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Genetics

Discrimination of odortypes determined by the major histocompatibility complex among outbred mice

(olfaction/chemosensory identity/reproductive behavior/population genetics)

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ABSTRACT **Genetically determined body odors that distinguish one mouse from another are termed odortypes. The best known odortypes, highly expressed in urine, are those specified by *H-2*, the major histocompatibility complex of the mouse, but other odortypes originate from unidentified loci in the rest of the genome, including both sex chromosomes. The definition of *H-2* odortypes and evidence that their perception affects reproductive behavior have so far depended on studies with inbred mouse strains whose genetic differences are confined to the *H-2* complex of genes. To simulate feral conditions more closely, a freely segregating population was bred from crosses involving four unrelated inbred strains contributing four different *H-2* haplotypes. After *H-2* typing, this outbred population was divided into four groups of freely segregating mice, comprising the four distinct *H-2* genotypes represented, to serve as conventional donors of urine for evaluation in the standard Y-maze system used in the training and testing of mice for *H-2* odortype discrimination. With respect to utility in training mice for *H-2* odortype discrimination, and to degrees of concordance attained in the Y-maze by trained mice, these urinary *H-2* odortype sources from outbred mice were no less effective than urines customarily obtained for those purposes from nonsegregating inbred donors. We conclude that discrimination of *H-2* odortypes is not appreciably affected or impaired by the usual concurrent segregation within the genome as a whole.**

The discovery of odortypes began with the fortuitous observation that a male mouse caged with two females, one of the same inbred strain and another differing at the *H-2* locus, generally preferred to keep company with the latter. Formal studies then showed that this preference extends to mating, the male generally favoring the female of different *H-2* type (1). The inference that this communication of *H-2* genotypic identity is olfactory was confirmed in a Y-maze system in which trained mice distinguished the scents of urine from mice of dissimilar *H-2* types (2), even differences as slight as a subdivision or single-gene mutation of the *H-2* complex (3, 4). *H-2*-selective mating is the result of familial imprinting (5). During pregnancy, outbred females acquire *H-2* odortypes of paternal *H-2* haplotypes carried by fetuses (6). Maintenance of early pregnancy depends substantially on the *H-2* odortypes to which the mother is exposed (7). Particularly in view of these striking effects of *H-2* odortypes on the reproductive life of the laboratory mouse, we need to establish, as others have remarked and explored (8), the extent to which such findings apply to this species in the wild. Since all definitive studies of *H-2* odortypes and their behavioral effects have so far of necessity involved inbred mouse strains that differ solely in their *H-2* genotypes (see ref. 9 for review), a prime question, addressed here, is whether the constitution of *H-2* odortypes and their perception are affected by the usual free segregation of the genome as a whole.

The present study is based entirely on the Y-maze test system. The two test systems that involve no laboratory training and which reveal inherent reproductive behavior influenced by H-2 odortypes—namely, the mating-preference (1) and pregnancy-block (7) systems—are unsuited to the routine definition and analysis of odortypes, for which the Y-maze and later the automated olfactometer (10) systems were specifically designed. It should be noted, however, that no *H-2* odortype distinction detected in the Y-maze, even from as slight a genetic distinction as a single mutant class I gene of the *H-2* complex, has failed to register a response on subsequent testing in these far more elaborate systems (9).

MATERIALS AND METHODS

Source of Odors. The derivation and particulars (source and number) of the odor-donor mice used in this study are illustrated in Fig. 1. Odor-donor mice were caged separately in the same animal room. They were individually numbered for use in rotation to provide sets of different sample pairs for each training and generalization trial (see below). Urine samples were obtained from individual mice by gentle abdominal pressure and were frozen at −20°C until needed. For testing, pairs of samples (each 0.3–0.4 ml) were defrosted and placed at room temperature in two 3.5-cm-diameter Petri dishes. Samples for each trial were assigned to the left or right odor chambers of the Y-maze according to a series of random numbers.

***H-2* Typing of the Four Heterozygous Segregants Identified in Fig. 1.** Tail DNA was prepared by digestion with proteinase K (250 μg/ml) in a 100-μl standard polymerase chain reaction (PCR) buffer plus 0.5% (vol/vol) Nonidet P-40 at 50°C overnight. Proteinase K was inactivated by incubation at 100°C for 15 min. After removal of debris by centrifugation, a 1-μl sample was used as a template. Three pairs of *H-2* haplotype-specific primers, corresponding to $H\text{-}2K^b$, $H\text{-}2L^d$, and $H\text{-}2K^k$, were used for PCR. Thus, $H\text{-}2K^{bk}$ is positive for both $H\text{-}2K^b$ and $H\text{-}2K^k$ primers and $H\text{-}2^{bd}$ is positive for both $H\text{-}2K^b$ and $H\text{-}2L^d$ primers, whereas $H\text{-}2K^{sk}$ is positive for only the $H\text{-}2K^k$ primer and $H\text{-}2K^{sd}$ is positive for only the $H\text{-}2L^d$ primer. The $T18^d$ (*Tla* region)-specific primers were used for monitoring crossovers within the *H-2* locus. Only those mice giving unambiguous results were used as odor donors.

Y-Maze. The design and operation of the Y-maze used in studying odortypes are detailed elsewhere (2). The two arms of the maze are scented by air currents conducted through chambers containing urines exposed in Petri dishes from pairs of *H-2* segregants.

Training and Testing Procedures. The 14 trained mice are identified in Tables 2 and 3. For training and testing in the Y-maze, gates are raised and lowered in timed sequence of up to 48 consecutive trials, the paired urine samples being

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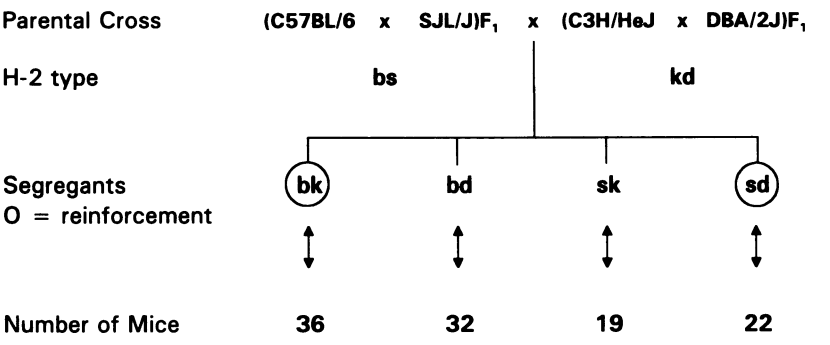

FIG. 1. Scheme used to generate a freely segregating population with four distinct *H-2* haplotypes. The F_1 mice were purchased from Jackson Laboratories. The male segregants were typed for *H-2* by using tail DNA and grouped into four panels identified as *bk, bd, sk,* and *sd*. These panels served as urine donors for training and generalization trials.

changed for each trial. Reward for correct response is a drop of water, the trainee mouse having been deprived of water for 23 hr. Preliminary training in the present study progressed from gross to fine distinctions in stages. Each mouse was first trained to discriminate between urine donors of two unrelated strains, then of *H-2*-congenic strains, and finally one group was rewarded for choosing *bk* (versus *sd*) odors whereas the second group was reinforced for the opposite response (see Table 1). Following successful training (>80% concordance), interspersed unrewarded trials (about one out of every four) were included to familiarize the mice with occasional absence of reward following a correct response. The trained mice performed on the unrewarded trials with the same accuracy as on rewarded trials. Training and testing continued as described above but samples from interspersed unrewarded (generalization) trials were now supplied from new panels (see Table 1) of *H-2* segregants.

RESULTS

Study I. The main object was to determine whether free segregation of the genome as a whole would substantially affect the perceptibility of *H-2* odortypes. For this purpose, urine donor panels representing segregants *bk* and *sd* (see Fig. 1) were assembled. Each of these panels was divided into two parts, the first for training and testing trials, and the second for generalization trials, in the Y-maze. Of these 14 mice trained, 10 were used in this first study, as Table 1 indicates, and 4 were reserved to take part only in the second study (see below), as indicated in Table 2 (mice 6 and 7) and Table 3 (mice 12 and 13).

Table 1 summarizes the data for 1324 trials, representing the three test phases: *rewarded*, representing degree of concordance in rewarded trials after mice in training had reached proficiency; *unrewarded*, sporadic interspersed unrewarded concordance trials, to accustom the trained mice to occasional lack of reward; *generalization*, similarly sporadic interspersed trials using the second panels of *bk* and *sd* urine donors not before encountered, all these unrewarded trials being coded for blind presentation by the Y-maze operators.

In all three categories these data indicate a high degree of proficiency in *H-2* odortype discrimination that matches all previous data pertaining to *H-2* odortype discrimination between mice of otherwise uniform genomes. Since results of generalization tests may deserve particular attention in the context of outbred populations, these data are given in more detail, for each of the 10 trained mice, as group 1 in Tables 2 and 3.The data are consistent: all 10 mice show more concordant than discordant trials.

Study II. A secondary aim, given the material at hand, was to determine whether trained perception of a given *H-2* heterozygote's odortype may extend to recognition of other *H-2* heterozygotes bearing one of the pertinent *H-2* haplotypes. This subsidiary study comprised a further 474 trials, all in the generalization mode, employing 11 mice, 7 of which had experienced generalization trials as above (*Study I*) and 4 of which had not. These data are shown as groups 2–5 in Tables 2 and 3.

Table 1. Discrimination in the Y-maze of *H-2* odortypes presented on the varied genetic background represented in Fig. 1

Reinforced alternative	No. of trained mice	Test phase*	No. of trials	Concordance, %	*U* value†	*P* value
bk (vs. *sd*)	5‡	Rewarded	554	87	17.55	$\ll$0.0001
		Unrewarded	98	90	7.78	$\ll$0.0001
		Generalization	100§	73	4.50	<0.0001
sd (vs. *bk*)	5¶	Rewarded	426	83	13.71	$\ll$0.0001
		Unrewarded	71	85	5.70	<0.0001
		Generalization	76‖	72	3.79	<0.001
Total	10	Rewarded	980	86	22.26	$\ll$0.0001
		Unrewarded	169	88	9.69	$\ll$0.0001
		Generalization	176	73	5.95	<0.0001

*Training panels comprised 20 *bk* and 14 *sd* male urine donors used in rewarded trials and in interspersed unrewarded trials. Generalization panels comprised of 16 *bk* and 8 *sd* male urine donors not encountered by the mice in training trials.

†Standardized normal deviate: $U = [\,|\,r - (n/2)\,|\, - (1/2)]/(n/4)^{1/2}$, where r is the number of concordant responses.

‡Identified in Table 2.

§Detailed in Table 2, group 1.

¶Identified in Table 3.

‖Detailed in Table 3, group 1.

Table 2. Responses of the seven mice* reinforced for *H-2* odortype *bk* versus *H-2* odortype *sd*, in generalization trials

	Alternative odortypes offered†		No. of unrewarded blind trials resulting in concordant (C) or discordant (D) choice														Total for 1–7	
			1 (♀)		2 (♂)		3 (♂)		4 (♂)		5 (♂)		6 (♂)		7 (♂)			
Group	C	D	C	D	C	D	C	D	C	D	C	D	C	D	C	D	C	D
1‡	*bk*	*sd*	19	10	18	3	12	4	11	7	13	3					73	27
2	(*b*)*k*	(*b*)*d*	9	4	10	2			8	3	11	3					38	12
3	*b*(*k*)	*s*(*k*)	5	3	5	3					6	1	5	1	6	2	27	10
4	*b*(*d*)	*s*(*d*)	2	13	6	12					11	9	9	8	8	6	§	§
5	(*s*)*k*	(*s*)*d*	8	0	7	6					12	9	8	10	7	11	§	§

*Mice 1–6, C57BL/6-*H-2*^{k}; mouse 7, C57BL/6.
†Parentheses indicate the shared *H-2* haplotype.
‡Combined data are given in Table 1.
§Unlike groups 1–3, in which all mice were consistent in showing preponderance of concordant choice, responses in Groups 4 and 5 were inconsistent, with preponderance of choice differing for different mice (see text).

Trained distinction of *bk* from *sd* entailed distinction also of *bk* from *bd* (Table 2, group 2), where the genetic difference is limited to *k* versus *d*, and of *bk* from *sk*, where the genetic difference is *b* versus *s* (Table 2, group 3). The same picture is seen for the reciprocal trained distinction of *sd* from *bk* (Table 3, groups 2 and 3), with genetic difference of *d* versus *k* and of *s* versus *b*. And again, as in *Study I*, the trained mice were consistent, each showing more concordant than discordant trials. Thus in this particular conformation it appears that either haplotype alone is readily "tracked."

But in the other conformation tested (groups 4 and 5 of Tables 2 and 3), which presents the same single-haplotype distinctions, but in combination with different *common* haplotypes (shared *d* instead of *b* in groups 4, and shared *s* instead of *k* in groups 5) there is no consistency, the responses of the 10 trained mice being seemingly concordant, discordant, or indifferent.

DISCUSSION

The *H-2* complex is not the only source of odortypes which distinguish individual mice. By the rough criteria of ease of training and proficiency of trained mice in the Y-maze, it can be surmised that the entire autosomal genome exclusive of the *H-2* complex may approach *H-2* in potency, if not variety, as a source of odortypes and that both sex chromosomes determine discriminable odortypes.

Despite extensive studies with analytical chemical methods such as gas chromatography that are generally employed in odorant chemistry, no *H-2*-related definitive features have been found in urine, the prime source of odortypes. We subscribe to the view that the potentially vast array of odortypes stemming from the extreme genetic polymorphism of the major histocompatibility complex are mostly compound odors, defined here, for simplicity, as odors whose differences depend solely on variation in the proportional representation of the same set of constituent odorants. It is inferred that these are normal metabolites, the output of each differing independently from one individual to another, this being simply a metabolic/olfactory corollary of the normal genetic variation which entails, for instance, that no two humans except identical twins are anatomically/visually indistinguishable. The validity of this hypothesis notwithstanding, it provides a useful context for questions arising from the present and other studies.

For instance, may different odortype-determining genes, *H-2* and non-*H-2* (say), affect output of the same odorants? While more than one gene might well influence output of a particular metabolite/odorant, and although this cannot be excluded, the evident indifference of *H-2* to concurrent genomic segregation, which probably involved several non-*H-2* odortype genes in the present study, leaves no doubt that *H-2* has sufficient independent control of an idiosyncratic odorant inventory, without reference to the rest of the genome.

On the other hand, concerted action of alleles of the same odortype gene might be expected to yield an odortype peculiar to the heterozygote. The upshot of previous studies is that training to recognize the odortype of a given *H-2* homozygote sometimes conferred ability to recognize that haplotype in heterozygotes, but the heterozygote's odortype could not be duplicated by combining the odortypes of the parental homozygotes (11). Since these studies involved the entire *H-2* complex, comprising several independent odortype loci (9), the conclusion that *H-2* heterozygote odortypes are in part similar and in part dissimilar to those of the respective homozygotes may be taken to conform to the hypothesis of odortype determination outlined above, but it is impossible at present to gauge the extent of similarity and

Table 3. Responses of seven mice* reinforced for *H-2* odortype *sd* versus *bk*, in generalization trials

	Alternative odortypes offered†		No. of unrewarded trials resulting in concordant (C) or discordant (D) choice														Total for 8–14	
			8 (♂)		9 (♀)		10 (♂)		11 (♂)		12 (♂)		13 (♂)		14 (♂)			
Group	C	D	C	D	C	D	C	D	C	D	C	D	C	D	C	D	C	D
1‡	*sd*	*bk*	14	2	12	5	8	4	8	4					13	6	55	21
2	(*b*)*d*	(*b*)*k*							9	3			7	1	7	1	23	5
3	*s*(*k*)	*b*(*k*)							11	7			6	2	7	0	24	9
4	*s*(*d*)	*b*(*d*)	1	10					1	9	0	7			10	5	§	§
5	(*s*)*d*	(*s*)*k*	14	17					23	11	12	11	8	5	7	13	§	§

*Mice 8–13, C57BL/6-*H-2*^{k}; mouse 14, C57BL/6.
†Parentheses indicate the shared *H-2* haplotype.
‡Combined data are given in Table 1.
§Unlike groups 1–3, in which all mice were consistent in showing preponderance of concordant choice, responses in groups 4 and 5 were inconsistent, with preponderance of choice differing for different mice (see text).

dissimilarity or the extent of variation with different *H-2* haplotypes.

In regard to unique elements of *H-2* heterozygote odortypes, and to instances in which trained recognition of such odortypes conferred adequate recognition of some haploidentical urine donors but not others (Tables 2 and 3, groups 2–5), it may be relevant that compound odors have often unpredictable perceptual attributes depending on the nature as well as the relative concentrations of the odorants. In a simple situation, as when two odorants are combined, it may be that with an equal mixture of the two, only one will be perceived, the other being completely masked; or they may fuse to form a seemingly unique third odor; or they may be perceived as a mixture of the two (see ref. 12).

From this viewpoint, results of the subsidiary part of this study, concerning whether trained recognition of the odortype of a given *H-2* heterozygote confers recognition of either haplotype in a different heterozygous combination, a so-far-unexplored permutation of odortypes, are not unexpected and generally conform to theory. Possible biological relevance could be signified by asking whether familial imprinting of males on an *H-2* heterozygote odortype would entail mating bias unfavorable also to females of each homozygote genotype or to haploidentical females.

In short, the data in Tables 2 and 3 (groups 2–5) concerning trained recognition of *H-2* heterozygote odortypes include clear instances of haplotype recognition in a different framework of presentation, and equally clear instances where haplotype recognition was not evident. In these latter data, there are suggestions that non-*H-2* odortypes are recognized by some mice, perhaps encountered and rewarded repeatedly by chance during training and testing with urines of segregating donors, and happening to outweigh a minor *H-2* odortype difference represented in this mode of presentation. Be that as it may, one is reminded that *H-2* is not the sole source of odortypes and surely not the only locus with a vested interest in odortype discrimination.

Finally, in considering how data obtained from training for *H-2* odortype distinction in the Y-maze may apply in nature, bearing in mind that all such tested distinctions have proved active in the mating-preference and/or pregnancy-block test systems (9), it may be taken into account that these two phenomena also have their equivalent of training, in the sense of past experience—namely, in familial imprinting (5) and the pregnant female's memory of her mate's odortype (7), respectively.

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