Proc. Natl. Acad. Sci. USA
Vol. 94, pp. 2210–2214, March 1997
Biochemistry

Volatile signals of the major histocompatibility complex in male mouse urine

(individual odor/organic acids)

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Communicated by Edward A. Boyse, University of Arizona College of Medicine, Tucson, AZ, December 31, 1996 (received for review October 15, 1996)

ABSTRACT **Variation in the genes of the major histocompatibility complex (MHC) contributes to unique individual odors (odortypes) in mice, as demonstrated by the ability of trained mice in a Y-maze olfactometer to discriminate nearly identical inbred mice that differ genetically only at the MHC (MHC congenic mice), while they cannot distinguish genetically identical inbred mice. Similar distinctions are possible with urine, a substance that is involved in many facets of mouse chemical communication. This paper reports results supporting the hypothesis that the MHC-determined urinary odor is composed of a mixture of volatile carboxylic acids occurring in relative concentrations that are characteristic of the odortype. Y-maze behavioral testing of urine fractions from anion exchange chromatography indicates that volatile acids are necessary and sufficient to convey MHC odortype information. Diethyl ether extracts, which are expected to contain the more volatile, less polar organic acids, were also discriminable in the Y-maze olfactometer. Ether extracts of 12 different urine samples from each of two panels of MHC congenic mice were analyzed by gas chromatography. No compounds unique to urine of either genotype were detected, but compounds did appear to occur in characteristic ratios in most of the samples of each type. Nonparametric statistical analysis of the gas chromatographic data showed that eight of the peaks occurred in significantly different relative concentrations in the congenic samples. One of the peaks was shown to represent phenylacetic acid, which has implications for the mechanism of the MHC specification of odortype.**

The genes of the major histocompatibility complex (MHC) were first recognized for their part in the rejection of tissue transplants between unrelated individuals, and then for their more general role in the immune response to pathogens and synthetic antigens (1). When given a choice, inbred mice prefer to mate with individuals of dissimilar MHC (2). Further investigation of this phenomenon demonstrated that mice were capable of discriminating the H-2 haplotypes of inbred mice by olfactory detection of the volatile compounds emanating from urine (3). In these experiments mice in a Y-maze olfactometer could be trained to discriminate urine samples from two groups of mice differing genetically only at the H-2 loci. Apparently this ability is used by mice to choose a mate differing at H-2, resulting in the avoidance of inbreeding and in the extreme diversity of the MHC-regulated cell surface proteins (4).

To understand how the MHC produces characteristic odors, presumably mixtures of small molecules specifically related to class I and class II proteins, we have undertaken to identify the compounds in mouse urine that are the source of the olfactory discriminability of samples of urine from MHC congenic mice. From the ability of the signal to travel in a slow stream of air in the Y-maze olfactometer (and in an automated olfactometer; ref. 5), we can infer that the discrimination is most probably based on the chemosensory detection of volatile compounds.

The obvious approach to the analysis of complex mixtures of volatile compounds would make use of gas chromatography. Earlier attempts to apply this technique to the analysis of mouse urine suggested that there are distinguishable patterns of neutral volatile compounds in different H-2 haplotypes (6, 7). However, these reported differences were not related to specific peaks in the chromatograms, which is necessary for the chemical characterization of the compounds involved in conveying odor information indicative of H-2 type.

For several reasons, neutral compounds are not necessarily the most likely class of molecules to signal the vast number of different H-2 types. First, the most constant and abundant products of metabolism that occur in human urine are acidic rather than neutral (8). Since these products are consistently related to fundamental metabolic processes, such as the catabolism of carbohydrates, the same compounds are likely to be equally plentiful in mouse urine. Second, some of these acids are normal metabolites of amino acids, which suggests that they plausibly could be derived from the peptides bound by MHC molecules. Third, in the mammalian chemical signal literature there is a history of suggestions that volatile acids occur in sufficient variety in various mammals to be useful markers of identity (9–11). Fourth, we previously found that acidification of mouse urine does not diminish its discriminability in the Y-maze (12). These reasons suggest that H-2 types could be signaled by variable mixtures of volatile organic acids.

As a direct test of this hypothesis, we have treated deproteinized mouse urine in an anion exchange procedure that reliably removes acidic compounds from urine (8). When the behavioral results, which are described below, supported our hypothesis, we proceeded to extract the acidified urine with ether to more selectively isolate the less polar, and presumably more volatile, acids to provide a sample that was amenable to gas chromatography. The details of this experiment as well as of the gas chromatographic analysis and behavioral testing of the resulting ether extracts are the other subjects of this paper.

METHODS

Panels of inbred male mice were used as urine donors. All panels of different inbred strains were maintained under uniform conditions in the same animal room. Urine was obtained from mice by gentle abdominal pressure. The urine

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0027-8424/97/942210-5$2.00/0
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Abbreviations: MHC, major histocompatibility complex; B6, C57BL/6.

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used for the chemical procedures was from two age-matched (3–12 months old) panels of congenic [C57BL/6 (B6) and B6-H-2^k] mice.

Collected mouse urine and all intermediate samples resulting from the chemical procedures were stored in a −20°C freezer. The mouse urine was pretreated by centrifugal ultrafiltration in Centricon-10 tubes (Amicon, 10,000 MW cutoff) at 5000 × *g* at 5°C to remove the abundant mouse major urinary proteins that would interfere with the subsequent chemical procedures. The urine filtrates prepared by this ultrafiltration procedure from B6 and B6-H-2^k mice are readily discriminable in the Y-maze olfactometer (12).

Anion exchange chromatography was performed on a 0.7 × 8.0 cm column of DEAE-Sephadex that had been freshly rinsed with 1.5 M pyridine-acetic acid followed by deionized water (8). Congenic H-2^b and H-2^k ultrafiltrate samples of 1.0 ml volume were each diluted with 9.0 ml of deionized water and applied to the ion exchange column, which was then eluted with water to give a first fraction of about 25 ml that had the typical odor of mouse urine. This was lyophilized and the dried residue was dissolved in 1.0 ml of water to prepare a stock solution of the unretained fraction. The second fraction of each H-2 type was prepared by elution of the column with 30 ml of 1.5 M pyridine-acetic acid, concentration of the eluant by vacuum evaporation at 20 Torr, 32°C to about 0.1 ml, and dilution to 1.0 ml with water to give a pair of stock solutions of the retained fraction for behavioral testing in the Y-maze.

Extractions were performed on samples of urine ultrafiltrate that had been prepared from two collection tubes, each containing about 0.6 ml of urine from one to three different mice. Thus each sample consisted of urine collected from two to six individual mice of the same H-2 haplotype; and each sample contained urine from different individuals. The urine samples had been collected from 1 to 7 weeks prior to the extraction. Immediately before extraction a 200 mg portion of KH_2PO_4-H_2O was dissolved in 1.0 ml of the ultrafiltrate, lowering the pH from 5.8–5.9 to 4.4–4.6. Twenty-six such samples, 13 of each H-2 type, were individually extracted with HPLC-grade diethyl ether (Aldrich) for 2 hr in a continuous liquid–liquid extractor at the reflux rate of ≈1 drop/sec. Extracts and the aqueous residues were stored separately.

One sample of extracted B6 urine and one sample of extracted B6-H-2^k urine were concentrated to about 0.5 ml by vacuum evaporation and then lyophilized. To provide stock solutions for the Y-maze olfactometer tests, the freeze-dried residues were each dissolved in 1.0 ml of 0.05 M succinic adjusted to pH 5.9 with sodium hydroxide. The ether extracts were concentrated by evaporating the ether to dryness at reduced pressure and room temperature; the dried samples were stored in the freezer until they were used for gas chromatography or behavioral testing. Gas chromatographic samples were prepared within 1 hr of the analysis (described below) by dissolving the dried extracts in 0.1 ml of methyl acetate. Stock solutions for Y-maze testing were prepared from a pair of the dried extracts from congenic mice by dissolving each in 1.0 ml of 0.05 *M* (pH 5.9) sodium succinate buffer.

Gas chromatography was carried out on a 30 m × 0.32 mm i.d. × 1.0 μm df Stabilwax column with a 4.5 m × 0.53 mm i.d. guard column (Restek, Bellefonte, PA). Samples were injected directly on the guard column in 1.7 μl of methyl acetate. After injection, the column temperature was held at 80°C for 2 min, then raised linearly at 5°C per min to 240°C. The helium carrier gas had a linear velocity of 37 cm/sec at a column temperature of 40°C. Compounds emerging from the column were detected by flame ionization. Quantitative calculations used peak heights.

In the Y-maze olfactometer (3) air is conducted through two odor chambers containing urine samples in open Petri dishes, one in each branch of the Y. Gates are raised and lowered in timed sequence to permit the training or testing of each mouse in a session of up to 48 consecutive trials. The reward for a correct response is a drop of water. Urine samples were collected, as described above for the chemical experiments, on the day of testing. Mice were first trained to discriminate urine from panels of unrelated inbred strains with the H-2 haplotypes: H-2^b and H-2^k (inbred strains B6 vs. AKR). Then they were trained to distinguish urine samples from the congenic H-2^b and H-2^k mice (inbred strains B6 vs. B6-H-2^k). Finally they were accustomed to detecting the urine after 5-fold dilution in water. Six mice were trained: four to select B6 and two to select B6-H-2^k.

Table 1. Y-maze responses in unrewarded trials

Chemical fraction	Number of trials	Urine score,* %	Concordance,† %
DEAE unretained	20	75‡	60
DEAE retained	26	88‡	73‡
Ether extract	32	82‡	72‡
Extracted urine	23	83‡	61

*Percent of correct responses to urine in unrewarded trials.
†Percent of trials in which the trained mice selected the fraction derived from urine of the H-2 type, which was concordant with their training on whole urine.
‡$P < 0.05$.

Stock solutions of chemical fractions at the same concentration as urine were diluted 5-fold with 0.05 *M* sodium succinate (pH 5.9) buffer. Mice were not rewarded for responding to chemical fractions, which were coded so that the operator of the test was not aware of the H-2 haplotype of the original urine donors. In a typical sequence of trials the mice were given three rewarded trials with 5× diluted urine samples, one unrewarded trial with the dilute urine samples, three rewarded trials with the urine samples, and one unrewarded trial with the congenic chemical fractions being tested. This sequence was repeated up to six times in one session with an individual trained mouse.

RESULTS

We used the DEAE-Sephadex column to separate the active mouse urine ultrafiltrate into two fractions: the material

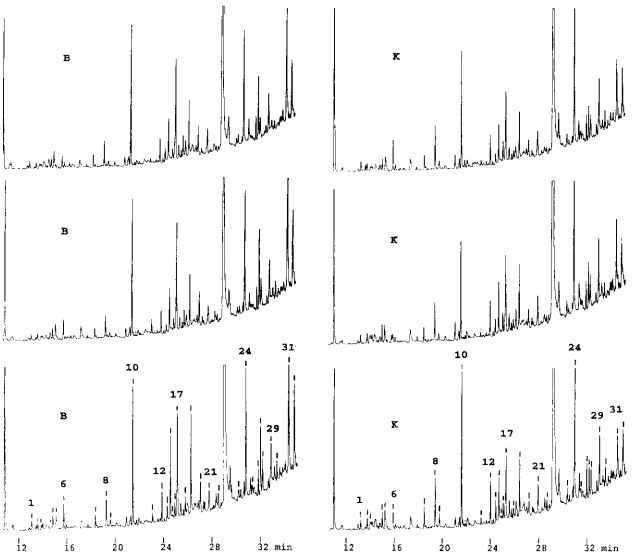

FIG. 1. Chromatograms from gas chromatographic analysis of ether extracts of three different urine samples from B6 mice (*B*), and three different urine samples from B6-H-2^k mice (*K*). Peaks included in Table 2 are indicated with tick marks and sequential numbers.

passing through the column and eluted with water, which had a pH of 6.2 (DEAE unretained), and the material eluted by the concentrated pyridine-acetic acid buffer (DEAE retained), with a pH of 4.4. Addition of the sodium succinate buffer to the DEAE fractions before Y-maze testing adjusted the acidity of the unretained fraction to pH 5.9, the usual value for the collected urine, but did not change the pH of the retained fraction. We made no further attempt to adjust the acidity of this material. The data in Table 1 clearly show that the behaviorally active compounds were retained by the column and eluted with the pyridine-acetic acid buffer.

Ether extraction of the urine after acidification to pH 4.4–4.6 with potassium phosphate likewise produced two fractions. The extract was active and the residue after extraction was not (Table 1).

The ether extracts from 12 H-2^b and 12 H-2^k urine ultrafiltrate samples were individually subjected to gas chromatography. On the basis of what appeared to be typical of the more consistent differences in chromatograms between the two H-2 types, we have selected three of the resulting chromatograms from each H-2 type for display in Fig. 1.

We recorded all the peaks that could be measured reliably in all of the chromatograms of urine extracts from either of the two congenic strains of mice. Although there were occasional anomalous peaks, there were no peaks that were reliably detectable in most of the urine samples from a single H-2 type of urine. In other words, there is no evidence that the two H-2 classes of urine are distinguished by unique volatile compounds. Since absolute levels of total compounds varied across individuals (presumably reflecting varying concentrations of the collected urine samples), and since it appears that the pattern of volatile differences is what distinguishes the odor of mice according to H-2 type, the data on peak height for each mouse were calculated as a percentage of the sum of all the measured peak heights. Nonparametric statistical analysis demonstrated that 8 of the 32 peaks had concentration values differing significantly ($P < 0.01$; 5 differed at $P < 0.002$, the value required by the Bonferroni correction for multiple tests) between the two H-2 types of urine (Table 2). This result is consistent with the hypothesis that it is differences in the relative concentrations of individual compounds in mixtures that signal the difference in H-2 types.

Several of the compounds were identified by gas chromatography–mass spectrometry and by the chromatographic retentions of authentic samples. One of these was acetic acid, which is the source of the lowest retention time major peak in the chromatograms in Fig. 1. Since all or most of this peak represented an impurity in the methyl acetate solvent used, it was not included in Table 2. The other seven identified compounds, which are listed in Table 2, were entirely from the mouse urine, as demonstrated by their absence in identically prepared ether extracts of water acidified with potassium dihydrogen phosphate (data not shown). Of the identified

Table 2. Gas chromatography results

Peak number*	Peak identity	H-2^b samples		H-2^k samples		Mann–Whitney *P*-level
		Median†	Interquartile range	Median†	Interquartile range	
1		0.57	0.41–0.74	0.51	0.39–0.88	
2		0.58	0.56–0.64	0.85	0.64–0.99	0.01
3		0.37	0.30–0.46	0.47	0.30–0.69	
4		1.02	0.69–1.13	1.10	0.81–1.41	
5		0.91	0.59–1.40	1.24	0.82–1.72	
6	Methylbutyric acids‡	12.5	1.64–30.1	11.9	1.52–32.1	
7		1.21	1.10–1.35	1.69	1.52–1.92	0.0005
8		2.38	2.03–2.61	3.30	2.94–4.19	0.0005
9		0.58	0.44–0.61	0.65	0.50–0.88	
10	Dimethyl sulfone	13.7	13.1–14.5	11.7	9.72–14.0	
11	Phenol	0.91	0.66–1.20	0.87	0.61–1.08	
12		2.06	1.55–2.34	2.52	2.06–3.14	
13		0.72	0.39–1.22	0.98	0.59–1.62	
14	*p*-cresol	3.16	2.24–4.25	3.48	2.55–4.10	
15		0.46	0.42–0.56	0.44	0.37–0.45	
16		1.10	0.95–1.17	1.37	0.86–1.73	
17		7.54	6.68–9.24	6.40	5.49–7.02	
18		1.43	1.18–1.76	1.04	0.97–1.10	0.0008
19	4-Ethylphenol	4.55	3.16–5.79	4.81	3.99–5.59	
20		2.10	1.72–2.75	1.45	1.20–1.77	0.008
21		1.22	0.54–1.86	1.55	0.58–2.53	
22		0.61	0.54–0.67	0.61	0.41–0.80	
23		0.64	0.51–0.79	0.96	0.56–1.57	
24	Benzoic acid	6.57	2.54–10.7	8.18	2.89–15.1	
25		0.69	0.50–0.78	0.85	0.43–1.02	
26		2.12	2.03–2.36	2.67	2.63–2.84	0.004
27		4.64	4.01–5.33	3.01	2.76–3.64	0.003
28		2.18	2.04–2.38	2.53	2.32–3.00	
29	Phenylacetic acid	3.77	3.39–4.09	5.86	5.03–7.21	0.0002
30		1.29	0.90–1.52	1.48	1.07–1.93	
31		9.11	7.99–11.9	5.61	4.70–6.49	0.001
32		3.93	2.93–4.87	3.71	2.81–4.10	

*Peak number corresponds to numbered peaks in Fig. 1.
†Median of peak height (as percent of total of the 32 peak heights measured in the sample) in the 12 samples analyzed.
‡Isovaleric and 2-methylbutyric acids are not separated under the gas chromatographic conditions used and are not readily distinguished in mixtures by mass spectrometry.

compounds, only phenylacetic acid was implicated in the signaling of H-2 type by its different relative concentration in samples of urine from these congenic mice.

DISCUSSION

The hypothesis that volatile acids in urine are sufficient to signal H-2 type is most directly supported by the discrimination by trained mice in the Y-maze of the compounds that are negatively charged at pH 6 (retained ion exchange fraction). Furthermore, the lack of activity of the neutral compounds (unretained ion exchange fraction) indicates that the negatively charged compounds are also necessary for discrimination (Table 1). The involvement of acids is further supported by the identification of one of the volatile acids, phenylacetic acid, as making up a significantly greater proportion of the entire fraction in B6-H-2^k relative to B6 (Table 2).

We chose continuous ether extraction at low pH to isolate the acids from urine for analysis because this is an established procedure for isolating the less polar of the acids and because evaporation is necessary to provide sufficient concentration of acids for gas chromatographic analysis. The ether solvent is more readily removed than the pyridine and acetic acid resulting from the ion exchange procedure. The ether extraction procedure, however, is much less selective for acids, as indicated by the identification of compounds neutral at pH 6: the three phenols and dimethylsulfone. We did not expect that these compounds would be involved in olfactory recognition of H-2, since they should not be part of the active material isolated by the anion exchange procedure. They might occur in this active fraction as their nonvolatile sulfates, and could conceivably contribute to the volatiles if the sulfates decomposed, but the data in Table 2 appear to confirm our expectation. The phenols do not differ significantly in the urine samples from these congenic mice.

Variability in relative peak heights found throughout these chromatograms is probably due to such factors as the variation in the time of storage of the urine before extraction and the difficulty of reproducible quantitative analysis of acids by gas chromatography. The extreme variability in the methylbutyric acids (peak 6) is possibly due also to their relatively high volatility and therefore variable loss during removal of the ether by vacuum evaporation. In spite of this variability, we were able see highly significant differences between H-2 types in the longer retained, less volatile compounds. It is noteworthy that variability of such magnitude may be typical of the natural situation under which these odors are detected in the context of mate choice and other social behaviors. H-2 odortypes in natural situations must be detected against a much more variable genetic background (13, 14) than occurs among these congenic mice, which are genetically identical at all loci except H-2.

We have not seen any evidence that differences in odortype are specified by compounds that are unique to mice of a particular H-2 type. Instead it appears that differences in H-2 are signaled by different relative amounts of the components of a mixture of acids (for a contrasting view, see ref. 7), or what we have called compound odors (15). From our analysis we can speculate that this odortype mixture contains at least eight components (Table 2, $P < 0.01$). The variability in our analysis probably obscures some of the peaks that are significant, and some of the peaks in the chromatogram, such as peak 6, may represent multiple compounds not resolved under these conditions. In addition, there may be significant odorous components that are not detected because the levels are too low to be seen at this sensitivity. Therefore, our analysis does not permit us to estimate the number of components distinguishing these H-2 types.

Knowledge of the chemical structures will also be necessary to test in the Y-maze whether the mice can distinguish the mixtures we have found. Our limited knowledge of the olfactory mechanisms for the detection of odor mixtures as well as of the chemical structures does not yet permit us to speculate whether the statistically significant differences we have found are sufficient for mice to distinguish these urine samples by olfaction. We do find that by visual inspection of the chromatograms—e.g., by comparing either of the ratios of peak heights (peak 20/peak 21 or peak 27/peak 28; Fig. 1)—we can distinguish B6 from B6-H-2^k urine samples at least as reliably as do the mice in the Y-maze olfactometer (80–90% of the trials). Similar discriminability can be obtained by inspection of the heights of some of the individual peaks.

In this study we used urine samples pooled from two to six individuals to provide the sample volume required for chemical and behavioral testing. In other studies (16) we have shown that animals can discriminate between two individual mice differing only at H-2. Thus we are confident that the H-2 differences in volatile acids reported here are responsible for differences not just in pooled samples, but also in individual urine odors. It remains to be determined whether these same compounds in the same ratios constitute the individual differences in other secretions that may be influenced by H-2 type (17).

The identification of phenylacetic acid as a significant component of the acid mixtures differing between H-2 types has implications for the question of the mechanism of the genetic control of H-2 odortype. In mouse urine, phenylacetic acid is excreted in relatively large quantities conjugated with the amino acids glycine and taurine (18). Such peptide-like derivatives could make use of the known specificity of MHC class I and class II molecules (19). It has been suggested that soluble derivatives of class I MHC molecules circulating in serum could select characteristic odorous molecules for excretion in the urine (20), but it has not been explained how an odorous molecule could be specifically bound to class I proteins that normally bind peptides, molecules of quite different chemical properties than the typical odorous molecule. Amino acid conjugates of odorous acids, however, contain peptide functional groups and, therefore, could very likely be bound by MHC proteins and might be derived from the normally bound peptides.

We thank Maryanne Curran for excellent technical assistance. This research was funded by a grant from the National Institutes of Health (GM-32096).

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