INDIVIDUAL VARIATION IN 3-METHYLBUTANAL: A PUTATIVE LINK BETWEEN HLA AND SKIN MICROFLORA

SERGEY U. SAVELEV,[1,3] SANJAY ANTONY-BABU,[3] S. CRAIG ROBERTS,[4] HUITU WANG,[1,2] ANTHONY S. CLARE,*[1] L. MORRIS GOSLING,[2] MARION PETRIE,[2] MICHAEL GOODFELLOW,[3] ANTHONY G. O'DONNELL,[3] and ALAN C. WARD[3]

[1]*School of Marine Science and Technology, University of Newcastle, Newcastle upon Tyne, NE1 7RU, UK*

[2]*Evolutionary Biology Group, School of Clinical Medical Sciences, Newcastle upon Tyne, NE1 7RU, UK*

[3]*School of Biology & Psychology, Newcastle upon Tyne, NE1 7RU, UK*

[4]*School of Biological Sciences, University of Liverpool, Liverpool, L69 3BX, UK*

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. Here, we use solid phase micro-extraction and GC-MS to identify 3-methylbutanal (3-MB) in human axillary odor, show that the abundance of this volatile compound varies significantly between individuals and demonstrate *in-vitro* that its formation may be influenced by interaction between human leukocyte antigen peptide and dermal microflora.

Key Words - 3-methylbutanal, human leukocyte antigen, human odor, skin microflora, solid phase micro-extraction.

* To whom correspondence should be addressed. E-mail: a.s.clare@ncl.ac.uk

INTRODUCTION

Human odor comprises many volatile compounds whose origin(s) is still largely unexplained. Penn & Potts (1998) reviewed hypotheses suggesting a role for the genes which encode human leukocyte antigens (HLA) in the origin of these compounds. The HLA complex enables the immune system to recognise 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 & Potts, 1998). HLA is specifically distributed across human skin (Imayama et al., 1992) and its 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). Our *in-vivo* study was designed to detect chemosignals which may be used as discriminative markers of individuality. An *in-vitro* experiment was also carried out to test the HLA–microflora hypothesis (Penn and Potts, 1998).

METHODS AND MATERIALS

Sampling of axillary odor. Eighteen volunteers comprised 8 females and 10 males (age range 22-51). The headspace of the left underarm region of each volunteer was sampled on three consecutive occasions, spaced 7 d apart, by solid phase micro-extraction (SPME). The SPME fiber selected was divinylbenzene-carboxen-polydimethylsiloxane (Supelco, UK). The fiber was positioned within a perforated (ID, 4 mm) 15 ml glass vial (Sigma-Aldrich) by piercing a septum in a cap with the SPME needle; this sampling device is the subject of UK Patent Application No. 0507950.4. 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 GC for sample desorption. The air in the sampling room was monitored for background volatiles using the same absorbent material.

Gas Chromatography-Mass Spectrometry analysis. GC-MS analysis of the odor compounds was performed using an Agilent 6890N GC. The GC injection port liner used was a SPME injection sleeve of 0.75 mm ID (Supelco). SPME desorption was at 230 ^{o}C, in pulsed splitless mode for 2 min at 25 psi. The GC was coupled to a 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 ^{o}C) Chemstation (Agilent Technology, UK). Separation of compounds was performed on a Zebron, ZB-Wax (30m × 0.25mm × 0.25 µm) capillary column coated with 100% polyethylene glycol (Phenomenex, UK). The carrier gas used was helium at a flow rate of 1ml min^{-1}. The oven temperature ramp program was 40 ^{o}C held for 5 min, then raised to 100 ^{o}C at 3 ^{o}C min^{-1} (5 min), followed by 5 ^{o}C min^{-1} to 150 ^{o}C (5 min) and reaching 230 ^{o}C at 5 ^{o}C min^{-1} (8 min).

Culturing of axillary bacteria. A sterile swab, pre-treated in 0.8% NaCl, was applied for 10 sec to the left axilla to harvest its bacterial flora. The swab was transferred into 20 ml of glucose-enriched media (glucose 1%, ammonium chloride 0.5%, sodium dihydrogen orthophosphate 0.2% with the final pH adjusted to 7.2) prepared in a screw cap bottle. The bottle was kept in an orbital shaker at 37 ^{o}C, 160 rpm, for 12 h. 1 ml of the culture was then transferred into a sterile 2 ml Eppendorf centrifuge tube which was spun for 10 min at 11500g. The supernatant was removed and the pellet was washed 8 times with sterile distilled water. The pellet was then re-suspended in

the sterile distilled water and a total bacterial count was equated to McFarland turbidity standard 10 (6 x 10^7cfu ml^{-1}).

Preparation of substrate solution. The LRGYYNQSED and GSHSMRYFST peptides were synthesized by Sigma Genosys Ltd, UK. To prepare a substrate solution, 1.5 mg of each 10 amino acid HLA peptide was dissolved in 1 ml filtered (0.25mm) sterile distilled water. 4.4 mg KH_2PO_4, 4.8 mg Na_2HPO_4, 1.0 mg NH_4Cl, 0.5 mg $MgSO_47H_2O$ were added to each substrate solution to make a final substrate solution.

Headspace analysis of medium. 20 µl of the re-suspended pellet and 115 µl of the final substrate solution were added to a 2 ml 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. The vial was capped and the mixture was incubated in an orbital shaker (160 rpm) at 37 ^oC for 48 h. 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 ^oC. After sampling, the fiber was immediately introduced into the GC for sample desorption. In all cases a negative control of the headspace of the bacterial culture without added peptide was analyzed. Each sample was analyzed in duplicate.

Data analysis. Ion 41 was selected as one of the main ions of 3-MB from the GC-MS profile of an individual and its abundance was read. Seven other compounds, across the same profile of the individual, containing ion 41 were also selected, their

abundances in the ion were read and the mean of their abundances was calculated. The abundance in the ion of 3-MB was divided by the mean to obtain a standardized value in ion 41 for 3-MB. The standardized value was calculated for every individual profile (Table 1). Comparisons of the abundance of 3-MB between individuals were performed using repeated-measures ANOVA; results at the <0.05 probability level were considered significant.

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 individual variability in abundance of 3-MB during three weeks of sampling is shown in Table 1. Since zero values do not represent a confirmed absence of the chemosignal, they were initially excluded from the analysis to test whether the variation in emission of 3-MB between the individuals was significant. ANOVA revealed significant variation in the signal between 13 subjects ($F_{1,12}$=22.9, P < 0.001), while variation across weeks was not significant ($F_{2,24}$=1.9, P > 0.05). There were also significant disparities in the signal between 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), while, again, there was 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 previous observation (Montag et al., 2001) that strains of environmentally controlled congenic mice, differing 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 10-amino-acid peptides, whose sequences are expressed in the α1 domain of the HLA class 1 region (Mason, 1998), namely LRGYYNQSED and GSHSMRYFST, were synthesized. The first sequence was selected because leucine (L) neighbors arginine (R) and because it has previously been shown that their 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). 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 3-MB in all 3 weekly samplings (Table 1), was incubated in two separate media; one contained the LRGYYNQSED peptide and the other the GSHSMRYFST peptide. The gaseous phase (headspace) above the surface of the medium, in each case, was sampled and analyzed for 3-MB. The compound was detected in the headspace over the LRGYYNQSED incubate (Figure 1A) but not for GSHSMRYFST (Figure 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. The subsequent analysis did not detect 3-MB in the headspace of the medium (Figure 1C). The results of the *in vitro* experiment were reproduced using the microflora cultured from ORE_8 and ORE_7. These findings are in agreement with the hypothesis that genes and microflora may influence formation of the odor (Penn &

Potts 1998), although it remains unclear whether the production of the chemosignal: i) is HLA-sequence specific, ii) depends on the L-R linkage, or iii) 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.

Acknowledgements - We thank Paul Miller and Daniel Farrelly for assistance in the *in vivo* experiment, Dr Catherine Dreanno and Yashawant Kumar for assistance in the *in vitro* experiment, and Dr George Wake for comments. This work was supported by the Wellcome Trust and by ARO grant DAAD19-03-1-0215. With respect to the ARO grant, opinions, interpretations, conclusions, and recommendations are those of the authors and are not necessarily endorsed by the United States Government.

REFERENCES

AULTMAN, D., ADAMASHVILI, I., YATURU, K., LANGFORD, M., GELDER, F., GAUTREAUX, M., GHALI, G.E., and MCDONALD, J. 1999. Soluble HLA in human body fluids. *Hum. Immunol*. 60:239-244.

IMAYAMA, S., YASHIMA, Y., and HORKI, Y. 1992. Differing cell surface distribution of human leukocyte antigen-DR molecules on epidermal langerhans cells and eccrine duct cells. *J. Histochem. Cytochem*. 40:1191-1196.

LEYDEN, J.J., MCGINLEY, K.J., HOLZLE, E., LABOWS, J.N., and KLIGMAN, A.M. 1981. The microbiology of the human axilla and its relationship to axillary odor. *J. Invest. Dermatol*. 77:413-416.

MASON, P.M. and PARHAM, P. 1998. HLA class I region sequences. *Tissue Antigens*. 51:417-466.

MONTAG, S., FRANK, M., ULMER, H., WERNET, D.W., and RAMMENSEE, H.G. 2001. Electronic nose detects major histocompatability complex-dependent prerenal and postrenal odor components. *Proc. Natl. Acad. Sci. USA* 98:9249-9254.

PENN, D. and POTTS, W. 1998. How do major histocompatibility complex genes influence odor and mating preferences? *Adv. Immunol*. 69:411-436.

SCHULZE, D.H., PEASE, L.R., GEIER, S.S., REYES, A.A., SARMIENTO, L.A., WALLACE, R.B., and NATHENSON, S.G. 1983. Comparison of the cloned H-2Kbm1 variant gene with the H-2Kb gene shows a cluster of seven nucleotide differences. *Proc. Natl. Acad. Sci. USA* 80:2007-2011.

ROBERTS, S.C., GOSLING, L.M., SPECTOR, T.D., MILLER, P., PENN, D.J., and PETRIE, M. 2005. Body odor similarity in non-cohabiting twins. *Chem. Senses* 30:651-656.

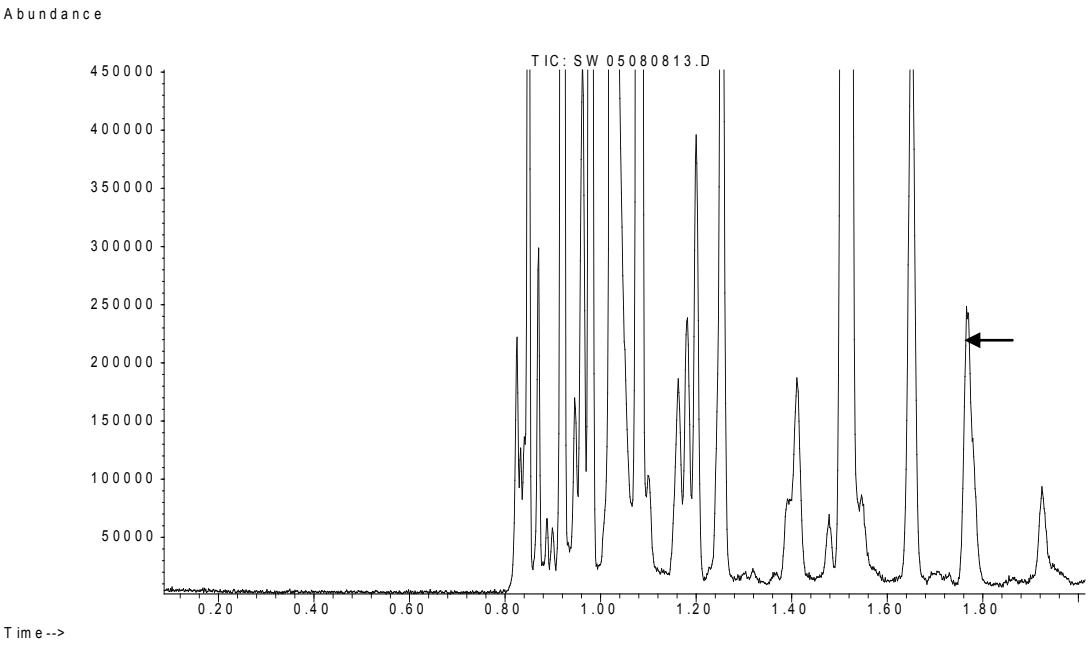
YAMAZAKI, K., BEAUCHAMP, G.K., EGOROV, I.K., BARD, J., THOMAS, L., and BOYSE, E.A. 1983. Sensory distinction between H-2^{b} and H-2^{bm1} mutant mice. *Proc. Natl. Acad. Sci. USA* 80:5685-5688.

ZAVAZAVA, N., WESTPHAL, E., and MULLER-RUCHHOLTZ, W. 1990. Characterization of soluble HLA molecules in sweat and quantitative HLA differences in serum of healthy individuals. *J. Immunogenet*. 17:387-394.

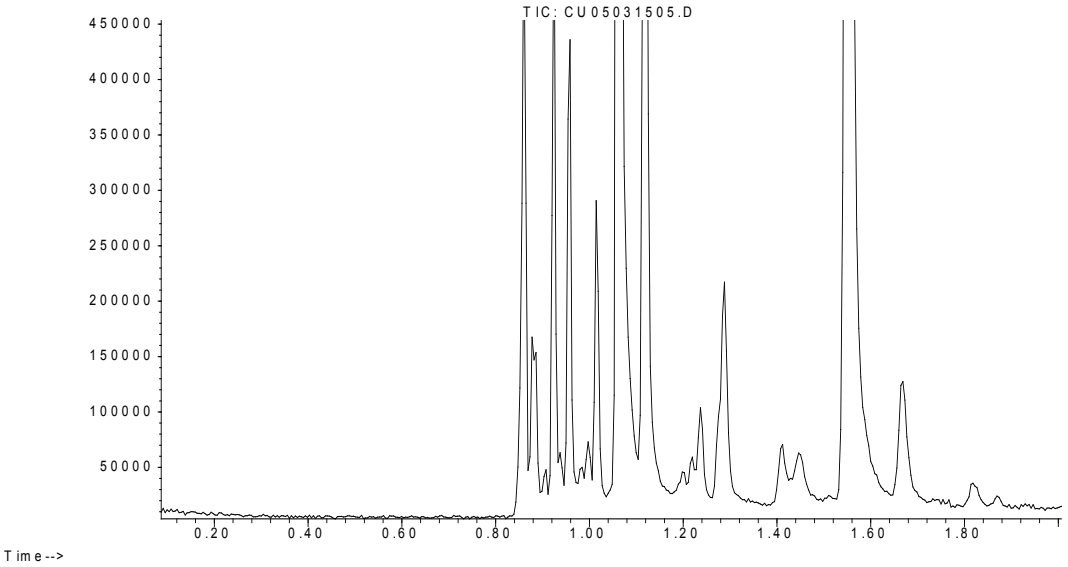
TABLE 1. AXILLIARY ABUNDANCE OF ION 41 OF 3-METHYLBUTANAL IN 18 HUMAN SUBJECTS SAMPLED OVER THREE WEEKS

Subjects	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


Abundance
TIC: SW 05080813.D
450000
400000
350000
300000
250000
200000
150000
100000
50000
0.20
0.40
0.60
0.80
1.00
1.20
1.40
1.60
1.80
Time-->

B


Abundance
TIC: CU05031505.D
450000
400000
350000
300000
250000
200000
150000
100000
50000
0.20
0.40
0.60
0.80
1.00
1.20
1.40
1.60
1.80
Time-->

C

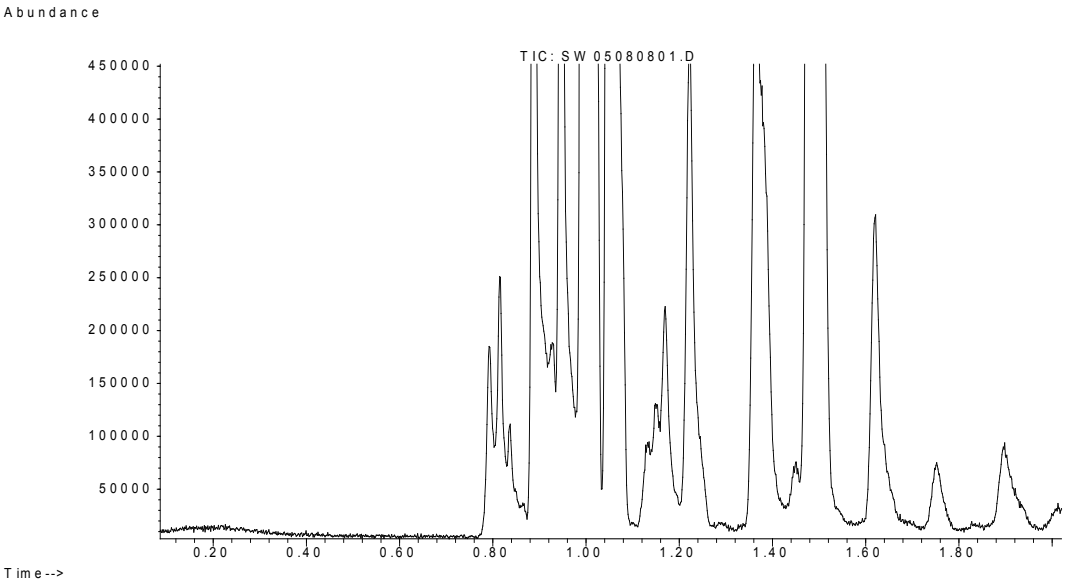


FIG. 1. GC-MS profiles of the gaseous phase of the incubation media. (A) Medium containing microflora cultured from the individual consistently emitting 3-MB and LRGYYNQSED; 3-MB was detected at 1.75 minutes (arrowed); (B) as in (A) except that GSHSMRYFST peptide was used as the substrate; 3-MB was not detected; (C) the medium containing microflora cultured from the individual with undetectable 3-MB and LRGYYNQSED; 3-MB was not detected.