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Individual Variation in 3-Methylbutanal: A Putative Link between Human Leukocyte Antigen and Skin Microflora

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Abstract The human derma emits volatile compounds whose interaction with a receiver's olfactory sensory system may affect individual recognition and mating preferences. Studies suggest that both genes and environmental factors determine characteristic odor of an individual. We used solid-phase microextraction and gas chromatography–mass spectrometry to identify 3-methylbutanal in human axillary odor; we showed that the abundance of this volatile compound varies significantly among individuals and demonstrated that its formation *in vitro* may be influenced by interaction between human leukocyte antigen peptide and dermal microflora.

Keywords Human leukocyte antigen · Human odor · 3-Methylbutanal · Skin microflora · Solid-phase microextraction

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Introduction

Human odor comprises many volatile compounds (Curran et al. 2005) whose origins are largely unexplained. Penn and Potts (1998) reviewed hypotheses suggesting a role for the genes that encode human leukocyte antigens (HLA) in the origin of these compounds. The HLA complex enables the immune system to recognize pathogens by capturing and presenting exogenous and endogenous antigen peptides to T cells.

HLA may also contribute to the development of a unique odor signature that influences mating preferences in humans (Penn and Potts 1998). HLA are specifically distributed across human skin (Imayama et al. 1992), and their fragments are present in sweat (Zavazava et al. 1990), thus providing an opportunity for the skin microflora to be involved in odor formation (Leyden et al. 1981). We speculate that HLA fragments, derived from the proteolytic cleavage of HLA, may serve as a nutrient to the microflora to contribute to a particular metabolite(s). We designed and executed an *in vivo* study to detect chemosignals that may be used as discriminative markers of individuality. We also carried out an *in vitro* experiment to test the HLA–microflora hypothesis (Penn and Potts 1998).

Methods and Materials

Sampling of Axillary Odor Eighteen volunteers comprised 8 women and 10 men (age, mean±s.e., 33.5±1.9 yr). The headspace of the left underarm region of each volunteer was sampled on three consecutive occasions, spaced 7 d apart during the same time of the day, by solid-phase microextraction (SPME) with a 50/30-μm divinylbenzene–carboxen–polydimethylsiloxane fiber (Supelco, Bellefonte, PA, USA). The fiber was positioned within a perforated

(4 mm inner diameter) 15-ml glass vial (Sigma-Aldrich, Dorset, UK) by piercing the septum in the cap with the SPME needle. The vial was kept under the armpit by an individual; this sampling device is the subject of UK Patent Application WO/2006/111748. To standardize environmental effects on odor profiles, the volunteers followed an established hygiene regime (Roberts et al. 2005). After 50 min of axillary sampling at room temperature, the fiber was introduced into the gas chromatograph for sample desorption. The air in the sampling room was monitored for background volatiles using the same absorbent material.

Gas Chromatography–Mass Spectrometry Analysis Separation and analysis of the odor compounds was performed by using an Agilent 6890N gas chromatograph. The gas chromatograph injection port liner used was a SPME injection sleeve of 0.75 mm inner diameter (Supelco). SPME desorption was at 230°C, in pulsed splitless mode for 2 min at 25 psi. The gas chromatograph was coupled to an Agilent 5973 mass-selective detector (EI+, electron voltage 70 eV, full scan mode in a range of *m*/*z* 35–400 amu, interface temperature of 300°C; Agilent Technology, Stockport, Cheshire, UK). Separation of compounds was performed on a Zebron, ZB-Wax (30 m× 0.25 mm×0.25 µm film thickness) capillary column coated with 100% polyethylene glycol (Phenomenex, Cheshire, UK). The carrier gas used was helium at a flow rate of 1 ml min^{-1}. The oven temperature program was 40°C (held for 5 min), then raised to 100°C at 3°C/min (5 min), followed by 5°C/min to 150°C (5 min), and reaching 230°C at 5°C/min (8 min).

Culturing of Axillary Microflora A sterile swab, pretreated in 0.8% NaCl, was applied for 10 sec to the left axilla to harvest its microbial flora. The swab was transferred into 20 ml of the glucose-enriched medium (glucose 1%, ammonium chloride 0.5%, sodium dihydrogen orthophosphate 0.2% with final pH adjusted to 7.2) (Sigma-Aldrich) prepared in a screw-cap bottle. The bottle was kept in an orbital shaker at 37°C, 160 rpm, for 12 hr. One milliliter of the culture was then transferred into a sterile 1.8-ml Eppendorf centrifuge tube that was spun for 10 min at 11,500×*g*. The supernatant was removed, and the pellet was washed eight times with sterile distilled water. The pellet was resuspended in the sterile distilled water, and a total microbial count was equated to McFarland turbidity standard 10 (6×10^7cfu/ml).

Preparation of Substrate Solution The LRGYYNQSED and GSHSMRYFST HLA decapeptides (97% purity) were synthesized by Sigma Genosys (Pampisford, Cambridge, UK). To prepare a substrate solution, 1.5 mg of each HLA peptide were dissolved in 1 ml filtered (0.25 mm) sterile distilled water. Each substrate solution also contained 4.4 mg KH_2PO_4, 4.8 mg Na_2HPO_4, 1.0 mg NH_4Cl, and 0.5 mg $MgSO_47H_2O$.

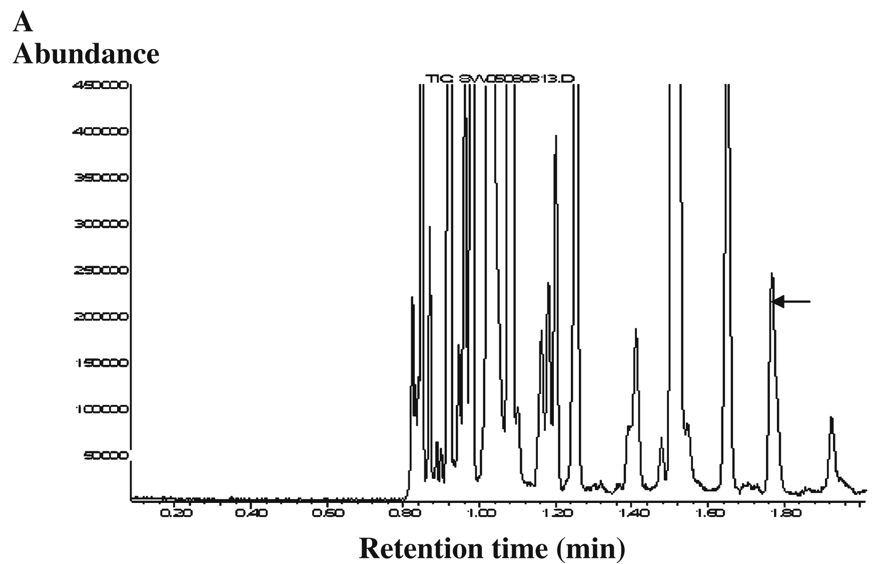
Headspace Analysis of Medium An aliquot (20 µl) of the resuspended pellet and 115 µl of the final substrate solution were added to a 2-ml gas chromatography–mass spectrometry (GC-MS) glass vial containing 2.5 µl of vitamin solution, made of 0.05% thiamine (aneurine) hydrochloride, 0.05% riboflavin, 0.05% niacin, 0.05% pyridine hydrochloride, 0.05% inositol, 0.05%, calcium pantothenate, 0.05% *p*-aminobenzoic acid, and 0.025% biotin (Sigma-Aldrich). The vial was capped, and the mixture was incubated in an orbital shaker (160 rpm) at 37°C for 48 hr. The cap septum was then pierced with the protective needle covering the SPME fiber, and the fiber was extruded to sample the headspace over the medium. The sampling was carried out for 30 min over a heating block set at 50°C. After sampling, the fiber was immediately introduced into the gas chromatograph for sample desorption. In all cases, a negative control of the headspace of the microbial culture without added peptide was analyzed. Each sample was analyzed in duplicate.

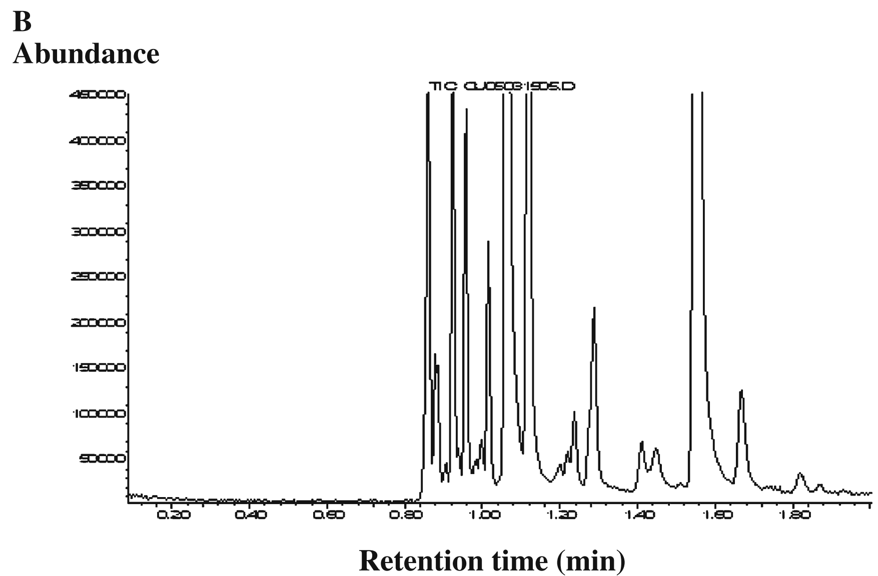
Data Analysis Ion 41 (*m*/*z* 41) was selected as one of the main diagnostic ions of 3-methylbutanal (3-MB) from the GC-MS profile of an individual, and its abundance was recorded. Six other compounds that also contained *m*/*z* 41 were selected from the same profile of the individual, their *m*/*z* 41 abundances were also measured, and the mean abundance across all six compounds was calculated. The abundance of *m*/*z* 41 for 3-MB was divided by this mean to obtain a standardized value of *m*/*z* 41 for 3-MB. The standardized value was calculated for every individual profile

Table 1 Standardized abundance of *m*/*z* 41 of 3-methylbutanal from axillary samples from 18 human subjects sampled over 3 wk[a]

Subject	Week 1	Week 2	Week 3
ORE_1	2.21	0.79	1.65
ORE_2	1.6	2.13	1.05
ORE_3	0.62	0.78	1.44
ORE_4	0	0.64	1.74
ORE_5	0	0	0
ORE_6	0.1	0.07	0.29
ORE_7	0	0	0
ORE_8	0.33	0.05	0.04
ORE_9	1.78	0.89	1.19
ORE_10	0.4	0.34	1.21
ORE_11	0.61	0.51	1.32
ORE_12	0.14	0.28	0.28
ORE_13	0.14	0.17	0.21
ORE_14	0.62	1.69	2.3
ORE_15	0	0	0
ORE_16	0	0.57	0.31
ORE_17	0.78	0.21	1.09
ORE_18	0.15	0.13	0.21

[a] ANOVA ($F_{1,\ 17}=40.125$, $P<0.001$) showed significant variation in abundance of 3-methylbutanal among 18 individuals.





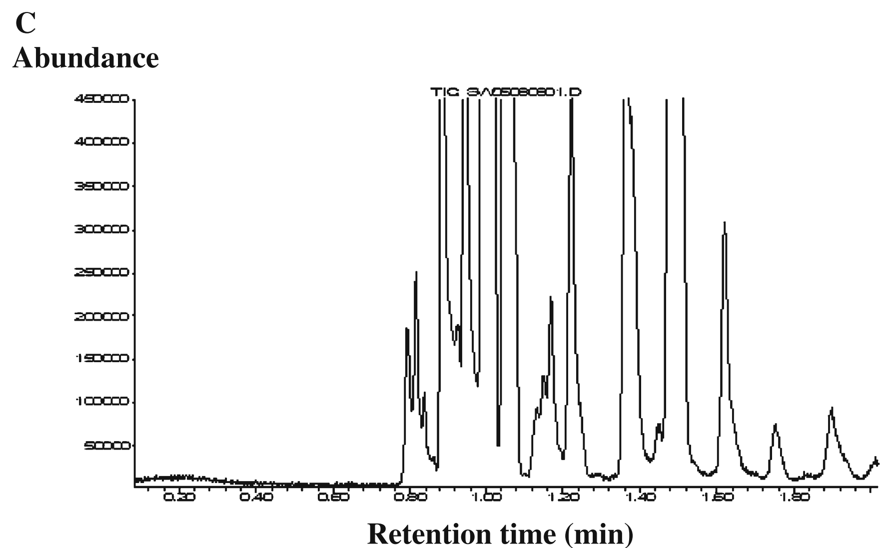


Fig. 1 GC-MS profiles of volatiles trapped above three incubation media including **a** medium containing LRGYYNQSED and microflora cultured from an individual (ORE_2), who consistently emitted 3-MB *in vivo* (*arrow* indicates 3-MB, which eluted at 1.75 min and was verified with the authentic standard from Sigma-Aldrich), **b** medium as in **a** except that the decapeptide GSHSMRYFST was used as the substrate (3-MB was not detected *in vitro*), and **c** medium containing LRGYYNQSED and microflora cultured from an individual (ORE_5), who did not emit detectable 3-MB *in vivo* (3-MB was not detected *in vitro*)

(Table 1). Comparisons of the standardized abundances of 3-MB among individuals were performed by using repeated-measures analysis of variance (ANOVA) with $\alpha=0.05$ as the threshold for significance (SPSS statistical software).

Results and Discussion

The GC-MS analysis of the axillary odor of 18 volunteers revealed a number of compounds among which 3-MB was noted as a potential link to HLA (Montag et al. 2001). The signal was not reported by Curran et al. (2005), who also used SPME for the analysis of the axillary odor. This discrepancy may be attributed to differences in sampling methodologies between the two studies. The abundance of 3-MB among individuals varied during 3 wk of sampling (Table 1) without a discernable general pattern. Five of 18 subjects yielded at least one zero measurement for 3-MB, and since zero values do not represent a confirmed absence of the chemosignal, abundance measurements from these individuals were excluded initially from the statistical analysis of the significance of the variation in emission of 3-MB among individuals. ANOVA revealed significant variation in the signal among the 13 subjects ($F_{1,\ 12}=22.9$, $P<0.001$), whereas variation among weeks was not significant ($F_{2,\ 24}=1.9$, $P>0.05$). There were also significant disparities in the signal among all 18 subjects when zero values were included in the analysis ($F_{1,\ 17}=40.125$, $P<0.001$; data were square root-transformed prior to analysis due to significant departures from normality). In this instance, there was also no significant variation in the signal across weeks ($F_{2,\ 34}=3.07$, $P>0.05$). These statistical analyses may indicate a possible genetic origin of 3-MB, especially after taking into account the observation of Montag et al. (2001) that strains of environmentally controlled congenic mice that differ only in the major histocompatibility complex (MHC or HLA in humans) expression can be distinguished on the basis of differences in the ratio of their urine volatile signals, including 3-MB.

An *in vitro* experiment was carried out to determine whether 3-MB may be formed from the interaction between HLA peptides and dermal microflora. Two decapeptides, whose sequences are expressed in the $\alpha1$ domain of the HLA class 1 region (Mason and Parham 1998), namely LRGYYNQSED and GSHSMRYFST, were synthesized. The first sequence was selected because it was shown previously that leucine and arginine substitutions in congenic mutant mice, differing only in three amino acids in the MHC region (Schulze et al. 1983), allowed olfactory distinction between the strains (Yamazaki et al. 1983) and that these two amino acids, L and R, were neighbors in the MHC sequence. The other peptide was chosen for the lack of L and L–R linkage in its sequence.

In the first instance, microflora cultured from individual ORE_2, whose axillary odor contained a relatively large abundance of 3-MB during all three weekly samplings (Table 1), was incubated in two separate media. One medium contained LRGYYNQSED, whereas the other contained GSHSMRYFST. The headspace above the surface of each medium was sampled and analyzed for 3-MB. The target compound was detected in the headspace above the LRGYYNQSED incubate (Fig. 1a) but not in the headspace above the GSHSMRYFST incubate (Fig. 1b). If the cutaneous microbial population is affected by an individual's genotype and possibly phenotype, we would predict that the sampled microflora from individual ORE_5, who was not associated with a positive 3-MB signal (Table 1), would not yield the signal when incubated with LRGYYNQSED. Subsequent analysis did not detect 3-MB in the headspace of the medium containing LRGYYNQSED or microflora from this individual (Fig. 1c). The results of the *in vitro* experiment were reproduced by using the microflora cultured from ORE_8 and ORE_7; that is, abundances of 3-MB were 123,560 and 0 (undetected), respectively. The negative controls provided evidence that different HLA peptides can alter production of the signal and that individuals possess differences in microbial populations that influence production of the signal. Based on Leyden et al. (1981), we speculate that aerobic diptheroids, *Corynebacterium*, were involved in the interaction with the HLA peptides. These findings are in agreement with the hypothesis that genes and microflora may influence formation of the odor (Penn and Potts 1998), although it remains unclear whether the production of the chemosignal: (1) is HLA sequence specific, (2) depends on the L–R linkage, or (3) is entirely due to the encoded presence of leucine in the peptide. A further study on the substitutions of the amino acids in the LRGYYNQSED may reveal the extent of HLA contribution to the production of the chemosignal.

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