A SEROLOGICALLY DETECTED VARIANT IN MOUSE SERUM: FURTHER EVIDENCE FOR GENETIC CONTROL BY THE HISTOCOMPATIBILITY-2 LOCUS

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IN a previous report (SHREFFLER and OWEN 1963) a new serologically detected serum protein variant system in the mouse, the Ss system, was described. Among the inbred strains tested, a difference of about 20-fold in the concentration of a specific serum protein was detected by the application of immunodiffusion techniques. The difference is controlled by a pair of additively acting alleles, Ss^h and Ss^l, determining high and low Ss protein levels respectively. Three phenotypes can be distinguished—the homozygous high (Ss-H), the heterozygous intermediate (Ss-HL), and the homozygous low (Ss-L). A genetic association between the *Ss* locus and the histocompatibility-2 (*H-2*) locus was indicated by the observation that among 21 inbred strains classified, all Ss-L strains were H-2k and all Ss-H strains were non-H-2k. Segregation analyses revealed a close genetic association between the two traits, but the results did not permit a conclusion as to whether they are controlled by separate, but closely linked, loci or by the same locus. This paper presents more extensive data pertaining to this association, which indicate that the Ss trait is controlled by the complex *H-2* locus.

MATERIALS AND METHODS

Crosses: The five backcross linkage tests analyzed are shown in Table 1. The female parent is given first in each case. The Ss and H-2 types of the three inbred stains employed are: C3H/HeJ—H-2k, Ss-L; C57BL/10J—H-2b, Ss-H; DBA/2J—H-2d, Ss-H. The stocks designated C3H-*T* in Cross 4 and DBA-*T* in Cross 5 were not inbred. The former was obtained by crossing C3H/HeJ to a tailless stock (T/t^1), then backcrossing the short-tailed progeny to C3H/HeJ and selecting animals carrying the desired recombinant chromosome (T $H\text{-}2^k$ Ss^l). The DBA-*T* stock was obtained in a similar manner, starting with DBA/2J and another tailless stock (T/t^0).

T is known to be linked to *H-2* (*see* ALLEN 1955), and was introduced into Crosses 4 and 5 to provide an outside marker for these studies of recombination in the *H-2* region. In all of the crosses except number 2, the female parent was the heterozygote. This ostensibly offered greater opportunity for the observation of recombination, since it has been noted (GORER and MIKULSKA 1959) that recombination occurs more frequently in the *H-2* region in females than in males. All backcrosses were made to an Ss-L parent to maximize the discrimination of the Ss typing procedure. The difference between Ss levels in the Ss-L and Ss-HL phenotypes is large and not subject to overlap or the possibility of misclassification which occasionally occurs when classifying Ss-H *vs.* Ss-HL.

Antisera: Rabbit anti-mouse globulin sera were used to classify Ss types by immunodiffusion. A specific anti-Ss, prepared by absorption of an antiglobulin serum, was utilized for classification

TABLE 1

Cross	Stocks crossed	*Crosses* Genotypes
1	(C3H/HeJ × C57BL/10J)F_1 × C3H/HeJ	$\frac{H\text{-}2^k\ Ss^l}{H\text{-}2^b\ Ss^h} \times \frac{H\text{-}2^k\ Ss^l}{H\text{-}2^k\ Ss^l}$
2	C3H/HeJ × Cross 1 progeny	$\frac{H\text{-}2^k\ Ss^l}{H\text{-}2^k\ Ss^l} \times \frac{H\text{-}2^k\ Ss^l}{H\text{-}2^b\ Ss^h}$
3	(C3H/HeJ × DBA/2J)F_1 × C3H/HeJ	$\frac{H\text{-}2^k\ Ss^l}{H\text{-}2^d\ Ss^h} \times \frac{H\text{-}2^k\ Ss^l}{H\text{-}2^k\ Ss^l}$
4	(C3H-*T* × DBA/2J)F_1 × C3H/HeJ	$\frac{T\ H\text{-}2^k\ Ss^l}{+\ H\text{-}2^d\ Ss^h} \times \frac{+\ H\text{-}2^k\ Ss^l}{+\ H\text{-}2^k\ Ss^l}$
5	(C3H/HeJ × DBA-*T*)F_1 × C3H/HeJ	$\frac{+\ H\text{-}2^k\ Ss^l}{T\ H\text{-}2^d\ Ss^h} \times \frac{+\ H\text{-}2^k\ Ss^l}{+\ H\text{-}2^k\ Ss^l}$

on microslides. The methods of preparation of antisera and of absorption have been previously described (SHREFFLER and OWEN 1963).

H-2 antigens of the red blood cell were detected by isoimmune sera which were obtained after four to six intraperitoneal injections at 10-day intervals of approximately one minced spleen per 30 animals. The immunized animals were bled from the tail artery at seven to ten days after the last injection. After the first immunization series, boosters of single injections of spleen cells were given. The antisera used in this study had titers ranging from 1/300 to 1/2000 against the cells being classified, but the sera were usually used at typing dilutions of 1/30 to 1/120. The following antisera were employed:

1. (C3H/HeJ × DBA/2J)F_1 anti-C57BL/10J (H-2d/H-2k anti-H-2b).
2. C3H/HeJ anti-C57BL/10J (H-2k anti-H-2b).
3. C3H/HeJ anti-DBA/2J (H-2k anti-H-2d).
4. CBA/J anti-A/HeJ (H-2k anti-H-2a).
5. (C3H/HeJ × C57BL/10J)F_1 anti-DBA/2J (H-2b/H-2k anti-H-2d).

Blood specimens: Blood was collected from the tail artery in 4 percent sodium citrate for red blood cells, and in dry tubes for serum. The cells were washed twice in buffered saline and always typed within two days after collection. Sera were either tested fresh, or frozen and stored at —20°C until testing.

Immunodiffusion: The technique for Ss classification was the same as that previously described (SHREFFLER and OWEN 1963). In addition, a microslide technique (RIDGWAY, KLONTZ and MATSUMOTO 1962) was employed with the specific anti-Ss serum to provide more rapid classifications. Each serum was independently classified for Ss type at least three times.

Hemagglutination: The PVP technique of STIMPFLING (1961) was employed with no essential modification. Each blood was tested with two to four different reagents. Antisera 1 and 2, each at two different typing dilutions, were used for classification of progeny from Crosses 1 and 2. Antisera 3 and 4 were used throughout the classification of progeny from Crosses 3, 4, and 5. In addition, for the latter half of the animals produced in Cross 3 and for almost all of those in Crosses 4 and 5, antisera 2 and 5 were also used.

RESULTS AND DISCUSSION

The Ss and H-2 classifications of the 802 progeny from the five crosses are presented in Table 2. The typing results for both traits were clear and consistent for most individuals, with a few exceptions. In Cross 1, all animals typed as H-2k were

TABLE 2

Results of tests for linkage of Ss *and* H-2

	Cross	Sex	Number of progeny		
			H-2bk Ss-HL	H-2k Ss-L	Total
1	$\frac{H\text{-}2^b\ Ss^h}{H\text{-}2^k\ Ss^l} \times \frac{H\text{-}2^k\ Ss^l}{H\text{-}2^k\ Ss^l}$	Male	55	47	102
		Female	31	36	67
		Total	86	83	169
2	$\frac{H\text{-}2^k\ Ss^l}{H\text{-}2^k\ Ss^l} \times \frac{H\text{-}2^b\ Ss^h}{H\text{-}2^k\ Ss^l}$	Male	28	21	49
		Female	14	26	40
		Total	42	47	89
			H-2dk Ss-HL	H-2k Ss-L	
3	$\frac{H\text{-}2^d\ Ss^h}{H\text{-}2^k\ Ss^l} \times \frac{H\text{-}2^k\ Ss^l}{H\text{-}2^k\ Ss^l}$	Male	81	81	162
		Female	78*	65	143
		Total	159	146	305
4	$\frac{+\ H\text{-}2^d\ Ss^h}{T\ H\text{-}2^k\ Ss^l} \times \frac{+\ H\text{-}2^k\ Ss^l}{+\ H\text{-}2^k\ Ss^l}$	Male	37(7)†	28(23)	65
		Female	47(7)	37(27)	84
		Total	84(14)	65(50)	149
5	$\frac{T\ H\text{-}2^d\ Ss^h}{+\ H\text{-}2^k\ Ss^l} \times \frac{+\ H\text{-}2^k\ Ss^l}{+\ H\text{-}2^k\ Ss^l}$	Male	20(18)	27(3)	47
		Female	24(20)	19(1)	43
		Total	44(38)	46(4)	90

* Includes one *H-2* recombinant (see text).
† Numbers in parentheses indicate number of short-tailed ($T/+$) individuals.

Ss-L. Thirty-one females and 42 males gave normal agglutination reactions with anti-H-2b sera, and all were typed as Ss-HL. However, seven males whose sera were typed as Ss-HL gave only weak agglutination reactions with anti-H-2b sera on repeated tests. These individuals were progeny-tested in matings to C3H/HeJ. The segregation of progeny reacting normally with anti-H-2b sera indicated that all of the seven males were H-2bk heterozygotes. Sufficient numbers of animals were produced in these progeny tests that the data have been pooled and presented in Table 2 as Cross 2. (No explanation can be offered at present for the significant dependence on sex in Cross 2 ($\chi^2 = 4.33$, $P = 0.03$). Additional crosses of this type have been made to determine whether this is a repeatable effect.)

In Cross 3, two males were progeny-tested by crossing to C3H/HeJ females, because, while their cells were consistently typed as H-2k, the Ss level of their sera was consistently higher than in the normal Ss-L phenotype. Thirteen progeny of one male and nine of the other were all classified as H-2k and normal Ss-L, so both must have been genotypically $H\text{-}2^kSs^l/H\text{-}2^kSs^l$. In Crosses 4 and 5 a number of animals were progeny-tested for T, which is not completely penetrant in these strains. From results of these tests, penetrance in Crosses 4 and 5 was estimated at approximately 90 percent. The frequencies of recombination between *H-2* and T observed in Crosses 4 and 5, 0.195 ± 0.03 and 0.111 ± 0.03 respectively, do not differ significantly from the 0.154 found by ALLEN (1955).

In Table 2, only the two parental phenotypic classes are listed, because only a single apparent recombination, noted in Cross 3, was detected. On repeated trials, the cells of this female (female 307) failed to react, or reacted very weakly, with typing sera 2 through 5, though her serum was consistently typed as Ss-HL. She was mated to a C3H/HeJ male, and six of her 13 progeny had the same exceptional phenotype. The other seven were typed as H-2k, Ss-L. Two Ss-HL male progeny of female 307 were subsequently mated to C3H/HeJ females, and produced 19 Ss-HL progeny, all with negative or weakly reacting cells, and 15 Ss-L, H-2k progeny.

These results suggested a simple recombination between *Ss* and *H-2*. However, further tests were conducted to determine whether the recombination had occurred *within* the *H-2* locus. A number of cases of recombination within the *H-2* region have been reported (ALLEN 1955; AMOS, GORER and MIKULSKA 1955; PIZARRO, HOECKER, RUBINSTEIN and RAMOS 1961; STIMPFLING and SNELL 1961). The frequency of recombination between the D and K antigenic factors controlled by the *H-2* region has been estimated at about 1.4 percent in females (PIZARRO *et al.*, 1961).

Detailed studies of the H-2 type of female 307 are still in progress. However, these studies thus far have provided convincing evidence that the recombination occurred *within* the *H-2* region. Tests of the cells of female 307 and her Ss-HL progeny with antisera specific for single H-2 antigenic factors have shown that factors D and M, which are controlled by the $H\text{-}2^d$ allele, have been lost, while factor E^d, also controlled by $H\text{-}2^d$, is still present. These results have been confirmed by *in vivo* absorptions according to the method of AMOS (1955). These observations suggest that female 307 represented a recombination within the *H-2* region. Contamination from another source is unlikely. No other strain or stock in our colony exhibits this pattern of red blood cell and serum types. The serum and H-2 types of female 307 and her progeny showed nothing to suggest contamination. A mammary tumor arising in female 307 grew in all of eight (C3H/HeJ $\times$ DBA/2J)F_1 hybrids into which it was inoculated, while failing to grow in four C3H/HeJ hosts. Mutation cannot be excluded, but if this was the origin of the change in H-2 specificity, it is significant that it was accompanied by a change in Ss type.

Further tests are in progress to determine more completely the combination of H-2 factors controlled by this presumed new *H-2* "allele", and results of these studies will be published later. It is clear at this point, however, that the new allele specifies the H-2 factor E^d and the high Ss type, but has lost H-2 factors D and M. Studies by GORER and MIKULSKA (1959) and by others (*see* STIMPFLING and SNELL 1961) have suggested that factors D and M are controlled by one end of the *H-2* region, while factors E, E^d and K are controlled by the other end. It would thus appear, on the basis of present evidence, that *Ss* lies either between the D and K ends of the region, or outside the K end. Additional data on more recombinants within the *H-2* region will probably be necessary before any firm conclusion can be drawn about the position of Ss relative to the various sub-loci of the *H-2* region. To provide such data, crosses have been set up to screen for recombination between the D and K factors.

Another means by which more information on the *Ss–H-2* relationship may be obtained is through studies of the association of Ss types with specific *H-2* alleles. Through the courtesy of DR. GEORGE D. SNELL, it has been possible to test this association in three more H-2 types not included in the previous report (SHREFFLER and OWEN 1963). A/Sn–*H-2*^{s} is Ss-H, RIII/WyJ (*H-2*^{r}) is Ss-H, and AKR/Sn–*H-2*^{m} is Ss-L. *H-2*^{m} thus becomes the only allele other than *H-2*^{k} which has to date been found to be associated with Ss-L. Since the two alleles are rather similar in the H-2 factors which they specify, this would constitute no apparent inconsistency. On the other hand, *H-2*^{r}, which is also very similar to *H-2*^{k}, is associated with Ss-H. This would suggest that whatever constitutes the antigenic difference between *H-2*^{k} and *H-2*^{r} might be of some significance with respect to the association between Ss and H-2. It is of interest that both *H-2*^{k} and *H-2*^{m} lack the antigenic factor F, which, with one possible exception, is determined by all of the alleles associated with Ss-H type. The possible exception is *H-2*^{r}, for which the presence or absence of F has not been clearly determined (STIMFLING and SNELL 1961; AMOS 1962). It is tempting to speculate that Ss might be specifically associated with a single H-2 factor, such as F, or a restricted group of factors, but much more information is necessary before this possibility can be realistically evaluated. The most important point with respect to the association of Ss types with *H-2* alleles is that in no instance has a specific *H-2* allele been associated with Ss-H in one inbred strain and Ss-L in another.

Discounting female 307, which clearly was not a simple recombinant between *Ss* and *H-2*, no recombination has been observed between the two traits among 802 segregants classified. The maximum probable frequency of recombination between *Ss* and *H-2*, if they are separable loci, can be calculated from $P = (1\text{-}r)^n$ to be 0.0037, where P is set at 0.05. Comparison of this value with the 0.01 to 0.015 frequency of recombination reported between the D and K antigenic factors can leave little doubt that *Ss* is closely associated with the *H-2* region. This conclusion is further strengthened by the association of Ss and H-2 types in inbred strains. Whether this genetic association reflects a direct functional role of the Ss protein in gene action of the *H-2* locus, a secondary physiological effect of H-2 differences, or a mere chance linkage between unrelated loci remains to be determined.

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SUMMARY

Several backcross linkage tests were conducted in an effort to define the nature of a genetic association between a quantitative serum protein variant trait, Ss, and the *H-2* locus. No recombination between *Ss* and *H-2* was observed among 802 individuals classified, although one individual was detected which showed a recombination within the complex *H-2* locus. These results are interpreted as

indicating that the Ss difference is controlled by a locus located either within the *H-2* region or immediately adjacent to it.

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