Carcinogenesis vol.18 no.9 pp.1739–1743, 1997

High acetaldehyde levels in saliva after ethanol consumption: Methodological aspects and pathogenetic implications

Nils Homann[1], Hannele Jousimies-Somer[2], Kalle Jokelainen[1], Riitta Heine[2] and Mikko Salaspuro[1,3]

[1]Research Unit of Alcohol Diseases, University Central Hospital of Helsinki, Tukholmankatu 8F, 00290 Helsinki, Finland and [2]Anaerobe Reference Laboratory, National Public Health Institute, Helsinki, Finland

[3]To whom correspondence should be addressed

Chronic ethanol ingestion leads to an enhanced risk of upper gastrointestinal tract cancer. Although many hypotheses for the tumor promoting effect of alcohol exist, the pathogenetic mechanisms remain unclear since alcohol in itself is not carcinogenic. Acetaldehyde, the first metabolite of ethanol, has been shown to have multiple mutagenic effects and to be carcinogenic to animals. Previous research has revealed that acetaldehyde can be formed from ethanol via microbial alcohol dehydrogenase. Thus, at least part of the proposed tumorigenic effect of ethanol may be linked to local production of acetaldehyde from ethanol by oral microflora. In this study we demonstrate the production of marked amounts of acetaldehyde in saliva after ingestion of moderate amounts of ethanol. Considerable inter individual variation in acetaldehyde production capacity is also shown. *In vivo* acetaldehyde production is significantly reduced after a 3-day use of an antiseptic mouthwash (chlorhexidine). *In vitro* acetaldehyde production was shown to be linear in time, inhibited by 4-methylpyrazole and it could not be saturated under ethanol conditions that are relevant *in vivo*. There was a significant positive correlation between salivary acetaldehyde production *in vitro* and *in vivo*. We conclude, that the microbial formation of acetaldehyde in saliva could be one explanation for the tumor promoting effect of ethanol on the upper gastrointestinal tract. Moreover, this may support the epidemiological finding, that poor oral hygiene is an independent risk factor for oral cavity cancer.

Introduction

Chronic ethanol ingestion leads to an enhanced cancer risk of the upper aerodigestive tract (1–4). However, although epidemiological data for the increased cancer risk in alcoholics are obvious, the tumor promoting effect of alcohol remains unclear since ethanol itself is not carcinogenic (2). In contrast, acetaldehyde, the first metabolite of ethanol, may produce mutagenic effects such as chromosomal aberrations, DNA-cross links, sister chromatid exchanges, aneuploidy and it can form stable adducts with DNA (5–11). The nucleotide sites of adduct formation have been identified and sensitive analytical methods developed (11–13). Moreover, acetaldehyde is carcinogenic in animals (14). Thus, acetaldehyde could, at least in part, be responsible for the tumor promoting effect of ethanol. Acetaldehyde is produced in the human body by all tissues with high alcohol dehydrogenase (ADH*) activity, e.g. the liver, and significant ADH levels are found also in the intestine, kidney and bone marrow (15–17). Furthermore, acetaldehyde can be formed from ethanol via microbial ADH in the gastrointestinal tract (18,19).

It has previously been described that acetaldehyde is formed *in vitro* from ethanol when incubating human bronchopulmonary washings and *in vivo* in the oral cavity of volunteers rinsing the mouth with ethanol (20–22). This production has been suggested to be of bacterial origin. Furthermore, we have recently demonstrated, that *in vitro* mouthwashings of patients with cancer of the upper aerodigestive tract possess a significantly higher acetaldehyde production capacity than those of the control group (23). However, neither the local acetaldehyde levels occurring in the oral cavity during ethanol ingestion nor other variables of these reactions have been investigated in the past.

The aim of the present *in vivo* study was to determine acetaldehyde levels that occur in the oral cavity during normal social drinking, to study whether this acetaldehyde is of bacterial origin and to elucidate the variables implicated in acetaldehyde production. Moreover, the comparison of acetaldehyde production *in vitro* to *in vivo* was performed.

***Abbreviations:** ADH, alcohol dehydrogenase, 4-MP, 4-methylpyrazole; PCA, perchloric acid; ALDH, aldehyde dehydrogenase.

Materials and methods

In vitro *acetaldehyde production in the oral cavity*

Different samples including mouthwashes, spontaneous saliva, paraffin wax stimulated saliva and swabs of buccal mucosa were tested for their acetaldehyde production capacity. Paraffin wax stimulated saliva was collected by chewing a commercially available wax chewing gum (Orion Diagnostics, Helsinki, Finland) for 1 min and volunteers were instructed to chew all sites of the jaw to effectively dislodge bacterial material from the gingival crevice. Thereafter, the produced saliva and the wax chewing gum were collected into a tube and mixed for 30 s. Mouthwashings were obtained by rinsing the mouth for 1 min with 10 ml of saline and then disgorging the mouth contents into a tube. Epithelial swabs were taken from the buccal mucosa and the material obtained was dissolved in 4 ml sterile saline. The influence of pH was tested in 100 mM potassium phosphate buffer (pH 6.0, 7.4 and 8.0) and in 100 mM glycine buffer (pH 9.6 and 11.0). To determine the effect of sterile filtration on acetaldehyde production, saliva was filter-sterilized through a 22-μm^2 sterile filter (Millipore, France). To study the effect of centrifugation, saliva was centrifuged at 1000 *g* for 15 min. Both the supernatant and the pellet, resuspended in saline, were used for testing. Influence of ethanol on acetaldehyde production was investigated at final ethanol concentrations of 11, 22, 44, 100, 250, 1000 and 2500 mM. The inhibition of ADH reaction by 4-methylpyrazole (4-MP) was evaluated at seven different 4-MP concentrations (0, 0.1, 1, 5, 10, 50, 100 mM). Samples were incubated for 5, 20, 60, 120 and 360 min. The influence of protein precipitation on acetaldehyde production was tested by adding 50 µl of 6 M perchloric acid (PCA) or 50 µl of water both after and before the incubation with ethanol. The standard procedure was performed by incubating 450 µl of paraffin wax stimulated saliva in 50 µl potassium phosphate buffer (final concentration 100 mM, pH 7.4) containing 22 mM ethanol (final concentration), without addition of inhibitors for 90 min in tightly closed vials at 37°C. Only one variable was changed during each experiment. Every investigation was repeated as six independent experiments with the saliva of different healthy, young volunteers. Acetaldehyde levels were analyzed immediately by head space gas chromatography as described previously (18). In all experiments, baseline acetaldehyde level was obtained by incubating samples without ethanol and this was subtracted from total acetaldehyde. To check the reliability of the *in vitro* acetaldehyde production

method, saliva from all volunteers participating in the *in vivo* study was collected before ethanol intake and an aliquot of 400 µl was incubated *in vitro* at 22 mM ethanol (dissolved in 50 µl potassium buffer, pH 7.4) for 90 min at 37°C. The reaction was stopped by injecting 50 µl of PCA (6 M) through the rubber septum of the closed vial. Acetaldehyde levels were analyzed as described above and results were compared with the values observed during the *in vivo* study.

In vivo *acetaldehyde production in the oral cavity*

Ten healthy young volunteers (age 18–30, six male, four female) took part in the study. After a light breakfast, spontaneous saliva and paraffin wax stimulated saliva were collected. A portion of it was immediately frozen at −80°C for later bacterial analyses. Baseline ethanol and acetaldehyde levels were measured in an aliquot of 500 µl in a tightly closed vial and an aliquot of 400 µl of saliva was used for the *in vitro* incubation as described above. Thereafter volunteers drank 0.5 g ethanol/kg body weight in a standardized 10% solution of absolute ethanol in orange juice within 20 min. After rinsing the mouth with water to remove local ethanol, spontaneous whole mixed saliva and paraffin-wax stimulated saliva were collected and 450 µl of each sample were transferred into a vial containing 50 µl of 6 M PCA. The vials were closed immediately and ethanol and acetaldehyde levels were analyzed by head space gas chromatography as described above. Salivary acetaldehyde and ethanol analyses were corrected for the baseline levels and the measurement was repeated every 20 min until the systemic ethanol level returned to baseline levels. To monitor systemic ethanol concentrations breath ethanol levels were measured simultaneously to saliva collection with an alcometer (Lion, UK). Collection of saliva was finished when breath ethanol level reached the pre-exposure level. Post-exposure spontaneous and paraffin wax stimulated saliva were collected for the determination of salivary bacterial count.

Chlorhexidine treatment

Volunteers rinsed their mouth twice daily for 60 s with 10 ml of 0.2% chlorhexidine solution (Corsodyl, SmithKline Beecham, UK) for 3 consecutive days and they were told not to change their oral hygiene habits. On day 4, the drinking experiment was repeated as described above, the last mouth rinse was performed 15 min before the ethanol ingestion started.

Bacterial analysis

Saliva samples were thawed, serially diluted in peptone yeast extract broth and 10 µl of undiluted saliva and the appropriate dilutions were inoculated on several non-selective and selective agar media for the enumeration and isolation of aerobic and anaerobic bacteria. Aerobic media blood agar base and chocolate agar were used for the determination of total aerobic counts. Anaerobic media Vitamin K1 and hemin supplemented *Brucella* blood agar were used for the determination of total anaerobic counts. The aerobic plates were incubated at 36°C in an atmosphere containing 5% CO_2 for 5–7 days and anaerobic plates in anaerobic jars filled with the evacuation replacement method with mixed gas (85% N_2, 10% CO_2, 5% H_2) for 7 days for the first inspection and further up to 14 days for the final inspection. Bacterial counts were determined by multiplying the number of colonies with the dilution factor, adjusted by inoculation volume (24,25).

Statistics

All results are expressed as mean ± standard error of the mean (SEM). Correlation was tested by using linear regression analysis. Statistical significance was tested by a paired non-parametric test. A P-value of <0.05 was considered statistically significant.

Results

In vitro *acetaldehyde production*

Comparable acetaldehyde production was seen for the two types of saliva (136 ± 14 µM for spontaneous saliva, 115 ± 28 µM for paraffin-forced saliva) but significantly lower values were detected for mouthwashes (54 ± 18 µM) and negligible values for mucosal swabs (3 ± 1 µM). The highest acetaldehyde production was seen at neutral pH with a decrease of 5% at acidic (pH 6.0) and 1%, 13% and 62% basic (pH 8.0, pH 9.6 and pH 11, respectively). Sterile filtration totally abolished the salivary acetaldehyde production capability and nearly all of the acetaldehyde production could be seen in the resuspended pellet of the centrifugate (98%), with only small traces of acetaldehyde production in the supernatant (2%). The salivary acetaldehyde production was enhanced linearly at ethanol concentrations of 11, 22, 44, 100, 250 and 1000 mM

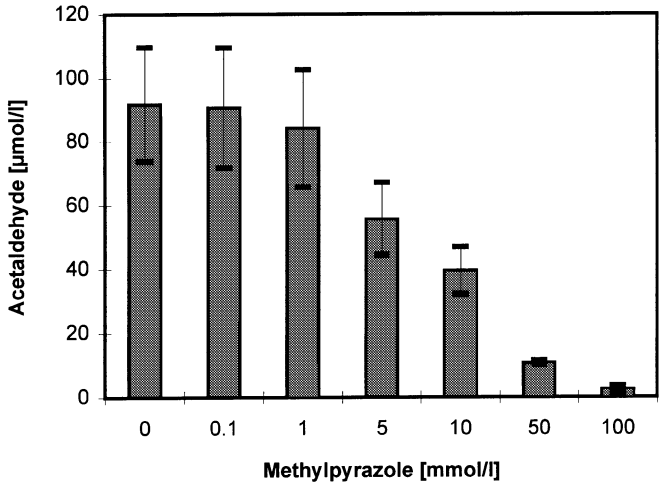

Fig. 1. Inhibition of salivary acetaldehyde production *in vitro* by 4-methylpyrazole (mean ± SEM). Samples were incubated in 22 mM ethanol for 60 min at 37°C

with a decrease at the highest ethanol concentration of 2.5 M (51% of the value at 1 M). It was inhibited by 5 mM 4-MP and totally abolished at 100 mM of 4-MP (Figure 1). Salivary acetaldehyde production increased with time with a linear positive correlation during the incubation of 4 h. Addition of PCA immediately after incubation decreased the acetaldehyde level determined, without protein precipitation, by 15%. PCA added before the incubation abolished acetaldehyde production even in the presence of ethanol. This indicates that the artefactual acetaldehyde formation was minimal. Chlorhexidine treatment led to a significant decrease in acetaldehyde production (149 ± 35 µM before versus 61 ± 25 µM after chlorhexidine treatment, $P = 0.0098$).

In vivo *salivary acetaldehyde production*

The breath ethanol analyses revealed a high positive correlation ($r = 0.98$) between breath ethanol and salivary ethanol levels (data not shown). All volunteers showed detectable amounts of acetaldehyde in their saliva after ethanol intake. The highest acetaldehyde levels detected before chlorhexidine treatment ranged between 18.7 and 143.4 µM (35.3 ± 6.3 µM, mean ± SEM) and in all volunteers the peak value was achieved within 40 min after ethanol ingestion. From this peak value, salivary acetaldehyde levels decreased to a mean value of 8.3 µM ± 2.4 after 240 min. The individual peak acetaldehyde levels observed after chlorhexidine treatment were significantly lower, ranging from 8.6 to 49 µM (21.5 ± 3.3, mean ± SEM) and baseline acetaldehyde levels 0.9 ± 0.6 µM were achieved after 240 min. The differences in salivary acetaldehyde levels pre- and post-chlorhexidine treatment were significant at every time point (Figure 2a). In contrast, mean ethanol peak level and ethanol elimination rates in saliva were not influenced by chlorhexidine treatment (Figure 2b). For salivary acetaldehyde and ethanol concentrations, there were no statistically significant differences between mixed and paraffin induced saliva (data not shown). There was a highly significant positive correlation for each individual between salivary ethanol and acetaldehyde level (Figure 3). Because of high inter individual differences in salivary acetaldehyde levels, the slopes of the regression lines varied between the different volunteers. By using the individual regression lines as a standard curve, we calculated the hypothetical *in vivo* acetaldehyde levels at 10 mM of ethanol and compared these data with the

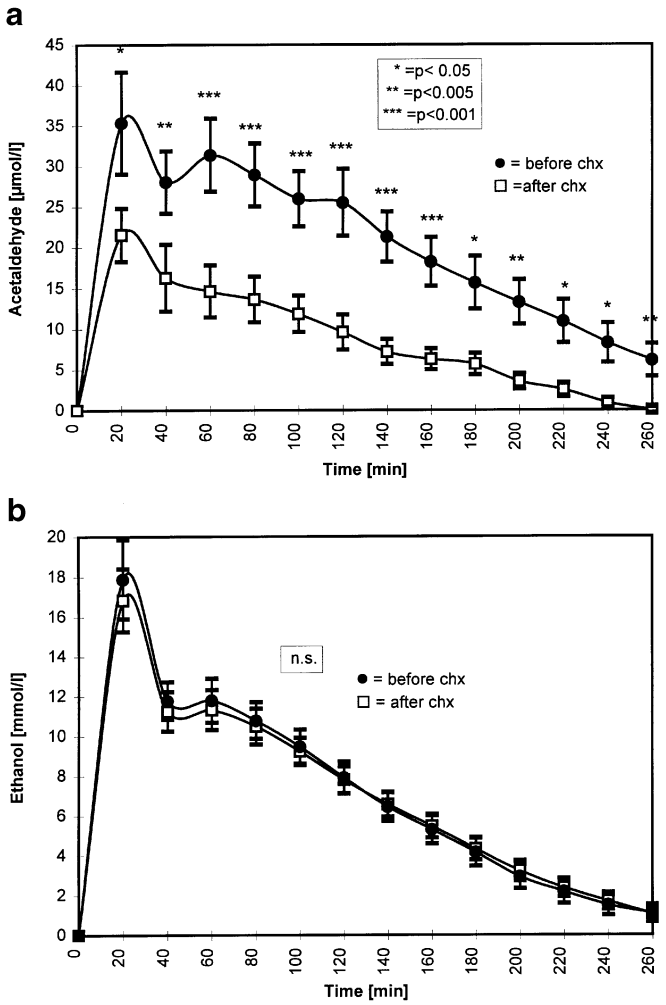

Fig. 2. (**a,b**) *In vivo* acetaldehyde and ethanol levels in the saliva after ethanol intake before or after chlorhexidine (chx) treatment in 10 healthy, young volunteers (means ± SEM).

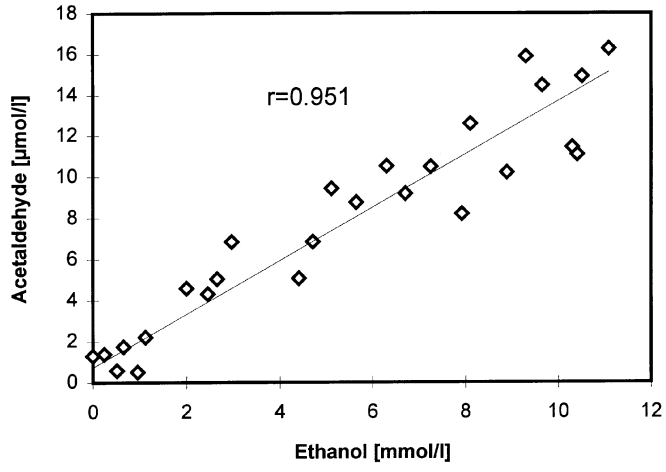

Fig. 3. Correlation between acetaldehyde and ethanol levels in saliva *in vivo*. This is an example in one volunteer, but a positive correlation could be demonstrated for each volunteer.

acetaldehyde levels observed *in vitro*. A highly significant positive correlation between acetaldehyde production *in vivo* and *in vitro* could be demonstrated (Figure 4).

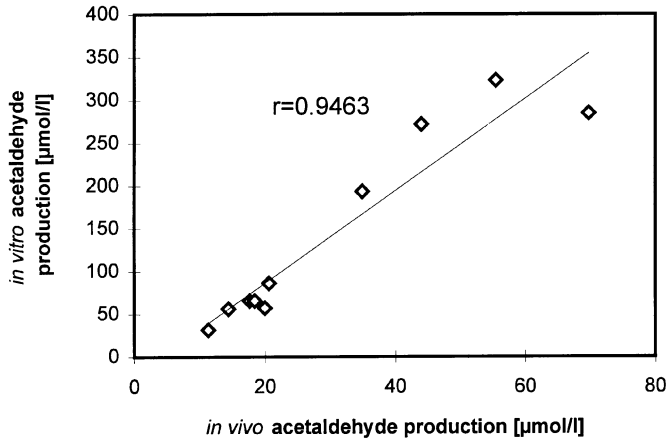

Fig. 4. Correlation between acetaldehyde production in saliva *in vivo* and *in vitro* in 10 healthy, young volunteers. *In vitro* data were obtained after the incubation at 22 mM ethanol and acetaldehyde levels *in vivo* were calculated at a hypothetical concentration of exactly 10 mM ethanol (see text).

Bacterial analysis

The baseline aerobic and anaerobic bacterial counts before alcohol intake and chlorhexidine treatment were $2.6 \times 10^8 \pm 1.4 \times 10^8$/ml saliva and $2 \times 10^8 \pm 5.5 \times 10^7$/ml saliva, respectively. There were no significant differences between the bacterial counts of spontaneous saliva and paraffin stimulated saliva or between baseline and post-drinking saliva (data not shown). However, a significant decrease in baseline aerobic ($2.4 \times 10^7 \pm 1.3 \times 10^7$/ml saliva, $P = 0.01$) and anaerobic ($3.1 \times 10^7 \pm 1.4 \times 10^7$/ml saliva, $P = 0.003$) bacterial counts was seen after chlorhexidine rinsing. There was no statistically significant correlation between the individual acetaldehyde levels *in vivo* or *in vitro* and the individual total salivary bacterial counts ($r = 0.02$, not significant).

Discussion

There are many hypothetical explanations for the tumor promoting effect of alcohol, e.g. enhanced solubility of ingested carcinogens, induction of tumor promoting microsomal enzymes, interference with detoxifying enzymes, impairment of nutrient metabolism, displacement of nutrients in the diet, alteration of hormonal status, increased oxidant exposure, carcinogenic components in alcoholic beverages and the immune suppressing function of alcohol (26–31). However, all these effects are systemic and do not explain the difference between the high relative risk of upper gastrointestinal tract cancer and the respectively low relative risk of other malignancies, such as cancer of the liver, breast and large bowel, associated with high ethanol consumption (2–4,32). Thus, the local tumor promoting effects of ethanol such as the altered membrane fluidity and direct toxic effect on the epithelia may be responsible for these epidemiological findings, but it is unclear to what extent (3).

Ethanol is present in saliva in concentrations comparable to blood ethanol levels after the consumption of alcoholic beverages (33). On the other hand, saliva is distributed over the upper gastrointestinal tract. In the present study, we have demonstrated the production of considerable amounts of acetaldehyde in saliva during normal social drinking. As acetaldehyde is mutagenic and carcinogenic (5–10,13–14) the long-term effects of locally produced acetaldehyde may be one

explanation for the enhanced cancer risk of the upper gastrointestinal tract among heavy drinkers (1–4,32).

Microbial acetaldehyde production after ethanol ingestion has been reported recently also in the large bowel (18,34,35). It has been demonstrated that alcohol intake caused mucosal hyper-regeneration in the rectum (35,36). This hyperproliferation leads to a higher susceptibility of the rectum to ingested carcinogens and could in part explain the tumor promoting effect of alcohol ingestion. In rodents, local acetaldehyde levels have been shown to correlate with an accelerated cell division in the colonic crypt (36). Accordingly, there is experimental evidence for the role of acetaldehyde in ethanol-related co-carcinogenesis (35). Mucosal hyperproliferation after ethanol ingestion has also been demonstrated in the floor of the mouth, the edge and base of the tongue and in the esophagus (37,38). Saliva is in close contact with the mucosa of the oral cavity and it is able to enter the esophagus. Accordingly, the increased cell regenerative activity observed in these tissues after ethanol ingestion could be due to acetaldehyde.

The analysis of acetaldehyde is most often afflicted with several methodological problems. It can be formed artificially in human biological samples that contain ethanol and it has been demonstrated, that protein precipitation in blood can cause non-enzymatic production of acetaldehyde from ethanol (39,40). However, in the present study we demonstrate the lack of acetaldehyde production from ethanol after the addition of PCA to the incubation medium, indicating that artefactual acetaldehyde formation in saliva is minimal. The addition of PCA immediately after incubation decreased acetaldehyde levels to some extent, presumably preventing the reaction during the heating period in the head space gas chromatograph. Accordingly, our acetaldehyde levels can be considered to be authentic.

Theoretically, acetaldehyde in saliva could be produced either by oral microflora via bacterial ADH or by ADHs of oral and esophageal mucosa. In gingival, lingual and esophageal mucosa, the presence of highly active and high K_m μ-ADH (also denoted as σ-ADH) has been described (41,42). These so called class IV ADHs have originally been identified and characterized from the human stomach (43). Due to their high K_m they contribute significantly to the first-pass metabolism of ethanol. Furthermore, the expression and activity studies on mucosal aldehyde dehydrogenases (ALDH) demonstrated that low activity, low K_m ALDH1 (in the esophagus) and high K_m ALDH3 (for oral cavity and esophagus) are present, and that highly active, low K_m ALDH (as mitochondrial ALDH2) is not expressed in these tissues. Consequently, it has been suggested that intracellular acetaldehyde may accumulate during ethanol ingestion (41,42). Our data obtained with centrifuged and sterile filtered saliva, as well as the significant reduction of acetaldehyde production after the use of an antiseptic agent (both *in vitro* and *in vivo*) associated with decreasing bacterial counts in saliva, further supports the concept that salivary acetaldehyde production is largely of bacterial origin.

Only small traces of acetaldehyde were produced by mucosal swabs and the acetaldehyde production was low in the mouthwashes. The higher production of acetaldehyde in saliva could be because of the higher concentration of bacteria in saliva as compared with the other samples. This was not investigated, as the main scope of the study was to evaluate acetaldehyde levels in a material representative for the oral cavity. In this study, no significant association between individual total salivary bacterial count and acetaldehyde level in saliva was found, which indicates that a higher count of bacteria will not lead consequently to higher acetaldehyde levels. Although paraffin induced saliva should contain more dislodged bacteria from the tooth surface and the gingival crevice there was no significant different between the two types of saliva. The lack of detailed bacteriological data and the fact that samples were not incubated under anaerobic conditions make our interpretations difficult. Our findings may indicate that acetaldehyde production capability of tooth- and mucosa-mounting bacteria is lower than that of saliva-soluble bacteria. This is supported by the fact that the activity of ADHs and acetaldehyde production capacity vary remarkably among different bacteria (44). Distinct bacterial species or even strains may be responsible for the major part of the acetaldehyde production.

It is well known from epidemiological studies that there exists a strong dose-dependency between the amount of ingested ethanol and the relative cancer risk (1–4). Furthermore, a poor dental status is an independent risk factor for oral cavity cancer (3,45). This study shows that acetaldehyde production in saliva is of bacterial origin, is linear in time and cannot be saturated under ethanol concentrations that are relevant *in vivo*. Accordingly, our study supports the epidemiological findings and the possible role of acetaldehyde in the pathogenesis of the upper gastrointestinal tract.

Systemic blood acetaldehyde levels, even after a high dose of ethanol, are very low (1–5 μM), i.e. scarcely above the detection limit (39). The observed local acetaldehyde levels in our volunteers reached values up to 143 μM after a moderate dose of ethanol (0.5 g/kg body weight). There was a positive correlation between ethanol and acetaldehyde levels *in vivo* and by using the regression lines as a standard curve we estimated that the local salivary acetaldehyde levels reach values of up to 450 μM at blood ethanol levels of 44 mM. Taking into consideration that our volunteers were young, with good dental health and moderate users of alcohol, it could be possible that heavy drinkers with poor dental status and an induced enzyme system could reach even much higher salivary acetaldehyde levels.

In most experimental studies on mammalian or human cell cultures, where mutations, sister chromatid exchanges and chromosomal aberrations were induced after acetaldehyde treatment with acetaldehyde concentrations ranging from 40 to 1000 μM and incubation times of 1–90 h, a strong dose-dependency was observed (6–9, see for review 5). Although the transferability of *in vitro* data obtained in cell cultures to an *in vivo* organism is difficult, it can at least be concluded, that under certain circumstances the *in vivo* salivary acetaldehyde levels would be sufficient to cause severe mutagenic damages.

We were able also to demonstrate a highly significant correlation between acetaldehyde production *in vitro* and *in vivo*. Consequently, acetaldehyde levels observed after incubating saliva with ethanol *in vitro* can be used to estimate salivary acetaldehyde levels that occur *in vivo* after ethanol intake. The high inter individual variations in the levels of acetaldehyde in saliva need to be elucidated. Studies on important predictors of the composition of oral microbial flora like ethanol, smoking habits or dental status, which all may influence the acetaldehyde production in saliva, need to be performed as well as the measurement of acetaldehyde formation capacity of different bacteria of the oral microflora on species or strain level.

Acknowledgements

The skilful technical assistance of Tuula Heiskanen is gratefully acknowledged. The work was supported in part by a grant of the Dr Mildred Scheel Stiftung, Deutsche Krebshilfe e.V., Germany (N.H.), Finnish Foundation for Studies on Alcohol and Sigrid Juselius Foundation.

References

1. Driver,H.E. and Swann,P.F. (1987) Alcohol and human cancer. *Anticancer Res*., **7**, 309–320.
2. Anonymous, (1988) Alcohol drinking. In *IARC Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Humans, Alcohol Drinking*, Vol. 44. International Agency for Research on Cancer, Lyon, France.
3. Blot,W.J. (1992) Alcohol and cancer. *Cancer Res*., **52** (Suppl.), 2119–2123.
4. Brugere,J., Guenel,P., Leclerc,A. and Rodriguez,J. (1986) Differential effects of tobacco and alcohol in cancer of the larynx, pharynx and mouth. *Cancer*, **57**, 391–395.
5. Anonymous (1995) Acetaldehyde. In *Environmental Health Criteria (No. 167), Acetaldehyde*. World Health Organization, Geneva, Switzerland.
6. Anonymous (1985) Acetaldehyde. In *IARC Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Humans, Allyl Components, Aldehydes, Epoxides and Peroxides*, Vol. 36. International Agency for Research on Cancer, Lyon, France, pp. 101–132.
7. De Raat,W.K., Davis,P.B. and Bakker,G.L. (1983) Induction of sister-chromatide exchanges by alcohol and alcoholic beverages after metabolic activation by rat-liver homogenate. *Mutat. Res*., **124**, 85–90.
8. Obe,G. and Ristow,H. (1977) Acetaldehyde, but not ethanol, induces sister chromatide exchanges in Chinese hamster cells *in vitro*. *Mutat Res*., **56**, 211–213.
9. Böhlke,J.U., Singh,S. and Goedde,H.W. (1983) Cytogenetic effects of acetaldehyde in lymphocytes of Germans and Japanese: SCE, clastogenic activity and cell cycle delay. *Human Genet*., **63**, 285–289.
10. Bird,R.P., Draper,H.H. and Basrur,P.K. (1982) Effects of malonaldehyde and acetaldehyde on cultured mammalian cells: Production of micronuclei and chromosomal aberrations. *Mutat. Res*., **101**, 237–246.
11. Hemminki,K. and Suni,R. (1984) Sites of reaction of glutaraldehyde and acetaldehyde with nucleosides. *Arch. Toxicol*., **55**, 186–190.
12. Fang,J.L. and Vaca,C.E. (1995) Development of a ^{32}P-postlabelling method for the analysis of adducts arising through the reaction of acetaldehyde with 2-deoxyguanosine-3-monophosphate and DNA. *Carcinogenesis*, **16**, 2177–2185.
13. Vaca,C.E., Löfgren,M. and Hemminki,K. (1992) Some quantitative considerations about DNA adduct enrichment procedures for ^{32}P-postlabelling. *Carcinogenesis*, **13**, 2463–2466.
14. Feron,V.J., Kuysse,A. and Woutersen,R.A. (1982) Respiratory tract tumours in hamsters exposed to acetaldehyde vapor alone or simultaneously to benz[*a*]pyrene or diethyl-nitrosamine. *Eur. J. Cancer Clin. Oncol*., **18**, 13–31.
15. Wickramasinghe,S.N., Bond,A., Sloviter,H.A., and Saunders,J.E. (1981) Metabolism of ethanol by human marrow cells. *Acta Haematol*., **4**, 238–243.
16. Julkunen,R.J.K., DiPadova,C. and Lieber,C,S. (1985) First pass metabolism of ethanol: a gastrointestinal barrier against the systemic toxicity of ethanol. *Life Sci.*, **6**, 567–573.
17. Leloir,L.F. and Munoz,J.M. (1938) Ethyl alcohol metabolism in animal tissues. *Biochem. J*., **32**, 299–307.
18. Jokelainen,K., Roine,R.P., Väänänen,H., Färkkila,M. and Salaspuro,M. (1994) *In vitro* acetaldehyde formation by human colonic bacteria. *Gut*, **35**, 1271–1274.
19. Salaspuro,M. (1996) Bacteriocolonic pathway for ethanol oxidation: characteristics and implications. *Ann. Med*., **28**, 195–200.
20. Pikkarainen,P.H., Baraona,E., Jauhonen,P., Seitz,K.H. and Lieber,C.S. (1981) Contribution of oropharynx flora and of lung microsomes to acetaldehyde in expired air after alcohol ingestion. *J. Lab. Clin. Med*., **97**, 631–636.
21. Miyakawa,H., Baraona,E., Chang,J.C., Lesser,M.D. and Lieber,C.S. (1986) Oxidation of ethanol to acetaldehyde by bronchopulmonary washings: role of bacteria. *Alcohol. Clin. Exp. Res*., **10**, 517–520.
22. Jauhonen,P., Baraona,E., Miyakawa,H. and Lieber,C.S. (1982) Origin of breath acetaldehyde during ethanol oxidation. Effect of long-term cigarette smoking. *J. Lab. Clin. Med*., **100**, 908–916.
23. Jokelainen,K., Heikkonen,E., Roine,R., Lehtonen,H. and Salaspuro,M. (1996) Increased acetaldehyde production by mouthwashings of patients with oral cavity, laryngeal or pharyngeal cancer. *Alcohol. Clin. Exp. Res*., **20**, 1206–1210.
24. Summanen,P., Baron,E.J., Citro,D.M., Strong,C.A., Wexler,H.M. and Finegold,S.M. (1993) *Wadsworth Anaerobic Bacteriology Manual*, 5th edn. Star Publishing Co., Belmont, California.
25. Jousimies-Somer,H.R., Summanen,P.H. and Finegold,S.M. (1995) *Bacteroides, Porphymons, Prerotella, Fusobacterium* and other anaerobic gram-negative bacteria. In Murray,P.R., Baron,E.J., Pfaller,M.A., Tenover,F.C. and Yolken,R.H. (eds) *Manual of Clinical Microbiology.* AMS Press, Washington DC, 6th edn. pp. 603–620.
26. McCoy,G.D. and Wynder,E.L. (1979) Etiological and preventive implications in alcohol carcinogenesis. *Cancer Res*., **39**, 2844–2850.
27. Gavaler,J.S. and Van Thiel,D.H. (1987) Reproductive consequences of alcohol abuse: males and females compared and contrasted. *Mutat. Res*., **186**, 269–277.
28. Walker,E.A., Castegnaro,M., Garren,L., Toussant,G. and Kowalski,B. (1979) Intake of volatile nitrosamines from consumption of alcohols. *J. Natl Cancer Inst*., **69**, 947–951.
29. Mikol,Y.B., Hoover,K.L., Creasia,D. and Poirier,L. (1983) Hepatocarcinogenesis in rats fed methyl-deficient, amino-acid defined diets. *Carcinogenesis*, **4**, 1619–1629.
30. Palmer,D.L. (1978) Alcohol consumption and cellular immunocompetence. *Laryngoscope*, **88**, 13–17.
31. Hamilton,S.R., Hyland,J., McAvinchey,D. *et al.* (1987) Effects of chronic dietary beer and ethanol consumption on experimental colonic carcinogenesis by azetoxymethane in rats. *Cancer Res*., **47**, 1551–1559.
32. Breslow,N.E. and Enstorm,J.E. (1974) Geographic correlations between mortality rates, alcohol and tobacco consumption in the United States. *J. Natl Cancer Inst*., **53**, 631–639.
33. Jones,A.W. (1979) Distribution of ethanol between saliva and blood in men. *Clin. Exp. Pharmacol. Physiol*., **6**, 53–59.
34. Jokelainen,K., Matysiak-Budnik,T., Mäkisalo,H., Höckerstedt,K. and Salaspuro,M. (1996) High intracolonic acetaldehyde levels produced by a bacteriocolonic pathway for ethanol oxidation in piglets. *Gut*, **39**, 100–104.
35. Seitz,H.K., Simanowski,U.A., Garzon,F.T., Rideout,J.M., Peters,T.J., Koch,A., Berger,M.R., Einecke,H. and Maiwald,M. (1990) Possible role of acetaldehyde in the ethanol-related cocarcinogenesis in the rat. *Gastroenterology*, **98**, 406–413.
36. Simanowski,U.A., Suter,P., Russell,R.M., Heller,M., Waldherr,R., Ward,R., Peters,T.J., Smith,D. and Seitz,H.K. (1994) Enhancement of ethanol induced rectal mucosal hyper regeneration with age in F344 rats. *Gut*, **35**, 1102–1106.
37. Maier,H., Weidauer,H., Zöller,J., Seitz,H.K., Flentje,M., Mall,G. and Born,I.A. (1994) Effect of chronic alcohol consumption on the morphology of the oral mucosa. *Alcohol. Clin. Exp. Res*., **18**, 387–391.
38. Simanowski,U.A., Suter,P., Stickel,F., Maier,H., Waldherr,R., Russel,R.K. and Seitz,H.K. (1993) Esophageal epithelial hyperregeneration following chronic ethanol ingestion: effect of age and salivary gland function. *J. Natl Cancer Inst*., **85**, 2030–2033.
39. Stowell,A.R. (1979) An improved method for the determination of acetaldehyde in human blood with minimal ethanol interference. *Clinica Chimica Acta*, **98**, 201–205.
40. Fukunaga,T., Sillanaukee,P. and Eriksson,C.J.P. (1993) Problems involved in the determination of endogenous acetaldehyde in human blood. *Alcohol Alcoholism*, **28**, 535–541.
41. Dong,Y.-J., Peng,T.-K. and Yin,S.-J. (1996) Expression and activities of class IV alcohol dehydrogenase and class III aldehyde dehydrogenase in human mouth. *Alcohol*, **13**, 257–262.
42. Yin,S.-J., Chou,F.-J., Chao,S.-F., Tsai,S.-F., Liao,C.-S., Wang,S.-L., Wu,C.-W. and Lee,S.-C. (1993) Alcohol and aldehyde dehydrogenases in human esophagus: Comparison with the stomach enzyme activities. *Alcoholism Clin. Exp. Res*., **17**, 376–381.
43. Moreno,A. and Pares,X. (1991) Purification and characterization of a new alcohol dehydrogenase from human stomach. *J. Biol. Chem*., **266**, 1128–1233.
44. Jokelainen,K., Siitonen,A., Jousimies-Somer,H., Nosova,T., Heine,R. and Salaspuro,M. (1996) *In vitro* alcohol dehydrogenase mediated acetaldehyde production by aerobic bacteria representing the normal colonic flora in man. *Alcohol. Clin. Exp. Res*., **20**, 967–972.
45. Maier,H., Zöller,J., Herrmann,A., Kreiss,M. and Heller,W.D. (1993) Dental status and oral hygiene in patients with head and neck cancer. *Otolaryngol. Head Neck Surg*., **108**, 655–661.

Received on November 28, 1996; revised on March 20, 1997; accepted on May 19, 1997