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Expression of urinary *H-2* odortypes by infant mice

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ABSTRACT **The extended *H-2* complex of genes in the mouse includes at least three loci that independently specify distinctive body odors, "odortypes," whose differential recognition influences mating choice and affects the maintenance of early pregnancy. A prime experimental method of identifying *H-2* odortypes is the specially designed Y-maze in which mice are trained, by water deprivation and reward, to distinguish odors conducted to the arms of the maze from *H-2*-dissimilar mice or their urines. It is confirmed that *H-2*-dissimilar infant mice, unlike adult mice, are not distinguished by trained mice in the Y-maze. However, a previous conclusion that infant mice do not express *H-2* odortypes is shown to be incorrect, because the urines of *H-2*-dissimilar infant mice, even at 1 day of age, were distinguished in the Y-maze. Thus urine, ingested by the mother, clearly could suffice for her to distinguish her own from other *H-2*-dissimilar pups. Further, urine would seem to be a unique source of *H-2* odortypes. If, as we believe, *H-2* odortypes represent mostly compound odors composed by *H-2* genetic variation in the urinary output of odorous metabolites, as distinct from simple odors that depend on chemical differences of single odorants, then the kidney, which is not responsible for *H-2* odortype specificity, may nevertheless impart a unique character to urinary odortypes by virtue of differential excretion/resorption processing of various constituent odorous metabolites. In that case, various organs and tissues, among which the hematopoietic/lymphoid system is known to contribute to *H-2* odortype specificity, may exhibit tissue-specific varieties of *H-2* odortypes, their products having not yet been subjected to renal processing.**

Odortypes, defined as genetically-determined body scents that enable individuals of a species to distinguish one another by scent, are specified by polymorphic genes of the major histocompatibility complex (MHC), called *H-2* in the mouse.

Perception of MHC odortypes causes preferential mating and also affects the maintenance of early pregnancy, thereby favoring the propagation of particular MHC genotypes in the mouse (reviewed in ref. 1).

MHC-selective mating depends on familial imprinting, as shown by the altered mating choices of appropriately fostered males (2). In the context of familial MHC imprinting, and in other reproductive contexts such as maternal identification of progeny in relation to nursing, it is necessary to know whether MHC odortypes are expressed in early life not only to elucidate further the reproductive significance of MHC odortypes but also because data on the early expression of MHC odortypes are essential to a number of potential studies on the generation of odortypes.

Our original studies with the Y-maze system of MHC odortype discrimination, before it was discovered that urine was the main and most convenient source of MHC-determined odors for discrimination in the Y-maze (3), involved the testing of alternative MHC-congenic mice placed in the odor chambers of the maze (4). Under these conditions, it appeared that infant mice could not be distinguished by trained mice that successfully distinguished adult mice of the same alternative MHC genotypes.

The present report concerns more recent studies indicating that these earlier studies, implying that infant mice lack MHC odortypes, were misleading and suggests reasons for the discrepancy, which are critical to better understanding of the nature and biological significance of MHC odortypes.

MATERIALS AND METHODS

The Y-Maze. As detailed elsewhere (3), air is conducted through two odor chambers, containing urine samples exposed in Petri dishes or perforated containers housing the infant mice, to the two arms of the maze. Gates are raised and lowered in timed sequence to permit the training or testing of each mouse in a series of up to 48 consecutive runs. As before (3), the samples were assigned to the left or right odor boxes of the Y-maze according to a series of random numbers. The reward for a correct response is a drop of water, the mouse having been deprived of water for 23 hr. The water dispenser in each arm of the maze is guarded by a fence, which is raised only if the mouse's choice is concordant with training.

Training. To test infant mice and their urines, the following testing paradigm was used: preliminary training progressed from gross to fine distinctions in stages as described (3). Adult mice were first trained to discriminate adult panels of unrelated strains [C57BL/6 (B6) vs. AKR]. When this was successfully completed, adult panels of congenic mice differing only in the MHC (B6 vs. B6-*H-2*^{k}) next served as urine donors. Once this was successfully accomplished (>80% correct in a block of 48 trials), testing of infant (B6 vs. B6-*H-2*^{k}) odor was initiated. For each testing session of infant odors, up to 24 reinforced trials were first conducted with adult odors. If, as occasionally happened, the mice did not attain approximately 80% or greater concordance in these preliminary trials, testing was deferred until another day. When this preliminary training was successful, a series of four trials was begun with adult urines followed by four trials with infant mice (study 1) or their urines (study 2) with all correct responses being reinforced. This schedule was repeated several times giving 16–36 trials of infant age groups with live infants or their urines.

Generalization. As fully described elsewhere (5), the purpose of this procedure is to test new urine-donor panels without reward and thereby rule out the possibility that incidental or genetically unrelated cues are being learned and responded to; if there is no reward, there can be no learning of adventitious cues. Generalization is conducted with blind testing of coded samples, which is possible because no reward is called for. To maintain the learned response, the unrewarded samples from new panels are interspersed with concurrent, unrewarded testing of the familiar sources to accustom the already trained mice to periodic withholding of reward even when their response is correct.

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Abbreviations: MHC, major histocompatibility complex; B6 mice, C57BL/6 mice.

Significant generalization is final proof that the trained mice are discriminating the odor sources on the basis of the class (*H-2*^{b} vs. *H-2*^{k}) to which the donors belong.

Source of Odors. To ensure that any odor difference found would be due to the infants and not some odor derived from the mothers, litters of B6 and B6-*H-2*^{k} mice, which would comprise the odor-donor panels, were removed from their mothers within 16 hr of birth and fostered onto lactating BALB/c females.

Six B6 mice (two males, four females) and six B6-*H-2*^{k} mice (four males, two females), were used as odor sources for tests of whole-animal odors (study 1). Previous work (3) has indicated that both male and female mice exhibit *H-2* odortypes.

The number of the infants providing urine for study 2 is shown in Table 1. Only male urine was collected. When picked up by the skin of the back, the pups emitted drops of urine that were drawn up into a test tube. Usually five or six infant mice provided sufficient urine (0.2–0.3 ml) to cover the bottom of a 3.5-cm diameter Petri dish, but sometimes more mice were needed. Urine samples were frozen at −20°C until needed. Freshly defrosted samples at room temperature from different donors were provided for each run and were assigned to the left or right odor chambers of the Y-maze according to a series of random numbers as has been described (3).

Order of Training and Testing. For study 1, the training began with the youngest pups tested (aged 4 days) and proceeded to successively older ages. For urines, training progressed in the reverse order, beginning with the oldest age group.

RESULTS

Study 1. Mice could not be trained in the Y-maze to distinguish B6 mice (*H-2*^{b}) of age 4 or 11 days from B6-*H-2*^{k} congenic mice of the same age.

As Fig. 1 indicates and as observed previously (unpublished data), under standard Y-maze conditions of training and testing that have revealed odortype distinctions due to MHC differences as fine as mutation of a single class I gene (6, 7), trained mice failed to distinguish 4-day-old or 11-day-old B6 mice from B6-*H-2*^{k} mice of the same ages, even though the genetic disparity in this case represents the entire homozygous extended MHC complex (*H-2–Qa–Tla*), which includes at least three loci that alone can independently specify a MHC-distinctive odortype (8).

Study 2. Mice were successfully trained in the Y-maze to distinguish the urine of B6 (*H-2*^{b}) mice of age 6 days, 3 days, 2 days, or 1 day from the urine of B6-*H-2*^{k} congenic mice of the same age.

Study 2 was similar to study 1 with the single difference that urine samples from 6-day-old, 3-day-old, 2-day-old, and 1-day-old mice were substituted for intact infant mice in the odor chambers.

As Fig. 2 indicates, in each case training was successful,

Table 1. Urine donor panels: studies 2 and 3

Age, days	Procedures*	Number of animals (litters) B6	B6-*H-2*^{k}
	Study 2		
1	Training	20 (6)	15 (4)
2	Training	11 (2)	8 (3)
3	Training	5 (1)	12 (3)
6	Training	9 (2)	8 (3)
	Study 3		
3	Training	9 (3)	24 (4)
3	Generalization	17 (5)	7 (2)

*See appropriate sections in *Materials* and *Methods*.

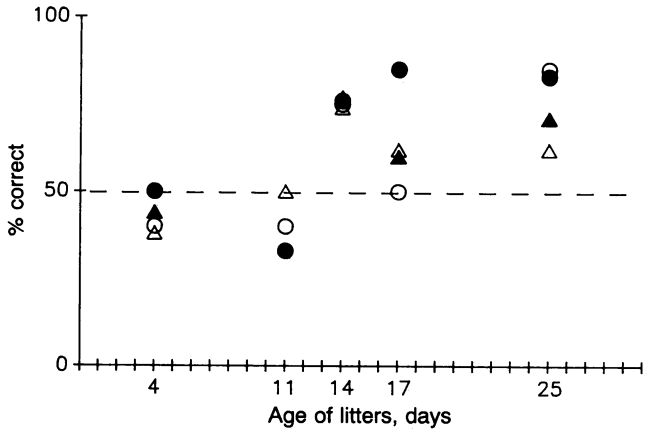

FIG. 1. Results of study 1. Percent correct choices for each of four adult mice [two females (○, △) and two males (●, ▲)] trained to differentiate odors of litters of B6 vs. B6-*H-2*^{k} infant mice in the Y-maze. Testing proceeded from younger to older litters. Trained animals were each given between 16 and 24 trials at each pup age. Overall mean percentage correct ± 95% confidence interval for the four test animals at different ages were 43 ± 11% at 4 days, 42 ± 14% at 11 days, 75 ± 9% at 14 days ($P < 0.05$), 64 ± 10% at 17 days ($P < 0.05$), and 74 ± 10% at 25 days ($P < 0.05$).

signifying the expression of MHC odortypes in urine by the age of 1 day at the latest.

Study 3. Generalization trials confirm the *H-2*-determined basis of infant urinary odortype distinction.

Three mice were successfully trained to distinguish the urines of 3-day-old B6 and B6-*H-2*^{k} pups, each attaining a concordance score (correct responses) of around 88% in 84 rewarded trials.

In 19 subsequent generalization trials, correct responses numbered 15 ($P < 0.05$), which attests to the *H-2*-determined basis of the odortype distinction and substantiates expression of *H-2*-determined odortypes by infant mice (Table 1).

DISCUSSION

The fact that infant mice manifest MHC-determined odortypes and the finding that urine appears to be the main or sole source are crucial not only to broader appreciation of the contexts in which odortype recognition affects behavior but

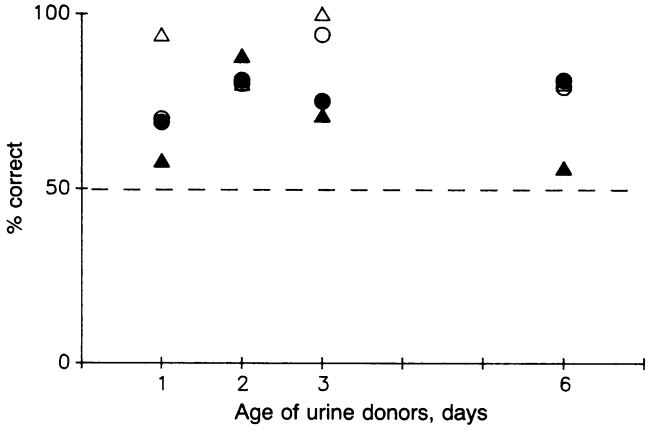

FIG. 2. Results of study 2. Percent correct choices for each of the same adult mice used in study 1, now trained to differentiate urine odors of B6 vs. B6-*H-2*^{k} pups in the Y-maze. Trained animals were each given 16–36 trials at each pup age. Testing proceeded from older to younger pup urines. Overall mean percentage correct ± 95% confidence intervals for the four test animals at different ages were 74 ± 8% at 1 day ($P < 0.05$), 82 ± 9% at 2 days ($P < 0.05$), 83 ± 8% at 3 days ($P < 0.05$), and 75 ± 10% at 6 days ($P < 0.05$). The same symbols are used to identify individual trained mice in Fig. 1.

also to improved understanding of how these odortypes are composed.

Our previous evidence (unpublished data) that contrasting MHC odortypes of infant mice less than 10 days of age are not distinguished in the Y-maze, even under conditions that suffice for distinction of a single class I gene disparity (6, 7) is confirmed. A probable reason why urinary *H-2* odortypes are not apparent when infant mice are tested in the Y-maze can be found in the fact that infant mice characteristically do not urinate spontaneously but are stimulated to do so when the mother licks them (9) and, thereby, undoubtedly ingests the odortype-expressing urine that serves to distinguish her own pups from others (10). Since urine serves as a chemical signal between pups and mother (11), it is likely that MHC-regulated odortypes provide a basis for this recognition.

The apparent absence of odortype from the cleaned nonurinating infant mouse affords further evidence that the urinary *H-2* odortype is the most potent source of these odors. The extraordinary precision and seemingly limitless range of odortypes, specified as they are not only by several parts of the *H-2* complex but also by other parts of the autosomal genome (12) and by each of the sex chromosomes (13), is best explained by proposing that odortypes represent mainly compound odors (defined as odors whose distinctive olfactory specificity depends on variation in the relative proportions of the same set of constitutive odorants) as opposed to odors that depend on structurally different odorants.

The most obvious sources of constituent odorants for composing urinary compound odors are odorous metabolites voided in urine. The relative output of such odorous metabolites could be geared to genetic polymorphism in many ways, such as genetic differences in the relative sizes of particular organs and tissues within individuals, which is known to be one of the manifestations of *H-2* polymorphism, and by normal genetic variation of metabolic mechanisms affecting the output of a given metabolite per cell, regardless of organ or tissue size. For the mouse, variation in commensal microorganisms does not play a role (14).

That the kidney itself might be the sole source of odorants would explain the apparent restriction of the odortype to urine, but this hypothesis is excluded by the finding that radiation chimeras acquire an added urinary *H-2* odortype typical of the *H-2*-dissimilar reconstituting donor (15). Thus, one kidney can subserve more than one *H-2* odortype, and the hematopoietic system is one proven source of odortype-determining odorants conveyed to the kidney by the bloodstream.

These considerations suggest that many body fluids, in particular plasma, may also possess MHC-regulated odortypes. The expectation that plasma expresses an odortype prior to renal processing (which is supported by preliminary unpublished evidence) has ramifications that extend to the circumstances of pregnancy, for circulating odorants of small molecular size should be transferred to the mother via the placenta from the fetus, which is likely to express its own urinary (and *ergo* plasma) odortype since the 1-day-old infant already does so, as reported here. Thus, if mother and fetus are *H-2*-dissimilar, which is the usual condition in all freely segregating populations, then the relative proportion of odorants, which by definition is the sole determinant of a compound odor, may be specifically altered and the maternal *H-2* odortype thereby modified temporarily in accord with the *H-2* genotype of the fetus. Similar considerations may apply to the suckling infant's odortype with respect to temporary acquisition of a maternal proportion of *H-2*-influenced odorants from the milk.

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