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A search for histocompatibility differences between irradiated sublines of inbred mice

By J. GODFREY* and A. G. SEARLE
Medical Research Council, Radiobiological Research Unit, Harwell, Berkshire, England

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1. INTRODUCTION

Studies on mutagenesis in mammals have so far been concerned mainly with estimations of the frequency of induced dominant and recessive lethal mutations and of mutation at a set of seven specific loci in the mouse (Russell, 1951; Carter *et al.*, 1956), six of which affect coat-colour and the seventh ear-length. The time seems ripe for extension of such work to include loci with entirely different functions, with effects which can be detected only indirectly, but which are nevertheless clear-cut and little influenced by environmental factors. The mouse histocompatibility loci, which control the immunological response of one individual to tissue transplanted from another and are also concerned in the erythrocyte antigenic system, seem very suitable candidates for any attempt to discover whether findings with respect to the seven specific loci are typical of the mouse genome as a whole. Extensive tumour and skin transplantation studies in mice have shown that at least four complex histocompatibility loci exist on the autosomes, with another one apparently on the Y chromosome. The total number of genes involved is unknown, but Barnes & Krohn (1957) have shown that the A and CBA strains differ with respect to at least fifteen independently segregating genes controlling the fate of transplants between them and their progeny.

Attempts have already been made to detect and to induce somatic mutations at mouse histocompatibility loci by the use of tumours and of the isogenic resistant lines developed by Snell. Mitchison (1956), Klein & Klein (1959) and Dhaliwal (1961) have described the method used, which is based on the supposition that a mutation towards a parental strain in a tumour cell from an F_1 hybrid between two isogenic-resistant strains differing at a single locus would make it able to multiply in that parental strain; it should be possible to detect the presence of such a cell in a large neoplastic population. Dhaliwal found, however, that a single sarcoma cell was unable to produce a tumour when injected subcutaneously into mice. About 100 cells were needed to do this. A number of variants, specifically compatible with one or other parental strain, were produced, but they seemed to be associated with chromosomal changes such as aneuploidy or polyploidy rather than with mutation at the histocompatibility locus concerned ($H-2$).

A different approach, involving reciprocal skin-grafts between individuals, might prove more rewarding. The genotype of individuals is more stable than that of

* Present address: Department of Zoology, University Museum, Oxford.

tumours; there would also be a greater chance that a change in histocompatibility relationships really involved a gene mutation, since the only aneuploid zygotes known to survive in mice are those involving the X chromosome. Moreover, the mode of inheritance can be tested by subsequent breeding. Barnes & Krohn (1957) have pointed out various other advantages of using skin-grafts rather than tumours for genetic analysis, such as the fact that the grafts are clearly visible, with a normal pattern of response which is well documented. Also, the behaviour of such a graft is a sensitive indicator of minor differences in compatibility, while the number of genes controlling the fate of such transplants seems larger than the number determining whether a tumour is rejected or not.

It was therefore decided to use skin-grafting as a tool for studying histocompatibility mutations and to start by testing for histocompatibility differences between closely related sublines of an inbred mouse strain kept in a radiation field.

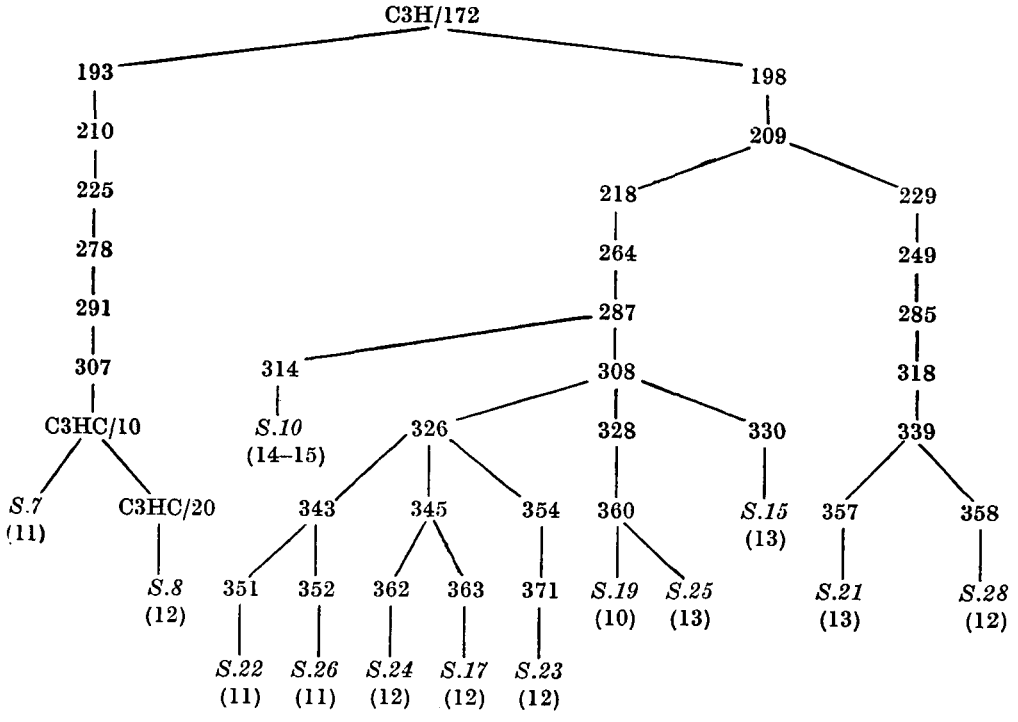
2. MATERIALS AND METHODS

The mice used belonged to thirteen sublines of the C3H/He/H strain, inbred by brother-sister mating since 1920. The foundation matings of these sublines were placed in a ^{60}Co gamma radiation field by Dr T. C. Carter in late 1957 and in 1958; since then they and their descendants have received a dose of 1 r. per night. Sibs of the foundation animals were used to start sister sublines in a control area. Each subline is kept as a single line without ramifications, any particular mating being stopped when there are grandchildren breeding. The breeding performance of the C3H strain in this environment was remarkably good, although litter-size tended to be somewhat reduced in later litters.

Fig. 1 shows the relationship between the subline foundation matings and the number of generations each subline had passed in the radiation field when removed for histocompatibility tests. The average cumulative dose of gamma radiation received by each C3H subline when mice were extracted for test was 1054 r., spread over an average of 12·2 generations. The foundation members of these sublines were separated from each other by a very variable number of generations, as Fig. 1 shows, but the average is about ten. Thus there were on the average about thirty-four generations between each pair of mice from different sublines tested for homograft rejection. In all these generations spontaneous mutation affecting histocompatibility loci was possible, while in twenty-four of them radiation induced mutation was also possible.

Mice for testing were removed from irradiation at around weaning age (3 weeks) and skin-grafting was carried out when they were mature, at the age of two months or later. The tail skin-grafting method of Bailey and Usama (1960) was used. The method is particularly suitable for mutation studies involving large numbers of mice because of its simplicity and its speed. It involves the exchange of approximately 5×2 mm. slices of skin taken from the dorsal (or ventral) side of the tail near its base, the graft being put on the host the wrong way round so that its hairs point forwards instead of backwards, which helps greatly in differentiating between

acceptance of the graft and regeneration of hair by the host. The grafts are protected by slipping a length of glass tubing (with diameter slightly larger than that of the tail) over the proximal part of the tail and holding it in place with a small Michel clip which is itself secured by surrounding it with collodion. This can be safely

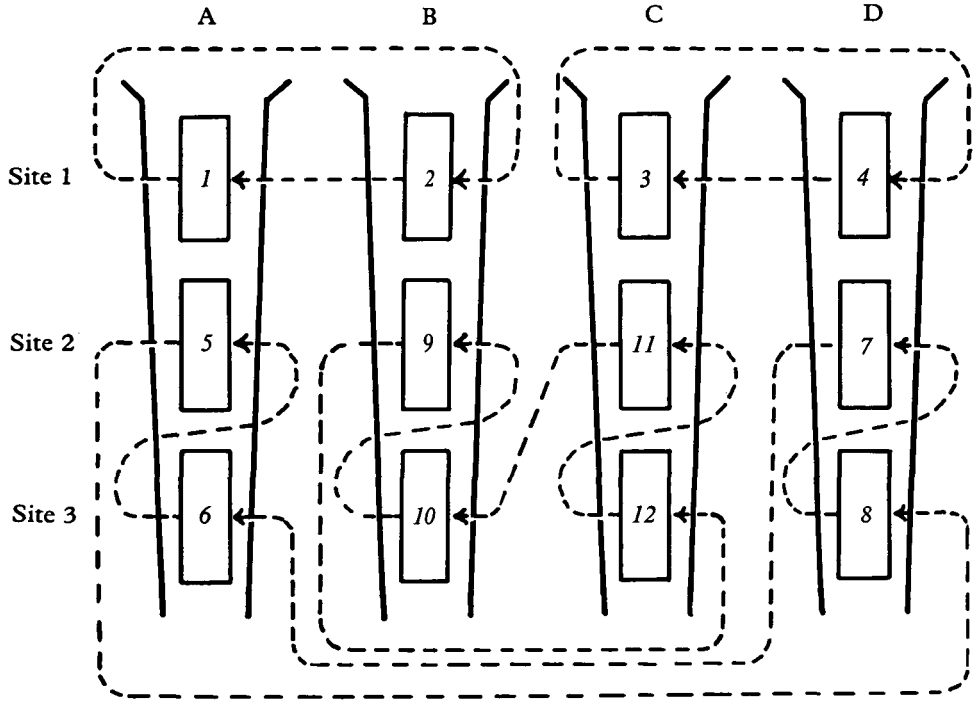


Text-fig. 1. Pedigree of C3H sublines, showing the relationships between the foundation matings of the irradiated sublines. The numbers (172, 193, etc.) are the record numbers of mated sib-pairs. The irradiated sublines originated from the matings marked *S.7*, *S.8*, etc. Below these, the numbers of generations of irradiation before extraction of individuals for histocompatibility tests are given in brackets.

removed in about 4 days. Bailey and Usama injected the mice with a penicillin–streptomycin mixture after the operation; we found it sufficient to sprinkle antibiotic powder ('Cicatrin') over the grafts. This also helped the grafts to dry, so that we did not need to hold them for 1–2 minutes under a warm lamp, as was recommended.

The grafting plan adopted is shown on Fig. 2 and, for each experiment, involved four mice each receiving three grafts. Usually one pair of mice belonged to the same subline, while the other two mice belonged to different sublines. Thus the procedure allowed each mouse to have an autograft and two homografts, one of the latter coming from a different subline and the other either from a different subline or from another member of the same subline. Thus we could detect heterogeneity within sublines as well as between them.

The dorsal side of the tail was used for most skin-grafts, but a few were done ventrally. If the dorsal grafts were a trifle thicker than normal there was the danger of cutting a caudal vein. This happened on several occasions, so that a ligature had to be applied briefly at the base of the tail, but the acceptance of the relevant graft was not affected, neither did the mouse suffer any obvious ill-effects. The danger of cutting a blood-vessel was much less with ventral grafts, but they could not be observed so easily.



Text-fig. 2. The plan adopted for exchanging skin grafts between and within mouse sublines (homografts being placed on tail sites **1** and **3**, autografts on site **2**). Tails A and B generally belonged to mice of the same subline, while C and D came from two other sublines. The numbers **1–12** show the order in which graft sites are prepared; when grafts have to wait they are placed in Ringer's saline.

To induce anaesthesia 0·06–0·066 mg. of pentobarbitone sodium per gram of body weight was given as an intraperitoneal injection in alcoholic saline. If necessary, ether vapour was used to supplement this.

The grafting operation was usually performed on Monday and the clip and tube removed from the mouse's tail on the following Thursday or Friday, after inspection of the grafts. The tube sometimes came off prematurely; if this happened in the first day or two after grafting the grafts were usually lost, but if later they generally survived. Grafts were examined approximately once a week to see whether or not they had been rejected, a final decision being delayed until 100 days after the

operation to allow for late rejections (Counce *et al.*, 1956; Barnes & Krohn, 1957). Whenever the donor graft could not be clearly seen a retest was made, with reciprocal homografts between donor and recipient, and autografts.

3. RESULTS

Some preliminary experiments were made to test the sensitivity of the method. Interstrain homograft rejection at about 12 days and accelerated second-set responses were clearly discernible, as was breakdown of grafts from strain C3H males on to their sisters, rejection occurring at about 18 days. Thus our results agreed well with those reported by Bailey and Usama.

Table 1. *Numbers of skin-graft compatibility tests between irradiated C3H sublines. R = retest made*

		DONOR SUBLINES												
		7	8	10	15	17	19	21	22	23	24	25	26	28
RECIPIENT SUBLINES	7	4	1				2		1	1	1R	1	1	
	8	1	2	1			2		1	1R	1R			
	10		1	2			2	1						1
	15						1			1				
	17					2			1			1		
	19	2	1	2	1		1	2	1	1		1		1
	21			1			2	2	1					
	22	1	1			1	1	1	2	2		2	2	
	23	1	2		1		1		2		1	1R		
	24	2	2				1			1	4		1	2
	25	1				1	1		2	1R		2		
	26								1R		1		1	1
	28			1			1				1R		1	2
	Total	12	10	7	2	4	15	6	12	8	9	8	6	7 = 106

Table 1 shows that in the main experiment 106 tests for histocompatibility were made, eighty-two between same-sexed members of different sublines and twenty-four between same-sexed members of the same subline. No retests were necessary in the latter category, but seven were made between members of different sublines when the original homograft had disappeared or was only doubtfully present. All the retest homografts were accepted though one donor mouse had to be killed two months after grafting because of a tumour. The reason for the failure of the first graft was usually clearly technical, for example premature removal of the glass tube protecting the graft. The homograft from subline 23 to 24 was lost (apparently due to a technical failure) but inadvertently not retested; that from 19 to 24 was doubtfully present but the donor was no longer available for restest. Thus these two homograft tests should be regarded as inconclusive, leaving eighty between sublines in which there was clear evidence of histocompatibility and none in which

incompatibility was proved. Fifty-two mice in all had satisfactory reciprocal tests with members of the same or different sublines.

Billingham *et al.* (1954) have reported the occurrence of 'cosmetic' abnormalities in homografts between individual mice some eight to thirteen generations apart within the highly inbred A and CBA strains. The homografts survived but each passed through an abnormal phase, with such symptoms as a transient weakness of the epithelial surface and slow hair growth. No specific search for such cosmetic reactions has been made in the present investigation, although anything abnormal about the state of the graft was noted, especially whether it was almost or completely devoid of hairs. Our records show, however, that long-continued baldness was just as common with autografts as with homografts. Thus it could not be regarded as evidence for small immunological differences in this experiment, being more probably the result of variability in the thickness of skin slices and in the stage of the hair cycle when the operation was performed.

4. DISCUSSION

The failure to detect any positive evidence for histocompatibility mutation having taken place in any of the irradiated inbred strains does suggest at first sight that either the mutation rates at the loci involved are not unusually high or the number of loci concerned is fairly small, or both. It is difficult to decide what would be expected on the basis of present knowledge, but we can make an approximate estimate of the probable effect of mutation rate in the following way.

Let us assume first that the average dose per generation of about 86 r given to irradiated sublines doubles the overall mutation-rate from μ to 2μ. This seems a reasonable estimate, since the 'doubling dose' with specific locus mutations after low dose-rate gamma irradiation of spermatogonia is not far removed from 100 r. (Carter *et al.*, 1958; Russell *et al.*, 1958), while that for oöcytes is probably lower. A histocompatibility mutation M, which we will suppose for the moment to be dominant, could occur in a gamete going to form one of the tested mice, or one of the ancestors, It could possibly have taken place in the original pair C3H/172 or in one of its ancestors, so that this pair carried both the M and + genes. This possibility would lead to only a small correction to the calculations which follow, so we will neglect it. There were fifty-two tested mice, of which forty-nine were derived from irradiated matings and three from non-irradiated matings, so that the expected number of mutant phenotypes among these due to fresh mutation to M is $2(49 \times 2\mu + 3 \times \mu) = 202\mu$, if μ is the mutation-rate to M. All would be detected if they occurred.

Let x be the actual number of fresh mutations occurring among ancestors of these tested mice, after the pair C3H/172. Since there were thirty-seven pairs of non-irradiated mice (thirty-four prior to irradiation and three after extraction from the irradiation field) and 157 pairs of irradiated mice, the expected value of x is $2 \times 2(37\mu + 157 \times 2\mu) = 1404\mu$. We could in principle make a precise estimate of the number which would be detected, using the matrix theory of inbreeding

(Fisher, 1949); but this would be very laborious and the following estimate seems sufficient for the purpose. Suppose that a fresh mutation to M appears in one of these ancestral pairs P. An inspection of Table 1 and Fig. 1 shows that a test has been made between at least one descendant of P and one mouse not descended from P. It is very unlikely that the mouse not descended from P will carry the mutation M, therefore a reaction will take place if and only if some tested descendant of P inherits M. Given that P carries M, this event has at most a probability 1 of occurring (and if many descendants of P are tested, as for example with the pair 326, the probability of detecting a mutation would be quite high). On the other hand, the probability will be least when only one remote descendant of P is tested. In any case the probability is at least 1/4. For the pair P carries four genes at the locus concerned, namely M, +, +, +. We can assume that they do not affect viability or fertility and therefore have an equal probability of surviving after several generations of inbreeding. At least one of these genes must still survive and any surviving gene has a probability of at least 1/4 of being the M gene. Hence, of the x mutations which have occurred, the expected number of those detected lies somewhere between $\frac{1}{4}x$ and x. Hence the total expected number detected lies between $202\mu + \frac{1}{4}(1404\mu)$ and $202\mu + 1404\mu$, i.e. between 553μ and 1606μ.

It is generally thought safe to assume that histocompatibility mutations can express themselves in single dose and there is plenty of evidence in support of this, Barnes & Krohn (1957) pointing out, for instance, that none of the many alleles revealed so far by analysis of the mouse $H-2$ locus has proved to be recessive. This would fit in with the idea that each histocompatibility gene determines a particular antigen, which can express itself whenever present. If one assumes, however, that recessive histocompatibility mutations can occur, then a similar calculation to the one above can be made for such a mutation m. Mutation in a gamete forming a particular tested individual would then give an $m/+$ genotype and no incompatibility would result. Neither would it with mutation in the previous generation, but those in generations before that would have a chance of at least 1/16 of being detected. With mutation in a distant ancestor the probability would approach 1/4. The number of fresh mutations to m which have a chance of becoming homozygous is $1404\mu - 2 \times 2(10 \times 2\mu + 3 \times \mu)$, since the generation prior to testing must be omitted, which equals 1312μ. The expected number detected is therefore at least 80μ and probably closer to 300μ.

No mutation was in fact observed. If the expected number of mutations was larger than 3 the probability of observing no mutants would be less than e^{-3} by the Poisson distribution formula; this $= 0{\cdot}05$, so we can reasonably infer that the expected number is less than 3. So the total mutation rate to dominant histocompatibility mutations at all loci is probably less than $3/553 = 5{\cdot}4 \times 10^{-3}$, while the rate to corresponding recessive mutations (if they exist) is probably less than 10^{-2}.

The natural mutation rate/gamete locus for dominant mutations in the mouse is not known, though Carter *et al.* (1958) found only two dominant visible mutants in 117,727 control mice; probably at least thirty loci are involved. The mutation

rate to specific locus recessives without irradiation seems to be around $1{\cdot}0 \times 10^{-5}$/ gamete locus (Carter *et al.*, 1958; Russell *et al.*, 1958). Comparing these figures with the results of our experiments makes it clear that the absence of evidence for mutations is not surprising, even if a fairly large number of different loci are involved in the production of changes detectable by the skin-grafting technique we used. However, our results do suggest that the natural and induced mutation rates at these loci are not unduly high. Billingham *et al.* (1962) have tabulated the various published estimates of the number of histocompatibility loci in the mouse, rat, etc., as judged chiefly from the results of crosses between unrelated inbred strains. Those for the mouse reach an upper figure of 17 in the work of Barnes & Krohn (1957) previously mentioned, but all are subject to a considerable degree of uncertainty.

The origin of the 'cosmetic abnormalities' observed by Billingham *et al.* (1954) after relatively few generations of separation remains problematical. If they are due to mutation then the number of loci involved must be very large indeed, unless some loci giving weak histocompatibility reactions are highly mutable. One is reminded of the high rate of subline differentiation with respect to 'threshold' skeletal characters which was reported by Deol *et al.* (1957). This was thought to be because a very large number of genes could affect the frequencies of the skeletal characters used. Other explanations are possible, however, and it is clear that this phenomenon of rapid subline differentiation with respect to minor differences needs further investigation. In any event, the present investigation suggests that the mutation rate of genes responsible for major immunological differences is not correspondingly high.

5. SUMMARY

(1) A tail-skin grafting method was used to test for histocompatibility differences between members of thirteen sublines of C3H inbred mice, kept in a 1 r./night gamma radiation field for twelve generations, on the average, and separated from each other by about thirty-four generations.

(2) No homograft rejections occurred, so there was no evidence to suggest that mutations at histocompatibility loci had taken place in any of these sublines. Calculations show that this finding does not conflict with the idea that a fairly large number of loci are involved, having mutation rates similar to those already known in the mouse.

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