LOCAL ACETALDEHYDE PRODUCTION AS A PATHOGENETIC FACTOR FOR UPPER DIGESTIVE TRACT CANCERS IN HUMANS

Satu Väkeväinen

Research Unit of Substance Abuse Medicine
University of Helsinki
Finland

ACADEMIC DISSERTATION

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Supervised by

Professor Mikko Salaspuro, M.D.

Research Unit of Substance Abuse Medicine
Department of Medicine
University of Helsinki

Reviewed by

Professor Onni Niemelä, M.D.

Department of Medicine
University of Tampere

and

Professor Markku Savolainen, M.D.

Department of Internal Medicine
University of Oulu

Opponent

Professor Helmut K. Seitz, M.D.

Laboratory of Alcohol Research, Liver Disease and Nutrition
Department of Medicine
University of Heidelberg, Germany

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To the memory of my father

CONTENTS

ABBREVIATIONS

ADH	alcohol dehydrogenase
ALDH	aldehyde dehydrogenase
CFU	colony forming units
CYP	cytochrome P450
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
IARC	International Agency for Research on Cancer
K_m	Michaelis constant
MEOS	microsomal ethanol oxidizing system
4-MP	4-methylpyrazole
NAD	nicotinamine adenine dinucleotide
NADH	reduced nicotinamine adenine dinucleotide
PCA	perchloric acid
PCR-RFLP	polymerase chain reaction/restriction fragment length polymorphism
SEM	standard error of the mean
V_{max}	maximal velocity

ORIGINAL PUBLICATIONS

This thesis is based on the following studies which are referred to in the text by their Roman numerals:

I. Väkeväinen S, Tillonen J, Agarwal DP, Srivastava N, Salaspuro M (2000) High salivary acetaldehyde after a moderate dose of alcohol in ALDH2-deficient subjects: strong evidence for the local carcinogenic action of acetaldehyde. Alcohol Clin Exp Res 24:873-877.

II. Väkeväinen S, Tillonen J, Salaspuro M (2001) 4-Methylpyrazole decreases salivary acetaldehyde levels in ALDH2-deficient subjects but not in subjects with normal ALDH2. Alcohol Clin Exp Res 25:829-834.

III. Väkeväinen S, Tillonen J, Salaspuro M, Jousimies-Somer H, Nuutinen H, Färkkilä M (2000) Hypochlorhydria induced by a proton pump inhibitor leads to intragastric microbial production of acetaldehyde from ethanol. Aliment Pharmacol Ther 14:1511-1518.

IV. Väkeväinen S, Mentula S, Nuutinen H, Salmela KS, Jousimies-Somer H, Färkkilä M, Salaspuro M (2002) Ethanol-derived microbial production of carcinogenic acetaldehyde in achlorhydric atrophic gastritis. Scand J Gastroenterol, in press.

V. Väkeväinen S, Tillonen J, Blom M, Jousimies-Somer H, Salaspuro M (2001) Acetaldehyde production and other ADH-related characteristics of aerobic bacteria isolated from hypochlorhydric human stomach. Alcohol Clin Exp Res 25:421-426.

ABSTRACT

Heavy alcohol consumption is a well-known risk factor for upper digestive tract cancers. The exact mechanism responsible for this is still obscure, but it has been suggested to be related to toxic effects of ethanol's first metabolite, acetaldehyde, which is a recognized animal carcinogen. Many microbes of the gastro-intestinal tract can oxidize ethanol to acetaldehyde via their alcohol dehydrogenase (ADH) enzymes. Acetaldehyde is further oxidized to less harmful acetate mainly by the mitochondrial aldehyde dehydrogenase-2 (ALDH2) enzyme. Genetic deficiency of ALDH2 strongly increases the risk of digestive tract cancers in heavy drinkers. Atrophic gastritis, a condition frequently associated with reduced gastric acidity and intragastric bacterial overgrowth, is also a risk factor for gastric cancer. Bacterial colonization of the stomach is also common during the use of medicines that inhibit gastric acid production, such as proton pump inhibitors.

The aim of this study was to investigate ethanol metabolism in the mouth and in the hypochlorhydric stomach both *in vivo* and *in vitro* in order to find more evidence for the local carcinogenic action of acetaldehyde in humans.

Firstly, we studied salivary acetaldehyde production in subjects with different *ALDH2* genotypes. Secondly, we examined whether the salivary acetaldehyde production can be reduced by using 4-methylpyrazole, an inhibitor of ADH. Thirdly, we studied the effect of proton pump inhibitors on gastric flora and acetaldehyde production from ethanol. Fourthly, we investigated both endogenous and exogenous ethanol metabolism in the stomach of patients with atrophic gastritis. Fifthly, we studied which microbes are responsible for acetaldehyde production in the hypochlorhydric stomach.

These studies revealed that after alcohol ingestion ALDH2-deficient subjects have significantly higher *in vivo* salivary acetaldehyde levels than subjects with normal ALDH2. In addition to oral microflora, parotid salivary glands may also produce acetaldehyde into saliva. A single dose of 4-methylpyrazole before ethanol ingestion reduced the flushing reaction and both blood and salivary acetaldehyde levels in ALDH2-deficient subjects, but not in subjects with the normal *ALDH2* genotype. Both iatrogenic hypochlorhydria and achlorhydria associated with atrophic gastritis led to intragastric bacterial overgrowth, and to marked microbial acetaldehyde production from ethanol both *in vivo* and *in vitro*. The most potent bacteria responsible for this seemed to be *Neisseria* species and *Streptococcus salivarius,* together with *Enterobacteriaceae* and yeasts, both of which have earlier been shown to be high acetaldehyde producers.

Together with previous epidemiological data, the findings of this study provide strong evidence for the local carcinogenic action of acetaldehyde in the upper digestive tract in humans, and open a new genetic and microbiological approach for the pathogenesis, screening, and prevention of digestive tract cancers.

1. INTRODUCTION

Ethanol in the form of various kinds of alcoholic beverages has been part of our social life for thousands of years. Unfortunately, alcohol is currently the most widely abused substance in the Western world, and can be regarded as one of the most severe public health problems of the modern society. Excessive drinking may lead to several liver diseases, such as "fatty liver", hepatitis, and cirrhosis, though heavy drinking can also harm nearly every organ and system in the human body.

Excessive alcohol consumption is one of the strongest risk factors for upper digestive tract cancers (Doll et al., 1999). Although the epidemiological data for this is convincing, the exact mechanism of ethanol-derived cancers has remained obscure, since ethanol itself is not a carcinogen (IARC, 1988). By contrast, the first metabolite of ethanol oxidation, acetaldehyde, has multiple carcinogenic effects according to cell culture and animal studies (IARC, 1999). Acetaldehyde has, in fact, been proposed to be the major factor behind ethanol-associated cancers. Recent epidemiological studies have reported an enhanced risk of upper digestive tract cancers among heavy-drinking Asian subjects with a genetically deficient ability to remove acetaldehyde (Yokoyama et al., 1998a).

The most important pathway for ethanol metabolism in the body involves two reactions and two enzymes catalyzing these reactions. In the first reaction, ethanol is converted to acetaldehyde by alcohol dehydrogenase (ADH), and in the second reaction acetaldehyde is oxidized to acetate by aldehyde dehydrogenase (ALDH):

$$\text{Ethanol} \xrightarrow{\text{ADH}} \text{Acetaldehyde} \xrightarrow{\text{ALDH}} \text{Acetate}$$

During the past few years it has been shown that microbes of the digestive tract can also participate in ethanol metabolism (Jokelainen, 1997; Salaspuro, 1996, 1997). Many aerobic bacteria of the gastro-intestinal tract possess ADH activity, and are thereby able to oxidize ethanol to acetaldehyde (Jokelainen et al., 1996a, Nosova et al., 1997), whereas opposite findings have been reported on ALDH activities of the alimentary tract microbes (Nosova et al., 1996, 1998; Muto et al., 2000). Since the capacity of both the digestive tract microflora and mucosa to metabolize acetaldehyde to acetate seems to be limited, there may be local accumulation of acetaldehyde in the gastrointestinal tract in the presence of microbes and exogenous or endogenous alcohol (Koivisto and Salaspuro, 1996). In the upper digestive tract, high acetaldehyde levels have been detected in saliva even after a moderate dose of alcohol (Homann et al., 1997a). This acetaldehyde production is strongly influenced by individual factors and differences in oral flora (Homann et al., 2000a). Considering its high reactivity, toxicity and carcinogenicity, the existence of acetaldehyde, especially at high concentrations, can be expected to have

deleterious effects. Therefore, studies on conditions associated with enhanced local production of acetaldehyde may provide important information for the understanding of the pathogenesis of alcohol-related diseases, and thus also contribute to the management and prevention of these diseases.

The aim of the present study was to investigate ethanol metabolism and the local production of acetaldehyde in the upper digestive tract and in the hypochlorhydric stomach in order to gather more evidence for the local carcinogenicity of acetaldehyde in humans.

2. REVIEW OF THE LITERATURE

2.1. ALCOHOL AND THE DIGESTIVE TRACT

Heavy and prolonged use of alcohol affects nearly every organ system of the human body. Liver damages, including "fatty liver", alcoholic hepatitis and cirrhosis, are the best known examples of the effects of chronic alcohol consumption on the digestive tract. Excessive drinking is, however, also associated with a wide variety of other gastrointestinal symptoms that may lead either to acute or chronic digestive tract diseases. In addition, excessive alcohol consumption has long been recognized as a risk factor for alimentary tract cancers.

Following oral alcohol intake the upper digestive tract, mouth, pharynx, larynx, esophagus, stomach and upper small intestine are exposed to high ethanol concentrations, and can thereby be directly affected by ethanol. However, since ethanol is rapidly and effectively transported through the circulation to more distal parts of the alimentary tract, chronic alcohol intake may also affect these parts. The most common gastrointestinal complaints among heavy drinkers are heartburn, nausea, vomiting, diarrhea and flatulence (Fields et al., 1994). These symptoms are associated with active alcohol use and are usually resolved after two weeks´ abstinence (Fields et al., 1994).

Poor nutritional status is a common finding among lower-income and homeless alcoholics. The etiology of this, as well as the above mentioned gastrointestinal symptoms, has generally been thought to be of multifactorial origin (Salaspuro, 1993). Ethanol accounts for about a half of the caloric intake of such alcoholics. It therefore displaces normal nutrients, causing malnutrition (Lieber, 1995). Structural and functional changes in the small intestine may also lead to malabsorption and cause malnutrition. Other possible factors responsible for malnutrition are pancreatic exocrine insufficiency, reduced biliary secretion, and impaired hepatic metabolism of nutrients (Lieber, 1995; Salaspuro, 1993).

Esophagus

According to some earlier studies, acute alcohol ingestion may impair the function of the lower esophageal sphincter and decrease the primary peristalsis of the distal esophagus (Hogan et al., 1972; Mayer et al., 1978). More recent studies have, however, reported opposite findings (Keshavarzian et al., 1987; Silver et al., 1986). There is also evidence indicating that alcohol may induce esophageal reflux and impair the acid clearance of the esophagus (Kaufman and Kaye, 1978; Vitale et al., 1987). This may explain the increased incidence of heartburn commonly reported among alcoholics. Other changes caused by excessive alcohol consumption in the same area are esophageal varices which are often responsible for upper gastrointestinal bleeding in alcoholics (Sutton and Shields, 1995). The majority of heavy drinkers with

liver cirrhosis develop esophageal varices as a consequence of portal hypertension (Feinman et al., 1992).

Stomach

Chronic alcohol intake affects the histology of the fundic and especially the antral mucosa of the stomach (Dinoso et al., 1972; Parl et al., 1979). According to the study of Dinoso et al., even 50% of chronic alcoholics show changes of fundic gastritis and 84% show changes of antral gastritis, 66% also having antral atrophic gastritis. In addition, chronic gastritis in alcoholic patients is known to develop into chronic atrophic gastritis at an earlier age than in non-alcoholic subjects (Parl et al., 1979). Bacterial overgrowth in the stomach is also associated with excessive drinking more often than with moderate alcohol consumption (Hauge et al., 1997). Furthermore, alcohol drinking may cause alterations in gastric emptying and gastric acid secretion. High intragastric ethanol concentration can delay gastric emptying and inhibit gastric acid secretion, whereas low concentrations can have the opposite effects (Feinman et al., 1992).

Small intestine

Acute ethanol ingestion causes histological changes, such as haemorrhagic erosions, subepithelial blebs, and infiltration of inflammatory cells in the lamina propria in the duodenum (Gottfried et al., 1978). Studies concerning chronic alcohol use have shown reduction in the villus height and a decreased mucosal surface area of villi in the small intestine (Bode et al., 1982a, Persson 1991, Seitz et al., 1985). Heavy drinking is also known to promote bacterial overgrowth in the small intestine, which has been thought to be a consequence of the increased pH of the gastric juice (Bode et al., 1984a). Bacterial overgrowth, in turn, together with temporary destabilization of intercellular junctions may lead to increased permeability of the small intestine (Bode et al., 1991; Draper et al., 1983). Elevated permeability of the gut wall may lead to either increased loss of substances from blood to the intestinal lumen or increased uptake of normally non-absorbable substances like bacterial endotoxins from the gut to the portal blood (Persson, 1991). Increased permeability of the gut has also been proposed to be one of the mechanisms of alcoholic liver diseases (Keshavarzian et al., 1999; Parlesak et al., 2000; Thurman, 1998). Interestingly, the paracellular permeability of the Caco-2 cell monolayer, a human colon adenocarcinoma cell line resembling normal small intestinal enterocytes, is reversibly increased by high acetaldehyde concentrations (Rao, 1998). Ethanol can also affect several enzymes that are located in the absorptive cells of the small intestine; decreased activities of disaccharidases, for example, have been demonstrated after chronic consumption of ethanol (Bode et al., 1982b). Reduced lactase activity as well as decreased oral-caecal time in alcoholics may contribute to the diarrhea commonly observed in heavy drinkers (Keshavarzian et al., 1986; Persson, 1991).

Large intestine

Sustained excessive consumption of alcohol has been shown to produce marked changes

in the rectal histology. These reversible changes include a decreased number of goblet cells, inflammatory changes and alterations in the cell organelles (Brozinsky et al., 1978). Chronic alcohol use reduces colorectal transit time and affects colonic motility, both of which have been suggested to be associated with diarrhea frequently seen in alcoholics (Bouchoucha et al., 1991).

2.2. ALCOHOL AND DIGESTIVE TRACT CANCERS

Cancer of the oropharynx and esophagus

Excessive alcohol consumption is a strong determinant of an enhanced risk of cancers of the upper digestive tract (IARC, 1988). The increased risk of cancers of the mouth, pharynx, larynx and esophagus among heavy drinkers has been confirmed by many epidemiological studies (Blot, 1992; Blot et al., 1988; Boffetta et al., 1992; Brugere et al., 1986; Doll et al., 1999; Franceschi et al., 1990; Mashberg et al., 1993). It has been estimated that alcohol consumption alone might account for about 25 to 50% of cancers of these regions (Franceschi et al, 1990). Tobacco smoking is another well-known strong risk factor for the upper digestive tract cancers, and together with alcohol consumption these factors are the major causes of cancers in the upper gastrointestinal tract, accounting for as much as 75% of all cases in Europe (La Vecchia et al., 1997). Smoking and alcohol drinking are independent risk factors for upper digestive tract cancers, but the combined effect of these agents seems to be more than additive (Blot et al., 1988; Brugere et al., 1986, La Vecchia et al., 1997). The risk of cancer increases proportionally with the number of cigarettes smoked and the amount of alcohol consumed. Even the regular use of mouthwash with a high alcohol content has been shown to increase the oral cancer risk (Winn et al., 1991).

Poor nutritional status and low intake of micronutrients, fruit and green vegetables, genetic factors, certain papilloma virus infections, occupational hazards as well as poor oral hygiene and dental status, tooth loss, and dentition are all factors associated with a higher risk of upper digestive tract cancers (Bundgaard et al., 1995; Graham et al., 1977; Harris, 1997; La Vecchia et al., 1997; Maier et al., 1993; Marshall et al., 1992). These factors may also contribute to the ethanol-associated carcinogenesis of the upper gastrointestinal tract. Interestingly, poor dental status has recently also been shown to increase salivary acetaldehyde production up to twofold as compared to good dental status (Homann et al., 2001).

Cancer of the stomach

The association between alcohol consumption and stomach cancer is not as clear as that with other upper gastrointestinal tract cancers. The epidemiological data concerning the role of alcohol consumption in gastric carcinogenesis is controversial. Many studies either supporting (Correa et al., 1985; Hoey et al., 1981; Wang et al., 1986;

Wu-Williams et al., 1990) or not supporting (Graham et al., 1967, 1972; Gray et al., 1992) this association have been published. The relative risk for a positive relationship has ranged from 1.5-1.7 in previous case-control studies. However, a relative risk as high as 3.05 has been reported in a study among the Japanese (Kato et al., 1992), who frequently have a genetically determined deficiency to metabolize acetaldehyde (Goedde et al., 1979). Yokoyama et al. (1998a) have found a high frequency of digestive tract cancers, including stomach cancer, in heavy-drinking individuals with deficient acetaldehyde removal. This supports the role of acetaldehyde in the carcinogenesis associated with alcohol use.

As the role of ethanol in gastric carcinogenesis remains unclear, the association between cancer of the gastric cardia and excessive alcohol consumption may prove to be clearer, since this cancer seems to resemble a specific type of cancer of the lower esophagus and may share common risk factors such as tobacco smoking and alcohol drinking (Vaughan et al., 1995). The importance of understanding the risk factors for cancer of the gastric cardia is increasing, because the incidence rate of this cancer, opposite to stomach cancer, has been rising during the last decades (Blot et al., 1991; Botterweck et al., 2000; Devesa and Fraumeni, 1999).

Cancer of the large intestine

The association between alcohol consumption and cancer of the large intestine, similar to gastric cancer, has long been discussed. Epidemiological studies both for and against such an association have been published (Doll et al., 1999). There is, however, some evidence showing that alcohol consumption leads to a slightly increased risk of colorectal cancer with an estimated relative risk of 1.1, and that the risk of rectal cancer is more increased than the risk of colon cancer (Kune and Vitetta et al., 1992; Longnecker et al., 1990). Furthermore, the World Health Organization Consensus Conference on Nutrition and Colorectal Cancer in 1999 declared that alcohol has a causal effect on colorectal carcinogenesis (Scheppach et al., 1999).

Possible pathogenetic mechanisms in carcinogenesis

It is clear that alcohol consumption is a risk factor for certain cancers discussed above. The exact mechanism responsible for this has been obscure, since there is no apparent evidence showing that ethanol itself is a carcinogen (Doll et al., 1999). However, many animal studies suggest that ethanol may act as a co-carcinogen at different sites of the body with a variety of chemical carcinogens (Griciūtė et al., 1982, 1984; Seitz et al., 1984).

Alcoholic beverages may contain congeners or contaminants that can be carcinogenic. Special attention has recently been paid to *N*-nitroso compounds, which have been related to colorectal cancer (Knekt et al., 1999). These compounds were found in high concentrations in some beers in the late 1970s (Walker et al., 1979). Some later studies have confirmed this finding (Riboli et al., 1991), whereas others have failed to support it (Potter and McMichael, 1986). According to Doll et al., 1999, the latest

consensus is that there is no difference in the cancer risk among different types of alcoholic beverages. However, an increased incidence of esophageal cancer has been reported in the area of France where calvados is a popular alcoholic beverage (Launoy et al., 1997). Interestingly, this type of alcoholic beverage has recently been shown to contain especially high amounts of acetaldehyde (Visapää et al., 2001a).

Prolonged alcohol intake induces microsomal cytochrome P450 enzymes, most importantly hepatic CYP2E1, which has a capacity to activate over 80 toxicologically important xenobiotics to potentially carcinogenic products (Lieber, 1997). Along with the activation of carcinogens, CYP2E1 mediates the breakdown of vitamin A (Leo and Lieber, 1982). Since vitamin A has an important role in the maintenance of normal growth and cell differentiation, this may also be a significant factor in the development of cancer (Sporn and Roberts, 1983). In addition, ethanol may block the hepatic inactivation of carcinogens, and thereby increase the exposure to these compounds (Blot, 1992).

Excessive alcohol consumption may either enhance nutritional deficiencies that increase the risk of cancer or reduce the intake and/or bioavailability of nutrients that may inhibit the development of cancer. Nutritional deprivation can lead to nutritional deficiencies that may alter epithelial cell chemistry and function, thus increasing susceptibility to carcinogens (Blot, 1992). An example of this is folate deficiency, which has been associated with an increased risk of colon cancer (Giovannucci et al., 1995). Folate acts as a methyl group donor in transmethylation reactions, e.g. in the methylation of DNA which is essential to normal gene expression. Decreased folate leads to the hypomethylation of DNA, which may initiate cancer development by impairing normal gene expression (Goelz et al., 1985; Kim et al., 1997). High levels of acetaldehyde have been reported to break down folate *in vitro* (Shaw et al., 1989). Moreover, it has been shown that alcohol administration to rats for two weeks leads to local folate deficiency of the colonic mucosa (Homann et al., 2000b). These findings indicate that high alcohol intake together with low folate can play a major role in the initiation of colorectal cancer (Giovannucci et al., 1995; Homann et al., 2000b).

Alcohol is recognized as an immuno-suppressant, and this effect has also been suggested to be a contributing factor in the increased rate of cancer in alcoholics. However, the role of ethanol-induced immunosuppression in alcohol-related cancers has remained questionable, since the incidence of cancers of the immune system itself, such as lymphoma, the most common cancer associated with depressed immune function, is not increased by alcohol consumption (Blot, 1992).

The possible mechanisms through which acetaldehyde can be related to ethanol-associated carcinogenesis will be discussed separately in chapter 2.8., as well as the effect of genetic factors, i.e. polymorphism of alcohol-metabolizing enzymes, in chapter 2.4.

2.3. UPPER GASTROINTESTINAL MICROFLORA

Microbes in saliva

The composition of the microflora varies greatly from site to site in the mouth. Studies regarding the microbial flora of the mouth often deal with the microbiota in the dental plaque and bacteria dislodged and exfoliated from oral sites to saliva. Ethanol is present in saliva in concentrations comparable to those in blood (Jones, 1979), and saliva is in close contact with the mucosa of the upper digestive tract. For the purposes of this thesis, the focus of the following overview is on the microbes in saliva.

Human saliva contains approximately 10^7-10^9 microorganisms per millilitre. The microbes in saliva originate from various parts of the oral cavity, i.e. the teeth, tongue, cheek, and pharyngeal mucous membranes (Herrera et al., 1988). Streptococci, especially viridans group streptococci, are the most common aerobic group of bacteria at all sites of the mouth. This group of Gram-positive cocci accounts for approximately 45% of the total cultivable microbes in saliva (Marsh, 1980). *Streptococcus salivarius*, *Streptococcus mutans,* and α-haemolytic streptococci, such as *Streptococcus sanguis, Streptococcus mitis, Streptococcus oralis* and anginosus group streptococci are the most numerous species and groups belonging to this group. Other aerobic microorganisms often isolated from saliva are Gram-positive *Stomatococcus, Staphylococcus*, *Micrococcus,* and *Corynebacterium* species and Gram-negative *Neisseria* and *Haemophilus* species (Jousimies-Somer et al., 2002; Marsh, 1980). Yeasts also belong to the aerobic microbial flora of the mouth, and can be found in about 40% of clinically healthy mouths (Marsh, 1980). *Candida albicans* species are the most numerous and prevalent yeasts in the oral cavity (Stenderup, 1990). Anaerobic bacteria in saliva are mainly comprised of Gram-positive rods like *Actinomyces, Lactobacillus, Bifidobacterium* and many novel *Eubacterium*-like genera and species, Gram-negative rods belonging to *Prevotella, Fusobacterium, Porphyromonas, Campylobacter* and *Bacteroides* genera, and Gram-negative cocci of *Veillonella* and *Capnocytophaga* species (Jousimies-Somer et al., 2002; Marsh, 1980).

Microbes in gastric juice

Since the normal pH of the gastric juice is below 3, the stomach is usually free of microbes. However, even in normochlorhydric persons, the stomach is not sterile all the time; e.g. during meals the acid in gastric juice is buffered, allowing the gastric pH to rise above 4 when the most acid resistant swallowed oral microbes can survive in the stomach (Drasar et al., 1969). The pH usually drops again below 3 quite fast after eating and the microbes are killed (Drasar et al., 1969; Hill, 1995). Consequently, permanent gastric flora can only occur when gastric acid secretion is impaired to the extent that the pH does not fall below 3-4. Microbial proliferation leading even to microbial overgrowth can be expected in the stomach if the pH of the gastric juice exceeds 5 (Gray and Shiner, 1967;

Stockbruegger, 1985). Gastric microbial overgrowth is a common finding in conditions with gastric hypo- or achlorhydria such as chronic atrophic gastritis, pernicious anemia and gastric surgery with vagotomy (Drasar et al., 1969; Stockbruegger et al., 1984). The prolonged use of drugs inhibiting gastric acid secretion, e.g. antacids, histamine-2-receptor antagonists and proton pump inhibitors, can also result in resident gastric colonization (Ruddell et al., 1990; Stockbruegger, 1985; Verdu et al., 1994). The primary source of the flora of the neutral stomach is the oral cavity. Contrary to the prevalence of aerobes and anaerobes in saliva, aerobes are usually more numerous in the gastric juice than anaerobes; the total counts for aerobes being 10^6-10^7 and for anaerobes 10^5-10^6 colony forming units per millilitre (Hill, 1985). The most commonly encountered aerobic bacteria in the gastric juice are viridans group streptococci, *Stomatococcus, Neisseria,* and *Corynebacterium* species, and the most prevalent and numerous anaerobes are *Actinomyces, Prevotella, Lactobacillus,* and *Veillonella* species (Hill, 1985, 1995). Occasionally, some bacteria belonging to *Enterobacteriaceae* can also be isolated from the neutral gastric juice (Drasar et al., 1969; Hill, 1985, 1995).

Helicobacter pylori infection in the stomach makes an exception to what was discussed above; it can survive and proliferate in the acidic stomach. *Helicobacter pylori* colonizes the mucosa of the stomach below the mucin barrier, and is thus protected from the luminal acid. Under the mucosal barrier it is still protected from the local acid production by its acid neutralizing urease activity, thus allowing the bacteria to proliferate (Marshall et al., 1990).

Alterations caused by chronic alcohol intake

So far, there are no well-controlled studies showing whether chronic alcohol consumption directly alters the oral flora. Studies done by Harris et al. (1996, 1997) suggest that alcohol abusers have not as good dental hygiene as abstainers or moderate alcohol consumers. Poor dental hygiene may lead to overgrowth of some microbes in the oral cavity. In addition, deficient diets and the suppressed immune defence system may favour microbial proliferation in the mouth (MacGregor, 1986; Oksala, 1990). Furthermore, many alcohol abusers are also heavy tobacco smokers (Harris et al., 1996), and smoking is known to increase the presence of yeasts and Gram-positive bacteria in the oral cavity (Colman, 1976; MacGregor, 1988; Sakki and Knuuttila, 1996).

Regarding gastric flora, mucosal bacterial overgrowth in the stomach is more prevalent in heavy drinkers and with higher microbial counts than in non-alcoholic controls (Hauge et al., 1997). This finding may result from the increased pH of the gastric juice.

2.4. ALCOHOL METABOLIZING ENZYMES

Alcohol dehydrogenase

Alcohol dehydrogenase (ADH) is the main enzyme catalyzing the oxidation of ethanol to acetaldehyde in mammals. The reversible reaction is as follows:

$$CH_3CH_2OH + NAD^+ \leftrightarrow CH_3CHO + NADH + H^+$$

ADH is an $NAD^+(NADP^+)$-dependent enzyme that is expressed as numerous isoenzymes with different kinetic properties and substrate preferences. Human ADHs can be grouped into five classes, I-V, based on the characteristics of their primary structure (Jörnvall and Höög, 1995). Functional ADH enzymes are dimers consisting of either two similar subunits or two distinct subunits belonging to the same class. ADHs are mostly present in the cytosolic fraction of the cells.

The most important enzymes in hepatic ethanol elimination are the class I ADHs. These enzymes have both a low K_m (about 1 mM) and a high V_{max} for ethanol, and consequently, they are capable of eliminating ethanol from the blood at a constant rate to very low ethanol concentrations. Since the K_m of ADH for acetaldehyde is only 0.6 mM, acetaldehyde needs to be rapidly oxidized further to acetate at the same time with ethanol oxidation to keep the reverse ADH-mediated reaction running in the right direction (Blair and Vallee, 1966). Ethanol oxidation to acetaldehyde via ADH increases the liver NADH/NAD ratio, which leads to a significant reduction in the redox state of this organ. This phenomenon also accounts for many acute metabolic effects of ethanol, such as the inhibition of hepatic gluconeogenesis, the decrease in citric acid cycle activity, and the impairment of fatty acid oxidation (Lieber, 1994).

Class I isoenzymes are expressed by three genes, *ADH1, ADH2,* and *ADH3*, which encode protein subunits α, β, and γ. *ADH2* and *ADH3* are polymorphic genes; three different allelic forms *(ADH2*1, ADH2*2,* and *ADH2*3)* have been found for *ADH2,* and two *(ADH3*1* and *ADH3*2)* for *ADH3.* The distribution of these alleles differs by race; the frequency of the *ADH2*1* allele, for example, has been estimated to be about 85% in Caucasian populations, but only 15% in Asian populations, whereas the *ADH2*2* allele is predominant in Asians (Bosron and Li, 1986; Goedde et al., 1992). The frequency of *ADH3*1* is approximately 50-60% in Caucasians and higher than 90% in Asians (Bosron and Li, 1986). Alleles *ADH2*2* and *ADH3*1* encode the most active enzymatic forms of the protein subunits, e.g. individuals having the *ADH3*1/*1* genotype metabolize ethanol to acetaldehyde 2.5 times faster than individuals with other *ADH3* genotypes, and individuals with the *ADH2*2/*2* genotype even 40 times faster than individuals with the *ADH2*1/*1* genotype (Bosron and Li, 1986). Interestingly, an enhanced risk of upper digestive tract cancers has been associated with the rapidly metabolizing *ADH3* genotype in some studies (Coutelle et al., 1997; Harty et al., 1997; Seitz et al., 2001), while two studies have reported opposite findings (Bouchardy et al., 2000; Olshan et al., 2001).

The mucosa of the gingiva and tongue expresses class III and class IV ADH isoenzymes. The estimated K_m value for ethanol of the gingival ADH is 27 mM (Dong et al., 1996). The main ADH isoenzyme of the esophagus belongs to class IV, although some other ADHs of class I have also been observed. The K_m value for ethanol of the esophageal class IV is 12 mM (Yin et al., 1993). Both of these high K_m values indicate that ethanol can be oxidized both in the mouth and in the esophagus during and after ethanol challenge. Additionally, the esophagus is known to possess the highest ADH activity of the organs in the digestive tract with a rate per milligram of protein similar to that of the liver, and about four times that of the stomach enzyme (Parés and Farrés, 1996).

The stomach expresses many ADH isoenzymes, of which classes I and IV are postulated to be the most important ones. Class I ADH's K_m value for ethanol is 1 mM and class IV ADH's 40 mM (Parés et al., 1992; Seitz and Oneta, 1998; Yin et al., 1997). Since class IV ADH is characteristic for the upper digestive tract and class I for the rest of the intestinal tract, the stomach seems to be the transition site for the expression of these ADH classes (Yin et al., 1997). The gastric ADHs have been suggested to play a marked role in the first-pass metabolism of ethanol. According to this theory, intragastric ethanol metabolism explains the differences in blood ethanol concentrations observed after either oral or intravenous ethanol administration (Julkunen et al., 1985). This theory has long been a subject of debate and its significance in total ethanol elimination still remains unclear. Seitz and Pöschl (1997) estimated that the first-pass metabolism of ethanol accounts for 1 to 20% of the total ethanol metabolism.

As mentioned earlier, the small and large intestine exhibit mainly class I ADH, with a K_m value for ethanol of 1-2 mM (Seitz and Oneta, 1998). This value corresponds to the ethanol concentrations commonly measured from the colon during alcohol consumption. The ADH activity of the colonic mucosa is similar to gastric ADH activity (Seitz et al., 1996). This suggests that ethanol may be effectively metabolized to acetaldehyde by the colonic mucosa as well.

The ADH-mediated reaction can be reduced by 4-methylpyrazole (4-MP), a drug that competitively inhibits the oxidation of ethanol to acetaldehyde by ADH (Li and Theorell, 1969; Salaspuro, 1985). The inhibitory effect of 4-MP can also be seen in the dose-dependent reduction of the total ethanol elimination rate (Salaspuro, 1985). 4-MP is used in the clinical practice in the treatment of methanol and ethylene glycol poisonings (Jacobsen and McMartin, 1997). In addition, it is efficient in the management of the disulfiram-alcohol reaction (Lindros et al., 1981) and the so-called flushing reaction of ALDH2-deficient subjects (Inoue et al., 1985).

Aldehyde dehydrogenase

The second reaction in alcohol metabolism, the oxidation of acetaldehyde to acetate, is catalyzed by aldehyde dehydrogenase (ALDH). Like ADH, ALDH needs NAD^+($NADP^+$) in order to act as a catalyst, and it is also expressed as many isoenzymes. In humans, at least 4-5 ALDH

isoenzyme classes have been isolated, and they are found both in the cytosolic and in the mitochondrial fraction of the cells (Agarwal, 1997). The isoenzyme mainly responsible for acetaldehyde oxidation is the mitochondrial class II ALDH (ALDH2), which has a micromolar K_m value and a high affinity for acetaldehyde (Lands, 1998). ALDH1 and ALDH5 also have micromolar K_m's for acetaldehyde, while most ALDH3 and ALDH4 isoenzymes possess millimolar K_m values.

The ALDH2 enzyme is polymorphic in humans, having two allelic forms, *ALDH2*1* and *ALDH2*2*. The *ALDH2*2* allele is a result of a single point mutation in the area of chromosome six coding the normal *ALDH2*1* allele. Individuals homozygous for this mutated *ALDH2*2* allele lack ALDH2 activity, whereas heterozygous individuals with the *ALDH2*1/*2* genotype have 30-50% of the activity of *ALDH2*1* homozygotes (Crabb et al., 1989). Certain Asian populations show relatively high frequencies of the *ALDH2*2* allele, e.g. about 50% of the Japanese express this ALDH2 variant, while it is extremely rare in Caucasian populations (Goedde et al., 1979, 1992). Partial or total inactivation of ALDH2 leads to the accumulation of acetaldehyde in the body. Blood acetaldehyde levels have been reported to be six and twenty times higher in subjects heterozygous and homozygous for the mutant allele, respectively, than in persons with normal ALDH2 activity (Yokoyama, 1996a). Elevated blood acetaldehyde levels can cause numerous unpleasant symptoms, such as flushing of the face and body, tachycardia, drop in blood pressure, headache, and nausea. Therefore, the homozygous form of the mutant *ALDH2*2* allele offers almost full protection against alcoholism, but despite the flushing symptoms, heterozygotic subjects may become heavy drinkers or even alcoholics (Chen et al., 1999; Higuchi et al., 1994; Peng et al., 1999). Alcohol-drinking individuals with low-activity ALDH2 can thereby be considered as human "knock-out models" for deficient acetaldehyde removal. Interestingly, many recent epidemiological studies have shown an increased risk of digestive tract cancers, and especially of upper digestive tract cancers, among heavy-drinking ALDH2-deficient subjects (Murata et al., 1999; Tanabe et al., 1999; Yokoyama et al., 1996a-c, 1998a,b).

Since the liver is the main organ for ethanol oxidation, the bulk of the ALDHs exist there. However, other organs also exhibit ALDH isoenzymes. ALDH3 has been detected in the mouth (Dong et al., 1996), and esophagus, which also exhibits ALDH1 (Yin et al., 1993). The stomach expresses ALDH classes 1, 2, and 3, which suggests that this organ could be a significant place for acetaldehyde oxidation (Yin et al., 1997). ALDH classes 1 and 2 have been found in the human duodenum (Liao et al., 1991) and classes 1, 2 and 3 in the colonic mucosa, but the expression of ALDH2, in particular, seems to be very low (Yin et al., 1994).

2.5. DISTRIBUTION OF ETHANOL IN THE BODY

Due to its small molecular size, good water solubility, but poor solubility in lipids, ethanol is absorbed from the gastrointestinal tract by simple diffusion (Wallgren and Barry III, 1970). Approximately 75 % of the ingested ethanol is absorbed from the proximal small intestine, duodenum and upper jejunum and about 25 % from the stomach. Delayed gastric emptying decreases the rate of ethanol absorption (Oneta et al., 1998). Eating, for example, is known to delay gastric emptying, and therefore slower rises and lower peak concentrations in blood ethanol levels can be detected after a meal (Jones et al., 1997).

After absorption, ethanol is distributed via circulation and diffusion throughout the body fluids. Alcohol rapidly equilibrates with the bloodstream in organs with dense vascularization and rich blood supply, such as the brain, lungs, and liver. Accordingly, the distribution of ethanol to the resting skeletal muscle is slow, since only part of the capillaries are functioning (Dundee et al., 1971). Due to ethanol's poor lipid solubility, tissue lipids can take up only about 4 % of the amount of alcohol dissolved in a corresponding volume of water. Thus women, having smaller total body water volumes than men, reach higher blood ethanol levels if both consume equal amounts of alcohol (Riveros-Rosas et al., 1997). The total volume of the body water is reduced with age, and changes similar to sex-related differences in blood ethanol levels can also be detected with ageing. The distribution of ethanol in the body is mainly related to the water content of various organs and tissues. Consequently, after alcohol consumption, ethanol concentrations in the terminal ileum (Halsted et al., 1973), colon (Levitt et al., 1982), and oral cavity (Jones et al., 1979) are equal to those in the blood. In contrast, the alcohol levels in urine are slightly higher than those in blood (Bendtsen et al., 1999).

About 90-95% of the absorbed ethanol is metabolized completely in the body, and excreted as CO_2 and water, so only a minor part of the ingested alcohol is excreted unaltered via expired air, sweat, and urine (Holford, 1987).

2.6. ETHANOL METABOLISM IN THE DIGESTIVE TRACT

Hepatic ethanol metabolism

It is generally agreed that most of the ethanol metabolism takes place in the liver. Under normal conditions the liver eliminates approximately 75-90% of ethanol (Agarwal and Goedde, 1990). In severe hepatic cirrhosis, the extrahepatic ethanol elimination can, however, rise up to 40% (Utne and Winkler, 1980). There are three metabolic pathways for ethanol oxidation in the liver: cytosolic alcohol dehydrogenase (ADH), microsomal ethanol oxidizing system (MEOS), and catalase, of

which the alcohol dehydrogenase pathway is the most important one. Because of the essential role of ADH in this thesis, the characteristics of the ADH-mediated pathways have been discussed separately in chapter 2.4.

The cytochrome P-450-dependent microsomal ethanol oxidizing system was first described by Lieber and DeCarli in 1968. It oxidizes ethanol to acetaldehyde as follows:

$$CH_3CH_2OH + NADPH^+ + H + O_2 \rightarrow CH_3CHO + NADP^+ + 2\ H_2O$$

In humans, CYP2E1 is the major cytochrome fraction responsible for ethanol oxidation. The MEOS contributes to ethanol elimination only at high blood ethanol levels since its K_m for ethanol is 7-10 mM. It has been estimated that the MEOS accounts only for about 1-5% of the total *in vivo* ethanol metabolism (Ingelman-Sundberg, 1997). The role of the adaptive CYP2E1 in the total ethanol elimination may, however, increase up to 10% in chronic alcohol consumers with constant high blood ethanol levels (Lieber, 1988).

Catalase, a haemoprotein located in the peroxisomes, can oxidize ethanol to acetaldehyde as follows:

$$CH_3CH_2OH + H_2O_2 \rightarrow CH_3CHO + 2\ H_2O$$

Since the presence of hydrogen peroxide is essential for catalase to be able to oxidize ethanol, the reaction is limited by the rate of its generation. The rate of hydrogen peroxide production in the liver is quite low (Boveris et al., 1972), which suggests that catalase plays only a minor role, less than 2%, in hepatic ethanol metabolism.

Other sites for ethanol metabolism

Other organs are also capable of oxidizing ethanol, although to a lesser extent than the liver. As already discussed in chapter 2.4., direct determinations of ADH activity in human tissues have revealed that ethanol may be actively metabolized in the digestive tract by the mucosa of the mouth (Dong et al., 1996), esophagus (Yin et al., 1993), stomach (Yin et al., 1997), and both the small and large intestine (Seitz et al., 1996; Seitz and Oneta, 1998). In addition, ethanol oxidation may occur in the kidneys (Leloir and Muñor, 1938), bone marrow cells (Wickramasinghe, 1981), lungs (Pikkarainen et al., 1981), testes (Boleda et al., 1989), and pancreas (Estival et al., 1981).

2.7. MICROBIAL ETHANOL METABOLISM

Alcoholic fermentation

Under anaerobic conditions, microbes cannot produce energy via respiration using oxygen as a terminal electron acceptor, so they derive energy via fermentation. In alcoholic fermentation, the pyruvate formed from glucose by glycolysis is converted anaerobically to ethanol and CO_2. The final step in alcoholic fermentation is the reduction of acetaldehyde to ethanol via microbial ADHs (Reid and Fewson, 1994). A detailed description of this phenomenon has been given for *Escherichia coli* (Clark,

1989; Dawes and Foster, 1956; Still, 1940; Wong and Barrett, 1983), group N streptococci (Lees and Jago, 1976), and *Enterobacteriaceae* in general (Salveson and Bergan, 1981).

Small amounts of endogenous ethanol can be found in the body fluids of mammals that have not received any alcohol. This phenomenon was first suggested to be a result of microbial alcohol fermentation by Krebs and Perkins in 1970. The finding was later confirmed in jejunal blind-loop rats with bacterial overgrowth (Baraona et al., 1986). In humans, marked endogenous ethanol levels have been measured in midjejunal aspirates of patients suffering from tropical sprue, a condition associated with intestinal overgrowth of *Entero-bacteriaceae* (Klipstein et al., 1973), and in the venous blood of patients after a jejunoileal bypass operation, a condition also known to lead to intestinal bacterial overgrowth (Mezey et al., 1975). In addition, small quantities of ethanol have been found in the gastric juice of patients receiving cimetidine or antacids. This has been suggested to result from the increased intragastric pH and microbial colonization of the stomach (Bode et al., 1984b). Moreover, microbial alcohol fermentation of ingested carbohydrates leading even to signs of ethanol intoxication has been reported to occur in Japanese patients (Kaji et al., 1984).

Ethanol oxidation

Under aerobic conditions, the reaction catalysed by microbial ADHs runs in the opposite direction of that described above (Maconi et al., 1988). In this reaction, ethanol is oxidized to acetaldehyde, and in that way used as an energy and carbon source. The characteristics of the microbially mediated acetaldehyde production from ethanol have been established in several *in vitro* and *in vivo* studies which will be reviewed in the following sections.

In the upper digestive tract, significant *in vitro* microbially mediated acetaldehyde production has been reported when human mouth and bronchopulmonary washings were incubated with ethanol (Jauhonen et al., 1982; Miyakawa et al., 1986; Pikkarainen et al., 1981). Furthermore, the mouth washings of patients with oropharyngeal cancer have been shown to produce increased amounts of acetaldehyde *in vitro*. This suggests that microbially mediated acetaldehyde production may be involved in ethanol-associated organ toxicity (Jokelainen et al., 1996b). Marked production of acetaldehyde has also been demonstrated in saliva in both *in vivo* and *in vitro* studies. This acetaldehyde production can be significantly reduced by using antiseptic chlorhexidine mouthwash, which indicates that acetaldehyde production is of microbial origin (Homann et al., 1997a). The same study also showed that there is a highly significant positive correlation between *in vivo* and *in vitro* salivary acetaldehyde production. Later *in vitro* studies have revealed that salivary acetaldehyde production is strongly influenced by individual factors, heavy tobacco smoking and alcohol drinking being the most important factors, which increase the production of acetaldehyde in saliva (Homann et al., 2000a). In addition, it has been demonstrated that especially

some oral *Candida albicans* strains have a high capacity to produce acetaldehyde from ethanol *in vitro* (Tillonen et al., 1999a). Marked cytosolic ADH activity has also been found in *Helicobacter pylori*, which can, consequently, produce significant amounts of acetaldehyde when incubated with ethanol *in vitro* (Roine et al., 1992, 1995; Salmela et al., 1993, 1994).

Regarding the small and large intestine, the first findings of microbial ethanol metabolism were reported as early as 1940 when Still showed that *Escherichia coli* possesses ADH activity. As mentioned earlier, this finding was later confirmed by many others. Baraona et al. showed in 1986 that microbial intraintestinal acetaldehyde production from ethanol also occurs *in vivo* in rats with a jejunal self-filling diverticulum and bacterial overgrowth. Furthermore, Seitz et al. (1990) found that the acetaldehyde concentration of the rectal mucosa was markedly higher in conventional rats than in germ-free rats after ethanol administration. A new microbiological approach for acetaldehyde production and the pathogenesis of ethanol-related gastrointestinal diseases was opened up by studies of Jokelainen et al., who first described a bacteriocolonic pathway for ethanol oxidation. These *in vitro* studies showed that human colonic contents can produce acetaldehyde from ethanol in a dose-dependent manner (Jokelainen et al. 1994), and that certain aerobic colonic bacteria can produce high amounts of acetaldehyde from ethanol by their ADH enzymes (Jokelainen et al. 1996a). In addition, it was demonstrated *in vivo* that both intragastric and intravenous ethanol administration to pigs lead to a marked increase in intracolonic acetaldehyde levels (Jokelainen et al. 1996c). Later *in vitro* studies have characterized the ADHs of human colonic bacteria in more detail (Nosova et al., 1997), and revealed that ethanol oxidation by *Escherichia coli* can also occur under microaerobic (6% O_2) conditions (Salaspuro et al., 1999). Moreover, high acetaldehyde levels have been detected in the caecal samples of rats after an acute intraperitoneal dose of ethanol (Visapää et al., 1998).

The bacteriocolonic pathway for ethanol oxidation can be modulated by treatment with antibiotics. Ciprofloxacin, which decreases the number of aerobic bacteria in the large intestine, also reduces the total ethanol elimination rate approximately by 9% and the faecal ADH activity both in rats (Jokelainen et al., 1997) and in humans (Tillonen et al., 1999b). Moreover, in rats treatment with ciprofloxacin totally abolishes the enhancement in the ethanol elimination rate caused by chronic ethanol administration (Nosova et al., 1999). The opposite effects can be found with metronidazole treatment, which is known to reduce the anaerobic flora of the large intestine, thus enhancing the growth of ADH-containing aerobes in the gut (Tillonen et al., 2000; Visapää et al., 2001b). The rats receiving metronidazole have five times higher intracolonic acetaldehyde levels than the rats receiving only ethanol (Tillonen et al., 2000).

Acetaldehyde oxidation

Yeasts and anaerobic bacteria possess aldehyde dehydrogenase activity (Steinman and Jakoby, 1968; Burdette and Zeikus,

1994). Furthermore, *Escherichia coli* (Dawes and Foster, 1956; Wong and Barrett, 1983) and many other bacteria belonging to *Enterobacteriaceae* (Nosova et al., 1996), as well as some oral *Neisseria* species (Muto et al., 2000) are known to exhibit ALDH activity. However, the ability of bacterial ALDHs to oxidize acetaldehyde to acetate seems to be rather low as compared to their ADH activity (Nosova et al., 1998, Muto et al., 2000). Considering the fact that ALDH activity e.g. in the colonic mucosa is rather low (Koivisto and Salaspuro, 1996), these studies suggest that acetaldehyde may accumulate in the gastrointestinal tract.

2.8. ORGAN TOXICITY OF ACETALDEHYDE

Cyto- and genotoxicity

Acetaldehyde has many mutagenic and carcinogenic effects both in cell culture conditions and in animal studies (IARC, 1999). It can induce chromosomal aberrations and micronuclei and/or sister chromatid exchanges in cultured mammalian cells (Dellarco, 1988; IARC, 1999), and gene mutations in human lymphocytes (He and Lambert, 1990). The ability of acetaldehyde to form DNA-DNA and/or DNA-protein cross-links may be responsible for the induction of these cytogenetic effects. *In vitro* studies with the human adenocarcinoma cell line Caco-2 show that acetaldehyde decreases some brush border enzyme activities and alters certain cell properties including an increase in the proliferation rate and disturbed cell differentiation. These results also suggest more aggressive and invasive tumour behaviour *in vivo* (Koivisto and Salaspuro, 1997, 1998).

Studies with experimental animals have provided sufficient evidence for the carcinogenicity of acetaldehyde in animals (IARC, 1999). An acetaldehyde inhalation experiment in rats showed an increased incidence of carcinomas in the nasal mucosa (Woutersen et al., 1984). Another inhalation study with hamsters resulted in an enhanced number of laryngeal carcinomas (Feron et al., 1982). In addition, a study where rats were given water with or without acetaldehyde showed marked histopathological hyperplastic and hyper-proliferative changes in the tongue, epiglottis, and forestomach in the animals receiving acetaldehyde (Homann et al., 1997b).

Recent studies on the associations between genotypes of ethanol- and acetaldehyde-metabolizing enzymes and cancer risk have provided strong epidemiological evidence for the carcinogenic action of acetaldehyde in humans. Some studies report an enhanced risk of upper gastrointestinal tract tumours to be associated with the rapid metabolizing *ADH3*1/*1* genotype, which leads to higher and quicker production of acetaldehyde (Coutelle et al., 1997; Harty et al., 1997; Seitz et al., 2001). Very recently, increased salivary acetaldehyde levels after alcohol consumption were detected in individuals with this genotype (Li et al., 2001).

Furthermore, ALDH2-deficiency, which leads to longer acetaldehyde exposure, increases the risk of alcohol-associated cancers in the oropharynx, larynx, esophagus, stomach, colon, and lungs, but not in the liver (Murata et al., 1999; Tanabe et al., 1999; Yokoyama et al., 1996a-c, 1998a,b). This phenomenon has so far been hypothesized to arise from the systemic effects of blood's elevated acetaldehyde concentration. Most interestingly, however, all the organs with enhanced cancer risk are covered with microbes. They are also places where microbial ethanol metabolism and acetaldehyde production have been described (Homann et al., 1997; Jokelainen et al., 1996c; Miyakawa et al., 1986; Pikkarainen et al., 1981). These findings, thus, suggest that local microbially mediated acetaldehyde production from ethanol might be involved in the pathogenesis of these cancers.

Acetaldehyde-protein adducts

The electrophilic nature of the carbonyl carbon of acetaldehyde makes it suitable for potential nucleophilic attacks (Sorrell and Tuma, 1987). As nucleophilic groups are commonly present in proteins, they are the natural binding targets for acetaldehyde in various tissues. The binding of acetaldehyde with proteins results in the formation of two types of products, which are classified as unstable and stable acetaldehyde-protein adducts (Sorrell and Tuma, 1985). Subsequently, the unstable adducts can either re-dissociate to acetaldehyde and protein or be stabilized by treatment with reducing agents such as NADH to stable acetaldehyde-protein adducts. The stable adducts appear to be the most likely candidates to produce toxic effects (Nicholls et al., 1992).

Acetaldehyde binds covalently to many cellular and extracellular proteins *in vitro* (Nicholls et al., 1992). *In vivo*, acetaldehyde forms multiple adducts with proteins, such as hemoglobin (Sillanaukee and Koivula, 1990). Furthermore, adduct formation occurs in the liver of experimental animals and humans (Lin et al., 1988, Niemelä et al., 1991). Immuno-histochemical techniques have been used to localize adducts in the liver. In these studies, acetaldehyde-protein adducts have been detected in the cytoplasm of the perivenular hepatocytes (Niemelä et al., 1991), in the areas of active fibrogenesis in alcoholic patients (Holstege et al., 1994), in the rough endoplasmic reticulum, and in some peroxisomes of hepatocytes, as well as in myofibroblasts and Ito cells (Paradis et al., 1996).

The exact role of acetaldehyde-protein adducts in the pathogenesis of alcohol-induced diseases has not been fully clarified, but several mechanisms have been proposed. Acetaldehyde adduct formation may alter the structure of the modified proteins, and thus interfere with their normal cellular functions (Sorrell and Tuma, 1987). Acetaldehyde also inhibits the function of the human DNA repair protein O^6-methylguanine transferase both *in vivo* and *in vitro*, which may occur even at nanomolar concentrations (Garro et al., 1986; Espina et al., 1988). In addition, acetaldehyde-protein adducts may be recognized as neoantigens by the immune system, and in this manner they may trigger harmful immune responses (Nicholls et al.,

1992). Circulating antibodies against acetaldehyde-protein adducts have indeed been detected in humans (Israel et al., 1986; Niemelä et al., 1987). These antibodies may contribute to the development and progression of liver injury, which suggests that immunological mechanisms are also involved in the pathogenesis of alcoholic liver damage (Tuma and Klassen 1992).

Both exogenous and metabolically derived acetaldehyde can bind with gastric mucosal proteins in rats (Salmela et al., 1997). This has been suggested to be one possible factor behind alcohol-associated gastric injury. So far, there is no evidence indicating that such adduct formation would occur at other sites of the digestive tract. Similar adduct formation could, however, also take place in the oral cavity or colon due to the high microbial production of acetaldehyde.

Acetaldehyde-DNA adducts

As a highly reactive agent, acetaldehyde can form adducts not only with proteins, but also with DNA bases (Hemminki and Suni, 1984; Vaca et al., 1995). In fact, the formation of acetaldehyde-DNA adducts is considered to be a critical event in the initiation of chemical carcinogenesis in alcohol consumers (Vaca et al., 1995). Acetaldehyde-DNA adducts have been identified in the liver of mice after chronic alcohol administration (Fang and Vaca, 1995). In humans, enhanced formation of these adducts has been detected in peripheral white blood cells of alcohol abusers (Fang and Vaca, 1997). Moreover, DNA adducts have been found in the colonic mucosa of patients with colorectal cancer (Pfohl-Leszkowicz et al., 1995), and in human buccal cells exposed to acetaldehyde *in vitro* (Vaca et al., 1998).

Lipid peroxidation

Lipid peroxidation is a degradative process caused by harmful actions of oxidizing free radicals, superoxide and hydroxyl radicals. Free radicals are molecules that contain one or more unpaired electrons, and thus are very reactive with a short half-life. These highly reactive molecules can abstract a hydrogen atom from a polyunsaturated fatty acid, and thereby initiate lipid peroxidation. Since lipids are major components of biological membranes, peroxidative loss of membrane integrity may lead to tissue injury (Mufti et al., 1993). Cells are normally protected against free radicals by glutathione, which is present in all animal cells in high concentrations. A severe reduction in glutathione levels increases lipid peroxidation *in vivo* (Wendel et al., 1979). Enhanced lipid peroxidation has been proposed to be one of the key mechanisms for ethanol-induced liver injury (Situnayake et al., 1990). One explanation for this could be acetaldehyde's capacity to reduce hepatic glutathione levels (Shaw et al., 1981), and so to induce lipid peroxidation, as demonstrated in isolated perfused livers (Müller and Sies, 1982). Furthermore, high acetaldehyde concentrations administered to rats have been reported to result in the formation of free radicals *in vivo* (Reinke et al., 1987). Lipid peroxidation products can also react with DNA and form adducts with known carcinogenicity and miscoding potential (Brooks, 1997). Accordingly, lipid peroxidation may play a prominent role in ethanol-associated carcinogenesis.

3. AIMS OF THE STUDY

Excessive alcohol consumption is associated with an increased risk of cancer of the upper digestive tract. However, the pathogenetic mechanisms responsible for the enhanced risk of cancer in alcoholics are not completely understood. Many recent studies have suggested that ethanol-associated digestive tract cancers might be caused by the local carcinogenic action of the first metabolite of ethanol oxidation, acetaldehyde. A lot of research has lately been carried out to explore the production and effects of this toxic compound in the digestive tract. It is now known that many microbes of the alimentary tract can produce acetaldehyde from ethanol in the gut. Acetaldehyde has been shown to be carcinogenic in animals, but so far there has not been enough evidence in humans.

The specific aims of this study were:

1. To examine salivary acetaldehyde production from ethanol in subjects with different *ALDH2* genotypes in order to find evidence for the local carcinogenic action of acetaldehyde in humans.

2. To investigate whether it is possible to reduce the local production of acetaldehyde in saliva by using 4-methylpyrazole prior to ethanol exposure.

3. To study ethanol metabolism in the hypochlorhydric stomach associated with the use of gastric proton pump inhibitors and atrophic gastritis, and to relate the findings to changes in gastric microbial flora.

4. To examine further which bacterial species and/or groups are responsible for acetaldehyde formation in the hypochlorhydric stomach, and to characterize their ADH enzymes.

4. MATERIALS AND METHODS

4.1. ETHICAL CONSIDERATIONS

All studies were approved by the Ethical Committee of the Department of Medicine, Helsinki University Central Hospital, and an informed consent to participate in the study was obtained from the subjects. The study with 4-methylpyrazole (II) was also approved by the Finnish National Agency for Medicines.

4.2. THE EFFECT OF ALDEHYDE DEHYDROGENASE-2 GENOTYPE ON SALIVARY ACETALDEHYDE PRODUCTION (I)

Subjects

Twenty subjects of Asian origin took part in the study (12 men, 8 women; age range 22-44 years, mean body weight 60±2 kg). All subjects were healthy, and none of them had received any antibiotics or used antiseptic mouthwashes for four weeks preceding the study. All subjects were told to refrain from alcohol for at least 36 hours before the study.

Study design

The studies started between 9.00 and 10.00 a.m. The volunteers were allowed to eat a light breakfast at least 90 minutes before the study. A commercially available paraffin wax chewing gum (Orion Diagnostics, Espoo, Finland) was used to stimulate the production of saliva. After baseline saliva collection, each volunteer ingested 0.5 g ethanol/kg body weight in a standardized 10% v/v solution of absolute ethanol in orange juice within 20 minutes. To remove local ethanol, the subjects rinsed their mouths with water, and thereafter the saliva samples were taken every 20 minutes until the systemic ethanol level returned to zero. Salivary acetaldehyde and ethanol levels were measured by using headspace gas chromatography. Blood samples for *ALDH2* genotyping and for gas chromatographic ethanol and acetaldehyde level determinations were taken from an antecubital vein 60 minutes after the start of alcohol ingestion.

In vitro and in vivo salivary acetaldehyde production

In vitro salivary acetaldehyde production was determined by incubating 400 µl of oral saliva with 50 µl of 22 mM ethanol (final concentration) for 90 minutes at 37°C. The reaction was stopped by injecting 50 µl of 6 M perchloric acid (PCA) through the rubber septum of the closed vial. To measure *in vivo* salivary acetaldehyde levels, 450 µl of saliva was immediately transferred into a vial containing 50 µl of PCA. Acetaldehyde and ethanol levels were analysed by using headspace gas chromatography.

Determination of blood acetaldehyde and ethanol

Samples for blood acetaldehyde measurement were collected into evacuated blood collecting tubes containing sodium fluoride and potassium oxalate as anticoagulants for 3 ml of blood. Immediately after sampling, 0.5 ml of blood was pipetted into 2 ml of ice-cold 0.6 M PCA made in saline and instantly deproteinized with a whirlimixer. The precipitated proteins were spun down by centrifugation at 4000 g; thereafter, 500 µl of the clear supernatants were transferred into glass vials, sealed with Teflon-coated rubber stoppers, and analysed by using headspace gas chromatography. For the measurement of blood ethanol concentration, blood was collected into 3 ml tubes containing ethylenediaminetetra-acetic acid (EDTA). 500 µl of blood was pipetted into vials, and thereafter blood ethanol levels were analysed by using headspace gas chromatography.

ALDH2 genotyping

ALDH2 genotyping was performed at the Institute of Human Genetics, University of Hamburg, Hamburg, Germany. Genomic deoxyribonucleic acid (DNA) was extracted from leukocytes isolated from EDTA-blood. *ALDH2* genotyping was carried out by using polymerase chain reaction/restriction fragment length polymorphism (PCR-RFLP) according to the method of Harada and Zhang (1993) with modifications suggested by Suzuki et al. (1994). 1 µl of genomic DNA was mixed with 0.5 µl (20 pmol) of each primer (5′-CAA ATT ACA GGG TCA AGG GCT-3′ sense; 5′-CCA CAC TCA CAG TTT TCT CTT-3′ antisense) in a total volume of 25 µl containing 0.5 µl of 10 mM deoxynucleotides (dNTPs), 0.8 µl of 50 mM $MgCl_2$, 1 unit of Taq DNA polymerase, 2.5 µl of PCR buffer and 19 µl of water. Thirty cycles of PCR (denaturation at 94°C for 90 sec, annealing at 58°C for 180 sec and extension at 72°C for 60 sec) were performed. Each PCR product was digested with the restriction enzyme Mbo II for 3-4 hours. Just before electrophoresis, the products were incubated at 60°C for 5 minutes and chilled on ice water. The digest was separated in 7% polyacrylamide gels under 10 V/cm for 1 hour. The gel was stained with silver nitrate solution as described by Budowle et al. (1991), and the genotypes were identified on the basis of the size of the restriction fragments.

4.3. PAROTID GLAND CANNULATION STUDY (I)

Subjects and study design

Three ALDH2-deficient Asians (males) and three Finnish volunteers (one female, two males) with normal ALDH2 participated in the study. All subjects were healthy, they had not received any antibiotics or used antiseptic mouthwashes for four weeks preceding the study, and had not used alcohol for at least 36 hours before the study.

At the beginning of the study all subjects ingested ethanol (0.5g/kg of body weight) in orange juice as 10% v/v solution within 20 minutes. In order to obtain sterile parotid gland saliva, the duct of the right parotid gland was cannulated with a sialography catheter. Parotid gland saliva was collected 60 to 80 minutes after ethanol ingestion, and its ethanol and acetaldehyde concentrations were determined by using headspace gas chromatography.

4.4. THE EFFECT OF 4-METHYLPYRAZOLE ON ETHANOL METABOLISM AND SALIVARY ACETALDEHYDE PRODUCTION (II)

Subjects

Five ALDH2-deficient Chinese (two females and three males; mean age 31±3 years; mean body weight 63±7 kg) and six Finnish volunteers (males; mean age 25±2 years; mean body weight 78±4 kg) with normal ALDH2 took part in the study. All subjects were moderate alcohol consumers (less than 70 g of ethanol per week). Two of the volunteers in both groups were light smokers, all others were nonsmokers. Other criteria for the study were the same as in study I (chapter 4.2.).

Study Design

In this study, we had two study days which were separated by a 1-week interval. The basic study design of the experiments on both study days was the same as in study I (chapter 4.2.). In the present study, we additionally followed the blood pressure, heart rate, and skin temperature of the volunteers. A bit lower dose of ethanol, 0.4 g/kg body weight, was also used. On the second study day, the volunteers received 4-methylpyrazole (4-MP) 10-15 mg/kg body weight orally two hours before other experiments. The liquid solution of 4-MP was specially prepared for the study at the Helsinki University Central Hospital Pharmacy.

In vitro and in vivo salivary acetaldehyde production

Salivary acetaldehyde and ethanol levels were determined as described in chapter 4.2.

Determination of blood acetaldehyde and ethanol

Blood acetaldehyde and ethanol were measured as described in chapter 4.2.

Determination of ethanol elimination rate

Salivary ethanol levels, which are known to be comparable to blood ethanol levels (Jones, 1979), were used in the determination of the ethanol elimination rate in this study. The concentration-time profiles of ethanol were evaluated according to zero order kinetics. This pharmacokinetic model assumes a rectilinear disappearance of ethanol from blood after the absorption and distribution of the dose is completed. The ethanol elimination rate from the body was obtained by dividing the dose given (0.4 g/kg) by the estimated time of reaching zero concentration of ethanol in blood.

4.5. THE EFFECT OF IATROGENIC HYPOCHLORHYDRIA ON INTRAGASTRIC ACETALDEHYDE PRODUCTION (III)

Subjects

Eight healthy men, with an age range of 21-25 years volunteered for the study. Their mean body weight was 73±3 kg and body mass index 22.7±0.7 kg/m². The subjects had not received any antibiotics for four weeks prior to the study neither did they use any other drugs during the study days. One of the volunteers was a light smoker and all were normal social drinkers, with an average consumption of 90 g or less (range 40-120 g) of ethanol per week.

Study design

A paired study design in which each subject served as his own control was used. Two study days were separated by a 1-week interval. The volunteers fasted for at least six hours before the study, and were admitted to the Department of Gastroenterology, Helsinki University Central Hospital, Helsinki, Finland, where all studies started between 1.30 and 2.00 p.m. Ethanol (0.6 g/kg body weight) was diluted in water at 15% v/v concentration. The volunteers ingested the dose within 20 minutes, and thereafter stayed on their left sides for 40 minutes to avoid total gastric emptying. At 60 minutes a paraffin-stimulated saliva sample was collected and gastroscopy performed. Gastric juice was aspirated into collectors immediately after the gastroscope (Olympus, GIF-Q140) entered the stomach. The pH of the obtained gastric juice was determined by using a glass electrode and a digital pH meter (WTW pH-521, Weilheim, Germany), and ethanol and acetaldehyde levels were measured by using headspace gas chromatography. Part of the gastric juice was frozen to -80°C for later microbial analysis. During the seven days between the experiments, the volunteers received 30 mg lansoprazole orally twice a day. Experiments were the same on both study days, except for routine gastric mucosal biopsies, which were only taken during the first endoscopy.

In vivo intragastric and salivary acetaldehyde production

Acetaldehyde and ethanol were measured from gastric juice and saliva by using headspace gas chromatography as described in chapter 4.2.

Microbial analysis

The microbiological analyses were performed at the Anaerobe Reference Laboratory of the National Public Health Institute, Helsinki, Finland. The gastric juice samples were thawed, and diluted in peptone yeast extract broth. An aliquot of 100 µl of the undiluted sample and its 100-fold dilutions were inoculated and spread on several selective and non-selective agar media for the enumeration and isolation of the total counts and main groups of aerobic and anaerobic bacteria and yeasts. The aerobic plates were incubated at 35°C in an atmosphere containing 5% CO_2 for up to five days; the anaerobic plates were

incubated in anaerobic jars which were filled by using the evacuation replacement method with a gas mixture (90% N_2, 5% CO_2, 5% H_2) for seven days for the first inspection and up to 14 days for the final inspection. The bacteria were enumerated and identified by using established methods (Murray et al., 1995; Summanen et al., 1993).

4.6. INTRAGASTRIC ETHANOL METABOLISM IN PATIENTS WITH ATROPHIC GASTRITIS (IV)

In vivo ethanol metabolism in the stomach

Seven patients (one male and six females) with achlorhydric atrophic gastritis and five healthy subjects (two males and three females) volunteered for the study. Six of the atrophic gastritis patients had also been diagnosed for pernicious anaemia. The mean age of the volunteers was 60±3 years for the atrophic gastritis patients, and 26±1 years for the controls. The mean body weights were 71±5 kg and 66±3 kg for the atrophic gastritis patients and controls, respectively. None of the subjects had used any antibiotics, proton pump inhibitors or histamine-2-receptor antagonists for four weeks preceding the study. All volunteers were nonsmokers and normal social drinkers, with an average consumption of 70 g or less of ethanol per week.

The volunteers fasted over night before the study days, which were separated by a 1-week interval. The participants were admitted to the Department of Gastroenterology, Helsinki University Central Hospital, Helsinki, Finland, and all studies started between 8 and 9 a.m. A nasogastric tube (Flocare CH8, Chatel Medical Devices SA, Chatel St Denis, Switzerland) was placed into the volunteers at the beginning of both study days, and the stomach was aspirated empty. On the first study day, glucose (3 ml/kg body weight) at 10% v/v solution was infused into the stomach of the subjects. On the second day, ethanol (0.3 g/kg body weight) diluted in water at 15% v/v concentration was used instead of glucose. After the infusions the volunteers stayed on their left sides to avoid total gastric emptying. 15 ml of gastric juice was aspirated from each volunteer into a collector at 30 and 60 minutes after both infusions. Thereafter, the gastric juice was transferred into gas chromatograph vials, and to cryovials which were frozen to -80°C for later microbial analysis. The pH of the obtained gastric juice was determined by using a glass electrode and a digital pH meter. At the end of both study days 5 ml of paraffin stimulated saliva was collected from each volunteer.

In vivo intragastric and salivary acetaldehyde production

Acetaldehyde and ethanol were measured from gastric juice and saliva immediately after sampling by using headspace gas chromatography as described in chapter 4.2.

Microbial analysis

The microbial analysis of the gastric juice and salivary samples was carried out as described in chapter 4.5.

In vitro intragastric acetaldehyde production

Samples of gastric juice were collected during elective upper gastrointestinal endoscopy from patients with different gastrointestinal diseases. Based on histological mucosal findings, 16 patients, five males and 11 females, mean age 49±2 years, had normal gastroduodenal mucosa, and 14 patients, six males and eight females, mean age 53±2 years, had chronic atrophic corpus gastritis. Patients using histamine-2-receptor antagonists or proton pump inhibitors were not included in the study. All subjects had fasted for at least 8 hours prior to the upper gastrointestinal endoscopy during which gastric juice was aspirated via endoscope. Due to the limited volume of gastric juice in some patients with corpus atrophy, a small amount of water was infused into the stomach before aspiration. The collected samples were stored refrigerated in sealed vials at +4°C for up to 20 hours until acetaldehyde analysis was performed. The pH of the samples was determined by using a glass electrode and a digital pH meter.

In vitro acetaldehyde production was studied by incubating 450 µl of the gastric juice with 50 µl of 1 M potassium phosphate buffer (pH 7.4) with or without ethanol (final concentration 1%) in sealed vials at 37°C for two hours. Acetaldehyde concentration was determined by using headspace gas chromatography.

4.7. ACETALDEHYDE PRODUCTION AND ALCOHOL DEHYDROGENASE CHARACTERISTICS OF AEROBIC GASTRIC BACTERIA (V)

Bacterial samples

Pure cultures of the aerobic bacteria isolated in study III (chapter 4.5) from hypochlorhydric gastric juices were used in this study. These bacterial samples, which had been frozen at –70°C after the isolation and identification of the bacteria, were revived on blood agar media. The cultures were then harvested into centrifuge tubes with 100 mM potassium phosphate buffer (pH 7.4) and washed with the buffer three times before bacterial suspensions, the turbidity of which had been adjusted to correspond to McFarland standard 3 (9 x 10^8 colony-forming units (cfu)/ml) for acetaldehyde and 6 (1.8 x 10^9 cfu/ml) for ADH activity determinations, were prepared. The actual number of viable bacteria, expressed as cfu/ml in the vials, was determined by quantitative bacterial culture.

Measurement of acetaldehyde production

The ability of different bacteria to produce acetaldehyde *in vitro* was determined by incubating 400 µl of the intact bacterial suspension in closed headspace vials with 50 µl of 100 mM potassium phosphate buffer (pH 7.4) containing ethanol (final concentration 22 mM) for 60 minutes at 37°C. The reactions were stopped by injecting 50 µl of 6 M perchloric acid (PCA) through the rubber septum of the vial, whereafter acetaldehyde was analysed by using headspace gas chromatography. The effect of different incubation times and ethanol and bacterial concentrations on acetaldehyde production was also studied by varying these parameters.

Measurement of ADH activity

An aliquot of the bacterial suspension was sonicated 8 x 20 sec in an ice bath and then centrifuged at 100,000 x g at 5°C for 65 minutes to obtain cytosol. Cytosolic ADH activities were determined spectrophotometrically, after addition of ethanol, by measuring the reduction of NAD (final concentration in the reaction mixture 1 mM) at 340 nm at 25°C in 100 mM glycine buffer (pH 9.6) containing 2 µM of rotenone, with ethanol concentrations ranging from 0.15 mM to 5 M. Enzyme activities were related to the protein concentrations of the supernatants, which were determined by using the Bio-Rad protein assay (Hercules, CA, USA).

4.8. GAS CHROMATOGRAPHIC MEASUREMENTS OF ETHANOL AND ACETALDEHYDE

Acetaldehyde production from ethanol for all studies was analysed by using headspace gas chromatography in which the vials were heated to a temperature of 37°C or 65°C (blood acetaldehyde determination, studies I, II), as reported earlier (Eriksson et al., 1982; Pikkarainen et al., 1979). The conditions for analysis were: Column 60/80 Carbopack B/5% Carbowax 20M, 2 m x 1/8" (Supelco Inc, Bellefonte, USA); oven temperature, 85°C; transfer line and detector temperature, 200°C; carrier gas flow rate (N_2), 20 ml/min.

An artifactual production of acetaldehyde from ethanol prior to headspace analysis is a problem associated with the measurement of acetaldehyde in biological fluids (Eriksson end Funaka, 1993). To control for this non-enzymatic artifactual acetaldehyde formation from ethanol during protein precipitation, perchloric acid was added simultaneously with ethanol into additional vials which were not incubated. The acetaldehyde concentrations of these control samples were subtracted from the acetaldehyde values obtained from the samples after the incubation periods (I-V).

4.9. STATISTICAL ANALYSIS

The results are expressed as means (V) or as mean ± SEM (I-IV). The statistical differences between the study groups were analysed by using Student's *t* test (I, II, IV), and the differences with and without the medications by using paired *t* test (II, III). Logarithmic transformation was performed when appropriate. The possible correlations were tested by using linear regression analysis (I, II) or Spearman Rank Order Correlation (III, V). Fisher's exact test was used to find out possible differences in confounding factors between the study groups (I). Values for K_m (Michaelis constant) were determined by using Lineweaver-Burk plots with a computerized data-analysis program (V).

5. RESULTS

5.1. THE EFFECT OF ALDEHYDE DEHYDROGENASE-2 GENOTYPE ON SALIVARY ACETALDEHYDE PRODUCTION (I)

ALDH2 genotyping showed that seven of the Asian volunteers were heterozygous for the mutant *ALDH2*2* allele (*ALDH2*2/ALDH2*1*) and thirteen had the normal *ALDH2*1/ALDH2*1* genotype. The groups did not differ with respect to gender, age, mean weight, smoking habits or alcohol consumption.

The mean *in vivo* salivary acetaldehyde levels were two to three times higher ($p<0.001$) in the ALDH2-deficient volunteers than in the subjects with normal ALDH2 throughout the whole follow-up period of 240 minutes (Fig. 1). Although the salivary ethanol levels were slightly higher in the subjects with the mutant *ALDH2*2* allele, the difference between the study groups was not statistically significant. The salivary acetaldehyde and ethanol levels correlated highly significantly ($p<0.001$) in both study groups. The mean capacity of the oral saliva to produce acetaldehyde from ethanol *in vitro* during the 90-minute incubation was equal in both groups; 86.2±6.5 µM and 84.1±13.1 µM for subjects with and without the *ALDH2*2* allele, respectively.

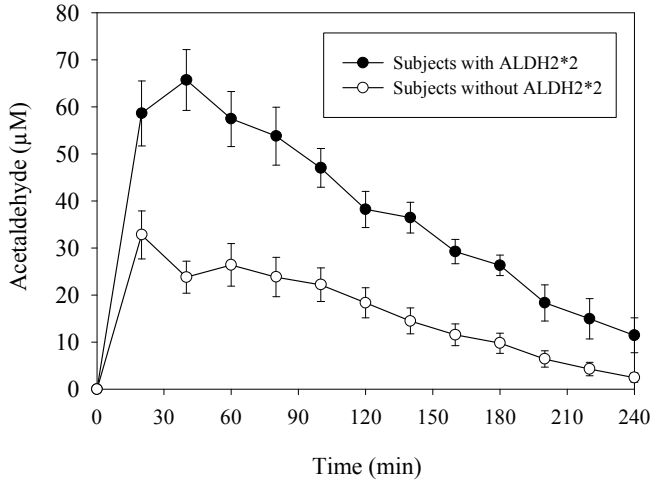

Fig. 1. *In vivo* acetaldehyde levels in oral saliva in seven subjects with the *ALDH2*2* allele and in 13 subjects with normal ALDH2 after a moderate dose of alcohol (0.5 g/kg of body weight). (Reproduced with permission from Lippincott Williams & Wilkins).

The mean acetaldehyde levels in the blood at 60 minutes were 6.6±2.6 μM in the ALDH2-deficient subjects and 0.3±0.2 μM in the subjects with normal ALDH2 (p=0.003). The mean blood ethanol levels at the same time were 12.3±1.3 mM and 10.9±0.5 mM for volunteers with and without the *ALDH2*2* allele, respectively (nonsignificant). The mean blood acetaldehyde level of the ALDH2-deficient volunteers at 60 minutes was only one ninth of the acetaldehyde level in the saliva *in vivo*.

Sixty minutes after the dose of alcohol, all three Asian subjects with the *ALDH2*2* allele had markedly elevated acetaldehyde levels in their sterile parotid gland saliva samples, whereas there was no measurable acetaldehyde in the parotid gland saliva samples of any of the Caucasians with the normal *ALDH2* genotype (Table 1).

Table 1. Parotid gland saliva in three subjects with and without the *ALDH2*2* allele 60 to 80 minutes after a moderate dose of alcohol (0.5 g/kg of body weight).

	Acetaldehyde (μM)	Ethanol (mM)
Subjects with *ALDH2*2*		
Case 1	75.0	8.8
Case 2	21.8	3.4
Case 3	3.9	9.9
Subjects without *ALDH2*2*		
Case 1	not detectable	6.2
Case 2	not detectable	13.9
Case 3	not detectable	13.2

5.2. THE EFFECT OF 4-METHYLPYRAZOLE ON ETHANOL METABOLISM AND SALIVARY ACETALDEHYDE PRODUCTION (II)

As expected, 4-MP decreased the ethanol elimination rate in both study groups, the reduction being 38 % in ALDH2-deficient volunteers and 46 % in subjects with normal ALDH2. The reduced ethanol elimination rate was also demonstrated by significant rises in the mean blood and salivary ethanol levels at 60 minutes in both study groups. The mean blood acetaldehyde levels at 60 minutes decreased in ALDH2-deficient subjects from 6.7±1.4 μM to 0.8±0.4 μM, p=0.013, but remained under the detection limit in subjects with normal ALDH2 on both study days.

Without 4-MP, the mean *in vivo* salivary acetaldehyde levels were approximately 1.5 times higher in ALDH2-deficient volunteers than in subjects with normal ALDH2 (Fig. 2). The difference between the groups disappeared, however, when 4-MP was used before ethanol administration (Fig. 2). The highest peak in the mean salivary acetaldehyde production in

subjects with the *ALDH2*2* allele at 40 minutes dropped significantly by using 4-MP, from 47.9±5.5 μM to 34.4±3.1 μM, p=0.035. At that time point there was no difference between the mean salivary ethanol levels with and without the medicine (11.9±1.5 mM without and 12.6±1.2 mM with 4-MP, nonsignificant). A marked reduction in the salivary acetaldehyde production with 4-MP in ALDH2-deficient subjects was found when the mean *in vivo* salivary acetaldehyde levels were expressed at different ethanol concentrations, whereas the medication did not have any effect on the salivary acetaldehyde production in the subjects with normal ALDH2 (Fig. 2). The correlations between individual salivary acetaldehyde and ethanol levels were positive and highly significant (r values ranged from 0.928 to 0.995 and all p values were less than 0.001) both with and without 4-MP in both groups. 4-MP did not change the mean *in vitro* salivary acetaldehyde production from ethanol in either of the study groups.

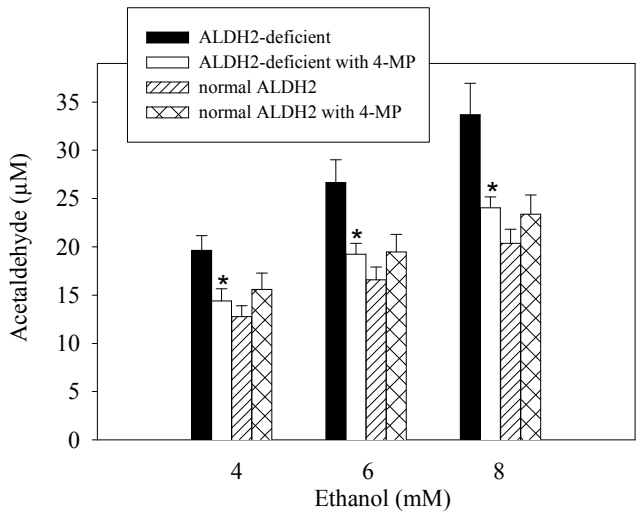

Fig. 2. The effect of 4-MP on the mean salivary acetaldehyde levels at certain ethanol concentrations in five ALDH2-deficient subjects and six subjects with normal ALDH2. *p<0.05 ALDH2-deficient volunteers with and without 4-MP. (Reproduced with permission).

Treatment with 4-MP markedly suppressed the flushing response of the ALDH2-deficient volunteers. The raises in heart rate and skin temperature as well as the drop in diastolic blood pressure disappeared when 4-MP was used before ethanol ingestion. In subjects with the normal *ALDH2* genotype there was no change in these parameters.

5.3. THE EFFECT OF IATROGENIC HYPOCHLORHYDRIA ON INTRAGASTRIC ACETALDEHYDE PRODUCTION (III)

The mean pH level of the gastric juice rose from 1.3±0.06 to 6.1±0.5, $p<0.001$ with lansoprazole treatment. After alcohol administration, this was associated with a significant increase in the mean intragastric acetaldehyde level from 22.1±2.3 µM to 55.4±8.0 µM, $p=0.003$. The intragastric acetaldehyde levels increased in all volunteers during the medication; the highest measured level was 100.5 µM (Fig. 3). The mean ethanol concentration of the gastric juice was 1.7% (range 0.7-3.4%) and 2.6% (range 1.2-4.1%), $p=0.054$, nonsignificant, before and during the treatment, respectively. Lansoprazole did not change the mean salivary acetaldehyde level (44.7±6.8 µM before the medication and 36.1±6.4µM during it).

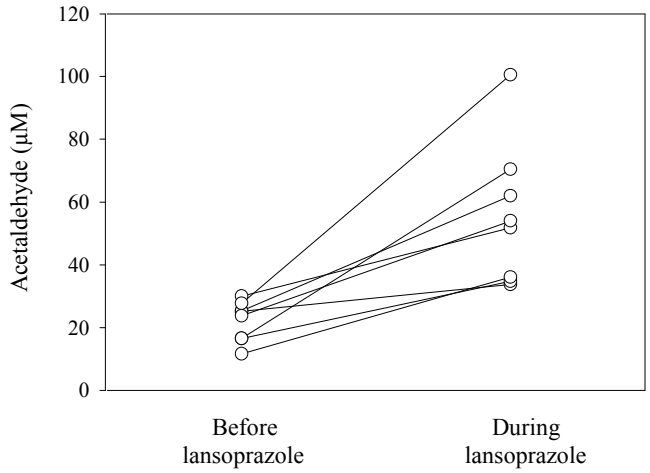

Fig. 3. The effect of lansoprazole treatment on gastric juice acetaldehyde concentrations in eight volunteers after ethanol (0.6 g/kg of body weight) ingestion. (Reproduced with permission from Aliment Pharmacol Ther).

Before the medication, minor growth of aerobic bacteria was detected in the gastric juice of two volunteers, 1×10^1 and 2×10^1 cfu/ml, respectively; the other bacterial cultures were negative. During lansoprazole, the mean total bacterial counts were 1.3±0.7 $\times 10^6$ cfu/ml for aerobes and 1.5±0.8 $\times 10^6$ cfu/ml for anaerobes. The increase in both total counts was highly significant ($p<0.001$). The cultures of yeasts were negative both before and during lansoprazole treatment. A vast selection of oral bacterial species was present; Table 2 summarizes the bacteriological results. There was also a highly significant positive correlation ($r=0.90$, $p<0.001$) between the individual total aerobic bacterial counts and the individual gastric juice acetaldehyde levels during the medication. The correlation between individual acetaldehyde levels and anaerobic bacterial counts was also positive ($r=0.76$, $p=0.021$).

Histologically, the gastric mucosal biopsies were within normal limits in all of the volunteers, and all of them were *Helicobacter pylori*-negative.

Table 2. The effect of lansoprazole treatment on the mean gastric juice bacterial counts (cfu/ml) in eight volunteers.

	Before lansoprazole	During lansoprazole	Prevalence
Aerobes			
Total counts	negative*	1.3×10^6	
Stomatococci spp.	negative	1.4×10^5	8/8
Viridans group Streptococci	negative	1.0×10^6	7/8
Neisseria spp.	negative	1.3×10^4	7/8
Corynebacterium spp.	negative	4.8×10^3	4/8
Staphylococcus aureus	negative	6.8×10^1	4/8
Coagulase-negative Staphylococci and Micrococci spp.	negative	1.0×10^3	1/8
Haemophilus parainfluenzae	negative	6.3×10^3	1/8
Bacillus spp.	negative	3.8×10^3	1/8
Anaerobes			
Total counts	negative	1.5×10^6	
Pigmented *Prevotella* spp.	negative	1.3×10^5	6/8
Actinomyces spp.	negative	5.5×10^4	6/8
Fusobacterium spp.	negative	1.0×10^4	5/8
Bacteroides ureolyticus-like group	negative	5.1×10^3	4/8
Lactobacillus spp.	negative	1.4×10^5	3/8
Nonpigmented *Prevotella* spp.	negative	1.4×10^4	2/8
Anaerobic cocci	negative	2.8×10^5	2/8
Capnocytophaga spp.	negative	3.4×10^1	1/8

* Detection threshold 10^1 cfu/ml

5.4. INTRAGASTRIC ETHANOL METABOLISM IN PATIENTS WITH ATROPHIC GASTRITIS (IV)

Endogenous ethanol and acetaldehyde production

The mean pH levels of the gastric juice of the *in vivo* study groups were 6.8±0.3 and 1.6±0.2 in atrophic gastritis patients and controls, respectively. Some endogenous intragastric ethanol production after glucose infusion was detected in four of the subjects with atrophic gastritis, whereas no endogenous ethanol was measured from the gastric juices of the control subjects. The mean ethanol levels and corresponding mean acetaldehyde levels of the gastric juice after intragastric glucose infusion in atrophic gastritis patients are summarized in table I, study IV.

In vivo acetaldehyde production from ethanol

After intragastric ethanol infusion, the mean acetaldehyde levels in the gastric juice of the study groups were 44.5±9.2 μM in atrophic gastritis patients vs. 9.8±0.9 μM in controls at 30 minutes and 33.7±7.9 μM vs. 5.1±1.5 μM at 60 minutes (Fig. 4). The difference between the study groups was 4.5-fold at 30 minutes (p=0.011) and 6.5-fold (p=0.01) at 60 minutes (Fig. 4). The mean ethanol concentrations of the gastric juice at 30 and 60 minutes were 2.8% (range 0.2-5.1%) and 1.5% (range 0.1-4.2%) in atrophic gastritis patients, and 4.3% (range 0.7-5.1%) and 0.8% (range 0.04-1.8%) in controls. The differences in gastric juice ethanol concentrations between the study groups were not statistically significant. Neither did the groups differ in their *in vivo* salivary acetaldehyde production; the mean salivary acetaldehyde levels at 60 minutes were 16.0±2.1 μM and 17.3±1.9 μM in atrophic gastritis patients and in controls, respectively.

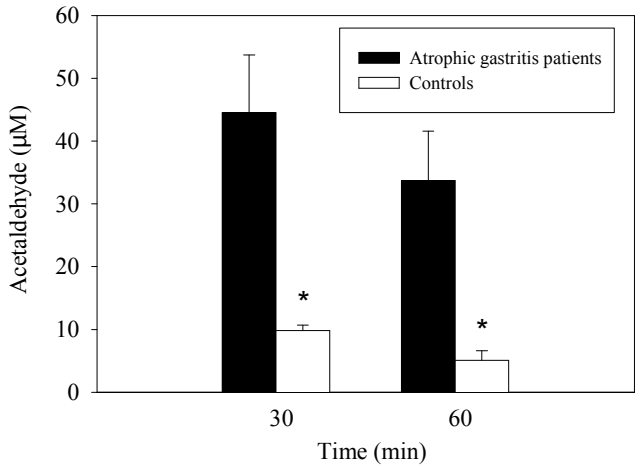

Fig. 4. The mean gastric juice acetaldehyde levels at 30 and 60 min after intragastric ethanol (0.3 g/kg of body weight) infusion in seven atrophic gastritis patients and five controls. *p<0.05 patients compared to controls. (Reproduced with permission from Scand J Gastroenterol).

Microbial analysis

Microbial analysis showed marked growth of both aerobic and anaerobic bacteria in the gastric juice of the atrophic gastritis patients, whereas only minor growth of anaerobic bacteria, 4.6 x 10^2 cfu/ml at 30 minutes and 2.0 x 10^1 cfu/ml at 60 minutes, was detected in one of the control subjects after ethanol infusion. The differences in bacterial counts between the study groups were highly significant (p<0.001) at all studied time points after both infusions. Most patients had a positive gastric juice culture for *Enterobacteriaceae* or yeasts on one or more sampling occasions; 2/7 of the patients harboured both groups. The most prevalent and numerous bacterial species were

Streptococcus, Stomatococcus, Neisseria, and *Corynebacterium* species of aerobes, and *Prevotella, Actinomyces, Fusobacterium,* and *Capnocytophaga* species of anaerobes. There were no significant changes in the composition of the gastric flora at 30 and 60 minutes after both ethanol and glucose infusions in the atrophic gastritis patients. However, the number of total anaerobes and *Prevotella* species was markedly higher ($p<0.05$) after glucose infusion compared to that after ethanol infusion. The microbial findings in the saliva were fairly similar in both study groups on both study days.

In vitro acetaldehyde production from ethanol

In vitro the achlorhydric, pH 6.7±0.2, gastric juice of the 14 atrophic gastritis patients produced very high acetaldehyde levels (maximum 694 μM) during the incubation with 1% ethanol. The mean acetaldehyde production was 7.6-fold higher ($p<0.001$) in this group than in the control group consisting of 16 subjects with histologically normal gastroduodenal mucosa and acidic gastric juice, pH 1.9±0.2. The mean acetaldehyde levels were 228±52 μM and 30±11 μM in atrophic gastritis patients and in controls, respectively (Fig. 2, study IV).

5.5. ACETALDEHYDE PRODUCTION AND ALCOHOL DEHYDROGENASE CHARACTERISTICS OF AEROBIC GASTRIC BACTERIA (V)

Altogether, 11 different aerobic bacterial species and 51 different strains were isolated from the hypochlorhydric gastric juices of the eight volunteers. The number of bacterial species or groups from one individual varied between one and 11. The *in vitro* acetaldehyde production of the isolated bacterial strains varied from less than one to 13,687 nmol acetaldehyde/10^9 cfu/hour. The acetaldehyde production capacity of the bacteria was in proportion to the length of the incubation time, the concentration of bacteria, and the concentration of ethanol.

From the 51 strains tested for their acetaldehyde production capacity, the strains that produced more than 100 nmol acetaldehyde/10^9 cfu/hour (n=23) were selected for ADH activity measurements. Exact K_m values for ethanol for 18 of the bacterial strains tested for ADH activity were obtained. The ADH activities of the strains varied from 3.9 to 1253 nmol NADH/min/mg protein, and K_m´s for ethanol ranged from 0.65 to 116 mM and from 0.5 to 3.1 M (high K_m). A statistically significant correlation ($r=0.64$, $p<0.001$) could be found between the acetaldehyde production and the ADH activity of the tested strains. The most potent acetaldehyde producers according to their acetaldehyde production, ADH activity, and K_m values for ethanol were *Neisseria* and *Rothia* species and *Streptococcus salivarius*, whereas nearly all *Stomatococcus*, *Staphylococcus,* and other *Streptococcus* species had very low capacities to produce acetaldehyde.

6. DISCUSSION

6.1. HIGH SALIVARY ACETALDEHYDE IN ALDEHYDE DEHYDROGENASE-2-DEFICIENT SUBJECTS: STRONG EVIDENCE FOR THE LOCAL CARCINOGENIC ACTION OF ACETALDEHYDE

Many epidemiological studies show that the risk of digestive tract and especially upper digestive tract cancers is markedly increased in ALDH2-deficient heavy drinkers (Murata et al., 1999; Tanabe et al., 1999; Yokoyama et al., 1996a-c, 1998a,b). ALDH2 deficiency is very common in certain Asian populations; 30-50% of Asians are known to be carriers of the mutated *ALDH2*2* allele (Goedde et al., 1992). So far, the observed ethanol-related cancer risk among these individuals has been hypothesized to originate from the systemic effects of elevated blood acetaldehyde concentrations (Yokoyama et al., 1996b).

In this study, we demonstrated that after a moderate dose of alcohol ALDH2-deficient subjects have two to three times higher acetaldehyde levels in their saliva than subjects with normal ALDH2. Since the blood acetaldehyde levels of the ALDH2-deficient volunteers were only one ninth of those in the saliva at the same time, the higher salivary acetaldehyde levels in these individuals cannot be derived from the blood. Subsequently, we found out for the first time that human parotid salivary glands are able to oxidize ethanol to acetaldehyde. However, the parotid glands of the ALDH2-deficient subjects did not appear to have a sufficient capacity to metabolize acetaldehyde further to acetate. Therefore, acetaldehyde as a very water-soluble compound diffuses together with ethanol into the saliva causing a marked increase in the acetaldehyde concentration of the mouth.

In accordance with our earlier findings (Homann et al., 1997a), this study confirmed that salivary acetaldehyde levels correlate positively and highly significantly with salivary and blood ethanol levels both in individuals with normal and in individuals with deficient ALDH2. Salivary acetaldehyde production in Caucasians with normal ALDH2 is mainly of microbial origin and it is strongly influenced by individual factors (Homann et al., 1997a). Tobacco smoking and alcohol drinking, the most potent external risk factors for upper digestive tract cancers, are the strongest factors increasing the microbial acetaldehyde production from ethanol (Homann et al., 2000a). Thus, it is likely that an ALDH2-deficient individual who smokes and drinks heavily produces very high levels of acetaldehyde in the oral cavity during alcohol consumption. The salivary samples of the volunteers with and without the mutant *ALDH2*2* allele did not differ with respect to their capacity to produce acetaldehyde from ethanol *in vitro*. This suggests that there are no major differences regarding this feature in the composition of the oral microflora between these two groups.

The present study with Asian volunteers representing different *ALDH2* genotypes showed that individuals with the mutant *ALDH2*2* allele have significantly higher *in vivo* acetaldehyde levels in their saliva after alcohol consumption than subjects with normal ALDH2. Together with earlier epidemiological findings these results suggest that enhanced alcohol-related cancers in ALDH2-deficient subjects originate from the local production of acetaldehyde in the digestive tract.

6.2. THE EFFECT OF 4-METHYLPYRAZOLE ON ETHANOL METABOLISM AND SALIVARY ACETALDEHYDE PRODUCTION

As already stated in chapter 2.4., 4-MP decreases the rate of ethanol elimination by inhibiting ADH competitively (Li and Theorell, 1969; Salaspuro, 1985). It can be used in the treatment of methanol and ethylene glycol poisonings (Jacobsen and McMartin, 1997; Jacobsen et al., 1988, 1990), and in the management of the disulfiram-alcohol reaction (Lindros et al., 1981) and the so-called flushing reaction of ALDH2-deficient individuals (Inoue et al., 1985). In these subjects, even a very small dose of alcohol causes marked cardiocirculatory stimulation with facial flushing, increased heart rate and decreased blood pressure (Kupari et al., 1983). Blood acetaldehyde levels, which during alcohol consumption are about six times higher in subjects with partially inactive ALDH2 than those in subjects with normal ALDH2 (Yokoyama et al., 1996a), can also be reduced by the use of 4-MP (Inoue et al., 1985). In accordance with these studies, all the unpleasant physiological effects as well as the blood acetaldehyde levels were also markedly suppressed in the present study with the oral pre-treatment with 4-MP.

In this study, the oral dose of 4-MP, before ethanol administration, reduced the salivary acetaldehyde production significantly in ALDH2-deficient subjects, but did not have any effect on the salivary acetaldehyde levels in subjects with normal ALDH2. As 4-MP is an effective inhibitor of human ADHs, but a poor inhibitor of bacterial ADHs (Jokelainen et al., 1994), these results suggest that salivary acetaldehyde production is mainly of bacterial origin in individuals with normal ALDH2, which supports earlier findings (Homann et al., 1997a). Since 4-MP did not decrease salivary acetaldehyde levels in subjects with the normal *ALDH2* genotype, these results, in addition, suggest that the role of oral mucosal ADHs in salivary acetaldehyde production is minimal.

The findings of study I concerning both *in vivo* and *in vitro* salivary acetaldehyde production were also confirmed by this study. The *in vivo* salivary acetaldehyde levels were significantly higher in subjects with the mutant *ALDH2*2* allele than in subjects without it, although the ethanol dose used in this study was lower than that in the first study. The *in vitro* salivary acetaldehyde production from ethanol was again equal in both study groups, which indicates that the composition of the oral microflora must have been fairly similar in these groups.

6.3. ETHANOL METABOLISM IN HYPOCHLORHYDRIC STOMACH

Due to its acidity, the stomach is usually sterile, but if the gastric pH rises over 5, microbial proliferation can be expected to occur in the stomach (Stockbruegger, 1985). Consequently, gastric microbial overgrowth is a common finding in achlorhydric atrophic gastritis and pernicious anaemia (Drasar et al., 1969; Stockbruegger et al., 1984), and during the use of gastric proton pump inhibitors or histamine-2-receptor antagonists (Thorens et al., 1996; Verdu et al., 1994).

Bacterial overgrowth and yeast infection are responsible for the endogenous ethanol production in the upper digestive tract via alcoholic fermentation carried out by microbial alcohol dehydrogenases (Bode et al., 1984b). Under aerobic and microaerobic conditions, and in the presence of exogenous ethanol, the ADH-mediated reaction can also run in the opposite direction and result in high local acetaldehyde production (Homann et al., 1997a; Jokelainen et al., 1996c; Salaspuro et al., 1999).

Atrophic gastritis has long been recognized as a risk factor for gastric cancer. Epidemiological follow-up studies have shown that the incidence of gastric cancer in patients with atrophic gastritis is about 4.6-10%, and approximately 20-40% of the gastric cancer cases are associated with atrophic gastritis (Morson et al., 1980; Siurala et al., 1974; Testoni et al., 1987; Walker et al., 1971). The risk of gastric cancer is exponentially correlated with the severity of the atrophy (Sipponen et al., 1985).

Heavy drinkers are also known to have more chronic gastritis than moderate drinkers (Dinoso et al., 1972; Parl et al., 1979). Furthermore, their evolution from superficial to atrophic gastritis occurs at an earlier age and they have more intestinal metaplasia than moderate alcohol consumers (Parl et al., 1979). It is clear that alcohol consumption is one of the major risk factors for upper digestive tract cancers (IARC, 1988). Nevertheless, the epidemiological data concerning the role of alcohol drinking in gastric carcinogenesis has been very controversial (Doll et al., 1999). However, the increased risk of stomach cancer has been reported to be associated with excessive drinking and the presence of the mutant *ALDH2*2* allele (Yokoyama et al., 1998a).

Study III showed that treatment with proton pump inhibitors leads to microbial colonization of the stomach and to a 2.5-fold increase in the gastric juice acetaldehyde levels after alcohol consumption. It can thereby be concluded that iatrogenic hypochlorhydria in the stomach leads to intragastric microbial acetaldehyde production from ingested ethanol in healthy volunteers. Moreover, a highly significant positive correlation between the individual total aerobic bacterial counts and the individual acetaldehyde levels during the medication was found in the study. Study IV demonstrated that microbial acetaldehyde formation also exists in the stomach of patients with achlorhydric atrophic gastritis. The acetaldehyde levels after intragastric

ethanol infusion in the gastric juice of the atrophic gastritis patients were 4.5-6.5–fold higher than those in controls. After 2 hours' *in vitro* incubation with ethanol, the difference in the acetaldehyde production of the gastric juice between atrophic gastritis patients and individuals with normal gastric mucosa was even more obvious, 7.6-fold. We also managed to detect minor endogenous ethanol production in the stomach of the atrophic gastritis patients after intragastric glucose infusion. In addition, the salivary acetaldehyde levels measured in these studies after alcohol ingestion or infusion correspond to those reported earlier (Homann et al., 1997a).

Both studies confirmed the finding that the oral cavity is the primary source of the microbial flora of the neutral stomach (Drasar et al., 1969; Hill, 1995). However, the present work additionally showed that *Enterobacteriaceae* and yeasts, well-known acetaldehyde producers (Jokelainen et al., 1996a; Tillonen et al., 1999a), are overrepresented in the gastric juice samples of patients with atrophic gastritis. The composition of the microbial flora in the saliva and in the gastric juice of the atrophic gastritis patients was very similar. The most numerous and prevalent aerobic bacterial species isolated from hypochlorhydric gastric juices in both studies were *Streptococcus*, *Stomatococcus*, *Neisseria* and *Corynebacterium* species, which is in accordance with earlier studies (Thorens et al., 1996; Verdu et al., 1994).

An interesting observation is that the gastric juice acetaldehyde levels found in these studies were equal to the salivary acetaldehyde levels found in ALDH2-deficient subjects after a moderate dose of alcohol (I, II). Moreover, many of the *in vitro* and animal studies dealing with the carcinogenic action of acetaldehyde have been carried out with acetaldehyde concentrations less than 500 μM (Homann et al., 1997b; Koivisto and Salaspuro, 1997, 1998). Thus, the intragastric acetaldehyde levels reported in these studies can be considered to be comparable to those suggested to be carcinogenic in other studies.

6.4. ACETALDEHYDE PRODUCTION AND ALCOHOL DEHYDROGENASE CHARACTERISTICS OF AEROBIC GASTRIC BACTERIA

As described in the previous chapter, acetaldehyde formation from ethanol occurs in the hypochlorhydric stomach by microbes mainly originating from the oral cavity. Our earlier studies have shown that some oral *Candida albicans* strains have a marked capacity to produce acetaldehyde (Tillonen et al., 1999a), but the role of other microbial species originating from the mouth in the production of ethanol-derived acetaldehyde had not been assessed before.

Marked differences in the capacity of aerobic gastric bacteria to produce carcinogenic acetaldehyde from ethanol were found in this study. The most potent acetaldehyde producers of the studied strains, according to their ADH activity,

K_m's for ethanol, and *in vitro* capacity to produce acetaldehyde from ethanol, were *Neisseria* and *Rothia* species and *Streptococcus salivarius*. A significant correlation between bacterial ADH activity and *in vitro* acetaldehyde production from ethanol by homologous strains was also found. Accordingly, *Neisseria* and *Rothia* species and *Streptococcus salivarius* had the highest ADH activities, and thereby, the highest capacities to produce acetaldehyde, whereas *Stomatococcus, Staphylococcus* and other *Streptococcus* species, which had low ADH activities, also produced minimal amounts of acetaldehyde. Many bacterial strains, especially *Neisseria* and *Rothia* species, had rather low K_m values for ethanol. These K_m values are comparable with the ethanol concentrations that are observed in the stomach after normal social drinking. Under these circumstances bacterial ADHs are able to metabolize ethanol to acetaldehyde with a velocity close to the maximum, and to produce marked amounts of acetaldehyde. The bacteria that had the lowest K_m values, practically less than 5 mM, are also most probably able to produce acetaldehyde from ethanol that reaches the stomach through blood circulation.

Study III showed that there is a highly significant correlation between the number of aerobic bacteria and the production of acetaldehyde from ingested ethanol in the hypochlorhydric stomach. The most prevalent and numerous aerobic bacteria isolated from the gastric juices in the same study were *Stomatococcus, Streptococcus* and *Neisseria* species. Although *Rothia* species of the present study were potent acetaldehyde producers according to their high *in vitro* acetaldehyde production and ADH activity, their role in the *in vivo* acetaldehyde production is most probably minimal since both the prevalence and the number of *Rothia* species were low in the *in vivo* study. On the other hand, *Neisseria* and *Streptococcus salivarius* species were both very prevalent and numerous and, in addition to high ADH activities and capacities to produce acetaldehyde from ethanol *in vitro*, they also had low K_m values. Thereby, it can be assumed that these bacterial species are probably also responsible for the major proportion of the *in vivo* acetaldehyde production in subjects with hypochlorhydric stomach.

7. SUMMARY AND CONCLUSIONS

The key findings of the present study were:

1. ALDH2-deficient subjects have two to three times higher *in vivo* salivary acetaldehyde levels than subjects with normal ALDH2 after a moderate dose of alcohol. Part of their "extra" acetaldehyde originated from parotid salivary glands, which indicates that in addition to oral microflora, parotid salivary glands may also produce acetaldehyde into saliva. When this information is combined with earlier epidemiological data reporting an increased upper digestive tract cancer risk in heavy-drinking ALDH2-deficient individuals, our results provide strong evidence that acetaldehyde produced from ethanol via microbes, mucosal cells and/or glands is a local and topical carcinogen in the digestive tract in humans.

2. A single dose of 4-MP before ethanol ingestion reduces the flushing reaction, and both blood and salivary acetaldehyde levels in ALDH2-deficient subjects, but not in subjects with the normal *ALDH2* genotype. Since 4-MP is an effective inhibitor of human ADHs, but a poor inhibitor of bacterial ADHs, these results indicate that the role of oral mucosal ADHs in salivary acetaldehyde production is minimal, and support earlier findings suggesting that salivary acetaldehyde production is mainly of bacterial origin in subjects with normal ALDH2.

3. The use of gastric proton pump inhibitors leads to hypochlorhydria and overgrowth of bacteria in the gastric juice. This associates with enhanced intragastric production of carcinogenic acetaldehyde via ADH mediated ethanol oxidation carried out by the aerobic bacteria representing normal oral microflora. These results indicate for the first time that microbial *in vivo* acetaldehyde production from ingested ethanol also takes place in the hypochlorhydric stomach. In addition, our results suggest that long term use of proton pump inhibitors may increase the risk of gastric cancer.

4. This study showed that high microbial acetaldehyde production from ethanol and minor endogenous ethanol and acetaldehyde formation from glucose also occurs in the stomach of patients with achlorhydric atrophic gastritis. Microbial analysis revealed that in addition to a vast selection of oral bacteria, *Enterobacteriaceae* and yeasts, both known to be high acetaldehyde producers, were also found in many of the gastric juice samples of the atrophic gastritis patients participating in the *in vivo* study. Since acetaldehyde is a local carcinogen in the digestive tract in humans, intragastric production of this compound could be one of the factors responsible for enhanced gastric cancer risk among patients with atrophic gastritis.

5. It has previously been demonstrated that oral yeasts have a high capacity to produce acetaldehyde from ethanol. In this study with oral aerobic bacteria isolated from hypochlorhydric gastric juices, we showed additionally that *Neisseria* and *Rothia* species and *Streptococcus salivarius* are also very potent acetaldehyde producers according to their ADH activities, K_m values for ethanol, and *in vitro* capacity to produce acetaldehyde from ethanol. Since *Neisseria* species and *Streptococcus salivarius* were also among the most prevalent and numerous bacteria found in the hypochlorhydric gastric juices, it can be assumed that these bacteria are also responsible for the bulk of the acetaldehyde production in the hypochlorhydric stomach. Thereby, these findings offer new and important information with respect to the local production of carcinogenic acetaldehyde in the upper digestive tract of achlorhydric human subjects.

Together with earlier epidemiological data and studies in this field, the findings of this thesis provide strong evidence for the local carcinogenic action of acetaldehyde in the upper digestive tract in humans. The carcinogenic effects of acetaldehyde can be modified by genetic factors, drinking and smoking habits, individual differences in oral and gastric microflora, and blood and intraintestinal ethanol levels. The present findings open a new genetic and microbiological approach for the pathogenesis, screening and prevention of digestive tract cancers.

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