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Inherited histocompatibility changes in progeny of irradiated and unirradiated inbred mice*

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1. INHERITED HISTOCOMPATIBILITY CHANGES

The histocompatibility (H) loci offer a promising system for the study of mutation in the mouse, since the entire group can be tested by a single isogenic tissue transplant. Unfortunately, the lack of a precise estimate of the total number of H loci necessitates treating observed mutations as occurring within a group, much as the lethal mutations in mice have been treated by Russell & Russell (1956), Carter (1957), Bateman (1958), Muramatsu *et al.* (1963) and Lyon *et al.* (1964).

Spontaneous mutations at the H-2 locus in mice, observed by chance (Borges *et al.*, 1952, 1954; Snell & Borges, 1953), have been questioned (Amos *et al.*, 1955; Gorer *et al.*, 1959). Mutations of H genes as a group have been detected through the accumulation of their effects in independently maintained sublines of various highly inbred strains (Bittner, 1930; Billingham *et al.*, 1954; Kindred, 1963; Linder, 1963).

Godfrey & Searle (1963) made the first attempt to induce H mutations in long separated sublines of C3H mice by chronically irradiating mated pairs throughout a number of generations before graft-testing, but found neither spontaneous nor induced heritable changes. Such an experiment takes advantage of the 'dichotomy' of sublines in that a number of mutations accumulated during past generations of separation can be detected by relatively few graft-tests. On the other hand, when graft-tests are restricted solely to between-line comparisons, sample size and potential reduction of error of estimate are limited at the outset by the number of available sublines and the number of generations of subline separation.

We have therefore taken a different approach. We decided to test a large number of mice from the same or closely related sublines, looking for the more recently originating mutations. The experimental sample size would be limited only by the number of animals tested. The present paper reports the results of our first exploratory attempt to carry out such determinations.

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2. MATERIALS AND METHODS

The two inbred strains, C57BL/6JN and BALB/cAnN, or B and C strains respectively, and their derived hybrids, (B♀ × C♂)F_1 and (C♀ × B♂)F_1, or BCF_1 and CBF_1 respectively, were used exclusively in this experiment. Parental mice in these crosses were from the Animal Production Section, National Institutes of Health. They were progeny of pedigreed full-sib mated mice that had been mated to mice of other pedigreed sibships within the strain.

Fifteen B males, 90 days of age, were irradiated, and 60 days later, mated with seventy-seven C females, producing test mice designated $CBF_1{}^X$. The reciprocal cross of eighteen irradiated C males also 90 days of age mated with seventy-two B females was also made, producing the test mice, $BCF_1{}^X$. A control group was produced by the cross of twenty unirradiated B males mated to forty C females and designated $CBF_1{}^O$. The reciprocal cross for controls was not made owing to a shortage of B females.

Only the posterior third of the body was irradiated; the dose of X-rays was 522 rads. The radiation factors were: 250 kVcp,H VL = 1·5 mmCu; dose rate of 36 rads per min. The method of restraining and shielding the animals was described by Kohn & Kallman (1956*b*).

The 60-day period between exposure and mating insured the testing of sperm from irradiated spermatogonia. Each male was mated with a set of females in several breeding cages of two or three females per cage. The combined progeny of sibs and half-sibs from one such cage are termed a 'progeny group'. Each male was rotated among assigned cages on approximately a weekly basis. The first litters sired by the irradiated C-strain males arrived 115 ± 9 days after irradiation; for the B-strain males the time was 178 ± 20 days. (It is of interest that the relative rates of recovery of the germinal epithelium of these two strains is just the reverse of that which might be expected from their established values for both acute and chronic X-ray LD_{50} (Kohn & Kallman, 1956*a*, Grahn & Hamilton, 1957; Roderick, 1963).

The F_1 progeny of these matings were tested for histocompatibility when 1 month old by orthotopic tail-skin transplantation (Bailey & Usama, 1960) modified as previously reported (Bailey, 1963). All grafts (6–12 mm.^{2}) were of dorsal tail skin so as to obtain maximum pigmentation. They were placed on the graft bed in reversed direction (hairs pointing forward), which helps to distinguish the graft from host skin. Grafts were observed at 1, 3, 5 and 9 weeks after grafting. Rejection was determined by onset of scaliness and loss of tail-scale pattern, the loss of pigment and the stubbing and loss of hair.

The grafts were exchanged in a 'reciprocal circle' system as illustrated in Fig. 1. In this system, graft rejections should indicate the existence of mutations, and also by their pattern of occurrence, as depicted in Fig. 2, whether a mutation has effected a gain, a loss, or both a gain and loss in tissue antigen specificity. These classes of mutants will be referred to as G, L and GL, respectively. Mice included

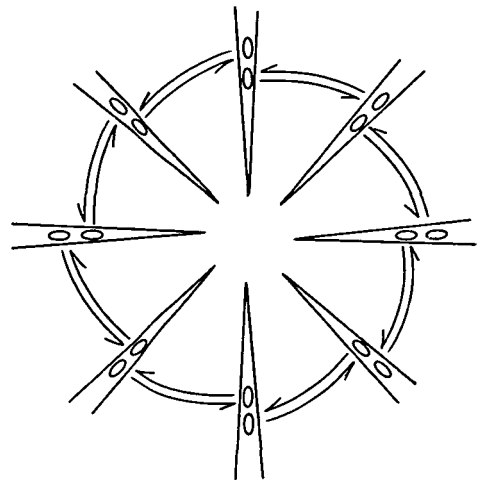

Fig. 1. Diagram of the 'reciprocal circle' system of skin-graft exchange on tails of mice. Large vees represent tails of mice, ovals within the vees represent grafts, and arrows indicate direction of donor to host. The number of mice may vary.

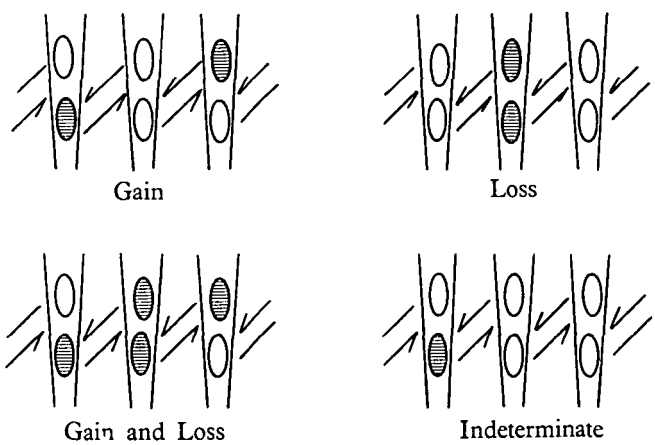

Fig. 2. Diagrams of the different patterns of graft rejection to be found in a segment of a 'reciprocal circle' when a mutant is included. Shaded ovals represent rejected grafts.

in any reciprocal circle were of the same sex and of the same reciprocal-type hybrid. Fifty-eight per cent of the exchanges were made within progeny groups.

The use of the F_1 hybrid makes possible the distinction between types G, L and GL, at least when the loss occurs at loci which differ in the two strains. A loss at a locus which has identical alleles in the two parents will pass undetected.

Independence of the origins of a few mutants was tested by comparing their specificities in second-set graft reactions. It was assumed to be highly unlikely that any two independently arising mutations would have effected identical specificities, for the minimal number of H-loci is estimated to be thirty (Bailey &

Mobraaten, 1964) and at each locus it is likely that any of several types of change might occur.

As a test of inheritance, skin grafts from female descendants of mutants backcrossed to the B strain were placed on CBF_1 or BCF_1 female hosts. One graft from each animal under test was placed on each of two hosts; each host carried grafts from up to four different experimental donors and one control isograft. The descendants tested were mainly from the second and third backcross generations of twenty-one of the detected G and GL mutants. Grafts were observed through 11 weeks.

3. RESULTS

The total number of mice tested for type G was 2641. However, some of these were not complete tests owing to accidental graft loss or to death of one of the hosts before completion of the observation period. To equalize all tests, only complete type G tests (i.e., two hosts) or their equivalents in half tests (one host) were considered here. These equal the total number of L tests, namely 2572 (Table 1). Thirty-two mutants were detected. Those retested (twenty-seven) were shown to retain their incompatibility. Thirty-one were of type G, one was of type GL, and none were of type L.

Table 1. *Histocompatibility mutants found in F_1 hybrid mice*

	Hybrid type						
	$BCF_1{}^X$		$CBF_1{}^X$		$CBF_1{}^O$		Total
	♂♂	♀♀	♂♂	♀♀	♂♂	♀♀	♂♂+♀♀
No. of complete tests	596	663	379	380	266	288	2572
Mutant type							
G	6	6	4	6	5	4	31
L	0	0	0	0	0	0	0
GL	0	1	0	0	0	0	1
Total	6	7	4	6	5	4	32
♂♂+♀♀	13		10		9		32
Relative frequency	0·0103		0·0132		0·0162		0·0124
95% confidence limits	0·0040		0·0063		0·0074		0·0077
	0·0175		0·0242		0·0309		0·0175

Originally, six suspected mutants were classified as L, but three of these from the $CBF_1{}^O$ group proved to be XO females. This was shown by graft tests of their X-linked histoincompatibility and by their chromosome counts which were consistently thirty-nine.

The remaining three mice (two male and one female) originally classified as type L were also excluded, since when retested with standard hybrid and parental strain grafts, they failed to reject them. It was thought that these were not L mutants but probably had been recipients of grafts concomitantly from two different

G mutants. This interpretation is strengthened by the fact that these events occurred in progeny groups in which several other type G mutants had been found. In the place of these so-called L mutants the appropriate number have been added to the G category in Table 1.

There were eleven incompatibilities of an indeterminate type. Five of these were shown by repeated graft tests on new CBF_1 hosts (at least three hosts each) to be type G and classified as such in Table 1, but with a low probability of eliciting a rejection. Six others did not elicit a rejection in these repeated tests and were considered nonimmunological in nature. We cannot exclude the possibility that these were extremely weak antigenicities of perhaps a relatively high incidence but with such a low probability of eliciting a rejection that our repeated tests were inadequate in number to confirm their existence.

X-irradiation of the fathers had no detectable effect on the incidence of histocompatibility mutants. The control group had, in fact, a greater frequency of

Table 2. *Tests of inheritance of detected incompatibilities. Each female, produced by backcrossing the original mutant or its proven carrier descendants to the B strain, was doubly tested for incompatibility by grafting her skin onto each of two* BCF_1 *or* CBF_1 *female hosts.*

	Backcross females tested					
		Fate of graft pair from each				
Mutant	Total	Both rejected	One rejected	None rejected	% proven carriers	Grafts rejected / Total grafts
H(z1)*	94	42	4	48	48·9	88/188
H(z2)	68	13	9	46	32·4	35/136
H(z17)	104	31	21	52	50·0	83/208
H(z18)	88	13	17	58	34·1	43/176
H(z20)	93	15	11	67	28·0	41/186
H(z22)	83	11	28	44	47·0	50/166
H(z23)	36	3	4	29	19·4	10/72
H(z24)	51	11	11	29	43·2	33/102
H(z25)	79	18	19	42	46·8	55/158
H(z31)	55	2	7	46	16·4	11/110
H(z37)	61	11	11	39	36·1	33/122
H(z40)	47	1	7	39	17·0	9/94
H(z43)	69	25	6	38	44·9	56/138
H(z44)	59	6	21	32	45·8	33/118
H(z45)	47	10	13	24	48·9	33/94
H(z46)	66	10	20	36	45·5	40/132
H(z47)	76	14	22	40	47·4	50/152
H(z49)	35	13	6	16	54·3	32/70
H(z50)	43	0	11	32	25·8	11/86
H(z53)	32	3	13	16	50·0	19/64
H(z54)	100	32	14	54	46·0	78/200
Control isografts						9/667

* Symbols of mutants are tentatively assigned until the locus of each is established.

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mutants but the difference was not statistically significant. There was no apparent difference between reciprocal type hybrids or between sexes with respect to mutation frequency.

The data from the tests of inheritance of detected incompatibilities are given in Table 2. In only one case was the proportion of female descendants of backcrossed mutants more than the expected 50% and that was not statistically significant. The difference in the proportion of incompatible grafts found between any one of the backcross lines and that of the control isografts was statistically significant ($P < 0{\cdot}001$ by Fisher's exact test). The detailed analysis of these data, together with those for later generations, will be published in a separate article.

In regard to the strengths of the tissue antigens determined by the thirty-two mutant genes, one was first noticed at 2 weeks after grafting, two at 3 weeks, ten at 5 weeks and nineteen at 9 weeks. The only GL mutant, H(z1) in Table 2, has consistently had, both originally and in subsequent generations, the shortest graft survival time of any of these mutants. Moreover, H(z1) was of spontaneous origin, since in subsequent tests the original mutant mouse rejected a skin graft from its maternal strain (B) but not one from its paternal strain (C).

The mutants were not randomly distributed among progeny groups; the clustering shown in Table 3 suggests that some mutants came from a parent that was a mutant carrier. However, owing to the caging system of the present experiments, mothers giving rise to clusters cannot be identified with certainty, since there were two or three mothers per cage. The data therefore cannot be corrected for such mutants until identity of mutants can be established by differences in their specificities. It is of interest to note in this regard, however, that the second-set reactions, thus far carried out in twenty-eight comparisons between mutants of different progeny groups, have indicated all of them to be distinct. In six comparisons of mutants from the same progeny group only one has indicated a difference in specificity.

Table 3. *Frequency distribution of progeny groups with respect to number of mutants per group**

	Mutants per progeny group									
Hybrid type	0	1	2	3	4	5	6	7	8	Total
$BCF_1{}^X$	33	7	1 (43)		1 (87)					42
$CBF_1{}^X$	33	2							1 (19)	36
$CBF_1{}^O$	17	1		1 (46)		1 (43)				20
Total	83	10	1	1	1	1			1	98

* Sizes of progeny groups with clusters are given in parentheses.

4. DISCUSSION AND CONCLUSIONS

In the design of the present study, graft rejection was presumed to indicate the presence of a mutant H gene. Assurance that this was more than a presumption was obtained when significant numbers of histoincompatible descendants were

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derived from the originally encountered histoincompatible mice (Table 2). The proportions of such descendants were never significantly greater, although often less, than the expected 50%. The deficient proportions could be attributed to a low probability of weak antigens to elicit a rejection during the limited graft observation period, and were therefore not considered to be inconsistent with our conclusion that the detected incompatibilities truly had a genetic origin.

Several of the mutants appeared in clusters, which implied an origin from carrier parents and therefore the existence of residual heterozygosity in one or both of the parental strains despite a long history of inbreeding. This is not surprising when the mutation rate is sufficiently high, for then past mutations will occasionally be in the segregating phase before their eventual fixation or loss. Residual heterozygosity, of course, can also result from heterozygote selection.

The presence of mutant carriers points out the fallacy of assuming highly inbred strains to be genetically homogeneous. Antigens that arise, although extremely weak, have the capacity of vitiating any investigation involving other weak antigens. Experimentally, this indicates that all parents entered in investigations like the present one should first be skin-graft tested.

Paternal irradiation had no apparent effect on the frequency of mutation. A small radiation effect may have been masked by the mutant progeny of carrier parents.

The presence of mutant carriers has prevented our estimating either the spontaneous or the induced mutation rates from the data of Table 1, for owing to the design of the experiment, we were unable to identify and correct for the products of such carriers. An independent estimate of the spontaneous mutation rate is obtainable, however, from the data on isografts in Table 2.

These isografts were from CBF_1 or BCF_1 hybrids derived from parents that were proven by progeny tests to be non-carriers. Nine mutants, all eliciting rejections by 9 weeks and all of the G type, were found in the 667 mice; the number of spontaneous mutations per zygote tested is thereby estimated as $13{\cdot}5 \times 10^{-3}$ (95% confidence limits of $6{\cdot}2$ to $25{\cdot}5 \times 10^{-3}$) and the number per gamete as $6{\cdot}75 \times 10^{-3}$.

A maximum estimate of the induced mutation rate can be made by comparing the data from the paternally irradiated groups with those from the controls in Table 2. With 2018 tested mice in the paternally irradiated groups and 667 in the controls, it can be shown that a mutation frequency twice the control frequency, or better, would have been detected (statistically significant), using Fisher's exact test. Since no augmentation of frequency by irradiation occurred, the estimate of maximum effect can be formulated as: $2\mu_s + 522\mu_i < 2(2\mu_s)$, where μ_s is the frequency of spontaneous mutations per gamete and μ_i is the frequency of induced mutations per gamete per rad. The estimate of μ_i, based on Table 2, as noted above is $6{\cdot}75 \times 10^{-3}$. Therefore, from the above inequality it follows that μ_i would be $< 2{\cdot}6 \times 10^{-5}$.

These estimates apply to the total of H loci, since all but the one GL mutation were of the G type. The estimates, however, may not rationally be put on a unit locus basis by applying our present best estimate of 30, for the number of H loci

(Bailey & Mobraaten, 1964), since the latter was a minimum estimate that included only those loci at which the B and C strains differ.

Since the mutation rate at the H loci could not be expressed on a per-locus basis, for purposes of comparison we have used the *doubling dose*, i.e., that dose of radiation that induces as many mutations as occur spontaneously. Russell & Russell (1959) have pointed out the fallacy of comparing doubling-dose estimates for mutations that were induced at different stages of gametogenesis, in different sexes, or under different dose rates. For the present purposes, therefore, only data on mutations induced by acute spermatogonial X-irradiation in the mouse are presented (Table 4). Data from the fractionated as well as single dose experiments of Russell (1963) are included to provide perspective on the influence of fractionation.

Table 4. *Estimates of spermatogonial mutation rates and doubling doses for various traits in the mouse*

Trait	Spontaneous mutations per gamete μ_s	Acute dose (rads)*	Induced mutations per gamete per rad μ_i	Doubling dose (rads) $= \mu_s/\mu_i$	Source of data
Average of seven specific loci	$7{\cdot}5 \times 10^{-6}$	600	$2{\cdot}1 \times 10^{-7}$	36	Russell, 1963
		600 + 400†	$2{\cdot}8 \times 10^{-7}$	26	Russell, 1963
Recessive visibles	7×10^{-3}	600 + 600‡	$1{\cdot}8 \times 10^{-5}$	400	Lyon *et al.*, 1964
Recessive lethals	$2{\cdot}10 \times 10^{-2}$	600 + 600‡	$2{\cdot}5 \times 10^{-4}$	80–400	Lyon, 1959; Searle, 1964; Lyon *et al.*, 1964
H loci	$6{\cdot}75 \times 10^{-3}$	522	$< 2{\cdot}6 \times 10^{-5}$	> 260	Present study

* Cited papers specified dose in roentgens, taken here equal to rads.
† Interval between fractions > 15 weeks.
‡ Interval between fractions > 8 weeks.

The estimates of doubling dose for the four classes of loci presented in the table range from a low of 26 R. to a high of 400 R. The minimum estimate derived in this study for the H loci is compatible with the estimates for recessive lethals and visibles all of which are at the higher end of the doubling-dose range. This is a reasonable outcome, since the H loci like the recessive lethals and visibles presumably involve relatively great numbers of unselected loci. The seven specific loci, chosen by Russell, on the other hand appear to be the most sensitive to X-irradiation. This perhaps reflects the point stressed much earlier by Russell (1951) that these seven loci are not a random sample, for they were deliberately chosen from those at which mutations had previously occurred in laboratory stocks.

Although Godfrey & Searle (1963) did not detect any mutations in their study, the present results are not at variance with theirs. If it is assumed that irradiation effects were practically nil in their study as in ours, they tested an equivalent of 298 gametes and found no mutations. We tested 1334 gametes by the isografts of Table 2 and found nine mutations. The difference between these frequencies is not significant ($P = 0{\cdot}16$).

In comparing mutation frequencies for histocompatibility in different laboratories, it should be emphasized that observed mutation frequency would be not only highly dependent on the sensitivity of the graft-testing techniques employed, but also upon the sensitivity of the immune reaction of the host and therefore upon the host's genotype. The strain(s) of the assayed mice, then, would be critical.

Mutation rate also would depend upon the length of the graft observation period. The antigens of the mutants found here had such a low probability of eliciting a rejection that grafts from derived mutant carriers were frequently not rejected by alternate hosts in 9 weeks (Table 2). No doubt the mutation rate would have been greater if the observation period had been extended.

One unexpected finding was the preponderance of the type G mutation. This type is the opposite of that found by Klein & Klein (1959) in tumor cells. However, their experimental design would permit detection of only type L changes, and such changes are suspected to be products of somatic crossing-over, not point mutation (Klein, 1961; Hellstrom, 1961).

The preponderance of G type in the present case, however, might be due to an artifact of our method. Recall that the purpose of using the F_1 generation for graft testing was to enable the detection of L type mutants. The potential for such detection holds only for those loci at which the two strains differ. For loci at which the two strains are alike, detection would be limited to G (or GL mutations without loss in specificity being revealed). Following this, if the two strains had more loci at which they were alike than at which they differed, there would then be less chance for detecting L mutations. This would mean that there are far more H loci in mice than any estimates based on strain differences to date indicate.

SUMMARY

(1) F_1-hybrid mice derived from a cross of the highly inbred strains: C57BL/6 and BALB/c, were tested for inherited changes of histocompatibility by an orthotopic inter-exchange of tail-skin grafts. The fathers of tested mice received either 522 rads of gonadal X-irradiation, or received no irradiation 2 months prior to mating.

(2) Thirty-two mice with altered histocompatibilities were found in a total of 2572 complete tests. All of those mutant mice (twenty-one) that produced an adequate number of offspring were shown to pass the incompatibility on to their progeny.

(3) Mutants were classified as to whether they effected a gain, a loss or both a gain and a loss in antigen specificity as determined by whether they rejected skin of donor mice or their skin was rejected by host mice. Twenty-six were clearly of the gain type, five were most likely gain type and only one showed both a loss and a gain effect. There was no clearcut evidence that loss types had occurred. The preponderance of gain types was tentatively explained as an artifact of the system used for the assay.

(4) Several of the detected mutants were probably from parents carrying mutations that originated in past generations, for some mutant mice occurred in clusters.

(5) There was no apparent effect of paternal irradiation (522 rads) on mutation frequency. The induced mutation rate was estimated to be less than $2{\cdot}6 \times 10^{-5}$/gamete/rad.

(6) Independent data on isografts from F_1 hybrids of proven non-carrier pedigreed parents provided an estimate of spontaneous mutation rate of $6{\cdot}75 \times 10^{-3}$/gamete.

(7) The estimate of doubling dose (greater than 260 rads) was consistent with the estimates for recessive lethals and visibles in mice.

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