed in sixty-seven F_1 animals produced during the early fertile period, or in eighty-two produced in the late period. Exp. I yielded one recessive visible mutation, 'crinkled', which has already been described fully (Auerbach & Falconer, 1949; Falconer, Fraser & King, 1951). This mutation was found in the progeny of an F_1 female of the Spätprobanden, the equivalent of 11·3 treated spermatozoa having been 'fully tested' (see Falconer, 1949). The three males which in Exp. III yielded Spätprobanden had no mutation in 20·7 fully tested spermatozoa, nor in 13·6 fully tested spermatozoa from Frühprobanden. Altogether, then, 'crinkled' was the only visible mutation in 45·6 fully tested spermatozoa from treated males. A number of other abnormalities were observed in the test litters, but all of these were proved by further tests to be non-genetic.

Fertility tests were carried out on six F_1 females, all of them Spätprobanden, and seventy-one F_1 males. One of the females was completely sterile; but as female sterility in inbred lines is fairly frequent, this case was not followed up. The tested males consisted of twenty-nine Frühprobanden and forty-two Spätprobanden. Two were completely sterile; one of them occurred among the Frühprobanden, the other—a cryptorchid—among the Spätprobanden. Both were examined cytologically (males S-1 and S-2). Among the

Frühprobanden of Exp. III, there was one clear case of semi-sterility. This male (S-8) sired twenty litters which ranged in size from one to five young. Fertility tests of his sons showed the hereditary nature of the reduced fertility: among eighteen tested sons, nine were clearly semi-sterile, eight clearly fertile and one doubtful. Some more semi-sterile males were found among the progeny of the semi-sterile sons of S-8. Male S-8 as well as a number of his descendants were examined cytologically. A second semi-sterile male occurred among the Spätprobanden of Exp. II. He produced seven litters ranging in size from two to seven, but unfortunately this male was lost before progeny for genetical and cytological tests had been secured. There were three cases—one among the Frühprobanden, two among the Spätprobanden—of males with only moderately reduced fertility, but they could not be subjected to further tests on account of shortage of space and labour.

(3) *Cytological results*

Of the two completely sterile F_1 males, one (male S-1) showed no chromosome abnormalities at pachytene, diplotene or diakinesis, and the proportion of abnormal sperm-heads in his tubuli—less than 5 %—was within the limits of variation typical for the CBA strain. The other, a cryptorchid (male S-2), showed no structural changes at pachytene, and the normal number of tetrads at diakinesis. Spermatogenesis, however, was not completed, and only a few abnormal-looking spermatid nuclei could be found. Since cryptorchism is not always incompatible with the formation of mature sperm, it cannot be decided whether the interruption of sperm development was a secondary effect of the existing cryptorchism.

The semi-sterile male S-8 and his semi-sterile progeny turned out to be very interesting objects of cytological studies. Male S-8 had only eighteen tetrads at diplotene, diakinesis and metaphase, an observation which suggested that two independent translocations were present. These were, in fact, revealed by closer examination. One of them, $T(10:13)$, involved the two autosomes 10 and 13; the other, $T(Y:A)$, an autosome of medium size, possibly 16, and the Y-chromosome.

In $T(10:13)$ (Pl. 3, figs. *a–d*) a very small segment of chromosome 10 has been exchanged for a medium-sized segment of chromosome 13. The breaking-point in chromosome 10 is near the end, in division 32 (Slizynski, 1949), while that in chromosome 13 is in division 44. The two chromosomes can thus be represented as follows:

(1) 29, 30, centromere of 10, 31, 32, break, 60/–63, and

(2) 58, centromere of 13, 59, 60, break, 32.

Translocation $T(Y:A)$ is shown in Pl. 3, figs. *e–j*. It obviously involves one of the two sex-chromosomes, which are characterized by the presence of the puffy region. The conclusion that it was the Y-chromosome rather than the X which took part in the translocation was made on genetical as well as cytological grounds. Genetically, a translocation involving the X-chromosome of a male will be handed on to all his daughters and none of his sons; whereas a translocation involving the Y will be handed on to none of his daughters and all his sons. Crossing-over between the centromere and the point of interchange may create some exceptions in either case, but will rarely be frequent enough to obscure the situation. When cytological examination of seven sons and one grandson of male S-8 revealed the presence of $T(Y:A)$ in all but one of them, the grandson, it was clear that it was the Y and not the X which took part in the translocation. This was corroborated by cytological evidence, which will be discussed in a separate paper on the sex-chromosomes

of the mouse. Here it is sufficient to say that the break in the *Y*-chromosome occurred between the centromere and the puffy region in a position which allows occasional crossing-over between breakage-point and centromere, such as must have occurred in the origin of the translocation-free grandson.

DISCUSSION

There can be little doubt that also in the mouse nitrogen mustard produces genetical effects. In the experiments reported here, the progeny of twelve treated males was found to contain one heterozygote for a visible recessive, two completely sterile males, two semi-sterile males and three suspected semi-steriles.

Undoubtedly, the best proof for an effect of the treatment comes from one of the semi-sterile males, S-8, which was analysed both genetically and cytologically. Genetically, he was found to transmit his semi-sterility to about half his sons; cytologically he was found to carry two independent translocations, one—$T(10:13)$—involving two autosomes, the other—$T(Y:A)$—involving a third autosome and the *Y*-chromosome. Unfortunately, the stock had to be discarded before the cytological analysis was carried out, and the decision as to which of the two translocations caused the observed semi-sterility had to be made on the basis of the available slides. Slides of the following individuals were examined: the original semi-sterile F_1 male, S-8; five semi-sterile F_2 males; one F_2 male and one F_3 male which had not been tested for fertility. The F_1 male and all F_2 males carried both translocations; the F_3 male carried neither. The presence of translocation $T(Y–A)$ is expected in all males of the line; it is rather its absence in the grandson which is unexpected. As discussed before, it can be explained by crossing-over. The $T(10:13)$ translocation, on the other hand, is expected in only half the F_2 males; its presence in all five semi-sterile ones makes a connexion between it and the semi-sterility probable. In any case, the fact remains that whichever translocation resulted in semi-sterility it must have arisen *de novo* in a treated spermatozoon; for the father as well as seven tested brothers of the semi-sterile male S-8 were fully fertile. Although nothing is known about the presence and rate of origin of translocations in untreated mouse stocks, it seems highly unlikely that the *de novo* origin of two different translocations in the same male, or of one translocation in a male which already happened to carry a spontaneous one, should have been unconnected with the mustard treatment. Moreover, one of us (B.S.) has cytologically examined between thirty and forty randomly chosen males of the same inbred line to which male S-8 belonged and has found neither of the translocations in any of them.

The other observed abnormalities in the progeny of the treated males furnish corroborative evidence for a genetical effect of the treatment, but are not in themselves sufficient to prove it. It is probable that the second clear case of semi-sterility was due to a third translocation, and possible that the same was true for one or more of the three cases of reduced fertility. The presence of two completely sterile males agrees with observations of X-ray workers, who also generally found some completely sterile individuals among the sons of treated males (Snell, 1935; Hertwig, 1940; Russell, 1950).

As regards the visible mutation to 'crinkled', the earlier discussion of its origin (Falconer, 1949) had overlooked the obvious fact that the method used does not distinguish between the source of the mutation from the treated or untreated gametes which produced the F_1, so that spontaneous mutations will be detected in either of these. When the method of test is by backcrossing F_2 progeny to the F_1 parent, an equal number of untreated and treated gametes are tested. When the method is by intercrossing the F_2, the number of untreated

gametes is three times the number of treated ones, because the two untreated gametes represented by the mate of the F_1 animal are tested equally with the one treated and one untreated gamete represented by the F_1 animal itself. In Exp. I several of the tests were by intercrosses, and the equivalent number of fully tested gametes was: treated 11·3, untreated 16·7, total 28·0. In Exp. II, no test crosses for the detection of visible recessives were carried out. In Exp. III all the tests were backcrosses, and a total of 68·6 gametes was fully tested, consisting of 34·3 treated and 34·3 untreated ones. Thus, although 'crinkled' was the only visible mutation detected in 45·6 fully tested treated gametes, it might have occurred spontaneously in a total of 96·6 fully tested gametes, treated or untreated. Too little is known about the frequency of visible recessive mutations in untreated stocks to decide whether these data suggest that the mutation had in fact been produced by the treatment. In any case, with the dose and method used, nitrogen mustard is not an efficient agent for the production of visible mutations in mice.

In this respect it does not differ from X-rays. It is true that Hertwig (1942) obtained several visible recessives in the progeny of irradiated mice, and attributes their origin to the effect of the treatment. But Snell (1935), from a quantitative study which was made more accurate than ours through routine application of autopsy to samples of the test-cross progeny, arrives at the conclusion that visible mutations are rarely, if ever, produced by X-rays. In about fifty fully tested gametes from males which had been treated with 600 r., he found one visible mutation, a dominant spleen abnormality of variable manifestation. Since his test method consisted exclusively in backcrossing the F_2 to the F_1, this number corresponds to one visible mutation, detectable only by autopsy, in a total of about 100 gametes.

The same X-ray dose produced translocations in about 35 % of the treated spermatozoa. It is here that our results differ greatly from those obtained with X-rays. In seventy-one treated spermatozoa two certain and one probable translocations were found, and even if three further doubtful cases are considered as translocations, the result falls still considerably short of the X-ray results. This does not, in our opinion, indicate that nitrogen mustard is less efficient than X-rays in breaking the chromosomes. There is no way of comparing X-ray doses with doses of nitrogen mustard except by their effects. As regards toxicity, the dose of nitrogen mustard was certainly much more effective than an X-ray dose of 600 r. which, according to Snell, produced no detectable injury in the majority of the exposed males. It seems that the noxious systemic effects of nitrogen mustard are so drastic that a dose able to produce genetical effects at a rate produced by moderate X-ray doses would be lethal to the treated animal. This is supported by the data on litter sizes in F_1. Whereas after irradiation the size of litters produced by the treated males falls off regularly with increasing dose and at 600 r. averages about three young per litter, only three out of eleven litters sired by mustard-treated males during their first period of fertility had less than six young, and none less than four. It seems that the dose, in spite of its pronounced toxicity, was not high enough to induce many of those chromosomal changes in the treated sperm which, by causing zygotic lethality (Brenneke, 1937), deplete the F_1 litters before birth.

Mustard compounds have recently come into use as means for producing new hereditary types. For many organisms this method seems to be as valuable as irradiation or even preferable to it. Our results show that this is not so for mice, although it is possible that different compounds and different methods of application might give better results.

SUMMARY

Male mice of the CBA strain were treated with intraperitoneal injections of nitrogen mustard (methyl-*bis* (betachloroethyl) amine hydrochloride), and their progeny was examined for the presence of visible recessive mutations and of heritable semi-sterility due to translocations. The progeny of twelve treated males contained one heterozygote for a visible recessive, two completely sterile males, two semi-sterile males, and three suspected semi-steriles. Only one of the two semi-sterile males could be analysed genetically and cytologically and was found to carry two independent translocations, one involving two autosomes, the second involving a third autosome and the *Y*-chromosome. The presence of one of these translocations, probably the autosomal one, resulted in transmission of the semi-sterility to half the progeny. At least one, probably both, of these translocations must have arisen *de novo* in treated sperm. The origin of the visible mutation by treatment cannot be proved. Although the results show that nitrogen mustard can produce structural changes in mouse chromosomes, their yield is low compared with that of even moderate X-ray doses. This low efficiency of the chemical treatment is probably due to the fact that the severe systemic effects on the treated animals do not allow the application of genetically more effective doses. The results of these experiments hold out no hope that the type of procedure used in them can be utilized for the routine production of new hereditary types in the mouse.

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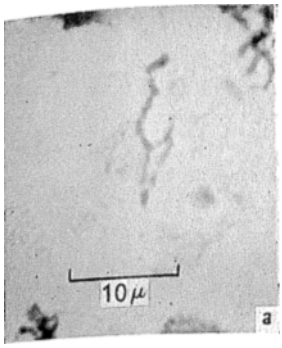
EXPLANATION OF PLATE

Translocation between chromosomes 10 and 13: *a*, *b* and *c*, pachytene configuration (*a*, photograph; *b*, camera lucida drawing; *c*, chromatid diagram); *d*, first meiotic metaphase.

Translocation between *Y*-chromosome and an autosome: *e*, *f* and *g*, diplotene configuration (*e*, photograph; *f*, camera lucida drawing; *g*, chromatid diagram); *h*, *i* and *j*, pachytene configuration; (*h*, photograph; *i*, camera lucida drawing; *j*, chromatid diagram).

In figures *c* and *j* the position of centromere is given by cross-lines, in *g* by a circle.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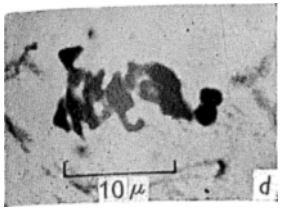
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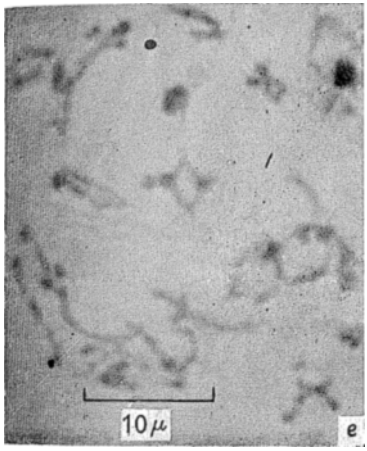
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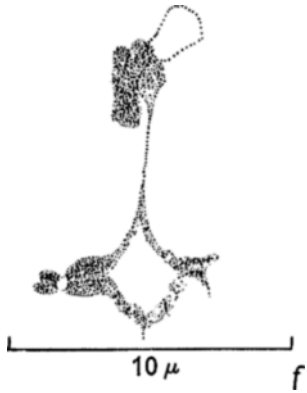
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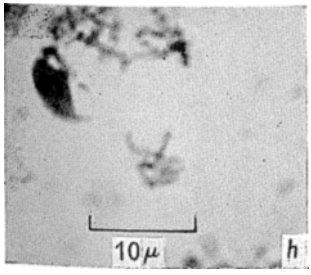
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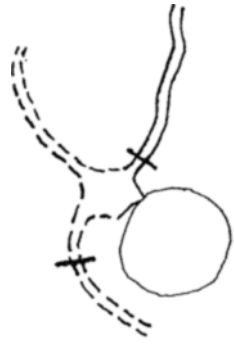
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